THE UNIVERSITY OF MANITOBA

BIOCHEMICAL CHARACTERIZATION OF THE *Pythium ultimum* NAD-GDH AND GENETIC ANALYSIS OF ASSOCIATED GENOMIC REGIONS IN *Pythium ultimum* AND *Achlya klebsiana*

BY

DOUGLAS SHAW BARKER

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BY

DOUGLAS SHAW BARKER

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

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"Little monk, little monk. You always take the hard road."

Frundsberg to Martin Luther
Diet of Worms, 1521

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Most of all, I would like to thank Bei for being there.

ABSTRACT

Investigation of an antisense gene pair previously identified in the oomycete *Achlya klebsiana* and of a NAD-specific glutamate dehydrogenase (NAD-GDH) and putative heat shock 70 stress response protein gene *(hsp70)* in the oomycete *Pythium ultimum* strain 471 were conducted. The transcriptionally active nature of *the A. klebsiana* antisense gene pair in both eukaryotic and prokaryotic host cell lines was confirmed although the specific identities and compositions of the transcripts produced were not. The *P. ultimum* NAD-GDH in crude cell extracts was characterized, and shown to be similar to the previously characterized NAD-GDH of *Pythium debaryanum*, but less so to the NAD-GDH of *A. klebsiana.* Biochemical characterization of the NAD-GDH included analyses of enzyme instability at several temperatures (counteracted with high concentrations of glycerol); pH optima of both the reductive and oxidative reactions of NAD-GDH (8.8 and 7.2, respectively); induction by L-glutamate; and allosteric activation (of up to 14 fold) by micromolar concentrations of $NADP^+$. As concentrations of $NADP^+$ are increased, K_m decreased over 10 fold and V_{max} increased over 2 fold with α-

ketoglutarate as substrate, while K_m decreased 30 fold and V_{max} increased 1.3 fold with L-glutamate as substrate. Partial sequencing of the putative *hsp70* gene showed it to have strong homology to other *hsp70* genes, and also to possess consensus sequences corresponding to transcriptional promoter regions (CCAAT boxes, TATAAT boxes, CTF/NF1 binding sites, heat shock elements) in the putative 5' untranslated region. The gene appears to consist of a single open reading frame, which is almost identical to another putative *hsp70* gene identified in *A. klebsiana* approximately 7.8 kb away from the *hsc70:nad-gdh* antisense gene pair.

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LIST OF ABBREVIATIONS

A	adenine
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
bp	base pair
Bis-acrylamide	N,N'-methylene bis-acrylamide
β-ME	β-mercaptoethanol
BSA	bovine serum albumin
C	cytosine
cDNA	complementary deoxyribose nucleic acid
CIP	calf intestinal phosphatase
CTF/NF1	CAAT-binding transcription factor
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytosine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DMEM	Dulbecco's modified Eagles medium
DMSO	dimethyl sulfoxide
DNA	deoxyribose nucleic acid
dTTP	2'-deoxythymidine 5'-triphosphate
EDTA	ethylenediamine-tetra-acetic acid
g	gram(s)

G	guanine
%GC	percent guanosine and cytosine content
GDH	Glutamate dehydrogenase
GTP	guanosine 5'-triphosphate
hr	hour(s)
hsc70	heat shock cognate 70 gene
HSC70	heat shock cognate 70 polypeptide
HSE	heat shock element
HSF	heat shock transcription factor
HSTF	heat shock transcription factor
hsp70	heat shock protein 70 gene
HSP70	heat shock protein 70 polypeptide
IgG	Immunoglobulin G
IPTG	isopropylthiogalactoside
kb	kilobase
kD	kilodalton
K_{mA}	Michaelis constant for an enzyme using substrate A
L	litre
LAC-ORF	long antisense complementary open reading frame
MCS	multiple cloning site
mg	milligrams
µg	micrograms
min	minutes
mL	millilitre
µL	microlitre
M	molar (moles per litre)
mM	millimolar (millimoles per litre)
µM	micromolar (micromoles per litre)

MOPS	morpholinepropanesulfonic acid
M_r	relative molecular mass
mRNA	messenger ribose nucleic acid
MRT	molecular recognition theory
$N_{2(l)}$	liquid nitrogen
NAD^+	nicotinamide adenine dinucleotide, oxidized form
NADH	nicotinamide adenine dinucleotide, reduced form
$NADP^+$	nicotinamide adenine dinucleotide phosphate, oxidized
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
NAD-GDH	nicotinamide adenine dinucleotide specific glutamate dehydrogenase protein
nad-gdh	nicotinamide adenine dinucleotide specific glutamate dehydrogenase gene
NADP-GDH	nicotinamide adenine dinucleotide phosphate specific glutamate dehydrogenase protein
NAD(P)-GDH	dual coenzyme specific glutamate dehydrogenase protein
NCS	newborn calf serum
ng	nanograms
NSF	non-stop frame
OD	optical density
ORF	open reading frame
P	probability
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
PPO	2,5 diphenyl oxazole
RNA	ribonucleic acid
$RNA_{POL}II$	ribonucleic acid polymerase II

rRNA	ribosomal RNA
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate denaturing polyacrylamide gel electrophoresis
T	thymine
TAME	Nα-p-tosyl-L-arginine methylester
TEMED	N,N,N',N'-tetramethylethylenediamine
TPCK	L-1-tosylamide-2-phenyl-ethylchloromethyl ketone
TRIS	trihydroxymethylaminomethane
U	enzyme units
UV	ultraviolet
V	volts
V_e	enzyme velocity at a given concentration of exogenous activator
V_o	enzyme velocity in the absence of exogenous activator
V_{maxA}	maximum velocity for an enzyme using substrate A
w/v	weight per volume
v/v	volume per volume
X-Gal	5-bromo-4-chloro-3-indoyl-β-D-galactoside

INTRODUCTION

The catalysis of the amination of α-ketoglutarate to L-glutamate and/or the reverse deamination reaction by glutamate dehydrogenase (GDH) are vital links between nitrogen metabolism and the tricarboxylic acid cycle. This family of enzymes is subcategorized based upon nucleotide coenzyme usage and associated function, including NAD-GDH, NADP-GDH and NAD(P)-GDH, the latter able to use both NAD^+ and $NADP^+$ as coenzymes (DiRuggiero and Robb, 1993; Rice, 1985; Smith *et al.*, 1975). A characteristic of an organism's phylogenetic grouping is which of these GDHs are present or if multiple isozymes are possessed (McDaniel, 1986; DeCastro *et al.*, 1970).

The NAD-GDHs of some members of the oomycota, a class of pseudofungal protists in the Kingdom Chromista, have been studied with a number found to be activeable by $NADP^+$ (LéJohn, 1975). One of these, the L-glutamine inducible, $NADP^+$ activeable NAD-GDH of *Achlya klebsiana*, was purified. Antibodies raised against this enzyme were used to probe a cDNA

library in order to obtain a clone used to identify and isolate a genomic clone containing the entire *A. klebsiana nad-gdh* gene (LéJohn *et al.*, 1994b). These anti-NAD-GDH antibodies not only recognized NAD-GDH, but also an immunologically cross-reacting peptide coinduced with NAD-GDH with an estimated M_r of 70000 Daltons. Analysis of the *nad-gdh* gene revealed a large open reading frame (ORF) on the antisense strand, encoding a predicted peptide of approximately M_r 70000 Daltons possessing several motifs or signatures characteristic of heat shock proteins (HSPs) (LéJohn *et al.*, 1994a).

In order to further investigate the significance of this "antisense gene pair" and nearby chromosomal regions, as well as the degree of conservation in other oomycetes of the antisense gene pair, a multi-faceted research strategy was employed. In order to demonstrate that the putative *hsc70* gene was indeed transcribable from the antisense gene pair, attempts to express the antisense gene pair in isolation from the rest of the *A. klebsiana* genome were made using recombinant DNA methodologies in both prokaryotic and eukaryotic host cell lines. The presence of a similar antisense gene pair in *Pythium ultimum* was predicted from previous genomic polymerase chain reaction (PCR) analysis, and characterization of the NAD-GDH from this organism was conducted to elucidate its similarity to the NAD-GDH of *A. klebsiana*. Finally part of a putative *hsp70* gene,

identified on the *A. klebsiana* genomic clone containing the antisense gene pair but distinct from and downstream of the putative *hsc70* gene was amplified by PCR from a *P. ultimum* genomic DNA library clone and sequenced.

These experiments have demonstrated that the *A. klebsiana* antisense gene pair can generate multiple transcripts in both prokaryotic and eukaryotic host cell lines. The specific identities of these transcripts were hypothesized but not confirmed. Production of overlapping transcripts from either the same or complementary DNA strands seems to have occurred, the specific identities of transcripts from either prokaryotic or eukaryotic host cell lines were not established.

The NAD-GDH of *Pythium ultimum* was demonstrated to share several characteristics with the NAD-GDH of *Pythium debaryanum*, including the role of $NADP^+$ as an activator but not as a cofactor; induction by L-glutamate; and different pH optima for the two reactions catalyzed by NAD-GDH. K_m and V_{max} values indicate a lower affinity and rate of substrate turnover for the oxidative deamination reaction compared to the reductive amination reaction catalyzed by NAD-GDH at the pH optima for these reactions. Stabilization of NAD-GDH at several temperatures was shown to be dependent upon glycerol concentration, as has been demonstrated previously for the *A. klebsiana* NAD-GDH. An ORF and associated 5' untranscribed regions

almost identical to analogous regions of the *A. klebsiana* genome were also identified and sequenced. These were shown to have several characteristics of, and high sequence homology to, *hsp70* genes from a wide variety of organisms.

HISTORICAL

1. Description of *Pythium ultimum* and *Achlya klebsiana*

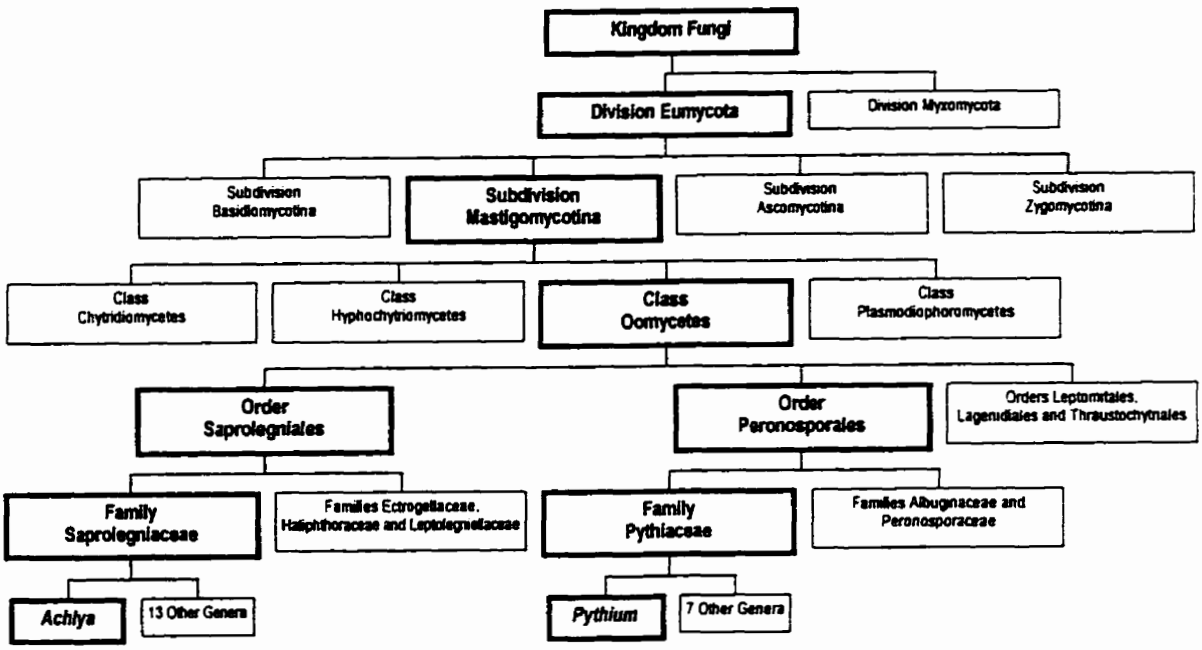
1.I. The Oomycota

Pythium ultimum and *Achlya klebsiana* are both oomycetes, one of four groupings of zoosporic fungi (Barr, 1983a) (Figure 1) which, although largely aquatic, can also be terrestrial. Members of the class Oomycetes are oogamous, producing two types of gametes termed eggs (contained in oogonia) and sperm, (Speer and Waggoner, 1995). Superficially, the filamentous growth of oomycetes resembles that of fungi, but they are physiologically distinct from true fungi in several ways. Oomycete cell walls are composed of β-glucans, hydroxyproline and cellulose instead of chitin although some, including the genus *Achlya*, are exceptions which do possess chitin in their cell walls (Campos-Takaki *et al.*, 1982) while oomycete mitochondria have tubular rather than plate-like cristae; oogamous reproduction involves gametangial contact to produce a sexual oospore (Alexopoulos *et al.*, 1996) with meiosis occurring prior to,

Figure 1: Classification of the Genera *Pythium* and *Achlya*.

Taxonomic categorization of the genera *Pythium* and *Achlya* are shown, using both **a.** the classical classification of the oomycota as fungi (Barr, 1983b; Dick, 1973; Waterhouse, 1973) and **b.** their placement in the new kingdom Chromista (Cavalier-Smith, 1993; Cavalier-Smith, 1989; Cavalier-Smith, 1986). Those classifications to which *Pythium* and/or *Achlya* belong are denoted with bold type and thick borders.

a.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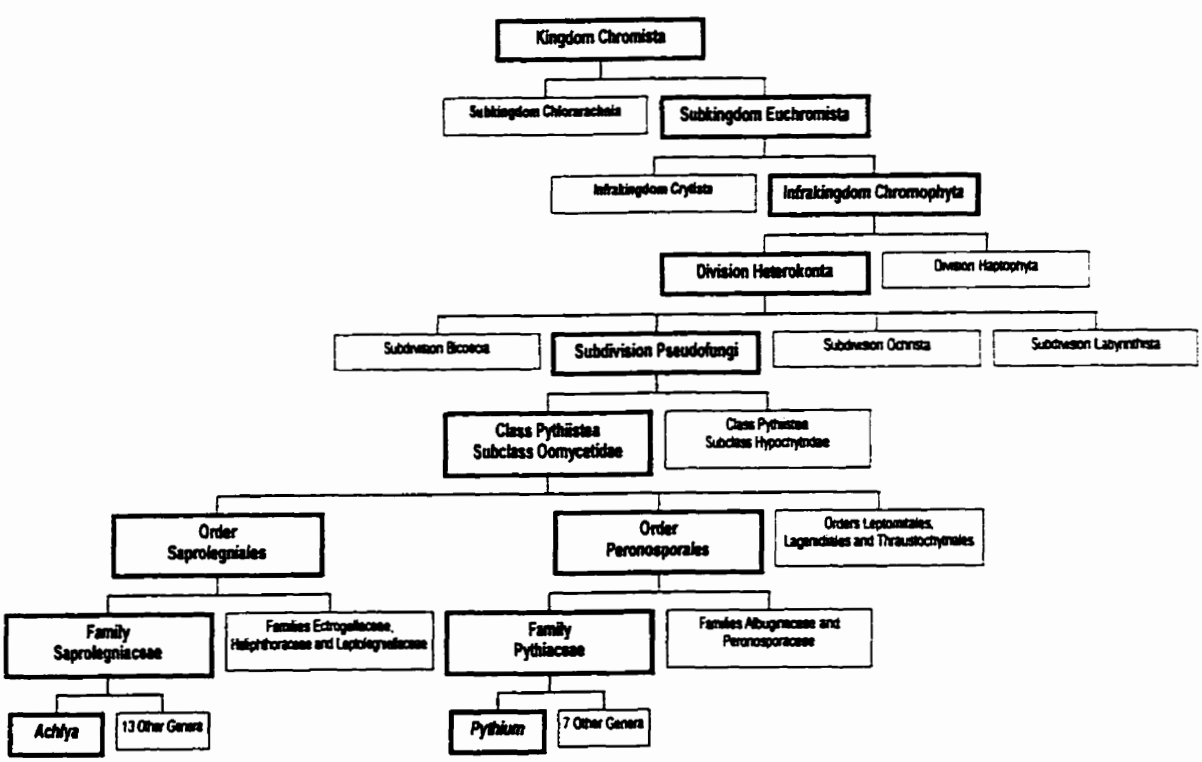
Division Eumycota
Division Myxomycota
Subdivision Basidiomycotina
Subdivision Mastigomycotina
Subdivision Ascomycotina
Subdivision Zygomycotina
Class Chytridiomycetes
Class Hyphochytriomycetes
Class Oomycetes
Class Plasmodiophoromycetes
Order Saprolegniales
Order Peronosporales
Orders Leptomitales, Lagenidiales and Thraustochytriales
Family Saprolegniaceae
Families Ectrogellaceae, Haliphthoraceae and Leptolegnellaceae
Family Pythiaceae
Families Albugnaceae and Peronosporaceae
Achlya
13 Other Genera
Pythium
7 Other Genera

b.


Kingdom Chromista
Subkingdom Chlorarachnia
Subkingdom Euchromista
Infrakingdom Cryptista
Infrakingdom Chromophyta
Division Heterokonta
Division Haptophyta
Subdivision Bicoeca
Subdivision Pseudofungi
Subdivision Ochrista
Subdivision Labyrinthista
Class Pythiistea Subclass Oomycetidae
Class Pythiistea Subclass Hyphochytridae
Order Saprolegniales
Order Peronosporales
Orders Leptomitales, Lagenidiales and Thraustochytriales
Family Saprolegniaceae
Families Ectrogellaceae, Haliphthoraceae and Leptolegnellaceae
Family Pythiaceae
Families Albugnaceae and Peronosporaceae
Achlya
13 Other Genera
Pythium
7 Other Genera

instead of following, fertilization (Alexopoulous and Mims, 1979; reviewed in Buchko, 1996). Asexual reproduction in oomycetes involves a characteristic biflagellate zoospore with dissimilar flagella and zoospore ultrastructure; oomycetes also have a diploid rather than haploid or dikaryotic somatic phase (Alexopoulos *et al.*, 1996). The use of a plant-like mechanism for lysine synthesis (Alexopoulos *et al.*, 1996) and the use of either a mixture of fucasterol, cholesterol and cholesterol derivatives (Köller, 1992; Griffith *et al.*, 1992; Weete 1989) or no sterols at all (Hendrix, 1970) rather than ergosterol during cell growth or as hormones, are both characteristics of oomycetes but not true fungi. Transcriptional regulatory sequences, particularly promoters but also to some extent transcriptional terminators, have been demonstrated to be non-interchangeable between higher fungi and oomycetes (Judelson *et al.*, 1992). Oomycetes also possess tandemly repeated DNA sequences in their genome, which are not present in higher fungi with the exception of telomeres (Mao and Tyler, 1996; Hudspeth *et al.*, 1977). Energy storage compounds (β-1,3-glucans termed mycolaminarans) used by the oomycetes are also distinct from those used by true fungi, and in fact appear to be related to the algal storage compounds leucosin and laminarin (Alexopoulos *et al.*, 1996; Bartnicki-Garcia and Wang, 1983).

The distant relationship between the oomycetes and other fungi has also been demonstrated through the molecular analysis of mitochondrial DNA and 18S rRNA. Cavalier-Smith (1993; 1989; 1986) has gone so far as to group them with diatoms and brown algae in a unique kingdom, the Chromista (Figure 1). The organisms which comprise the kingdom Chromista include brown algae and other protists which possess chloroplasts within the endoplasmic reticulum and/or tubular ciliary mastigonemes (Cavalier-Smith, 1986), with the oomycetes and other members of the subdivision Pseudofungi apparently arising from the heterokont Chromista, via the loss of photosynthesis and the acquisition of a cell wall, into fungus-like organisms (Cavalier-Smith, 1993).

1.II. The Genus *Pythium* and the Species *Pythium ultimum*

Whereas aquatic oomycetes are generally saprotrophic (Dick, 1990) with some being parasitic (Alderman and Polglase, 1988), most terrestrial oomycetes are plant parasites, including species causing rusts (Speer and Waggoner, 1995; Waterhouse, 1973), downy mildews (Waterhouse, 1973), root rotting fungi and seedling dampening mold (reviewed in Buchko, 1996). *Pythium ultimum* is a prevalent plant pathogen with a worldwide distribution, and has been isolated from plants as varied as tulip, apple, watermelon, coffee and sugar beet (reviewed in Buchko, 1996). The genus *Pythium* was initially placed in the family Saprolegniaceae by Pringsheim (1858; reviewed

in Buchko, 1996), but was later placed in its current family, the Pythiaceae, order Peronosporales (Buchko, 1996; Barr, 1983b). *P. ultimum* was first described, but misidentified as *Pythium debaryanum*, by deBary (1881; reviewed in Buchko, 1996), and later isolated by Trow (1901). *P. ultimum* rarely produces zoospores, but this characteristic is by no means unique amongst *Pythium*, and may be an adaptation to terrestrial existence. Members of the genus *Pythium* are, in fact, difficult to distinguish from one another, due to the overlap of morphological characteristics between species. Therefore, molecular methods have been used to better identify particular species within this gene and to elucidate their phylogeny (Buchko, 1996; Klassen *et al.*, 1996; Lévesque *et al.*, 1994; Martin and Kistler, 1990).

1.III. The Genus *Achlya* and the Species *Achlya klebsiana*

The genus *Achlya,* first established by Nees von Esenbeck in 1823 (Johnson, 1956), is a member of the family Saprolegniaceae, order Saprolegniales (Barr, 1983b; Dick, 1973). This genus appears to be the most primitive member of the most primitive order of oomycetes (Barr, 1983a). Terrestrial members of this genus inhabit very wet soils, and are opportunistic pathogens which parasitize plants that are already under some form of stress. Some aquatic members are also known to parasitize fish and fish eggs (Alexopoulos *et al.*, 1996). *Achlya klebsiana* was initially described by Pieters (1915), with synonomous isolates described as *Achlya oryzae* and

Achlya michiganensis (Johnson, 1956). *A. klebsiana* has been reported as an opportunistic parasite of rice which has been subjected to the environmental stresses of poor aeration and low temperatures (Barr, 1983b; Johnson, 1983).

2. Antisense Systems

2.I. Nomenclature

The molecular structure of deoxyribonucleic acid (DNA) is comprised of a double helix formed by two antiparallel backbones of alternating deoxyribose and phosphate residues, with purine and pyrimidine residues bound to the deoxyribose 1' carbon interacting with one another in the core of the molecules (Watson and Crick, 1953). In nature, one of these two strands will typically possess, at some location, a sequence which can be transcribed to produce a messenger ribonucleic acid (mRNA), which is then subsequently translated into a protein (Watson *et al.*, 1992; Stryer, 1988; Zubay, 1988). For the purposes of this work, the DNA strand which is complementary to the mRNA transcription product is termed the "sense" strand, while the corresponding region of the other strand of the DNA duplex is termed the "antisense" strand. This complementarity is based upon nucleotide sequence, with adenine residues complemented by thymine (or uracil in RNA), and guanine complemented by cytosine. The terminology of sense and antisense can also be applied to any pair of polynucleotides able

to form a duplex wherein at least one of the strands can be transcribed or translated (Stryer, 1988). It is of importance to note that there has been a certain degree of confusion and conflict concerning sense and antisense terminology regarding DNA, leading to the proposition that the terms sense and antisense be replaced with either transcribed and non-transcribed (Hengen, 1996) or non-coding and coding (Lewin, 1990), respectively.

Amino acid pairs analogous to nucleotide pairs have been described, but this pairing only occurs between amino acids found in particular protein secondary structures (Root-Bernstein, 1982). Antisense relationships which may exist between proteins have also been described as "complementary proteins" (Blalock, 1990; Blalock and Bost, 1986), with the application of the concepts of complementarity and sense/antisense to peptides being in a completely different context from their nucleic acid counterparts. A sense peptide has been defined as a peptide resulting from the transcription and translation of the sense strand of a DNA in the 5'→3' direction. In contrast, an antisense peptide is entirely hypothetical, and would arise if the same DNA sequence encoding the sense peptide was reversed, that is read in the 3'→5' direction. In a similar manner, transcription and translation of the antisense DNA strand will result in sense complementary and antisense complementary peptides (Jarpe and Blalock, 1994), with the

assumption that all sense and antisense messages use the same reading frame for translation (Figure 2).

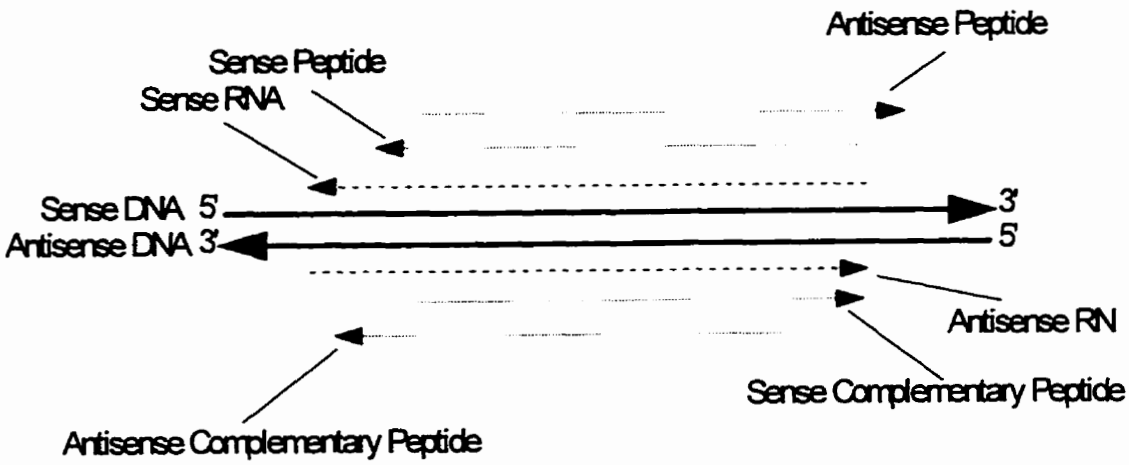
One feature of this arrangement is that complementary peptide pairs (sense:sense complementary or antisense:antisense complementary) will tend to possess hydrophilic and hydrophobic amino acid arrangements which are inverted. Therefore, wherever one peptide tends towards being primarily hydrophobic, the complementary peptide will tend to be primarily hydrophilic. It is this hydropathic complementarity which is the basis of protein complementarity (Jarpe and Blalock, 1994; Blalock, 1990; Blalock and Bost, 1986), the implications of this which will be discussed in more detail in *2.III Complementary Proteins* below.

2.II. Polynucleotide Antisense Systems

2.II.a. <u>Overlapping genes</u> Overlapping genes have long been well characterized in a wide variety of organisms (Normark *et al.*, 1983), including several eubacteria (Wan *et al.*, 1997; Ramaswamy *et al.*, 1997; Sloan *et al.*, 1994; Kiino *et al.*, 1993; Thisted and Gerdes, 1992; Scholz *et al.*, 1989), Archaebacteria (Jones *et al.*, 1995; Barany *et al.*, 1992) and eukaryotic organelles (Fearnley and Walker, 1986; Krebbers *et al.*,1982; Anderson *et al.*, 1982), but most commonly in bacteriophages and viruses (Sherman *et al.*, 1994; Dinesh-Kumar and Miller, 1993; Keese and Gibbs, 1992; Li and Graur, 1991; Stryer, 1988; Cohen *et al.*, 1988; Barrel *et al.*,

Figure 2: Diagrammatic representation of the relationship between sense, antisense, complementarity and anti-complementarity.

Arrowheads indicate 5'→3' directionality of nucleic acids and amino→carboxyl directionality of peptides. DNA, RNA and polypeptide molecules are as indicated.


Antisense Peptide
Sense Peptide
Sense RNA
Sense DNA 5'
3'
Antisense DNA 3'
5'
Antisense RN
Sense Complementary Peptide
Antisense Complementary Peptide

1976). Overlapping genes consist of two or more genes encoded by the same region of a single DNA strand. The presence of several genes at the same locus using a single sense can be facilitated through the use of different start codons and reading frames for each gene (Barany *et al.*, 1992; Fearnley and Walker, 1986; Thisted and Gerdes, 1992), or through differential splicing (Gilbert, 1978).

2.II.b <u>Antisense overlapping genes</u> Genes located on complementary DNA strands can also overlap at the same location in either the same (LéJohn *et al.*, 1994a; Xuan *et al.*, 1990) or in different reading frames (Hahn, 1988; Anderson *et al.*, 1982). Such an overlap can be between the 5' untranslated regions (Farnham *et al.*, 1985), 3' untranslated regions (Svaren *et al.*, 1997; Swalla and Jeffery, 1996; Spencer *et al.*, 1986; Williams and Fried, 1986) or the coding regions of the two genes (Konstantopoulou *et al.*, 1998; Zhang and Chou, 1996; Konstantopoulou *et al.*, 1995; Heinrich *et al.*, 1995; Bedford *et al.*, 1995; LéJohn *et al.*, 1994a and 1994b; Klaer *et al.*, 1981). Examples of antisense overlapping reading frames have been identified in viruses (Ward *et al.*, 1996; Heinrich *et al.*, 1995; Green *et al.*, 1986; Hoopes and McClure, 1985), eubacteria (Yomo *et al.*, 1992; Mizuno *et al.*, 1984; Simons and Kleckner, 1983; Rak *et al.*, 1982), yeast (Boles and Zimmermann, 1994; Xuan *et al.*, 1990; Hahn *et al.*, 1988; Kreike *et al.*, 1987), algae (Gilson and McFadden, 1996), oomycetes (LéJohn *et al.*, 1994b), plants (Schmitz and

Theres, 1992; van der Krol *et al.*, 1988), nematodes (Erttmann *et al.*, 1995), insects (Konstantopoulou *et al.*, 1998 and 1995), amphibians (Kimelman and Kirschner, 1989) and mammals (Li *et al.*, 1997; Murashov and Wolgemuth, 1996; Li *et al.*, 1996; Bedford *et al.*, 1995; Noguchi *et al.*, 1994; Knee *et al.*, 1994, Dolnick, 1993; Celano *et al.*, 1992; Miyajima *et al.*, 1989; Adelman *et al.*, 1987; Nepevu and Marcu, 1986; Anderson *et al.*, 1982; Anderson *et al.*, 1981).

Antisense overlapping reading frames can be sub-categorized based upon the transcriptional and translational status of the two open reading frames (ORFs) involved. Non-stop frames (NSFs) are characteristically comprised of a single gene complemented by an antisense strand with long regions lacking stop words in one or more reading frames (Yomo and Urabe, 1994; Ikehara and Okazawa, 1993; Yomo *et al.*, 1992). Specific examples of these have been identified in eubacteria (Ikehara *et al.*, 1996, Ikehara and Okazawa, 1993; Yomo *et al.*, 1992), *Saccharomyces cerevisiae* (Boles and Zimmerman, 1994), humans (Zhang and Chou, 1996; Goldgaber, 1991) and a variety of other eukaryotes (Merino *et al.*, 1994). The presence of NSFs in a genome is highly dependent upon the genomic GC content (%GC), with NSFs rapidly becoming more common in genomes with a %GC of 60% or higher (Ikehara *et al.*, 1996, Merino *et al.*, 1994), although codon usage and average gene size may also have an effect upon NSF frequency (Merino *et*

al., 1994). Initial investigations led to the conclusion that the likelihood of NSFs occurring randomly in a typical gene was extremely small (P=1x10^{-4} to 8x10^{-3}) (Yomo *et al.*, 1992), but further analysis has demonstrated that the probability of an NSF occurring in an organism with a sufficiently high %GC is so large (approximately 200 fold that of Yomo's estimations) that NSFs can be expected to reside opposite some proportion of genes in these genomes (Ikehara *et al.*, 1996; Ikehara and Okazawa, 1993). This has been confirmed by computer analyses of various public genetic databases, with 180 of 2681 human protein coding sequences examined possessing NSFs on their antisense strand (Zhang and Chou, 1996), as do 110 *E. coli* protein coding sequences (Chou *et al.*, 1996).

Antisense transcripts (also termed long antisense complementary open reading frames (LAC-ORFs) (Konstantopoulou *et al.*, 1995)) comprise the second category of overlapping antisense reading frames, consisting of a gene complemented by an ORF on the antisense strand which is transcribed but not necessarily translated. Short antisense transcripts of this nature (Celano *et al.*, 1992; Simons, 1988; Green *et al.*, 1986) as well as antisense transcripts encoded at different genomic loci (Delihas, 1995) have been postulated to have a regulatory function, either by modifying sense transcript processing (Tasheva and Roufa, 1995; Kimelman and Kirschner, 1989), interfering with message translation (Athanasopoulos *et al.*, 1995;

Ehrenberg and Sverredol, 1995; Hartmann *et al.*, 1995), or by affecting RNA stability (Simons, 1988). Several transcripts with the characteristics of a translatable gene have also been identified, but their translation into a protein product remains unconfirmed (Ward *et al.*, 1996; Dolfini *et al.*, 1993; Scholz *et al.*, 1989; Kreike *et al.*, 1987; Grindley and Joyce, 1980), and therefore must be classified as antisense transcripts rather than in the final category of antisense overlapping reading frames. This category consists of two transcribable regions at the same genomic locus which are both translated into functional protein products, and are termed antisense gene pairs (LéJohn *et al.*, 1994a and 1994b). Antisense gene pairs have been identified in viruses (Chang *et al.*, 1998), bacterial transposons (Simons and Kleckner, 1983; Rak *et al.*, 1982), eubacteria (Noriega, Genbank accession #U35656), oomycetes (LéJohn *et al.*, 1994a and 1994b), insects (Henikoff *et al.*, 1986) amphibians (Kimelman and Kirschner, 1989) and other animals (Li *et al.*, 1997; Li *et al.*, 1996; Knee *et al.*, 1994; Fu and Maniatis, 1992; Miyajima *et al.*, 1989). Most known antisense gene pairs have overlapping coding regions, although it is possible for one member of the antisense gene pair to reside complementary to an intron of the other (Henikoff *et al.*, 1986). In some cases, antisense gene pair members are coexpressed (LéJohn *et al.*, 1994a and 1994b), but in others the genes are expressed at different times during the lifecycle of, or in different tissues belonging to, the same

organism (Murashov and Wolgemuth, 1996). It is also noteworthy that in many cases at least one member of many antisense gene pairs encodes a stress response protein, including heat shock proteins (Murashov and Wolgemuth, 1996; LéJohn *et al.*, 1994a and 1994b) chemokines (Erttman *et al.*, 1995) and antimutator proteins (Li *et al.*, 1997 and 1996).

Several other potential antisense gene pairs in *E.coli* have been identified through comparisons of protein coding and peptide sequences in public databases (Chou *et al.*, 1996). These potential antisense gene pairs encode cytosine specific DNA methyl transferase (Sohail *et al.*, 1990) and a complementary ORF (Hanck *et al.*, 1989); *cysX* (Tei *et al.*, 1990) and serine O-acetyl transferase (Denk *et al.*, 1987); *oxa2* β-lactam resistance gene (Hall and Vockler, 1987) and an integrase-like protein (Sundstroem *et al.*, 1988); and finally antisense gene pairs involving the *mob* genes in the plasmid RSF1010 (Scholz *et al.*, 1989; Derbyshire *et al.*, 1987). It was also noted that *oxa2* could also form an antisense pair with a *Pseudomonas aeruginosa* integrase (Bissonnette and Roy, 1992) and a *Klebsiella pneumoniae* integrase (Radstroem *et al.*, PIR accession #S32184). These final two findings highlight the caution which should be exercised when inferring the existence of an antisense gene pair based solely upon computer database searches, since genes with complementary sequences may not necessarily share the same locus.

2.II.c <u>Rationale for the existence of antisense overlapping genes</u> An analysis of overlapping genes in a broad range of viruses has demonstrated a tendency for one member of an overlapping gene pair to be much less evolutionarily conserved than the other (Keese and Gibbs, 1992). One example of this is the overlapping protein (*op*) gene unique to tymoviruses, which overlaps a replicase protein (*rp*) gene identified in a far wider variety of viral families (Bozarth *et al.*, 1992; Keese and Gibbs, 1992). Intuitively, the more broadly distributed gene (in this case *rp*) must have existed in an ancestral form before the divergence of the viral families bearing that gene. In contrast, the overlapping *op* gene must have developed after this divergence. The maintenance of such multiple overlapping genes in a functional form is unnecessary in organisms where gene duplication is common, such as in eukaryotes (Loomis *et al.*, 1990; Hentschel and Birnsteil, 1981; Efstratiadis *et al.*, 1980). This is due to the absence of any selective advantage conferred upon an organism by the possession of extra copies of such an overlapping gene arrangement over those which lose some copies of one or the other overlapping gene, while still possessing sufficient functional copies of both to retain function (Keese and Gibbs, 1992; Watson *et al.*, 1992). Those organisms unable to frequently duplicate genes can maintain overlapping genes through alternative splicing (Lees-Miller *et al.*, 1990) or through biased codon/nucleotide usage (Sharp, 1985; Nussinov,

1984) in order to minimize the occurrence of nonsense mutations in overlapping genes.

Overlapping antisense open reading frames may have arisen from a capacity in the standard genetic code to retain information concurrently on both the sense and antisense nucleotide strands (Konecny *et al.*, 1993). Such a capacity would have been necessary in a protobiotic RNA-dominated world, wherein at some early stage any translational mechanism decoding RNA into a peptide sequence would have likely been unable to differentiate an RNA strand from the template sequence necessary for replication. Analysis of the redundancy of the standard genetic code indicates that there is a certain degree of flexibility in what can be encoded by an antisense strand while retaining the same sense peptide sequence. It has also been observed that silent mutations in one strand tend to be complemented by conservative mutations in the other strand, assuming that the two strands are in codon register (Konecny *et al.*, 1993). The standard genetic code's double-strand coding superiority over that of random codes has been attributed both to this type of arrangement and the stability of the middle base of a codon (Konecny *et al.*, 1993; Alff-Steinberger, 1969; Woese, 1965). This has been qualified in that a predisposition for double-stranded coding tends to be greatest in those organisms with the least codon usage bias, as well as bias in favour of amino acids which are encoded by multiple

codons (Konecny *et al.*, 1993). Related to this, study of rat globin antisense RNA has also identified novel 3'-terminal sequences complementary to the 5'-UTR of globin sense mRNA, which has led to the hypothesis that the antisense RNA could be involved in RNA-dependent globin mRNA synthesis (Volloch *et al.*, 1996).

Another hypothesis which may explain the presence of both overlapping genes and NSFs is that both could represent methods to amplify the rate of evolution. This would be done through the provision of the genome with a pool of potential genes of novel function which could arise *de novo* as has been described in detail elsewhere (Yomo and Urabe, 1994; Keese and Gibbs, 1992; Yomo *et al.*, 1992), rather than by the gradual mutation of function of existing genes. This would enable a genome to bypass constraints put upon genetic evolution by the small number of exons, calculated at between one and seven thousand unique sequences, available to modern genes (Dorit *et al.*, 1990).

2.II.d <u>How can antisense gene pairs exist?</u> Antisense gene pair arrangements have interesting characteristics which may have both evolutionary and functional implications. In the majority of cases, the members of the gene pair are in codon register (Noriega, Genbank accession #U35656; Li *et al.*, 1996; Knee *at al.*, 1994; LéJohn *et al.*, 1994a and 1994b; Kimelman and Kirschner, 1989; Miyajima *et al.*, 1989; Rak *et al.*,

1982), with a codon encoding the sense amino acid A complemented by three nucleotides in the antisense gene which form a codon encoding antisense amino acid B (Figure 3). Since the first two nucleotides in a codon (sites 1 and 2) are associated with correct amino acid coding but the third base (site 3) can usually be altered without effect (Stryer, 1988; Zubay, 1988), alterations in the codon for amino acid A which will not alter the encoded amino acid tend to cause drastic changes in the identity of the amino acid encoded by the complementary codon B. This is due to the fact that, when in codon register, the nucleotide at site 1 in the sense codon complements the nucleotide at site 3 of the corresponding antisense codon, and vice-versa. Thus mutations which do not tend to alter the sense codon (those at site 3), will be complemented by corresponding nucleotides at the antisense codon's site 1, tending to cause alterations to the identity of the amino acid encoded by the antisense codon. Further analysis of what can be termed codon architecture has also indicated that the genetic code has evolved to permit a minimization of error not only through the flexibility of nucleotide identity at site 3 in a codon, but also by conservation of amino acid character at site 2 (Haig and Hurst, 1991). While site 1 nucleotides seem to be responsible for the determination of amino acid identity and site 3 nucleotides tend to be generally unrelated to the amino acid encoded by a particular codon, site 2 nucleotides tend to be a

Figure 3: Diagrammatic representation of two overlapping antisense open reading frames which are in codon register.

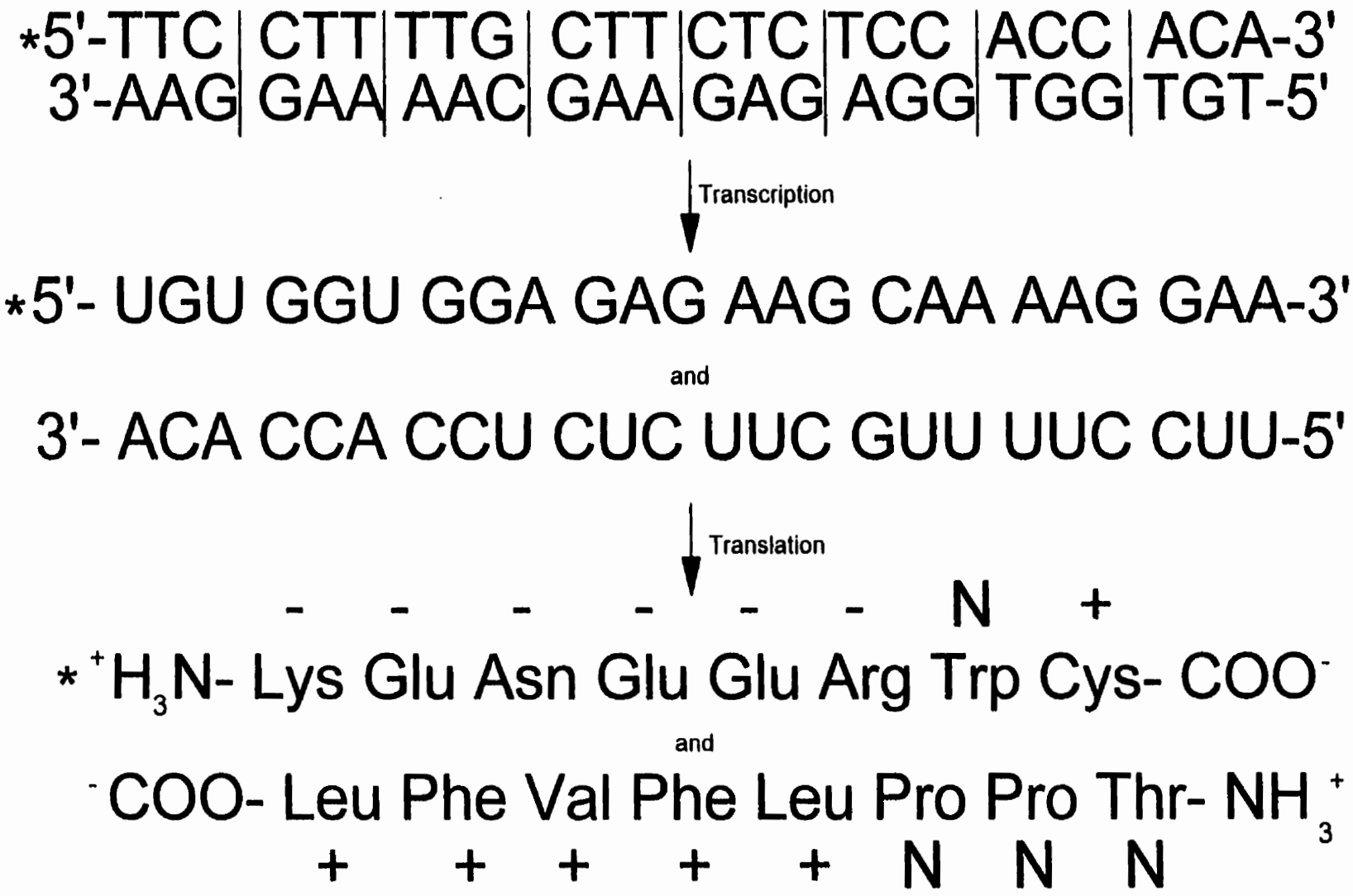
The original DNA sequences are shown in their antiparallel arrangement at top, whereas the transcription and translation products for both strands are shown with the same orientations at the middle and the bottom, respectively.

*: DNA sense strand, sense mRNA and sense peptide.

-: Hydrophilic amino acid.

+: Hydrophobic amino acid.

N: Neutral amino acid.


*5'-TTC CTT TTG CTT CTC TCC ACC ACA-3'
3'-AAG GAA AAC GAA GAG AGG TGG TGT-5'
Transcription
*5'- UGU GGU GGA GAG AAG CAA AAG GAA-3'
and
3'- ACA CCA CCU CUC UUC GUU UUC CUU-5'
Translation
- - - - - - N +
* +H3N- Lys Glu Asn Glu Glu Arg Trp Cys- COO-
and
-COO- Leu Phe Val Phe Leu Pro Pro Thr- NH3+
+ + + + + N N N

gauge for amino acid hydrophobicity (Figure 4). It is also this site which corresponds to the least error-prone nucleotide in a codon (Alff-Steinberger, 1969; Woese, 1965). In an mRNA, site 2 of a codon which encodes a hydrophilic amino acid will tend to be adenine, whereas uracil tends to code for hydrophobic amino acids. In a similar fashion, cytosine and guanine tend to encode uncharged or slightly hydrophilic amino acids (Table 1 and Table 2) (Haig and Hurst, 1991; Blalock and Bost, 1986). This phenomenon also has the effect that, should two proteins be encoded directly opposite one another in codon register (as is predominantly the case in antisense gene pairs), wherever one protein would tend to be hydrophobic, the protein encoded by the antisense strand would tend towards hydrophilicity (Haig and Hurst, 1991; Blalock and Bost, 1986; Blalock and Smith, 1984). Thus, proteins encoded by complementary nucleotide sequences should tend to be complementary hydropathically as well. Observations of this were recorded as early as 1969 (Mekler, 1969; reviewed in Root-Bernstein and Holsworth, 1998). It also becomes apparent that alterations to sites 1 or 3 in the sense codon of an antisense gene pair by random mutation have little effect upon the hydropathy of the amino acid encoded by either the sense or the antisense mRNA, and should therefore tend to leave the hydropathic indices of the protein products largely unaffected. In the case of an alteration to the second base in the sense codon, any alteration

Figure 4: Typical codon architecture.

The 5'-most (first) nucleotide (N_1) is responsible for determining encoded amino acid identity. The second nucleotide (N_2) can be correlated with the hydrophobicity of encoded amino acid, such that if N_2=adenine, 86% of codons will be hydrophilic and the remainder neutral; if N_2=uracil all encoded amino acids are hydrophobic; if N_2=cytosine, 75% of codons encode neutral amino acids, and the remainder hydrophobic; if N_2=guanine, 47% of amino acids encoded are neutral, 40% are hydrophilic and 13% are hydrophobic. The identity of the nucleotide at the N_3 position has little influence upon the amino acid encoded.

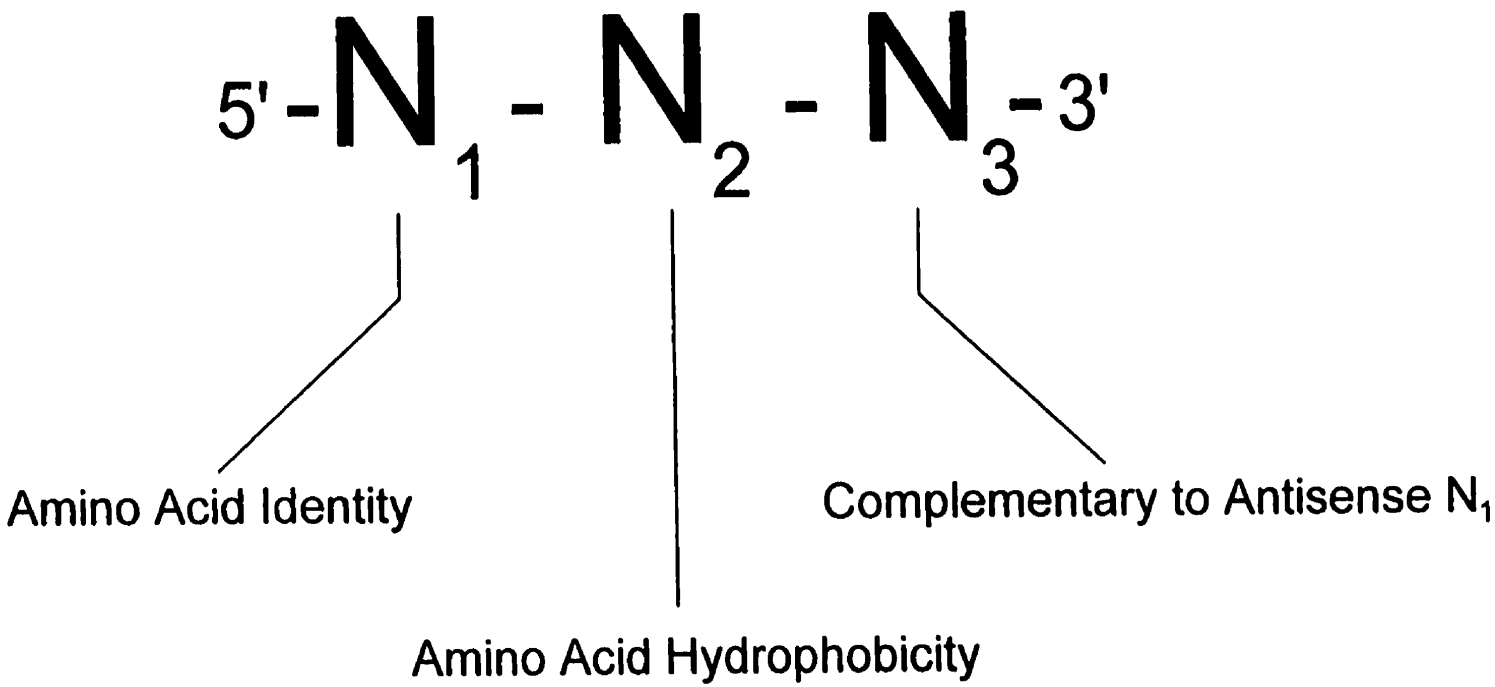

5'-N1-N2-N3-3'
Amino Acid Identity
Amino Acid Hydrophobicity
Complementary to Antisense N1

Table 1: Relative hydrophobicities of the twenty natural amino acids.

Hydrophobicities are based upon the scoring system of Kyte and Doolittle (1982). Increasingly positive values indicate increasingly hydrophobic amino acids, whereas increasingly negative values represent increasingly hydrophilic amino acids. Amino acids with hydropathic indices of between 0 and -2 have been termed "neutral" amino acids after Blalock and Smith (1984).

Amino Acid	Hydropathic Score	Hydropathic Categorization
Isoleucine (Ile)	+4.5	Hydrophobic
Valine (Val)	+4.2	Hydrophobic
Leucine (Leu)	+3.7	Hydrophobic
Phenylalanine (Phe)	+2.7	Hydrophobic
Cysteine (Cys)	+2.5	Hydrophobic
Methionine (Met)	+1.9	Hydrophobic
Alanine (Ala)	+1.8	Hydrophobic
Glycine (Gly)	-0.4	Neutral
Threonine (Thr)	-0.7	Neutral
Serine (Ser)	-0.9	Neutral
Tryptophan (Trp)	-0.9	Neutral
Tyrosine (Tyr)	-1.3	Neutral
Proline (Pro)	-1.6	Neutral
Histidine (His)	-3.2	Hydrophilic
Asparagine (Asn)	-3.5	Hydrophilic
Aspartic Acid (Asp)	-3.5	Hydrophilic
Glutamic Acid (Glu)	-3.5	Hydrophilic
Glutamine (Gln)	-3.5	Hydrophilic
Lysine (Lys)	-3.9	Hydrophilic
Arginine (Arg)	-4.5	Hydrophilic

Table 2: The relationship between the second nucleotide in a codon and encoded amino acid hydrophobicity/hydrophilicity.

All codons of the standard genetic code, except stop codons, are shown here in the form found in mRNA. The amino acids encoded by these codons are given in parentheses in the standard three letter format.

Amino Acid Class	2nd Base A	2nd Base C	2nd Base G	2nd Base U
Hydrophobic	nil	GCA (Ala) GCC (Ala) GCG (Ala) GCU (Ala)	UGC (Cys) UGU (Cys)	AUA (Ile) AUC (Ile) AUG (Met) AUU (Ile) CUA (Leu) CUC (Leu) CUG (Leu) CUU (Leu) GUA (Val) GUC (Val) GUG (Val) GUU (Val) UUA (Leu) UUC (Phe) UUG (Leu) UUU (Phe)
Hydrophilic	AAA (Lys) AAC (Asn) AAG (Lys) AAU (Asn) CAA (Gln) CAC (His) CAG (Gln) CAU (His) GAA (Glu) GAC (Asp) GAG (Glu) GAU (Asp)	nil	AGA (Arg) AGG (Arg) CGA (Arg) CGC (Arg) CGG (Arg) CGU (Arg)	nil
Neutral (Slightly Hydrophilic)	UAC (Tyr) UAU (Tyr)	ACA (Thr) ACC (Thr) ACG (Thr) ACU (Thr) CCA (Pro) CCC (Pro) CCG (Pro) CCU (Pro) UCA (Ser) UCC (Ser) UCG (Ser) UCU (Ser)	AGC (Ser) AGU (Ser) GGA (Gly) GGC (Gly) GGG (Gly) GGU (Gly) UGG (Trp)	nil

to the hydropathic nature of a protein tends to be reflected by corresponding alterations in the antisense protein, such that complementarity between the two is maintained (Blalock, 1990; Blalock and Bost, 1988).

2.II.e <u>The antisense gene pair of *Achlya klebsiana*</u> An example of an antisense gene pair is the $NADP^+$-activeable, NAD^+-dependent glutamate dehydrogenase gene (*nad-gdh*) paired with a putative heat shock cognate 70 protein gene (*hsc70*) in *Achlya klebsiana* (LéJohn *et al.*, 1994a; LéJohn *et al.*, 1994b). The enzymology of the GDHs of *A. klebsiana* and related organisms has been extensively studied (LéJohn, 1975; Stevenson and LéJohn, 1971; Stevenson and LéJohn, 1970; LéJohn *et al.*, 1970; LéJohn *et al.*, 1969a; LéJohn *et al.*, 1969b; LéJohn and Jackson, 1968), but only a small number of *gdh* genes of any type of fungal or pseudofungal origin have been characterized (Schaap *et al.*, 1996; LéJohn *et al.*, 1994b; Kapoor *et al.*, 1993; De Zoysa *et al.*, 1991; Hawkins *et al.*, 1989; Moye *et al.*, 1985; Nagasu and Hall, 1985). This antisense gene pair was identified when purification of NAD-GDH from *A. klebsiana* led to the fortuitous discovery of a 74kD protein which was recognized by polyclonal anti-GDH antibodies even after purification of the antibodies by affinity chromatography on a column composed of purified *A. klebsiana* NAD-GDH linked to an inert matrix (LéJohn *et al.*, 1994a and 1994b). These same antibodies were also used to probe an expression cDNA library for the *nad-gdh* message, from which

several positive cDNA clones were isolated and subsequently used to probe a genomic library for the *nad-gdh* gene. Study of a genomic clone of 17 kb isolated in this manner identified a sequence consisting of ten exons and nine introns encoding *nad-gdh*, encompassing the entirety of a 5.2 kb XbaI restriction endonuclease fragment. The final exon in the *nad-gdh* gene was substantially larger than the others, comprising approximately 40% of the entire gene. Subsequent analysis of the antisense strand of *nad-gdh* revealed the presence of an ORF in codon register with and directly opposite to the majority of the sequence of the final *nad-gdh* exon. This ORF was shown to have significant amino acid sequence similarity (varying from 69 to 87%) to a wide variety of heat shock 70 proteins, and also to have typical heat shock gene sequences. Thus, this sequence was posited to encode a heat shock 70 cognate protein (LéJohn *et al.*, 1994a). Such an evolutionarily restrictive arrangement is unlikely to be fortuitous, particularly in view of the ability of oomycetes to duplicate portions of their genome (LéJohn *et al.*, submitted for publication; Bhattacharya and Stickel, 1994; Hudspeth *et al.*, 1977). In fact, subsequent work with *Drosophila auraria* (Konstantopoulou *et al.*, 1995) demonstrated the presence of a similar arrangement between an *hsp70* and a LAC-ORF sharing 32% nucleotide sequence identity with the tenth exon of the *A. klebsiana nad-gdh* gene. Similar levels of sequence identity are also shared by the complementary strands of several other heat

shock protein genes in a wide variety of organisms. In addition to this, the mRNA of an *hsp70*-type gene of *Neurospora crassa* comprised of five exons and four introns (the glucose regulated protein *grp78* gene) has an antisense ORF with 40% encoded amino acid sequence identity with the *A. klebsiana* NAD-GDH protein (Techel *et al.*, 1998).

The transcription of an RNA corresponding to the expected size of the *A. klebsiana hsp70* gene was shown to be induced concurrently with *nad-gdh* induction with L-glutamine, and was speculated to be acting as a stress-induced protein influenced by nutritional stress involving nitrogen imbalances (LéJohn *et al.*, 1994a), and to possibly interact as a chaperone for the NAD-GDH encoded by the complementary DNA strand (LéJohn *et al.*, 1994b). This type of interaction between complementary proteins produced by complementary DNA strands has never been demonstrated *in vivo*, but compelling *in vitro* work concerning complementary proteins has been conducted by several investigators.

2.III Complementary Proteins

2.III.a Theoretical basis of interaction between complementary proteins

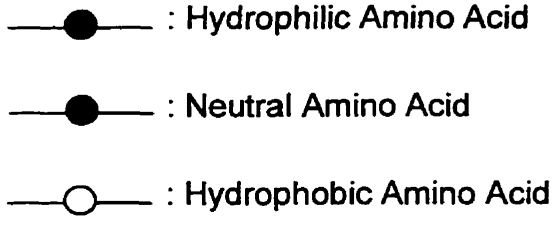
The hydropathic complementarity of two proteins encoded by the members of an antisense gene pair should give these proteins a (theoretical) capacity to form an amphiphilic complex if they were to interact, which would tend to be more stable than either of the two proteins by themselves in an aqueous

environment (Blalock, 1990; Markus *et al.*, 1989; Kaiser and Kézdy, 1984). The formation of hydrophilic extrusions in one peptide complementary to corresponding hydrophobic intrusions in the other are driven by entropy, which promotes a maximization of interactions between non-polar (hydrophobic) and other non-polar amino acids concurrently with corresponding interactions between polar (hydrophilic) amino acids and water (Stryer, 1988; Zubay, 1988). These complementary structural features provide an opportunity for hydrophilic regions of two proteins to move closer together in three dimensional space, permitting a greater level of interaction between the hydrophilic residues of the two proteins than would otherwise be possible (Blalock, 1990; Markus *et al.*, 1989) (Figure 5).

The concept of hydropathy being the basis of complementary protein interactions is not without it's detractors, who question the structural viability of such a mechanism due to the implication of interactions between some unlikely amino acid pairs. An alternative explanation (Root-Bernstein and Holsworth, 1998; Root Bernstein, 1982a) takes advantage of the limitation of amino acid pairing possibilities by reading the antisense nucleic acid strand in a 3'→5' direction to give more understandable pairings. One point not mentioned by Root-Bernstein is that, as mentioned above (*2.II.d How can antisense gene pairs exist?*), even 3'→5' reading of a gene's antisense strand will still tend to yield a peptide hydropathically complementary to the

Figure 5: Schematic representation of the interaction between segments of two peptides encoded by a hypothetical antisense gene pair.

Both (**a**) the DNA sequence used and (**b**) the resulting amino acid sequences are given in addition to (**c**) a simple diagram showing the hydrophobic intrusions and hydrophilic extrusions formed by the two peptides which facilitate easier interactions between hydrophilic residues of the two peptides. The sense DNA strand and sense peptide are uppermost and the antisense DNA strand and complementary sense peptide are lowermost in all cases.



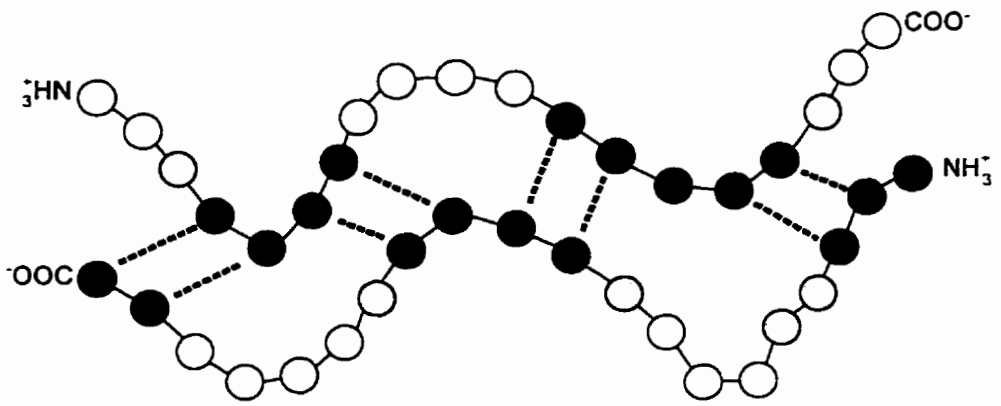
a.

5'-TTT GCG GGC CAA CAA GAT CGG GTG GCG GTT ATT AGG AAA CGC CGT CGA TCG TTG CTG-3'
3'-AAA CGC CCG GTT GTT CTA GCC CAC CGC CAA TAA TCC TTT GCG GCA GCT AGC AAC GAC-5

b.

H_2N-Phe Ala Gly Gln Gln Asp Arg Val Ala Val Ile Arg Lys Arg Arg Arg Ser Leu Leu-COOH
HOOC-Lys Arg Ala Leu Leu Ile Pro His Arg Asn Asn Pro Phe Ala Thr Ser Arg Gln Gln-NH_2

c.



sense protein. Root-Bernstein's methodology is also limited to the explanation of amino acid pairing in parallel β-ribbon secondary structures (Markus, 1989; Root-Bernstein, 1982a), whereas complementarity described by Blalock would be a force in such macromolecular functions as protein folding and receptor binding.

2.III.b <u>The study of complementary protein interactions</u> The interactions between complementary proteins produced from antisense DNA sequences form the foundation of the molecular recognition theory (MRT) (Jarpe and Blalock, 1994; Blalock, 1990; Kaiser and Kézdy, 1984), which postulates that complementary peptides generated by complementary DNA sequences will interact with one another. This theory has been studied primarily *in vitro* through the use of artificial complementary peptides, which are generated by decoding the antisense strand of a well characterized gene in a region which is highly conserved between species (Araga *et al.*, 1993; de Souza and Brentani, 1992; Dillon *et al.*, 1991; Clarke and Blalock, 1990; Ghiso *et al.*, 1990; Brentani *et al.*, 1988; Elton *et al.*, 1988; Knutson, 1988; Mulchahey *et al.*, 1986). These studies involve the chemical synthesis of short antisense peptides (usually about ten to twenty amino acids in length), including control peptides which possess similar amino acid contents to the test peptides, but with randomized sequences (Brentani *et al.*, 1988; Elton *et al.*, 1988; Knutson, 1988). These peptides are sometimes grouped together as

octapeptides on a polylysine core as is done for multiple antigenic peptides in order to increase their binding efficiency (Zhou and Whitaker, 1996; Fassina *et al.*, 1992a). In both the cases of monomeric and octameric peptides, peptides are bound to an inert matrix for use during column chromatography. Analysis of proteins retained by the column after a crude protein extract is eluted through this matrix can identify which antisense peptide(s) most effectively and selectively bind the target protein (de Souza *et al.*, 1994; Fassina, 1994; Fassina *et al.*, 1995; Fassina *et al.*, 1992b and 1992c; Fassina and Cassani, 1992; Lu *et al.*, 1991; Fassina *et al.*, 1989a and 1989b). Although this methodology has been used to successfully identify a number of antisense peptides able to bind to the target protein, such peptides have not been identified in all cases tested (Goldstein and Brutlag, 1989; Guillemette *et al.*, 1989, Rasmussen and Hesch, 1987).

Interaction between complementary peptides has also been inferred using several other methods, including the effect of antisense peptides upon target enzyme or receptor binding activity either positively (Misra *et al.*, 1993; Dillon *et al.*, 1991) or negatively (Fassina *et al.*, 1995; de Souza *et al.*, 1994; Budisaviljevic *et al.*, 1992; Ghiso *et al.*, 1990); identification of putative receptor proteins with antibodies generated against a peptide complementary to a ligand (Martins *et al.*, 1997) or with nucleic acid primers targeting the genes for hypothetical proteins interacting with known proteins

(Ruiz-Opazo *et al.*, 1995); competition between antisense peptides and natural receptor molecules for target protein binding (Wu *et al.*, 1997; Fassina *et al.*, 1995; McGuignan and Campbell-Thompson, 1992; Bajpai *et al.*, 1991); mimicry of protein function by anti-antisense peptide antibodies (McGuignan and Campbell-Thompson, 1992); the binding to receptors of the original sense protein by such antibodies (Bret-Dibat *et al.*, 1994; Swords *et al.*, 1990; Clarke and Bost, 1990; Elton *et al.*, 1988); and protein target molecule characteristics being held by the antisense protein itself (Zhou *et al.*, 1994; Dillon *et al.*, 1991). In addition to these, interaction between complementary peptides has been demonstrated using mass spectrometry (Loo *et al.*, 1994), circular dichroism and nuclear magnetic resonance (Fassina *et al.*, 1992b) and can be predicted with computer modeling (Radulescu *et al.*, 1995; Borovsky *et al.*, 1994; Fassina and Melli, 1994).

Intramolecular interactions between complementary regions of the same protein can also occur, as may be the case for the antisense homology boxes identified in endothelin receptor type A (Baranyi *et al.*, 1998; Baranyi *et al.*, 1995) as well as in C5a receptor and C5a anaphylatoxin (Baranyi *et al.*, 1996), although these findings may not be statistically relevant (Segersteen *et al.*, 1986), and have been used to generate vaccines against autoimmune diseases (Zhou and Whitaker, 1996; Araga and Blalock, 1994; Araga *et al.*, 1993). It should be noted that interaction between

complementary proteins is far from universal (Holsworth *et al.*, 1994; Beattie and Flint, 1992; Goldstein and Brutlag, 1989; Guillemette *et al.*, 1989; Rasmussen and Hesch, 1987), and because of this there has been some dispute concerning the validity of the molecular recognition theory (Root-Bernstein and Holsworth, 1998; Blalock, 1990). However, it would appear that these instances may be exceptions to a general rule, and could be due to peculiarities of the proteins being tested or the specific systems used rather than to any underlying fault in the molecular recognition theory (Blalock, 1990).

3. Glutamate Dehydrogenases and Heat Shock 70 Proteins

3.I. Glutamate Dehydrogenases

The glutamate dehydrogenases (GDH, EC 1.4.1.2-4) are vital, multimeric enzymes providing a linkage between nitrogen uptake (in the form of ammonia), amino acid catabolism and the tricarboxylic acid cycle (Figure 6) (Zubay, 1988; Marzluf, 1981; Smith *et al.*, 1975; Stadtman, 1966). GDH protein has been purified from several sources (Table 3), with a number of *gdh* genes also being isolated and sequenced (Table 4). Together with glutamine synthase (Legrain *et al.*, 1982) and glutamate synthase (Roon *et al.*, 1974; Tempest *et al.*,1970), GDH is a vital of nitrogen metabolism. Nitrogen uptake into several types of cellular macromolecules during *de novo* synthesis using either L-glutamate (amino acids only) or L-

Figure 6: Schematic representation of the linkage of ammonia assimilation with carbon metabolism via the glutamate dehydrogenases

Enzymes involved in the interconversions of α-ketoglutarate, glutamate and glutamine are indicated by roman numerals.

I: NADP-specific glutamate dehydrogenase (NADP-GDH)

II: NAD-specific glutamate dehydrogenase (NAD-GDH)

III: Glutamine synthase

IV: Glutamate synthase

Modified from Yang (1991).

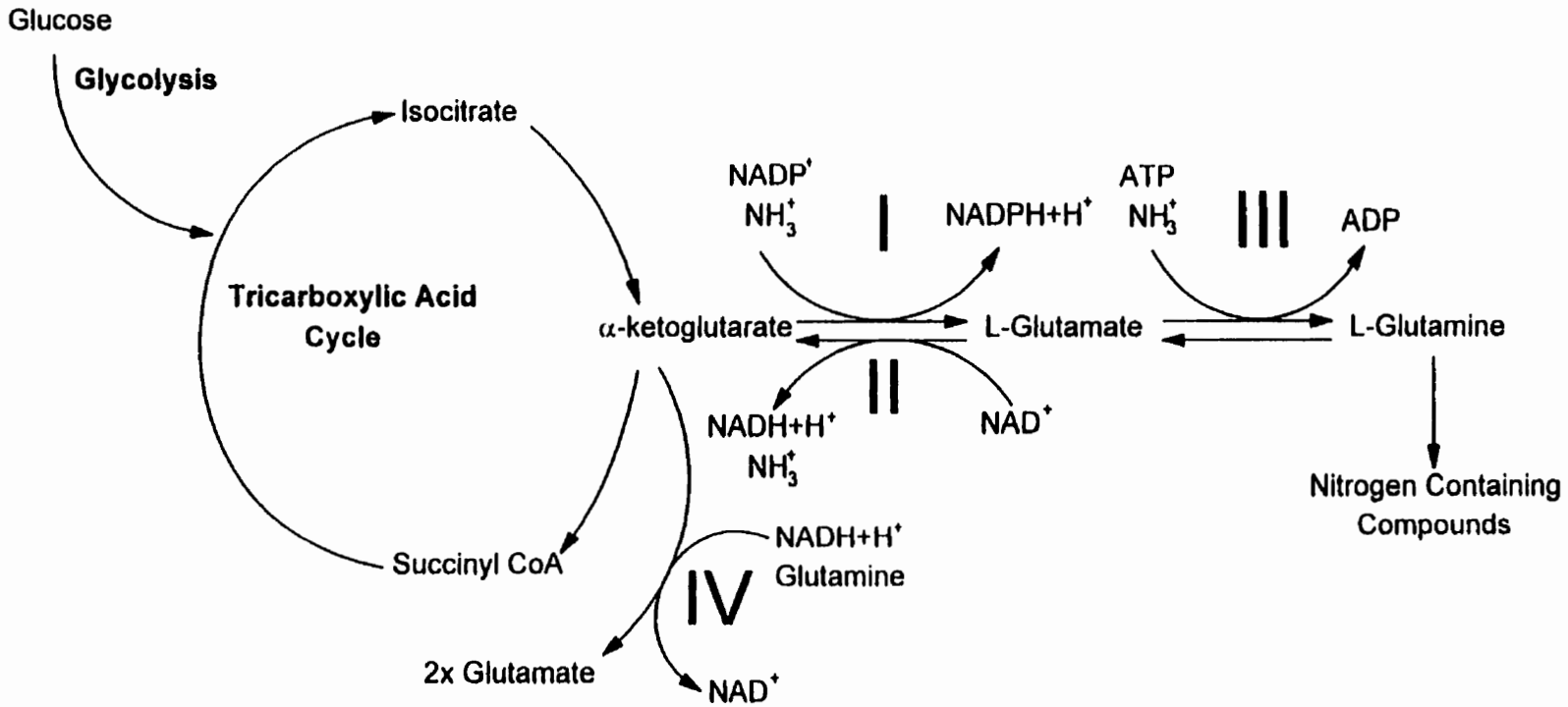

Glucose
Glycolysis
Isocitrate
Tricarboxylic Acid Cycle
Succinyl CoA
α-ketoglutarate
NADP+
NH3+
I
NADPH+H+
L-Glutamate
ATP
NH3+
III
ADP
L-Glutamine
Nitrogen Containing Compounds
II
NADH+H+
NH3+
NAD+
NADH+H+
Glutamine
IV
2x Glutamate
NAD+

Table 3: Sources of purified glutamate dehydrogenase

A selection of organisms from which glutamate dehydrogenase has been purified.

Organsim	Reference
Archaeoglobus fulgidus	Aalen *et al.*, 1997
laccaria bicolor (Maire) *orton.*	Garnier et al., 1997
Bryopsis maxima	Inokuchi *et al.*, 1997
Haloferax mediterranei	Ferrer *et al.*, 1996
Thermococcus litoralis	Ma *et al.*, 1994
Achlya Klebsiana	Yang and LéJohn, 1994
Clostridium difficile	Anderson *et al.*, 1993
Ruminococcus flavefaciens	Duncan et al., 1992
Paracoccus denitrificans	Kremeckova *et al.*, 1992
Chlamydomonas reinhardtii	Moyano *et al.*, 1992
Sulfolobus solfataricus	Schinkinger *et al.*, 1991
Dictyostelium discoideum	Pamula and Wheldrake, 1991a and 1991b
Bacillus fastidiosus	Op den Camp *et al.*, 1989
Aspergillus nidulans	Stevens *et al.*, 1989
Streptomyces fradiae	Vancurova *et al.*, 1989
Phycomyces sp.	Van Laere, 1988
Azospirillum brasilense	Maulik and Ghosh, 1986
Rat brain	Colon *et al.*, 1986
Rat heart mitochondria	McDaniel *et al.*, 1984
Pseudomonas aeruginosa	Smits *et al.*, 1984
Bacteroides thetaiotaomicron	Glass and Hylemon, 1980
Ox liver	McCarthy *et al.*, 1980
Human placenta	Julliard and Crastes de Paulet, 1978
Loligo pealeii	Storey *et al.*, 1978
Bacillus megaterium	Hemmila and Mantsala, 1978
Chlorella sorokiniana.	Gronostajski *et al.*, 1978
Yeast	Camardella *et al.*, 1976
Thiobacillus thioparus	Adachi and Suzuki, 1977
Eschericia coli	Sakamoto *et al.*, 1975; Veronese *et al.*, 1975
Neurospora sp.	Veronese *et al.*, 1974; Stachow and Sanwal, 1967
Ox liver	di Prisco and Garofano, 1974; Strecker, 1951
Salmonella typhimurium	Coulton and Kapoor, 1973
Peptococcus aerogenes.	Johnson and Westlake, 1972
Bacillus licheniformis.	Phibbs and Bernlohr, 1971
Rat liver	King and Frieden, 1970
Dogfish liver	Corman *et al.*, 1967

Table 4: A selection of complete, unique DNA sequences encoding glutamate dehydrogenase

All sequences were initially obtained through a computer search of GenBank *(<<http://www.ncbi.nlm.nih.gov/Entrez/nucleotide.html>>)*. All references are published except those otherwise noted.

@: in press

*: unpublished

Organism	Reference
Leishmania tarentolae	Bringaud *et al.*, 1997
Thermotoga maritima	Kort *et al.*, 1997
Thermococcus profundus	Higuchi *et al.*, 1997
Agaricus bisporus	Schaap *et al.*, 1996
Arabidopsis thaliana	Melo-Oliveira *et al.*, 1996
Bacteroides fragilis	Abrahams *et al.*, 1996*
Bacteroides thetaiotamicron	Baggio and Morrison, 1996@
Prevotella ruminicola	Wen and Morrison, 1996
Solanum lycopersicum	Botella *et al.*, 1996*
Vitis vinifera	Syntichaki *et al.*, 1996
Synechocystis sp.	Chavez *et al.*, 1995
Achlya klebsiana	LéJohn *et al.*, 1994b
Homo sapiens	Shashidharan *et al.*, 1994
Sulfolobus shibatae	Benachenhou-Lahfa *et al.*, 1994
Neurospora crassa	Kapoor *et al.*, 1993; Kinnaird and Fincham, 1983
Pseudomonas putida	Kim *et al.*, 1993*
Pyrococcus endeavori	DiRuggiero *et al.*, 1993
Pyrococcus furiosus	Eggen *et al.*, 1993
Rattus norvegius	Das *et al.*, 1993
Clostridium symbiosum	Teller *et al.*, 1992
Corynebacterium glutamicum	Bormann *et al.*, 1992
Clostridium difficile	Lyerly *et al.*, 1991*
Chlorella sorokiniana	Cock *et al.*, 1991
Debaryomyces occidentalis	De Zoysa *et al.*, 1991
Halobacterium salinarium	Benachenhou and Baldacci, 1991
Peptostreptococcus asaccharolyticus	Snedecor *et al.*, 1991
Porphyromonas gingivalis	McBride *et al.*, 1990
Drosophila melanogaster	Papadopoulou and Louis, 1990
Aspergillus nidulans	Hawkins *et al.*, 1989
Salmonella typhimurium	Bansal *et al.*, 1989
Klebsiella aerogenes	Mountain *et al.*, 1985
Saccharomyces cerevisiae	Moye *et al.*, 1985; Nagasu and Hall, 1985
Eschericia coli	Valle *et al.*, 1984; McPherson and Wootton, 1983

glutamine (nucleic acids, some amino acids and NAD^+) as a nitrogen source (Wolheuter *et al.*, 1973) is possible, with glutamate having the primary role. However, the function of GDH will vary between different organisms, as will the type(s) of GDH(s) those organisms possess (DiRuggiero and Robb, 1993; Rice *et al.*, 1985; Smith *et al.*, 1975).

3.I.a. Categorization of the glutamate dehydrogenases Generally, GDH is localized in the cytoplasm of microorganisms, the mitochondria of mammals and the chloroplasts of plants (Stewart *et al.*, 1980; Hollenberg *et al.*, 1970; de Duve, 1962). Oomycetes may be an exception to this rule, however, with some GDH located in the mitochondria (Stevenson and LéJohn, 1971; LéJohn and Stevenson, 1970). As well as differences in distribution within a cell, different groups of organisms possess different classes of GDHs. The basis of the classification system used involves the nucleotide cofactor(s) (NAD, NADP or both) used by the GDH in question. Some organisms, however, may possess more than one class of GDH (DeCastro *et al.*, 1970; Sanwal and Lata, 1961), or may have multiple isozymes (McDaniel, 1986; LéJohn, 1975).

The NADP-GDHs (EC 1.4.1.4) comprise the first of the category of GDHs, and are used for ammonia assimilation by bacteria, fungi and algae (Smith *et al.*, 1975). In contrast to this, NAD-GDHs (EC 1.4.1.2) have a catabolic role in fungi and anaerobic bacteria (Rice *et al.*, 1985; Smith *et al.*, 1975; Buckel

and Barker, 1974), while the dual cofactor specific NAD(P)-GDHs (EC 1.4.1.3) are used in both a catabolic and a metabolic manner in animals, plants (Smith *et al.*, 1975) and archaebacteria (DiRuggiero and Robb, 1993). The favouring of the catabolism of L-glutamate to α-ketoglutarate, by NAD-GDH in oomycetes has been indicated by the increase of GDH levels in cells grown with L-glutamate or L-glutamine as a nitrogen source compared to those grown with ammonia (Yang, 1991; Stevenson, 1974; LéJohn and Stevenson, 1971). Intracellular NAD(H) will also tend to exist mainly in the oxidized form, helping drive the conversion of L-glutamate to α-ketoglutarate (Lehninger, 1982). Cofactor specificity is also related to enzyme quaternary structure and subunit size. NADP and dual cofactor specific enzymes have homohexameric structures, with monomers in the 48-55kD range, whereas NAD-GDHs can be homohexameric (Rice *et al.*, 1985) or homotetrameric with much larger monomers of over 100kD (Yang and LéJohn, 1994; Veronese, 1974). The primary structures of homohexamers and homotetramers are very different (Britton, 1992), but do share some sequence similarities.

The NAD-GDHs used by the Phycomycetes can be further subcategorized regarding the activators and inhibitors used to regulate the enzyme of a particular species (LéJohn, 1975). This categorization includes Type I GDHs which are unregulated; Type II GDHs which are activated by

AMP, Ca^{2+} and Mn^{2+} and inhibited by ATP, GTP, citrate and fructose-1,6-bisphosphate; and Type III GDHs which are activated by $NADP^{+}$, ATP, GTP and short chain acyl-CoA derivatives (likely through cooperative binding at more than one allosteric site (LéJohn *et al.*, 1970)) but are inhibited by Ca^{2+} and Mn^{2+} (LéJohn, 1975; Stevenson, 1974; Stevenson and LéJohn, 1971; LéJohn and Stevenson, 1971; LéJohn *et al.*, 1970). This final class of NAD-GDH is the type possessed by *A. klebsiana* and other oomycetes (LéJohn, 1975).

3.I.b. <u>Structure of *gdh* genes</u> A wide variety in gene structure can be observed amongst eukaryotic *gdh* gene sequences. Unfortunately, some eukaryotic sequences are available only as cDNA sequences (Melo-Oliveira *et al.*, 1996; Das *et al.*, 1993), precluding any analysis of the number and locations of any introns which may be present. Of those recorded eukaryotic *gdh* gene sequences which are genomic, the NAD-GDH and NADP-GDH of *S. cerevisiae* are both intronless (Moye *et al.*, 1985; Nagasu and Hall, 1985), as is the *gdh* gene of *Leishmania tarentolae* (Bringaud *et al.*, 1997). *Drosophila melanogaster gdh* gene contains a single large intron (Papadopoulou and Louis, 1990), whereas the *Aspergillus nidulans gdh* (Hawkins *et al.*, 1989), human *gdh* (Amuro *et al.*, 1990) and both the *nad-gdh* and *nadp-gdh* of *Neurospora crassa* (Kapoor *et al.*, 1993; Kinnaird and Fincham, 1983) all consist of three exons and two introns. *Agaricus bisporus*

(Schaap *et al.*, 1996) is much more complex with seven exons and six introns. The most complex *gdh* sequenced to date is the $NADP^+$-activeable, *nad-gdh* of *Achlya klebsiana* with none introns and ten exons (LéJohn *et al.*, 1994b) which, as noted above (*2.II.e The Antisense Gene Pair of* Achlya klebsiana), is associated with a putative *hsc70* gene in an antisense gene pair (LéJohn *et al.*, 1994a and 1994b).

3.II. Heat Shock 70 Proteins

Heat shock proteins (HSPs) comprise a broad category of proteins encoded by conserved genes which are often, but not always, induced by one or more forms of environmental stress. HSPs have been subcategorized on the basis of molecular size, with HSP70s (M_r of about 68 to 74kD) forming the largest class of HSP. HSP70s are highly conserved, and have been identified in prokaryotes as well as eukaryotes (Nagao *et al.*, 1990). Some HSP70s are constitutively expressed and are termed heat shock cognate (HSC) proteins (LéJohn *et al.*, 1994a; Nagao *et al.*, 1990) to differentiate them from other, inducible, HSP70s. Still other HSP70s are constitutively expressed at low levels, but are induced in response to one or more environmental stresses (LéJohn *et al.*, 1994a; Burel *et al.*, 1992; Ang *et al.*, 1991). Typical of the conditions inducing *hsp70* gene expression and protein synthesis are heat shock (Lindquist and Craig, 1988), nutritional stress (LéJohn *et al.*, 1994a; Munro and Pelham, 1986; LéJohn and Braithwaite,

1984), heavy metals and amino acid analogs (Mosser *et al.*, 1988), hormonal treatment (Silver *et al.*, 1993) and cell differentiation (Heikkila, 1993; Zimmerman *et al.*, 1983). Often, more than one member will be expressed in the same organism (Ang *et al.*, 1991).

Members of the *hsp70* gene family share a very high degree of encoded amino acid sequence identity, with a highly conserved N-terminal region of approximately 44kD conferring ATPase activity upon the protein (Bukau and Horwich, 1998) and a more variable C-terminal, 25kD region. The C-terminal region can be further subdivided into a 15kD substrate binding domain and a C-terminal 10kD domain of unknown function (Bukau and Horwich, 1998). The 44kD N-terminal and 25kD C-terminal domains are separated from one another by a central protease sensitive site (Gething and Sambrook, 1992). Similarity between *hsp70* genes at the nucleotide level is also high, generally with 50% to 90% identity between *hsp70*s of different organisms. The highly conserved nature of *hsp70* genes has allowed the isolation of *hsp70*s from a wide variety of organisms using known *hsp70* genes as probes for cross-hybridization (Roberts and Key, 1991; Rochester *et al.*, 1986).

3.II.a. <u>The roles of heat shock 70 proteins</u> HSP70s are thought to act as intracellular chaperones (Morimoto *et al.*, 1990), in order to guide protein folding, unfolding, oligomerization and oligomer disassembly (Bukau and Horwich, 1998; Ang *et al.*, 1991), disaggregation (Hwang and Kornberg,

1990; Zylicz *et al.*, 1989), the control of the biological activity of some regulatory proteins (Bukau and Horwich, 1998) and cross-membrane protein transport (Waters *et al.*, 1989; Chirico *et al.*, 1988; Deshaies *et al.*, 1988). All of these functions have been observed to involve both the disruption of normal intra- and inter-protein interactions (Bienz and Pelham, 1987) and ATP-hydrolysis associated binding to hydrophobic regions of the target protein (Rüdiger *et al.*, 1997a; Flynn *et al.*, 1991; Lindquist and Craig, 1988). Proteins targeted for interaction with HSP70s may be specified by the extended conformation of short hydrophobic sequences in unfolded proteins (Zhu et al., 1996; Schmid *et al.*, 1994; Palleros *et al.*, 1994; Landry *et al.*, 1991). The specific binding of HSP70s to peptide sequences enriched with aliphatic amino acids has also been demonstrated (Gragerov and Gottesman, 1994; Richarme and Kohiyama, 1993; Flynn *et al.*, 1991), leading to the hypothesis that HSP70s interact with internal peptide sequences not normally available for binding after post-translational folding (Gragerov and Gottesman, 1994; Ang *et al.*, 1991; Rothman, 1989). Rüdiger *et al.* (1997b) have identified a consensus motif for proteins targeted by the *E. coli* HSP70 DnaK, which consists of a heptapeptide most often found in β strands buried within a properly folded protein. These same internal sequences would also be available for binding if the structure of the protein was disrupted, such as during heat shock. Therefore, it has been proposed

that HSP70s could bind the extended conformation of abnormal or disrupted proteins in order to prevent unwanted aggregation. Proteins which are otherwise identical but fail to bind to HSP70s would then be targeted for degradation (Bienz and Pelham, 1987).

3.II.b. Heat shock 70 protein mode of action The HSP70 ATPase domain appears to be involved in controlling the affinity of the substrate binding domain for the target protein (Bukau and Horwich, 1998). ATP binding "opens" the substrate binding domain to permit interaction with the target protein(s). ATP hydrolysis to ADP and inorganic phosphate will "close" this domain, and the binding of a new molecule of ATP subsequent to the release of ADP reopens the substrate binding domain. This renews the cycle with the release of the bound substrate molecule and replacement with another (Bukau and Horwich, 1998). However, the speed with which protein folding occurs is significantly greater than the turnover rate of the HSP70 ATPase activity (Theyssen *et al.*, 1996; Gao *et al.*, 1994; Flynn *et al.*, 1989). Therefore, co-chaperones, such as members of the DnaJ family of proteins (Laufen *et al.*, 1997; McCarty *et al.*, 1995) in prokaryotes and eukaryotes, and GrpE in prokaryotes (Bukau and Horwich, 1998) are required. Taking *E. coli* DnaK as an example, the co-chaperone DnaJ is thought to bind the substrate protein, stimulating ATPase activity of DnaK as soon as the substrate protein is transferred from DnaJ to the DnaK:ATP complex. DnaJ is

released upon this transfer, and the DnaK:substrate:ADP complex is then targeted by GrpE, which stimulates ADP release. ATP then rapidly binds the DnaK:substrate complex, causing the release of the substrate protein and readying DnaK for binding to another molecule of substrate protein.

3.II.c. <u>Control of *hsp70* gene expression</u> Prokaryotic control of *hsp70* gene expression is primarily at the transcriptional level, due to the short half life of mRNA in prokaryotes compared to that in eukaryotes (minutes in *E. coli* compared to hours in plants and *D. melanogaster*) (Nagao *et al.*, 1990). This control involves the use of alternative σ factors through interaction with untranslated sequences characteristic of *hsp70* genes. Transcription factor σ^{32} is specific for cytoplasmic heat shock gene transcription in *E. coli*, with levels increasing greatly in response to heat shock, due in part to increased protein synthesis and also in part to decreased protein turnover (Grossman *et al.*, 1987; Erickson *et al.*, 1987). Similar transcription factors have been identified in several other eubacteria (Nakahigashi *et al.*, 1995), and are involved in other stress responses, including σ^{s} (σ^{38}) (nutrient starvation, heat shock, acid and hyperosmotic stress) (Muffler *et al.*, 1997) and σ^{E} (σ^{24}) (for the extracytoplasmic stress response) (de las Peñas *et al.*, 1997). DnaK is the primary HSP70 in *E. coli* and has been shown to repress its own production as well as the production of other HSP70s (Chappell *et al.*, 1986; Craig, 1985; Craig and Jacobsen, 1984; Tilly *et al.*, 1983), indicating that the

heat shock response may be autoregulated to some degree. Similar observations have also been made for heat shock response regulation in eukaryotic cells (Nagao *et al.*, 1990).

Eukaryotic *hsp 70* genes are characterized by the presence of one or more copies of (to an extent) interchangeable transcriptional enhancers termed heat shock elements (HSEs) (Spena and Schell, 1987; Rochester *et al.*, 1986; McMahon *et al.*, 1984). These cis-acting sequences are located from 15 bp to 400 bp 5' of a heat shock protein gene's TATA box (Bienz and Pelham, 1987; Pelham, 1985; Pelham, 1982), in both *hsp70* genes and genes for heat shock proteins of other classes (Czarnecka *et al.*, 1989; Baumann *et al.*, 1987). The HSE consensus sequence of 8 bases in a stretch of 14 bp (CnnGAAnnTTCnnG) (Nagao *et al.*, 1990; Bienz and Pelham, 1987) can occur as doublets and triplets as well as singly. HSEs can also be described as arrays of a series of 5 bp sequences (nGAAn) in alternating orientations (Lis *et al.*, 1989). Depending upon the specific gene, more than one HSE may be necessary for complete induction (Xiao and Lis, 1988; Bienz and Pelham, 1987; Gurley *et al.*, 1986; Amin *et al.*, 1985). This could be indicative of inter-species variance in either the strength of transcription factor:HSE interactions, or the number of transcription factors required (Bienz and Pelham, 1987).

The primary factors interacting with HSEs are known as heat shock transcription factors (HSFs), although other factors are also involved (Judelson *et al.*, 1992; Greene and Kingston, 1990). HSFs in eukaryotes are very similar (Wu *et al.*, 1987; Sorger *et al.*, 1987; Kingston *et al.*, 1987), but can activate *hsp70* gene transcription in different ways (Nagao *et al.*, 1990): by either enhancing $RNA_{POL}II$ binding to transcriptional promoters (Rougvie and Lis, 1988; Gilmour and Lis, 1986), or by enhancing *hsp* gene activation by variations in the phosphorylated state of HSFs (Sorger and Pelham, 1988). The 5 bp motif described by Lis *et al.* (1989) is sufficient for HSF binding, although longer sequences are necessary for stable interactions. Other motifs present in transcriptional promoters, such as any CCAAT boxes located in the immediate 3' region of an HSE, may also influence HSF binding, particularly in the absence of environmental stresses (Bienz, 1986; Beinz and Pelham, 1986).

4. Summary

In summary, the oomycetes are pseudofungal chromists which form a phylogenetic group biochemically and physiologically distinct from the true fungi. One of the oomycetes, *Achlya klebsiana* (Order Saprolegniales, Family Saprolegniaceae) has been found to have an antisense gene pair arrangement comprised of an NAD-dependent glutamate dehydrogenase gene (*nad-gdh*) and a heat shock cognate 70 gene (*hsc70*). The former gene

encodes a vital, multimeric enzyme linking nitrogen and carbon metabolism, while the latter encodes a typical "chaperone" protein, thought to help guide correct protein folding. It has been demonstrated *in vitro* that peptides derived from complementary strands of DNA are themselves complementary and capable of interacting with each another. It follows that there could be a potential for the NAD-GDH and HSC70 encoded by the *A. klebsiana* antisense gene pair to interact with one another. In this thesis, the potential of both members of this antisense gene pair to be transcribed concurrently was further studied. In addition to this, both the NAD-GDH of another oomycete predicted to have an *A. klebsiana*-like antisense gene pair, *P. ultimum* (Order Peronosporales, Family Pythiaceae) was characterized. Lastly, a non-antisense gene pair, putative *hsp70* gene in *P. ultimum* similar to an analogous *hsp70* from *A. klebsiana* was identified and characterized.

METHODS AND MATERIALS

METHODS

1. Cell Culture

1.I. Mammalian Cell Culture

1.I.a. <u>Culture for RNA extraction</u> COS7 monkey cells (supplied by Dr. B. Yang, University of Toronto) were grown in 25 cm^2 culture flasks using 10 mL Dulbecco's modified Eagle's medium (DMEM) containing 584 mg/L L-glutamine and 110 mg/L pyruvate (Gibco BRL) supplemented with 10%(v/v) newborn calf serum (NCS) (Gibco BRL) and 1.5%(v/v) antibiotic solution (5 mg/mL streptomycin sulfate and 5000 U/mL penicillin G (sodium salt) in sterile water) (Gibco BRL). COS7 cells to be transfected prior to RNA recovery were cultured in 24 well culture plates, using 2 mL growth medium per well. All COS7 cells were subcultured in fresh medium using a split ratio of 1:4 after incubation at 37°C in a 5% CO_2 atmosphere for four days or until cells reached confluence. All cell transfers and manipulations of cells and growth medium were conducted in a Labgard laminar flow biological safety

cabinet (Model Number NU-408FM-600) sterilized with UV light from a Westinghouse sterilamp (Model 782L-30) for at least 10 minutes.

1.I.b. <u>COS7 cell recovery</u> COS7 cells were recovered by trypsinization for subculturing, transfection or medium renewal. Two hundred and fifty microlitres 0.25%(w/v) trypsin in ice cold, sterile phosphate buffered saline (PBS) (137 mM NaCl, 1.4 mM KH_2PO_4, 4.3 mM K_2HPO_4, 2.7 mM KCl, pH 7.4) was added to 25 cm^2 culture flasks containing a confluent monolayer of COS7 cells which had been gently washed with a large excess of ice cold, sterile PBS. Cells were monitored at room temperature until most no longer adhered to the growth surface, at which point trypsinization was arrested with 10 mL ice cold PBS supplemented with 10%(v/v) NCS. This cell suspension was divided into 2.5 mL aliquots which were centrifuged at 4°C for 5 minutes in a clinical centrifuge. The supernatant was discarded and the cells were gently resuspended in 10 mL mammalian growth medium prior to introduction into a new 25 cm^2 culture flask.

1.I.c. <u>Long term mammalian cell storage</u> COS7 cells were stored in $N_{2(l)}$ in 1.5 mL aliquots of 70:20:10 DMEM:NCS:DMSO after slow freezing to -70°C (1 hour at 4°C, 1 hour at -20°C, 3 hours at -70°C while surrounded by a thick covering of cotton in a styrofoam box). Cells were recovered by rapid thawing in a 37°C water bath, an entire vial being used to inoculate 10 mL freshly prepared mammalian growth medium (Fresney, 1987). Cell survival

was verified microscopically through viability staining with 0.2%(w/v) trypan blue dye in PBS at room temperature.

1.II. Oomycete Culture

1.II.a. Short Term Culture The oomycete *Pythium ultimum* strain BR 471 (obtained from Dr. G.R. Klassen, University of Manitoba) cultures were maintained at room temperature for up to 30 days in 100x15 mM petri plates containing approximately 25 mL GY medium (5.0g/L glucose, 0.5g/L yeast extract, 1 mM $CaCl_2$, 1 mM $MgCl_2$) or 25 mL Czapek Dox liquid medium (Oxoid Ltd) supplemented with 100 μg/mL ampicillin. Oomycetes were subcultured in a Labgard laminar flow biological safety cabinet sterilized as for mammalian cell work, using a small (<1 cm^2) piece of mycelial mat as an inoculum for each fresh plate.

Larger scale cultures were obtained using a single overnight oomycete culture in a petri plate as an inoculum (shredded with scissors and tweezers sterilized with ethanol and flame) for every 500 mL of GY medium to be inoculated. These cultures were incubated overnight in 1L flasks at 28°C with shaking (~170rpm). Oomycete cultures of 10L volume using modified GY medium were incubated in sterile carboys at room temperature for 48 hours, with vigorous aeration provided by two spargers using an air supply forced through sterilized cotton.

1.II.b. Long term storage *P. ultimum* strain BR 471 stock cultures were preserved for up to 6 months on potato dextrose agar (Gibco BRL) slant cultures in six inch screw-capped culture tubes at 4°C, using small (<1 cm^2) pieces of mycelial mat as inoculum. The medium was supplemented with 100 μg/mL ampicillin to prevent bacterial contamination. Dessication of the cultures was inhibited by using parafilm wrapped around the cap prior to storage. Historically, storage of oomycetes in $N_{2(l)}$ has met with limited success, and appears to be species specific (Hohl and Iselin, 1987). Attempts to store *P. ultimum* strain BR 471 in $N_{2(l)}$ in this manner (using 8.5%(w/v) skim milk powder supplemented with 10%(v/v) glycerol, or LB broth (see *1.III.a General culture conditions* below) supplemented with 50%(v/v) glycerol), both with and without pre-incubation of cultures at 4°C to induce cold hardening, were unsuccessful.

1.III. Bacterial Cell Culture and Storage

1.III.a. General culture conditions Unless otherwise noted, *E. coli* cultures were maintained in 10 mL LB broth (10g/L tryptone, 5g/L yeast extract, 5g/L NaCl) cultures grown overnight at 37°C and stored for up to 2 weeks at 4°C. Cultures were also kept on LB agar (LB broth supplemented with 15g/L agar prior to autoclaving) after overnight growth at 37°C for up to one month at 4°C.

1.III.b. Long term bacterial storage Bacterial stock cultures were derived from 10 mL cultures grown to late log phase. Cells were pelleted by centrifugation for 10 minutes at 5000g at 4°C, the growth medium discarded and the cells resuspended in 5 mL ice cold LBG medium (10g/L tryptone, 5g/L yeast extract, 5g/L NaCl, 50%(v/v) glycerol) by vortexing. The cell suspension was divided into 1 mL aliquots and stored at -70°C until needed.

2. Antisense Gene Pair Co-transcription

2.I. Recombinant Molecule Construction

2.I.a. Insert and vector preparation Both the insert (a 5.2 kb XbaI restriction endonuclease fragment ligated into the pM13 (Stratagene) MCS provided by LéJohn) and pBK-RSV vector (Stratagene) were prepared for ligation in a similar manner. Both molecules were digested at 37°C with 2U XbaI restriction endonuclease per μg DNA for three hours, after which the reaction was supplemented with an additional 1U XbaI restriction endonuclease per μg DNA and digestion allowed to proceed for another hour. Digestion was halted with a one-sixth volume of 6x agarose gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 15% Ficoll (Type 400), 0.1M EDTA) and electrophoresed in a horizontal submerged gel electrophoresis apparatus (Gibco BRL) overnight at 25V on a 1% (w/v) agarose gel (11 cm x 13 cm) in TAE buffer (40 mM TRIS acetate, pH 8.5, 2 mM EDTA). Gel running buffer consisted of 1 L TAE buffer supplemented

with 40 μL 10 mg/mL ethidium bromide. Appropriate bands representing linearized vector and insert (4.5 kb for vector, 5.2 kb for insert) were identified, excised and placed in dialysis tubing prepared as described (Maniatis *et al.*, 1982) The DNA fragments were electroeluted in TAE buffer for three hours at 100V, followed by reversal of the current for two minutes. The agarose was removed and examined on a UV transilluminator to verify migration of the DNA out of the fragment. The solution containing the DNA was dialyzed against 4L TE buffer, pH 7.5 for four hours at 4°C, after which the buffer was replaced and dialysis continued overnight at 4°C. The contents of the dialysis bag were emptied into sterile glass centrifuge tubes, and the DNA concentrated using sec-butanol extractions until the final volume was less than 300 μL. The solution was combined with an equal volume of 7.5 M ammonium acetate, pH 5.8, and precipitated with the addition of three volumes ice cold anhydrous ethanol at -70°C for 30 minutes, followed by centrifugation in a microcentrifuge for 30 minutes at 4°C. The supernatant was removed with a pipette, and the DNA pellet washed twice with ice-cold 80%(v/v) ethanol prior to dessication under vacuum for 15 minutes. The pellet was resuspended in 250 μL TE buffer, pH 7.5. DNA and stored at -20°C prior to further manipulation.

2.I.b. <u>Vector dephosphorylation, ligation and bacterial transformation</u>

Digested vector was dephosphorylated using 1U calf intestinal

phosphorylase (CIP) (Gibco BRL) per μg DNA in 1x phosphorylase buffer (20 mM TRIS HCl, pH 8.0, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, 50 μg/mL BSA) for thirty minutes at 37°C. The reaction was brought to a final volume of 250 μL with TE buffer, pH 7.5, and vortexed one minute with 250μL LTSPC buffer (20 μL 8M LiCl, 10 μL 1M TRIS HCl, pH 9.5, 4 μL 20%(w/v) SDS, 250 μL TE saturated phenol:chloroform:isoamyl alcohol 25:24:1 (v/v)). The mixture was microcentrifuged ten minutes at room temperature, and the aqueous phase recovered and reextracted with 24:1 (v/v) chloroform:isoamyl alcohol. The aqueous phase was recovered and the dephosphorylated vector ethanol precipitated as described (*2.I.a. Insert and Vector Preparation*, above).

The Rapid DNA Ligation Kit™ (Boehringer-Mannheim) was used to ligate purified 5.2 kb XbaI restriction endonuclease fragment to the dephosphorylated pBK-RSV vector, using 2 μL T4 DNA ligase and an insert:vector ratio of 3:1 μg in a final reaction volume of 22 μL. The reaction mixture was incubated 30 minutes at room temperature, after which a 5 μL aliquot was electrophoresed as described (*2.I.a. Insert and Vector Preparation*, above) to verify that ligation had occurred.

E. coli XL-1 Blue MRF' was used as a recipient cell line for transformation by $CaCl_2$ as described Ausubel *et al* (1992), using 12.5 μg/mL tetracycline as selective agent for the F-episome, and 50 μg/mL kanamycin as selective agent for the recombinant plasmid. Transformants were picked

and transferred to fresh selective plates, and then spotted with 2 μL 100 mM IPTG:2% (w/v) 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) (10:75 (v/v) ratio) and grown overnight at 37°C. White and pale blue colonies were then picked and replated prior to transfer to 10 mL LB medium supplemented with 50 μg/mL kanamycin and growth overnight at 37°C. The presence of insert bearing plasmid was verified through a crude plasmid extraction by pelleting and suspension of a 500 μL aliquot of cells in 60 μL STE buffer (100 mM NaCl, 20 mM TRIS HCl, pH 7.5, 10 mM EDTA). This mixture was vortexed 60 seconds in the presence of 40 μL TE saturated phenol:chloroform:isoamyl alcohol (25:24:1 (v/v)). The aqueous phase was recovered and incubated 2 minutes at room temperature with 1μg RNase. Thirty microlitres 6x agarose gel loading buffer was added, and the presence of recombinant plasmids demonstrated by agarose gel electrophoresis as described (*2.I.a. Insert and Vector Preparation*, above), using pBK-RSV transformed XL-1 Blue MRF' *E. coli* as a negative control.

2.I.c. <u>Plasmid amplification and recovery</u> Amplification of insert bearing plasmid for use in insert orientation determination within the multiple cloning site was conducted using 10 mL LB broth cultures supplemented with 50 μg/mL kanamycin grown at overnight with shaking 37°C as described (*2.I.d. Insert Orientation Determination* below). Larger scale cultures were grown in 500 mL LB plus 50 μg/mL kanamycin overnight at 37°C with shaking, using 5

mL inocula grown overnight at 37°C with shaking. Subsequently, only one clone of each insert orientation was maintained, with stock cultures stored as described (*1.III.a. Long Term Bacterial Storage* above). In all cases, plasmids were extracted by the lysozyme sphaeroplasting:boiling method of Holmes and Quigley (1981), with reactions being scaled up or down as necessary to accomodate larger or smaller starting bacterial cultures. Plasmid DNA was quantified spectrophotometrically, with one OD unit at A_{260} equivalent to 50 μg double stranded DNA in the aliquot measured (Ausubel *et al.*, 1992)

2.I.d. Insert orientation determination The orientation of the 5.2 kb XbaI restriction endonuclease fragment inserted into the pBK-RSV MCS for each clone was determined by restriction endonuclease digestion using EcoRI restriction endonuclease and KpnI restriction endonuclease and visualized by agarose gel electrophoresis, as described (*2.I.a. Insert and Vector Preparation*, above).

2.II. Transient Transfection of Mammalian Cells

COS7 cells were transfected by lipofection using LipofectAMINE™ reagent (Gibco BRL) according to the manufacturer's instructions and the procedure of Yang *et al.* (1998) in 24 well culture dishes. Five micrograms of plasmid DNA was incubated with 10 μL LipofectAMINE™ and 200 μL DMEM for 15 minutes at room temperature, followed by an additional 800 μL

DMEM. Two hundred microlitres of the lipofection mixture was incubated per well, with each well containing cells grown to 70% confluence previously washed with 2 mL DMEM. Lipofection was allowed to proceed for 10 hours at 37°C, after which cells were grown under normal culture conditions for three days prior to harvesting for RNA recovery. Harvesting was conducted by washing cells with two volumes cold PBS, followed by incubation with 250 μL 0.5 mM EDTA in PBS for 15 minutes under normal incubation conditions as described (Ausubel *et al.*, 1992; Warren and Shields, 1984).

2.III. Total RNA Extraction

Total mammalian, oomycete and *E. coli* RNAs were extracted using the alkaline phenol method of LéJohn (1985). This procedure was modified for mammalian cells by initially rupturing cells with repeated flash freezing in $N_{2(l)}$ and thawing on ice, instead of grinding in $N_{2(l)}$ with a mortar and pestle. *E. coli* cells were not treated in any way prior to treatment with alkaline extraction buffer and phenol. RNA recovered was quantified by spectrophotometric determination of A_{260}, with one OD unit equivalent to 40 μg RNA in the aliquot measured (Ausubel *et al.*, 1992).

2.IV. Analysis of Total RNA

2.IV.a. <u>RNA electrophoresis and Northern capillary blotting</u> Total RNA was combined with 8 μL deionized formaldehyde, 25 μL deionized formamide and 5μL 10xMOPS buffer (4 mM 3-(N- morpholino)propane

sulfonic acid (MOPS), pH7.0, 100 mM sodium acetate, 10 mM EDTA) in a final volume of 50 μL (Maniatis *et al.*, 1982) and denatured for 5 minutes at 65°C. Samples were then combined with 10 μL agarose gel loading buffer and loaded onto denaturing agarose gels (1.0%(w/v) agarose and 17%(v/v) formaldehyde (40%(v/v)) in 1xMOPS buffer) and electrophoresed in a chamber containing 1L RNA gel running buffer (5 mM sodium acetate, 1 mM EDTA, 20 mM TRIS-acetate, pH 7.5) at 40V and room temperature for 4 hours. A 10 μg aliquot of 1 kb DNA ladder for use as a molecular weight standard was treated in the same manner as described for RNA samples with the addition of 1 μL 10 mg/mL ethidium bromide added directly to the sample after denaturation to allow visualization under UV light after electrophoresis.

Samples were transferred onto Hybond-N^+ nylon membranes (Amersham), via capillary transfer conducted at 4°C overnight (12-16 hours) with 20xSSC (3M NaCl, 0.3M Na-citrate) as transfer buffer. Immediately after transfer, membranes were briefly washed in 2xSSC and allowed to air-dry completely at room temperature. RNA was fixed to the dried membranes by exposure to UV light on a Foto/Prep I transilluminator (Bio/Can Scientific) for 6 minutes. Membranes were then stored at room temperature under vacuum until needed.

2.IV.b. Probe construction Probes were designed to identify the *nad-gdh* and *hsc70* transcripts (produced either concurrently or separately) from mammalian, oomycete and *E. coli* total RNAs. The complete *Achlya klebsiana* antisense gene pair contained on the 5.2 kb XbaI restriction endonuclease fragment was initially used to identify any transcripts which would correspond to any region of either the *nad-gdh* or *hsc70* genes. A 3.2 kb XbaI restriction endonuclease fragment containing a duplication of the first nine exons of *A. klebsiana nad-gdh* was also used to identify transcripts corresponding to *nad-gdh*, as well as transcripts corresponding to the 3' untranslated region of the *hsc70*. The remaining probes were derived by polymerase chain reaction (PCR) from particular regions of the *A. klebsiana* antisense gene pair (Figure 7).

2.IV.c. Polymerase chain reaction PCR reactions were conducted using 200 ng of each primer (Figure 7), 10ng of template DNA, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 200 μM dTTP, 18 mM $(NH_4)_2SO_4$, 60 mM Tris-SO_4 (pH 9.1), and 1.6 mM $MgCl_2$. One μL Taq polymerase (provided by Dr. P.C. Loewen) was added to the reaction mixes immediately prior to amplification using a Perkin Elmer Cetus DNA thermal cycler. The PCR reaction consisted of a strand separation incubation at 94°C for 1 minute, followed by primer annealing at 50°C for 1 minute, and strand extension at 72°C for 2 minutes for 40 cycles, and then concluded by a single extension step at 72°C for 10

Figure 7: Probes used for antisense gene pair transcript identification during Northern RNA capillary blotting

Names and transcript targets for each probe are given beneath their diagrammatic representation. Location and directionality of any primers used to generate PCR products for probes are shown by labeled arrows. Sequences for primers as well as a key for the identification of specific characteristic regions of the probes and PCR templates are given below.

Primer PH3': 5'-TCA TTA ACT GGC TCG ACC AC-3'

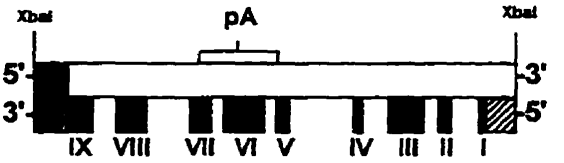
Primer I9: 5'-TGA ACG AGT CTA TGC CTA-3'

Key to probe sequence regions of note

: *gdh* promoter region.
: Unique 470bp region.
: *hsc70* open reading frame.
: *gdh* open reading frame.
I-X : *gdh* exon number.
pA : *hsc70* polyadenylation site.

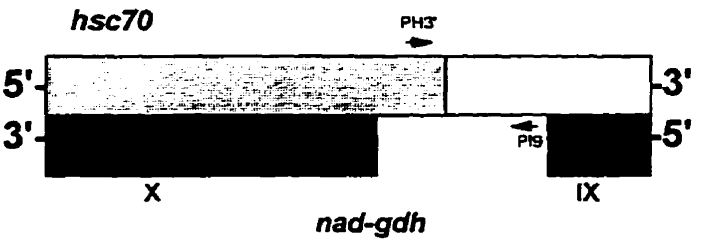
5.2kb XbaI Fragment

(Targets *nad-gdh* and *hsc70* Transcript)



3.5kb XbaI Fragment

(Targets *nad-gdh* Transcript and *hsc70* 3' UTR)



340bp PCR Fragment of Intron 9

(Targets *hsc70* Transcript and Unprocessed *nad-gdh* Transcript)

minutes. PCR reactions were halted with 15 μL agarose gel loading buffer,and the entire mixture loaded and electrophoresed on a 1%(w/v) agarose gel in TAE buffer for 4 hours at 70V constant voltage as described (*2.I.a. Insert and Vector Preparation* above).

Bands corresponding to PCR products to be used for radiolabeling as probes were excised, and these gel plugs were incubated at room temperature for one hour with shaking in freeze-squeeze buffer (0.3M sodium acetate, pH7.0; 1 mM EDTA) before being frozen in $N_{2(l)}$.

The frozen gel plugs were crushed to a fine powder and placed in a clean 1 mL syringe packed at the tip with sialinized glass wool. The syringe was placed in a 15 mL sterile glass test tube, with the flanges at the top of the syringe suspending it above the bottom of the tube. This apparatus was centrifuged at 10000g for 15 minutes at 4°C, and the volume of the liquid recovered in the test tube measured. Ethanol precipitation of nucleic acids from this liquid with three volumes ice-cold absolute ethanol -70°C for one hour followed by centrifugation in a microcentrifuge for 30 minutes was conducted. The DNA was then washed with 80%(v/v) ethanol and dried under vacuum for 15 minutes prior to resuspension in sterile water.

2.IV.d. <u>Probe radiolabeling</u> Nucleotide probes of greater than 1 kb in length were radiolabeled by random primer labeling (Ausubel *et al.*, 1992), using 500 ng template DNA, 150 ng random primer and 6 μL (approximately 60

μCi) α^{32}PdATP in a final reaction volume of 40 μL. Polynucleotide probes of less than 1 kb in length were radiolabeled by nick translation (Ausubel *et al.*, 1992) using 1 μg template DNA and from 5 to 20 μL (approximately 50 to 200 μCi) α^{32}PdATP (depending upon the age of the isotope) in a final volume of 50 μL. Radiolabeled probe was ethanol precipitated for 15 minutes at -70°C with an equal volume of stop buffer (1.33 mM EDTA, pH 8.0, 0.8 μg/mL tRNA, 0.1% SDS) and 2.5 volumes of absolute ethanol, followed by centrifugation in a microcentrifuge for 15 minutes. The radioactive supernatant was disposed of and the pellet tested for radioactivity as an indication of isotope incorporation. The pellet was dried in a 65°C waterbath for 10 minutes, after which it was resuspended in 100 μL sterile water and denatured by boiling five minutes and immediate placement on ice. The denatured probe was used immediately for hybridization to membranes (*2.IV.e. Probe Hybridization and Autoradiography*, below).

2.IV.e. <u>Probe hybridization and autoradiography</u> Stored Northern blots were prehybridized for a period of 4 to 8 hours at 65°C in a Turbospeed hybridization oven (Bio/Can). Six times SSC, 5x Denhardt's solution (1.0g/L Ficoll 400, 1.0 g/L polyvinylpyrrolidone, 1.0 g/L BSA), 0.5% (w/v) SDS and 0.2 mg/100 mL denatured salmon sperm DNA was used as a prehybridization mixture. Radiolabeled probe was added to this mixture after prehybridization and incubated overnight at 65°C. Membranes were washed

at room temperature in glass trays for 15 minutes in 5xSSPE (0.9M NaCl, 50 mM NaH_2PO_4, pH7.4, 50 mM EDTA, pH7.4)) and then 30 minutes with 2xSSPE+0.1% (w/v) SDS. Radiolabeled Northern blots were exposed to Kodak X-ray film overnight at -70°C with an intensification screen.

3. NAD-GDH Characterization

3.I. Enzyme Stability

The effect of temperature upon NAD-GDH activity was tested using TPED medium (50 mM TRIS-HCl, pH7.3, 10 mM K_2HPO_4, 1 mM EDTA, 1 mM dithiothreitol) supplemented with a variety of glycerol concentrations. Enzyme inactivation at -20°C, 4°C and at 37°C was tested using TPED with 0, 10, 20, 30 or 50%(v/v) glycerol. Aliquots of enzyme were assayed at the following time points: 37°C: 0, 2, 4, 8 and 16 minutes; 4°C: 0, 1, 3, 6 and 24 hours; -20°C: 0, 6, 15, 22 and 32 days.

3.II. pH Optima Determination

P. ultimum NAD-GDH activity was measured in buffers for every one fifth pH unit between pH 6.0 and 9.8, inclusive, using 1M TRIS-acetate as buffering agent to a final concentration of 67 mM. The same protein source as in *3.III.a Activation by NADP*$^+$ below was used for these experiments.

3.III. NADP$^+$ Effects

3.III.a. <u>Activation by NADP$^+$</u> Mycelia were induced as described above (*3.IV.c. Time Course Induction*) for 4 hours to provide protein for these

experiments. NAD-GDH activities and protein quantities were determined as described (*3.VI. NAD-GDH Activity Assay* and *3.VII. Protein Quantification* below), using $NADP^+$ as enzyme activator in final concentrations of 0 μM, 0.23 μM, 0.83 μM, 1.4 μM, 8 μM, 2.3 μM, 5.6 μM, 11 μM, 23 μM, 55.5 μM, 83 μM, 111 μM, and 138 μM.

3.III.b. Confirmation of $NADP^+$ as an activator Verification of $NADP^+$ function as an activator and not as a cofactor was conducted through double reciprocal analysis of enzyme specific activity for both the reductive and oxidative reactions of *P. ultimum* NAD-GDH at several substrate concentrations (0.33 mM, 0.66 mM, 1.32, 2 mM, 2.64 mM and 3.3 mM α-ketoglutarate or 4 mM, 8 mM, 24 mM, 40 mM, 60 mM, 80 mM and 160 mM L-glutamate, respectively) and activator concentrations (0 μM, 0.484 μM, 4.84 μM and 181.6 μM for the reductive reaction and 0 μM, 4.84 μM, 48.4 μM and 181.6 μM for the oxidative reaction).

3.IV. Enzyme Induction

NAD-GDH expression was induced using several L-glutamate concentrations as supplements to either starvation or GY media, or for different times with a constant L-glutamate concentration. Mycelia were recovered after induction in each case by filtration under vacuum through 3MM Wattman filter paper. Mycelial mats were immediately plunged into $N_{2(l)}$ and subsequently stored at -70°C until used for RNA and protein extractions

as described (*2.III. Total RNA Extraction* above and *3.VII. Protein Extraction* below). Proteins were then quantified and NAD-GDH activities assayed as described below (*3.VI. NAD-GDH Activity Assay* and *3.VII. Protein Quantification*).

3.IV.a. Induction without starvation Fungal mycelia were grown in medium scale cultures, as described above (*1.II.a. Short Term Culture*). Growth medium was supplemented with sterile L-glutamate to final concentrations of 0g/L, 1g/L, 2g/L, 5g/L, 10g/L and 15g/L, and the cells reincubated for 2 hours at 28°C with shaking at ~170rpm.

3.IV.b. Induction during starvation Oomycetes were grown overnight as described (*1.II.a. Short Term Culture*, above) and recovered by filtration through nylon mesh in a Labgard laminar flow biological safety cabinet (Model Number NU-408FM-600). The mycelia mats were washed with sterile starvation medium (1 mM TRIS-acetate, pH 6.8, 100 μM $MgCl_2$, 100 μM $CaCl_2$) and then resuspended in starvation medium equivalent to one half the volume of the initial cell culture. The mycelial mats were incubated for 30 minutes at 28°C with shaking at ~170rpm. Sterile L-glutamate was added to final concentrations of 0 mM, 1 mM, 2 mM, 4 mM, 8 mM and 16 mM and the mycelia were then incubated for 4 hours at 28°C with shaking at 170rpm.

3.IV.c. Time course induction *P. ultimum* mycelia were prepared as described (*3.IV.b. Induction During Starvation*, above), but induced with 10 mM L-glutamate for 0, 1, 2, 4 or 8 hours at 28°C with shaking at ~170rpm.

3.V. Protein Extraction

Mycelia were prepared as described in (*3.IV. Enzyme Induction*, above) and frozen after recovery in $N_{2(l)}$. The mycelia were crushed to a fine powder using a pre-chilled mortar and pestle and suspended in 2 volumes TPED medium supplemented with 50%(v/v) glycerol and 0.1 mM (phenyl-methylsulfonyl fluoride) PMSF as described by Yang (1991), except as noted in *3.I. Enzyme Stability* above. The slurry was sonicated thrice for 20 seconds each time with a model 300 sonic dismembrator (Fisher), using either a small sized probe (3.5 mm diameter) set at 35% maximum output, a medium sized probe (9.0 mm diameter) set at 60% maximum output, or a large probe (19 mm diameter) set at 90% maximum output. Alternatively, proteins from large scale cultures were recovered after crushing of the mycelial mats by shaking for five hours on ice in the presence of TPED medium supplemented with 50%(v/v) glycerol (Yang, 1991). In all cases, cell debris was removed by centrifugation, supernatant recovery and recentrifugation as described by Yang (1991). Protein samples were stored at -20°C for up to a maximum of 7 days until needed.

3.V.a. <u>Protein radiolabeling</u> Proteins generated by *P. ultimum* were radiolabeled with a total of 10μCi of a combination of L-^{35}S-labeled methionine and cysteine (Express Protein Labeling Mix, NEN) per 20 mL culture volume for one hour immediately prior to harvesting and protein retrieval by sonication as described above (*3.V. Protein Extraction*) using 100 μL extraction buffer (1 mM TRIS-HCl, pH 7.4, 0.25 mM $MgCl_2$, 0.1 μg/μL benzamidine, 1 μg/μL L-1-tosylamide-2-phenyl-ethylchloromethyl ketone (TPCK) and 1 μg/μL Nα-p-tosyl-L-arginine methylester (TAME)) per 20mL culture (Braithwaite, 1987; LéJohn, 1982). The proteins were quantified (*3.VII. Protein Quantification*, below) and assayed for specific radioactivity with a Model 1215 Rackbeta liquid scintillation counter (LKB Wallace) in counts per minute per μL (cpm μL^{-1}) and per μg (cpm μg^{-1}) protein. A few grains of urea were then added to the samples prior to storage at -70°C. Aliquots containing an equal number of cpm for each sample were combined with an equal volume of urea loading buffer (9.5M urea, 2% SDS, 5% β-mercaptoethanol (β-ME)) and 1 μL 1%(w/v) bromophenol blue and electrophoresed (*3.V.b. SDS-polyacrylamide gel electrophoresis (PAGE)*, below).

3.V.b. <u>SDS-polyacrylamide gel electrophoresis (PAGE)</u> SDS-PAGE was conducted using a Bio-RAD Mini-Protean™ II apparatus (separating gel volume of 5 mL). Separating gels with a final acrylamide concentration of 8%

were produced by combining 2.4 mL water with 1.3 mL 30% acrylamide (30%(w/v) acrylamide, 0.8% (w/v) N,N'-methylene bis-acrylamide (bis-acrylamide)), 1.25 mL separating gel buffer (1% (w/v) SDS, 1.5 M TRIS base, pH 8.8), 8 μL N,N,N',N'-tetramethylethylenediamine (TEMED) and 25 μL 10%(w/v) ammonium persulfate. Ethanol was layered on top of the separating gel until it had polymerized. The ethanol was poured off and the top of the gel rinsed with distilled water, excess water being removed with a piece of filter paper. The stacking gel (750 μL water, 600μL 30% acrylamide, 1.25 mL stacking buffer (1% (w/v) SDS, 0.5M TRIS-HCl, pH 6.8), 5 μL TEMED, and 10 μL 10% (w/v) ammonium persulfate) was layered on top of the polymerized separating gel and a comb inserted. Concurrently with stacking gel polymerization, protein samples were prepared as described (*3.V.a. Protein radiolabeling*, above) The gels were electrophoresed at 25mA for one hour or until the dye exited the gel, using an electrophoresis buffer containing 0.1% (w/v) SDS, 192 mM glycine and 25 mM TRIS base. Larger gels of 16x16 cm were also used in an identical manner, with six times the volume of both separating (30 mL final volume) and stacking (15 mL final volume) gels used, and electrophoresis proceeding for 4-5 hours at 50 mA.

3.V.c. <u>SDS-PAGE gel staining and fluorography</u> SDS-PAGE gels were stained with coomassie brilliant blue stain (0.125%(w/v) coomassie blue R-250, 50%(v/v) methanol, 10%(v/v) glacial acetic acid) for 20 minutes after

electrophoresis and destained by alternating one hour washes with destain solution (10%(v/v) glacial acetic acid, 35%(v/v) methanol) and 30 minute washes with 70% methanol prior to incubation for 30 minutes with 1M sodium salicylate as described by Chamberlain (1979) or saturation with PPO in DMSO as described elsewhere (Hames, 1981). The latter was the preferred method, as diffusion of bands is noticeable using sodium salicyclate, markedly decreasing resolution (Chamberlain, 1979). After drying, the gels were autoradiographed at -70°C for 3 to 5 days.

3.VI. NAD-GDH Activity Assay

The reductive amination of α-ketoglutarate to L-glutamate and the oxidative amination of L-glutamate to α-ketoglutarate were assayed to quantify NAD-GDH activity as has been described by Stevenson (1974). Exceptions to this procedure are noted where appropriate in the text. Enzyme assays were conducted at λ=340nm using an MR3000 Spectronic spectrophotometer, with a change of 0.6OD units equivalent to the metabolism of 1.0 mmoles substrate.

3.VII. Protein Quantification

Protein quantification was conducted according to the method of Lowry (1951) as outlined by Ausubel *et al.* (1992). BSA was used to generate a standard curve in association with the standard curve program of a MR3000 Spectronic spectrophotometer.

4. Characterization of *P. ultimum hsp70*

4.I. Morphological and Growth Analysis of *P. ultimum*

A 0.5 cm^2 plug of *P. ultimum* mycelia was incubated at 28°C on a potato-dextrose agar (Gibco-BRL) petri plate supplemented with 100 μg/mL ampicillin until mycelia had overgrown the plate. An ethanol sterilized 1.5 mL Eppendorf tube (without cap) was used to cut out circular agar plugs from this plate for use as inocula for six fresh potato-dextrose agar petri plates with agar plugs removed from the centre in an identical manner. These plates were pre-incubated at room temperature (approximately 22°C) before growing at room temperature, 28°C, 31°C, 34°C, 37°C and 42°C for 24 hours, after which the mycelia were photographed with a Nikon FX35 camera attached to a Nikon Optiphot light microscope using Kodak Ektachrome 160 tungsten-treated film. Rate of mycelial growth was measured on plates pre-incubated at the desired growth temperature, with inocula immediately placed at this temperature without pre-incubation. Survival after incubation at 34°C, 37°C and 42°C was also tested by inoculating two sets of petri plates per temperature as described above, growing for one for 24 hours and the other for 48 hours at the temperatures being tested. After this incubation, the plates were placed at room temperature for one week, at which time they were examined for mycelial growth.

4.II. DNA Sequencing

4.II.a. Production of *hsp70* DNA template for sequencing An unsequenced region of a previously identified *hsp70* gene from a *P. ultimum* genomic library clone was amplified by polymerase chain reaction, which was conducted as described in section *2.IV.b. Probe Construction* above, and sequenced.

4.II.b. DNA sequencing DNA template concentrations were quantified as described in section *2.Ic. Plasmid Amplification and Recovery* above, and distributed into 5 μg aliquots, which were frozen at -70°C for 30 minutes lyophilized overnight. Primers were prepared in an identical manner, but were distributed in 200 ng aliquots. Primers and DNA template were then sent to either the University Core DNA Services, University of Calgary for sequencing.

4.III. Heat Induction of Stress Proteins

Mycelia were grown overnight in petri plates at 28°C as described (*1.II.a. Short Term Culture*, above) and harvested by filtration through nylon mesh. After washing with a copious amount of starvation medium, mycelia from four plates were transferred to a single plate containing starvation medium and grown at either room temperature, 28°C, 31°C, 34°C, 37°C or 42°C for one hour. The mycelia were then transferred to fresh starvation medium placed at the same temperature in the presence of 10μCi L-^{35}S-labeled methionine and cysteine (Express Protein Labeling Mix, NEN) per 20 mL medium as

described (*3.V.a. Protein radiolabeling*, above). The mycelia were then harvested by vacuum filtration through 3MM Whatman paper, proteins extracted and electrophoresed as described (*3.V. Protein Extraction* and *3.V.b. SDS-polyacrylamide gel electrophoresis (PAGE)*, above).

5. Radioisotope Safety

All radioisotope usage, storage and monitoring was conducted within the guidelines set by the University of Manitoba Environmental Health and Safety Office Radiation Safety Manual.

MATERIALS

1. Chemicals

Media were produced in the laboratory using chemicals from Sigma Chemical Company, Difco Laboratories, Gibco BRL and Fisher Scientific Company. Exceptions to this are noted in the text.

Chemicals used for protein denaturing polyacrylamide gel electrophoresis were obtained from Bio-Rad Laboratories. Chemicals used for other purposes were obtained from Sigma Chemical Company and Fisher Scientific.

Hybond-N^+ and Hybond-ECL membranes were obtained from Amersham.

X-ray film was obtained from Kodak.

2. Computer Hardware and Software

Computer equipment consisted of a Pentium 166 megaHertz microprocessor with 32 megabytes random access memory. Access to GenBank (<<*http://www.ncbi.nlm.nih.gov/*>>) was obtained using Netscape

Navigator™ Gold v.3.01 (Netscape Communications Corporation). Access to Medline, Biological Abstracts and Current Contents at the University of Manitoba was obtained using Windows NetDoc on the University of Manitoba local area network. DNA sequence manipulation was conducted with Gene Runner v.3.00 (Hastings Software, Inc.). Figures and graphs were produced using Freelance Graphics for Windows v.2.1 (Lotus Development Corporation). All other tables and wordprocessing were produced using Microsoft Word v.6.0a (Microsoft, Inc.).

3. Equipment

Specific types, models and manufacturers of equipment used are listed in the text where relevant.

4. Enzymes, Antibodies, Molecular Biologicals and Radioisotopes

Enzymes were obtained from Boehringer-Mannheim, Gibco BRL, Pharmacia and Promega. Radioisotope was obtained from DuPont. Sources of other molecular biologicals are listed where necessary in the text.

5. Kits

Kits for various procedures were obtained from Amersham, Boehringer-Mannheim, Invitrogen, Stratagene and Promega. Sources for specific kits are listed in the text where relevant.

RESULTS AND DISCUSSION

1. Transcriptional Co-expression of Members of an Antisense Gene Pair

The original analysis of the *nad-gdh:hsc70* antisense gene pair in *Achlya klebsiana* by LéJohn *et al.* (1994a and 1994b; Yang and LéJohn, 1994) indicated the presence of two distinct RNAs of approximately 3.4 kb and 2.4 kb which were recognized by the same probe complementary to the antisense gene pair. These transcripts not only corresponded in size to the expected *nad-gdh* and *hsc70* transcripts, but were also coinduced concurrently with NAD-GDH activity as well as the NAD-GDH protein and an undescribed 70kD polypeptide (LéJohn *et al.*, 1994a and 1994b; Yang and LéJohn, 1994), which were proposed to be the transcription products of the antisense gene pair. However, the possibility exists that either or both of the members of the antisense gene pair are in fact untranscribed, with another transcript of similar size but encoded at a different locus being fortuitously expressed to give the RNAs observed. In order to demonstrate that both members of the antisense gene pair can be transcribed concurrently

(although not necessarily simultaneously), antisense gene pair transcription was analyzed in isolation from the rest of the *A. klebsiana* genome using both prokaryotic *E. coli* XL-1 Blue and eukaryotic COS7 monkey cells as host systems.

1.I. Production of Recombinant Plasmids

Two recombinant plasmids, designated pBKNADGDH and pBKHSC70, were constructed using the pBK-RSV shuttle vector and the *A. klebsiana* antisense gene pair as described in detail in Materials and Methods *2.I. Recombinant Molecule Construction* above. These molecules can be differentiated by the orientation of the insert within the pBK-RSV MCS (Figure 8), and can be discerned via characteristic restriction endonuclease digestion patterns with the restriction endonucleases EcoRI restriction endonuclease and KpnI restriction endonuclease. Specifically, a 8.75 kb KpnI restriction endonuclease and a 7.85 kb EcoRI restriction endonuclease fragment are unique to pBKNADGDH and pBKHSC70, respectively (Figure 9). Clones of both recombinant molecules were maintained in *E. coli* XL-1 Blue (*endA1, hsdR17 (rk^-, mk^+), supE44, thi-1, lambda$^-$, recA1, gyrA96, relA1, (lac^-)[F', proAB, $lacI^qZ\Delta M15$, $Tn10(tet^R)$]* for large scale plasmid amplification and long term storage.

1.II. Analysis of RNA Derived from an Antisense Gene Pair

1.II.a. <u>Prokaryotic expression</u> Prokaryotic transcription of the antisense

Figure 8: Orientation of the antisense gene pair insert in the recombinant plasmids pBKNADGDH and pBKHSC70

The *A. klebsiana* antisense gene pair on a 5.2 kb XbaI restriction endonuclease fragment was inserted into the XbaI restriction endonuclease site of the pBK-RSV shuttle vector MCS (Methods and Materials *2.I. Recombinant Molecule Construction*, above) to produce the recombinant molecules pBKNADGDH and pBKHSC70. These differ only in their orientation with relation to the RSV and *lacZ* transcriptional promoters in the vector's expression cassette. Directionality of transcription for the inserts of both molecules are as indicated in the figure. Within the insert, the *nad-gdh* exons are in black , *hsc70* coding region in grey, and the non-coding regions in white. Components of the vector's expression cassette are as labeled in the figure.

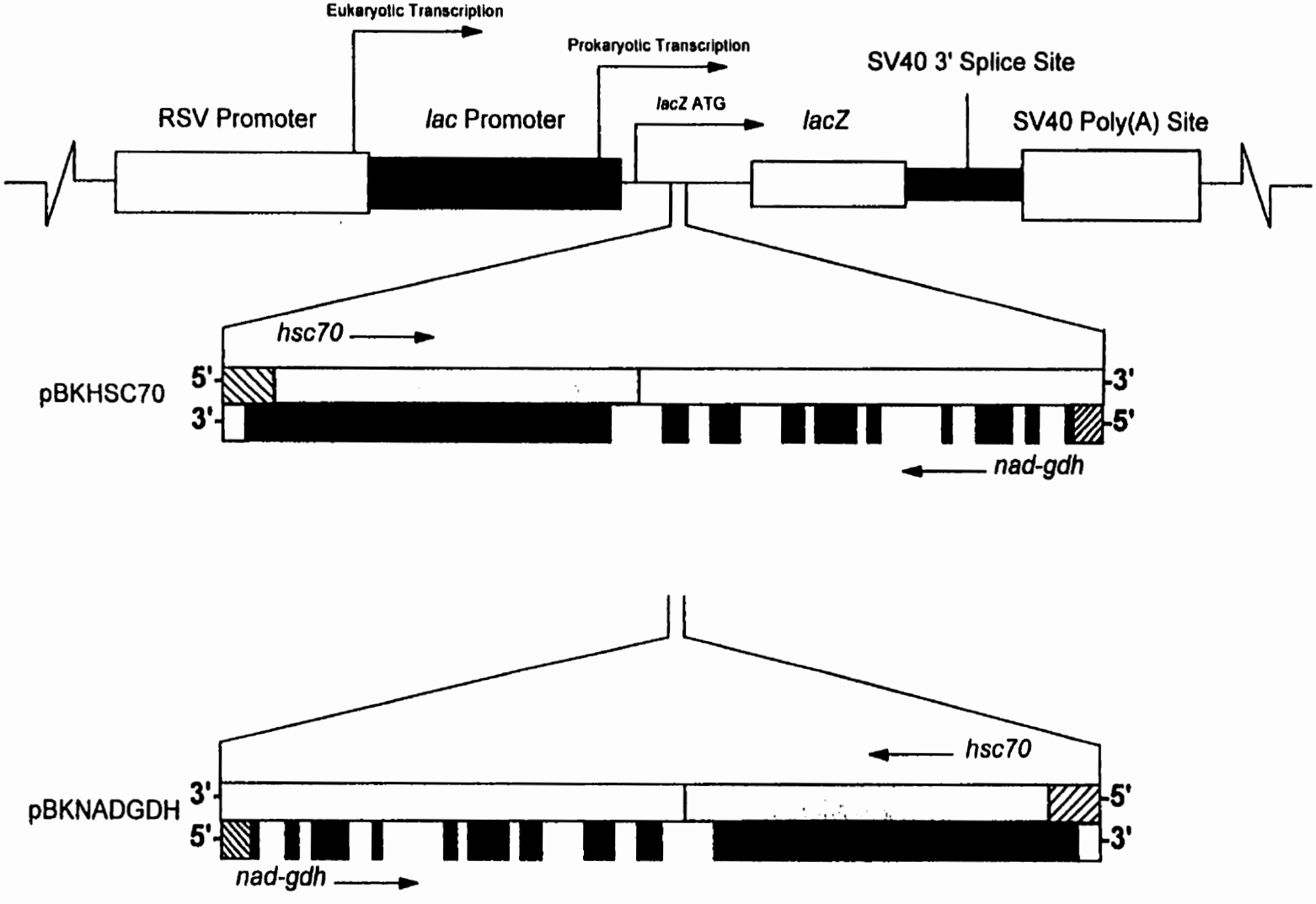
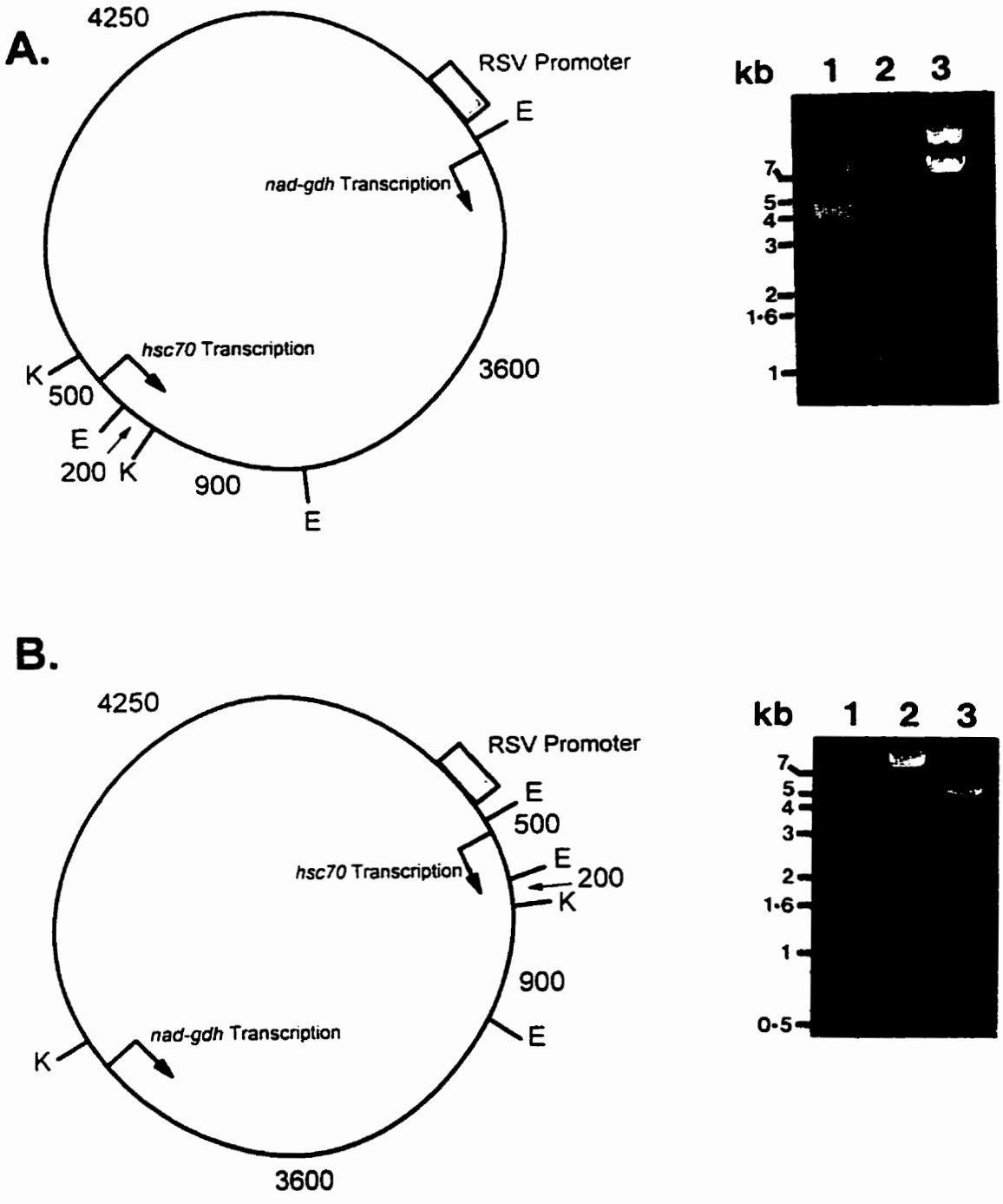

Eukaryotic Transcription
Prokaryotic Transcription
SV40 3' Splice Site
RSV Promoter
lac Promoter
lacZ ATG
lacZ
SV40 Poly(A) Site
hsc70
pBKHSC70
5'
3'
3'
5'
nad-gdh
hsc70
pBKNADGDH
3'
5'
5'
3'
nad-gdh

Figure 9: Restriction endonuclease digestion analysis of pBKNADGDH and pBKHSC70

Predicted restriction endonuclease digestion maps and agarose gel electrophoresis of restriction digestion products are illustrated for **A.**, pBKNADGDH and **B.**, pBKHSC70. Predicted restriction endonuclease fragment sizes on these maps are given in bp. Restriction endonucleases used were EcoRI (E) and KpnI (K). Restriction enzyme digestion and agarose gel electrophoresis were carried out as described (Materials and Methods *2.I.d. Insert Orientation Determination*, above). Lanes illustrated for each gel contain 1 μg of: 1. undigested recombinant molecule, 2. recombinant molecule digested with EcoRI restriction endonuclease, and 3. recombinant molecule digested with KpnI restriction endonuclease. Standard molecular sizes for these lanes are given in kb.


A.
4250
RSV Promoter
E
nad-gdh Transcription
hsc70 Transcription
3600
K
500
E
200
K
900
E
kb
1
2
3
7
5
4
3
2
1·6
1
B.
4250
RSV Promoter
E
500
E
200
K
hsc70 Transcription
900
E
nad-gdh Transcription
K
3600
kb
1
2
3
7
5
4
3
2
1·6
1
0·5

gene pair from within the pBK-RSV vector was expected to occur from the *lacZ* transcriptional promoter to generate a fusion product with the β-galactosidase gene, although the likelihood of transcription originating from within the 5.2 kb XbaI restriction endonuclease fragment insert itself, particularly the eukaryotic promoter sequences was unknown. Total RNA recovered from *E. coli* XL-1 Blue containing either pBKNADGDH or pBKHSC70 and induced with 0.4 mM IPTG were found to produce major transcripts of approximately 2.3 kb and 0.75 kb not present in untransformed control cells when probed with radiolabeled 5.2 kb XbaI restriction endonuclease fragment insert (Figure 10). Other minor transcripts could also be visualized in both *E.coli* transformed with pBKNADGDH (1.8 kb) and pBKHSC70 (1.5 kb), although they were not very distinct from background signals.

1.II.b. Eukaryotic Expression Analysis of total RNA derived from COS7 monkey cells transfected with either pBKNADGDH or pBKHSC70 indicated the presence of two distinct RNAs not present in untransfected cells which were recognized by the 5.2 kb XbaI restriction endonuclease fragment containing the *A. klebsiana* antisense gene pair (Figure 11). These RNAs, with molecular sizes of 4.5 kb and 2.2 kb, correspond to the predicted lengths of unprocessed *nad-gdh* and *hsc70* transcripts from the antisense gene pair which when processed have lengths of 3.4 kb and 2.4 kb, resp-

Figure 10: Analysis of transcript production from the recombinant molecules pBKNADGDH and pBKHSC70 in *E. coli* XL-1 Blue

Total RNA was recovered, electrophoresed and transferred to nylon membranes by Northern capillary blotting as described (Methods and Materials *2.IV.a. RNA Electrophoresis and Northern Capillary Blotting,* above) and probed with the 5.2 kb XbaI restriction endonuclease fragment containing the antisense gene pair. Hybridization was not observed after autoradiography in untransformed cells (Lane 1), whereas cells transformed with pBKNADGDH (Lane 2) or pBKHSC70 (Lane 3) both produced major transcripts of 2.3 kb (□) and 0.75 kb (■). Other, minor transcripts of 1.8 kb (<) and 1.5 kb (*) were also observed. Ten micrograms RNA were loaded on each lane. Molecular standards are given in kb on the left.

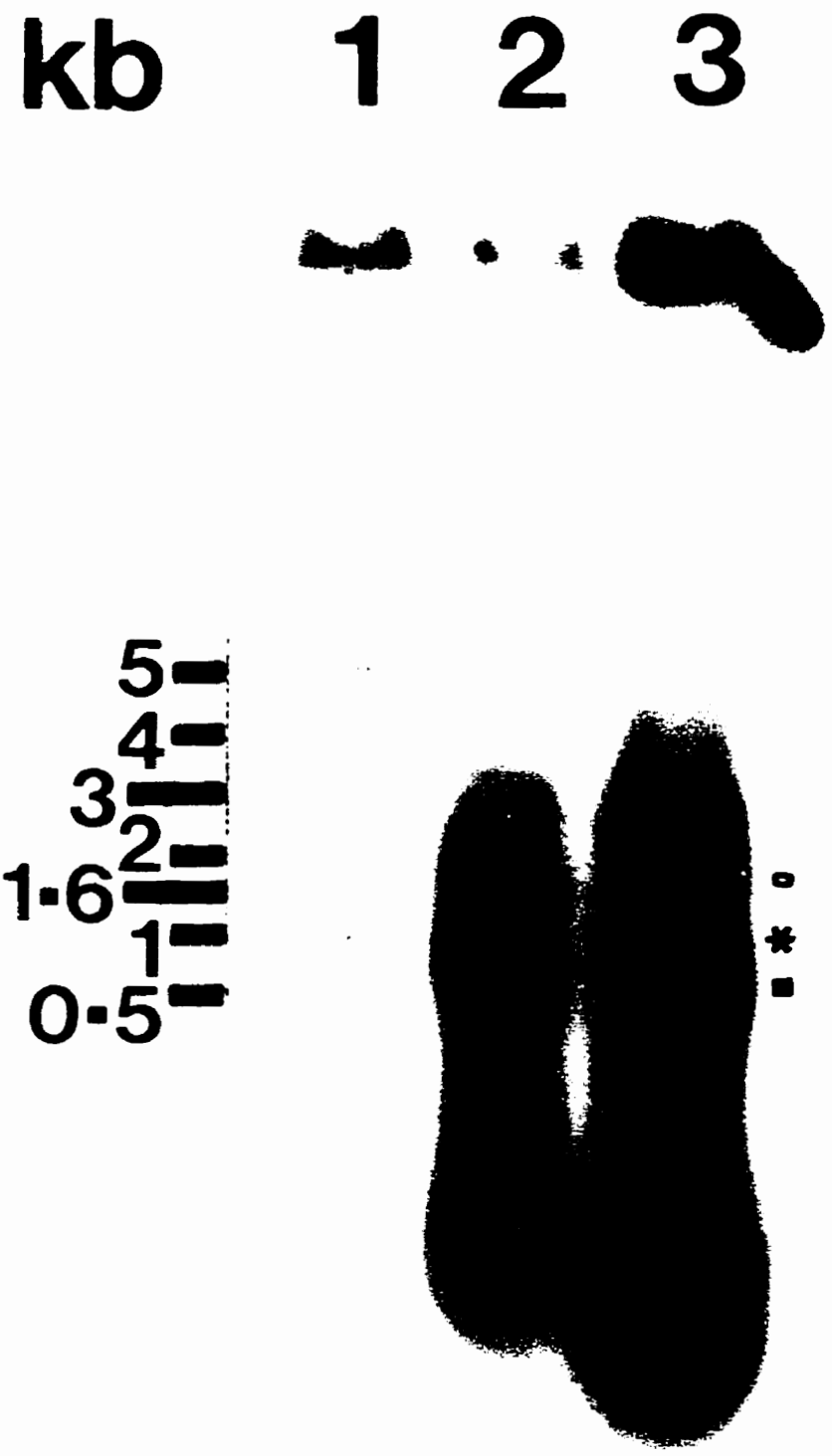

kb
1
2
3
5
4
3
2
1·6
1
0·5

Figure 11: Analysis of transcript production from the recombinant molecules pBKNADGDH and pBKHSC70 in COS7 cells with the *A. klebsiana* antisense gene pair

RNA was recovered, electrophoresed and transferred to nylon membranes by Northern capillary blotting as described (Methods and Materials *2.IV.a. RNA Electrophoresis and Northern Capillary Blotting,* above) and probed with the 5.2 kb XbaI restriction endonuclease fragment containing the antisense gene pair. Hybridization was not observed after autoradiography in untransformed cells (Lane 1), whereas cells transformed with pBKNADGDH (Lane 2) or pBKHSC70 (Lane 3) both produced major transcripts of 4.5 kb (□) and 2.2 kb (■). Ten μg RNA were loaded on each lane. Molecular standards are given in kb on the left.

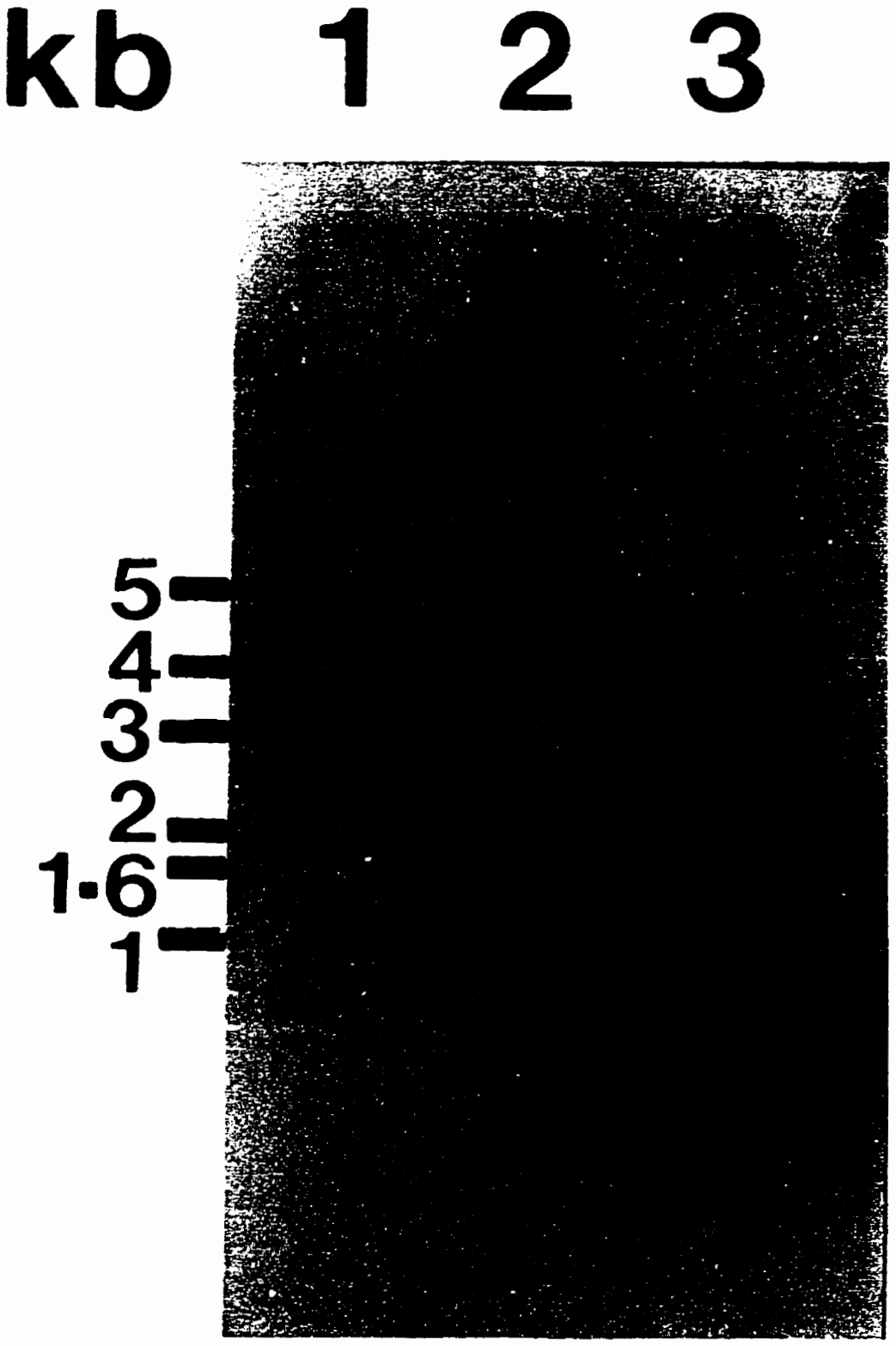
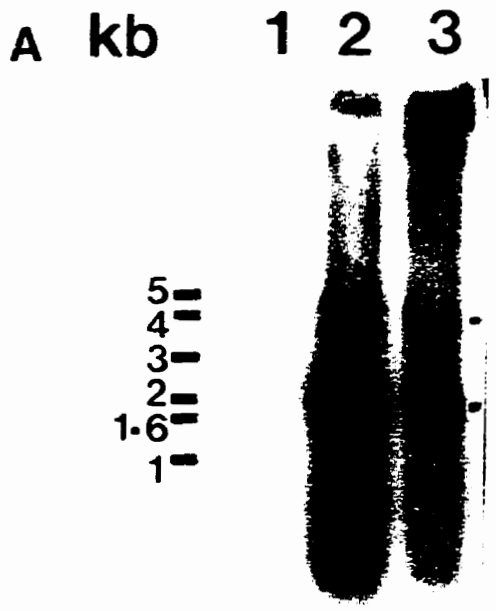
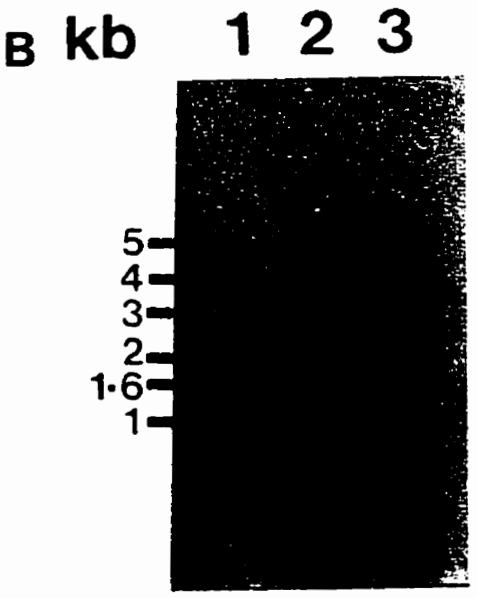

kb
1
2
3
5
4
3
2
1.6
1

Figure 12: Analysis of transcript production from the recombinant molecules pBKNADGDH and pBKHSC70 in COS7 cells with intronic and non-antisense gene pair regions of the *A. klebsiana nad-gdh* gene

RNA was recovered, electrophoresed and transferred to nylon membranes by Northern capillary blotting as described (Methods and Materials *2.IV.a. RNA Electrophoresis and Northern Capillary Blotting,* above) and probed with the either (**A.**) a 3.2 kb XbaI restriction endonuclease fragment corresponding to exons I-IX and introns 1-8 of the *A. klebsiana nad-gdh* from the antisense gene pair, or (**B.**) a PCR product corresponding to intron 9 of the same gene (bottom) as illustrated in Figure 7. In both cases, hybridization was not observed after autoradiography in untransformed cells (Lane 1), whereas cells transformed with pBKNADGDH (Lane 2) or pBKHSC70 (Lane 3) both produced major transcripts of 4.5 kb (□) and 2.2 kb (■). Ten micrograms RNA were loaded on each lane. Molecular standards are given in kb on the left.


A kb
1 2 3
5
4
3
2
1·6
1


B kb
1 2 3
5
4
3
2
1·6
1

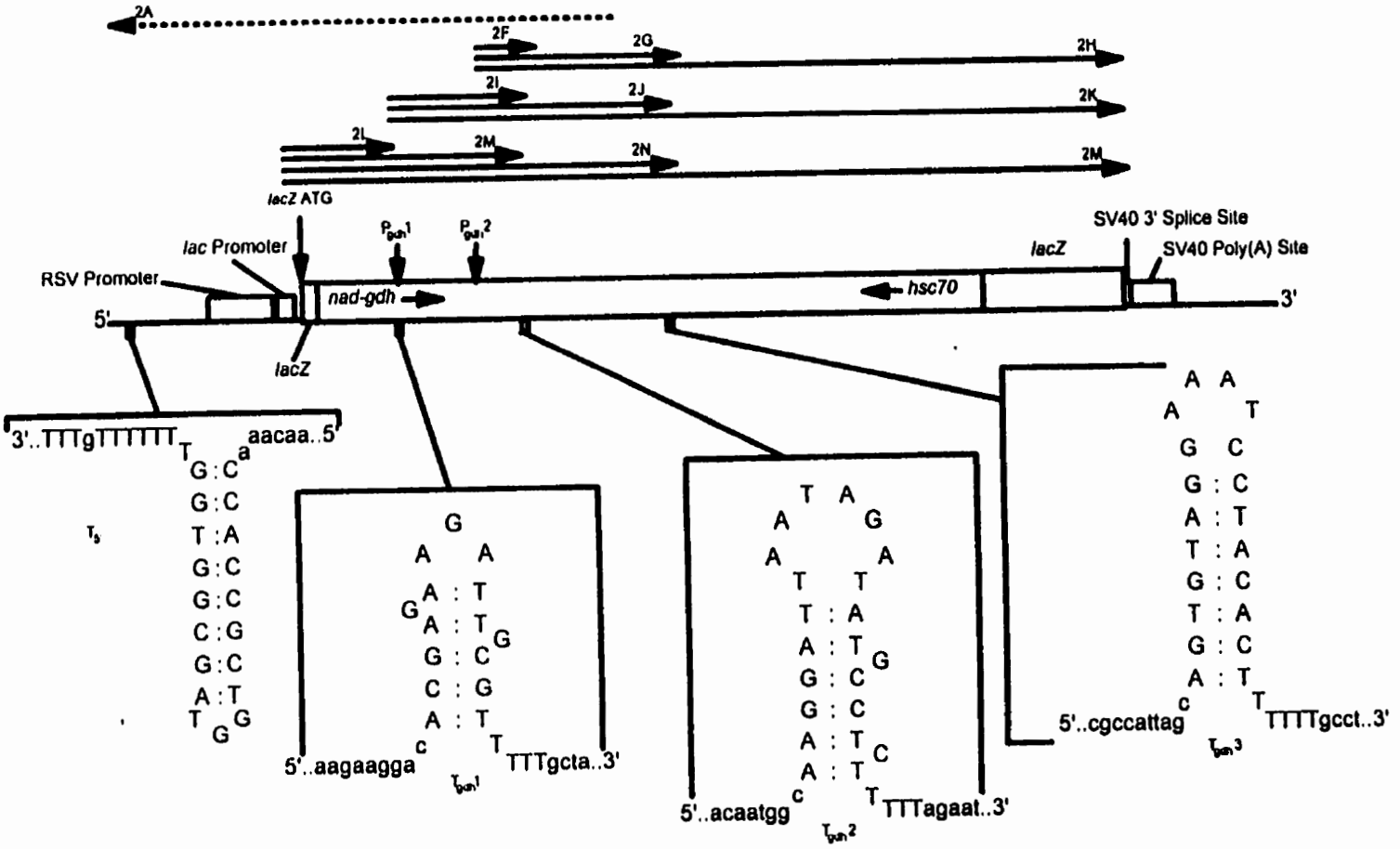
ectively (LéJohn *et al.*, 1994a and 1994b). Use of the 3.2 kb XbaI restriction endonuclease fragment and the intron 9 PCR product gave identical results (Figure 12), once more corresponding to unprocessed transcripts generated by the antisense gene pair.

1.III. Discussion of Antisense Gene Pair Expression

1.III.a. Production of multiple transcripts from the antisense gene pair is possible in *E. coli* The generation of two pairs of similarly sized transcripts from pBKNADGDH and pBKHSC70 in *E. coli* (Figure 10) is indicative of the presence of at least two functional prokaryotic transcriptional promoters within these recombinant molecules. A detailed analysis of the nucleotide sequence of the *A. klebsiana* antisense gene pair reveals the presence of several regions corresponding to cryptic prokaryotic transcriptional promoter and ρ-independent terminator consensus sequences on both the *hsc70* and *nad-gdh* encoding strands (Figures 13 and 14). The various combinations of these and vector borne promoters and terminators give a total of 15 possible distinct transcripts for both pBKNADGDH and pBKHSC70, with transcript sizes ranging from 350 bp to 5680 bp (Table 5). Of these, the *nad-gdh* strand transcripts 2I or 2L (980 bp and 830 bp, respectively) from pBKNADGDH or 1I (980 bp) from pBKHSC70 are closest in size to the 750 bp transcript identified by Northern hybridization, while the 2.3 kb transcript could be the *nad-gdh* strand transcripts 2J (2100 bp) or 2M

Figure 13: Cryptic prokaryotic promoters, terminators and their possible transcripts arising from pBKNADGDH

Sequences and locations for prokaryotic terminators and promoters present on the β-galactosidase sense strand in pBKNADGDH other than those from the pBK-RSV *lacZ*:β-galactosidase expression system are as indicated. All possible transcripts are indicated by arrows originating and ending at the appropriate promoter and terminator locations (solid for β-galactosidase sense strand, dotted for β-galactosidase antisense strand). Lengths of transcripts are summarized in Table 5.

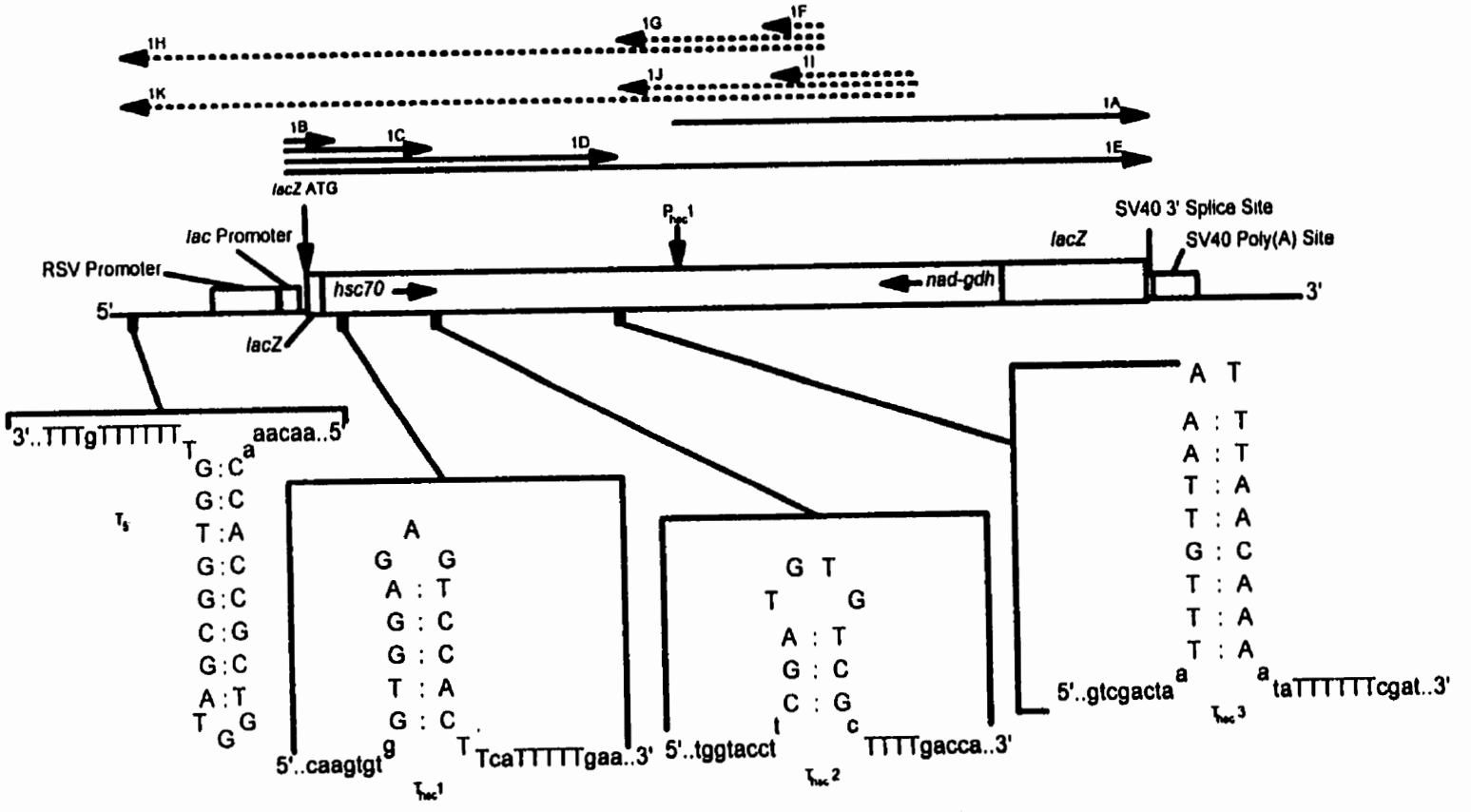


$P_{gdh}1$: 5'....agAAGGACAcgagaagattgcgttttgcTATAATcagaaaa....3'

$P_{gdh}2$: 5'....gcTTGACAgtagacTATATTcgaaatt....3'

Figure 14: Cryptic prokaryotic promoters, terminators and their possible transcripts arising from pBKHSC70

Sequences and locations for prokaryotic terminators and promoters present on the β-galactosidase sense strand in pBKHSC70 other than those from the pBK-RSV *lacZ*:β-galactosidase expression system are as indicated. All possible transcripts are indicated by arrows originating and ending at the appropriate promoter and terminator locations (solid for β-galactosidase sense strand, dotted for β-galactosidase antisense strand). Lengths of transcripts are summarized in Table 5.



$P_{hsc}1$: 5'....atcgTAGACAacaagtgtcaccagaggggaTATAA....3'

(1810 bp) from pBKNADGDH or the pBKHSC70 transcripts 1J (*nad-gdh* strand transcript of 2100 bp) or 1D (*hsc70* strand transcript of 2400 bp). As the two major transcripts appear to be the same for both types of transformant, it would seem reasonable that the transcripts observed are ones of appropriate size that could arise from both pBKNADGDH and pBKHSC70. If this is indeed the case, then the smaller major transcript (750 bp) is likely a transcriptional product from promoter $P_{gdh}1$ extending to terminator $T_{gdh}2$ (transcripts 1I and 2I, Figures 13 and 14). In a similar manner, the larger 2.3 kb major transcript appears to originate from the same promoter as the 750 bp major transcript, but extending to terminator $T_{gdh}3$ (transcripts 1J and 2J, Figures 13 and 14).

Analysis of the potential prokaryotic *A. klebsiana nad-gdh* and *hsc70* transcripts to determine their theoretical translation products indicates that, of the 15 unique transcripts which could arise from pBKNADGDH and pBKHSC70, all arising from $P_{gdh}1$ (transcripts 1I, 1J, 1K, 2I, 2J and 2K) will terminate translation after only nine amino acids, while the six $P_{gdh}2$ transcripts (transcripts 1F, 1G, 1H, 2F, 2G and 2H) terminate immediately after the initial methionine. The two transcripts arising from the $P_{hsc}1$ promoter (1A and 2A) generate predicted 75 amino acid peptides, which are entirely outside of the *hsc70* ORF. The remaining eight transcripts (four from pBKNADGDH and another four from pBKHSC70) are initiated from the *lacZ*

Table 5: Summary of possible transcripts arising from pBKNADGDH and pBKHSC70 prokaryotic host cell lines

The possible combinations of potential prokaryotic promoters and terminators as presented in Figures 13 and 14 are given, including lengths of hypothesized transcription and translation products. Transcripts, transcriptional promoters and transcriptional terminators are named in a fashion identical to that given in Figures 13 and 14.

Transcript Name	Transcriptional Start	Transcriptional Stop	Transcript Length (bp)	Predicted Peptide Product Length (aa)
1A	$P_{hsc}1$	T_{lacZ}	2886	75
1B	$lacZ_{hsc}$	$T_{hsc}1$	386	65
1C	$lacZ_{hsc}$	$T_{hsc}2$	1035	65
1D	$lacZ_{hsc}$	$T_{hsc}3$	2394	65
1E	$lacZ_{hsc}$	T_{lacZ}	5280	65
2A	$P_{hsc}1$	$T_{5'}$	4116	75
1F	$P_{gdh}2$	$T_{gdh}2$	350	1
1G	$P_{gdh}2$	$T_{gdh}3$	1470	1
1H	$P_{gdh}2$	$T_{5'}$	5050	1
1I	$P_{gdh}1$	$T_{gdh}2$	980	9
1J	$P_{gdh}1$	$T_{gdh}3$	2100	9
1K	$P_{gdh}1$	$T_{5'}$	5680	9
2F	$P_{gdh}2$	$T_{gdh}2$	350	1
2G	$P_{gdh}2$	$T_{gdh}3$	1470	1
2H	$P_{gdh}2$	T_{lacZ}	3820	1
2I	$P_{gdh}1$	$T_{gdh}2$	980	9
2J	$P_{gdh}1$	$T_{gdh}3$	2100	9
2K	$P_{gdh}1$	T_{lacZ}	4450	9
2L	$lacZ_{gdh}$	$T_{gdh}1$	830	75
2M	$lacZ_{gdh}$	$T_{gdh}2$	1809	75
2N	$lacZ_{gdh}$	$T_{gdh}3$	2930	75
2O	$lacZ_{gdh}$	T_{lacZ}	5280	75

promoter. Due to the location of the XbaI restriction endonuclease insertion site in the MCS the insert is not in the same reading frame as the vector's β-galactosidase gene, causing the premature termination of translation 30 bp into the 5' untranslated region of the pBKNADGDH transcripts 2L, 2M, 2N, 2O giving predicted peptides of approximately 75 amino acids in length. In the case of the pBKHSC70 transcripts, the ρ-dependent transcriptional terminator $T_{hsc}1$ is about 60 bp into the *hsc70* 5' untranslated region (transcripts 1B, 1C, 1D and 1E), giving transcripts encoding proteins of approximately 65 amino acids in length.

Briefly then, the promoter from which the two major transcripts produced from the antisense gene pair (2.3 kb and 0.75 kb) (Figure 10) in *E. coli* may arise may be a cryptic promoter located within the second *nad-gdh* intron on the *nad-gdh* strand of the antisense gene pair from *A. klebsiana*. These transcripts would terminate either in the ninth exon or in the fourth intron of *nad-gdh*, respectively. A number of other transcripts were also observed (Figure 10), indicative to some degree of activity of other cryptic prokaryotic promoters within the antisense gene pair. Analysis of potential translation products for each transcript indicates that the only translation products of significant size would be generated from the transcript arising from $P_{hsc}1$ in pBKNADGDH (75 amino acids) and pBKHSC70 (65 amino acids). In neither of these cases does a potential protein product overlap any portion of the

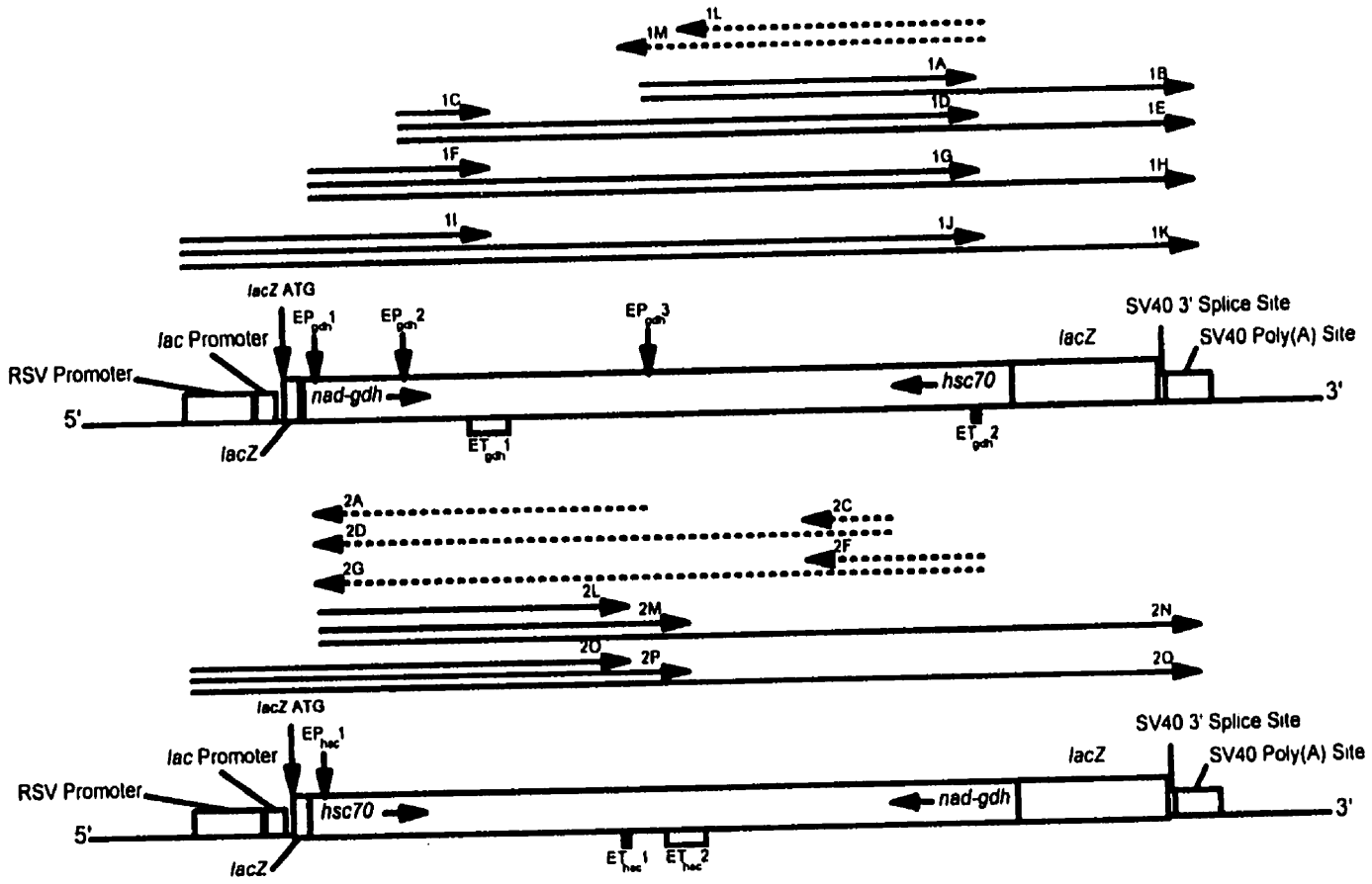
ORFs for either the *nad-gdh* or the *hsc70* of the *A. klebsiana* antisense gene pair.

The high levels of background signal observed in the Northern blotting experiments conducted for both *E. coli* and mammalian cells (*1.III.b. Generation of multiple transcripts occurs in a mammalian cell*, below) transformed with pBKNADGDH anf pBKHSC70 must be due to some transcription occurring from the plasmids introduced into the host cells, as neither prokaryotic nor eukaryotic total RNA negative controls indicated any hybridization to the probes used. There is the possibility that the background is the result of an inherent instability of the RNAs which form distinct bands on the Northern blots, but experiments to confirm or refute this were not conducted.

1.III.b. Generation of multiple transcripts occurs in a mammalian cell line The consistent production of two RNAs unique to COS7 cell lines transiently transfected with plasmids containing the *A. klebsiana* antisense gene pair (Figures 15) is indicative of transcription from multiple sites within the antisense gene pair region within a eukaryotic host, similar to what has been speculated to occur in *E. coli*. A detailed sequence analysis of the antisense gene pair region identified the existence of hitherto unpredicted regions resembling eukaryotic transcriptional start and stop consensus sequences other than those predicted for the *A. klebsiana nad-gdh* or *hsc70*

Figure 15: Cryptic eukaryotic promoters, terminators and their possible transcripts arising from pBKNADGDH and pBKHSC70

Sequences and locations for eukaryotic terminators and promoters present on the β-galactosidase sense strand in pBKHSC70 other than those from the pBK-RSV *lacZ*:β-galactosidase expression system are as indicated. All possible transcripts are indicated by arrows originating and ending at the appropriate promoter and terminator locations (solid for β-galactosidase sense strand, dotted for β-galactosidase antisense strand). Lengths of transcripts are summarized in Table 6.



$EP_{gdh}1$: 5'....tctagaTATAtt...n_{34}..aaTATAaa...n_{88}..ttCCAATat...n_{78}..ctTATAATtatTATAtctta...3'
$EP_{gdh}2$: 5'....ttaCCAATttaccgaaagtagcgccaTATActg...3'
$EP_{gdh}3$: 5'....ttgCCAATactcgtggacgcgtacaccgccgttgctcgTAATTtct...3'
$EP_{hsc}1$: 5'....ggaTAATATtcc...n_{129}..gagCCAATcaa....3'
$ET_{gdh}1$: 5'....ttgAATAAAAtgt...n_{125}..gatAAATAAAtag...n_{110}..tgtAATAAAtct...3'
$ET_{gdh}2$: 5'....aaacttccAAATAAAActtttttt...3'
$ET_{hsc}1$: 5'....aggctAAAATATcctt...3'
$ET_{hsc}2$: 5'....tgtAAAGTAAAgct...n_{50}..tttAAATAAAAAtgc...n_{220}..ttAAATAAAAt...3'

Table 6: Summary of possible transcripts arising from pBKNADGDH and pBKHSC70 in COS7 cells

The possible combinations of eukaryotic transcriptional promoter and terminator consensus sequences as presented in Figure 15 are given, including lengths of hypothesized transcription and translation products. Transcripts, transcriptional promoters and transcriptional terminators are named in a fashion identical to that given in Figure 15. Different translation product lengths are given for those transcripts arising from $EP_{hsc}1$ and $Ep_{gdh}2$ for each of the two or three start codons (respectively) within 120 bp 3' of the promoter consensus sequence.

Transcript Name	Transcriptional Start	Transcriptional Stop	Transcript Length (bp)	Predicted Peptide Product Length (aa)
1A	$EP_{gdh}3$	$ET_{gdh}2$	2550	18
1B	$EP_{gdh}3$	T_{SV40}	3050	18
1C	$EP_{gdh}2$	$ET_{gdh}1$	500	2/18/9
1D	$EP_{gdh}2$	$ET_{gdh}2$	4100	2/18/9
1E	$EP_{gdh}2$	T_{SV40}	4600	2/18/9
1F	$EP_{gdh}1$	$ET_{gdh}1$	1300	20
1G	$EP_{gdh}1$	$ET_{gdh}2$	4900	20
1H	$EP_{gdh}1$	T_{SV40}	5400	20
1I	EP_{RSV}	$ET_{gdh}1$	1600	50
1J	EP_{RSV}	$ET_{gdh}2$	5200	50
1K	EP_{RSV}	T_{SV40}	5700	50
1L	$EP_{hsc}1$	$ET_{hsc}1$	2200	82/652
1M	$EP_{hsc}1$	$ET_{hsc}2$	3000	82/652
2A	$EP_{gdh}3$	$ET_{gdh}2$	2550	18
2C	$EP_{gdh}2$	$ET_{gdh}1$	500	2/18/9
2D	$EP_{gdh}2$	$ET_{gdh}2$	4100	2/18/9
2F	$EP_{gdh}1$	$ET_{gdh}1$	1300	20
2G	$EP_{gdh}1$	$ET_{gdh}2$	4900	20
2L	$EP_{hsc}1$	$ET_{hsc}1$	2200	82/652
2M	$EP_{hsc}1$	$ET_{hsc}2$	3000	82/652
2N	$EP_{hsc}1$	T_{SV40}	5400	82/652
2O	EP_{RSV}	$ET_{hsc}1$	2500	50
2P	EP_{RSV}	$ET_{hsc}2$	3300	50
2Q	EP_{RSV}	T_{SV40}	5700	50

genes (Figures 15). On the *hsc70* strand, a poly-adenylation signal ($ET_{hsc}1$) was observed just 3' to the *hsc70* gene (Figure 15), while the *nad-gdh* strand contains a previously unpredicted poly-adenylation signal ($ET_{gdh}2$) and two eukaryotic promoters ($EP_{gdh}2$ and $EP_{gdh}3$). $ET_{gdh}2$ is intronic (Intron 4), while $EP_{gdh}2$ is within exon III and $EP_{gdh}3$ is within exon VIII (Figure 15).

The transcripts which could arise from each combination of vector and antisense gene pair eukaryotic transcriptional promoters and terminators (Table 6) indicate that, assuming no post-transcriptional modifications occur, only transcripts 1D, 2D, 1E, 1G and 2G are likely the larger 4.5 kb transcript, while transcripts 1A, 2A, 1M and 2M are likely the 2.2 kb transcript. The discrepancy between the observed (4.5 kb and 2.2 kb) and expected (3.4 kb and 2.4 kb) transcript sizes (Figure 15) may indicate that COS7 cells cannot properly edit the *nad-gdh* transcript. Problems with transcript processing could be the result of an incompatibility between the transcriptional machinery and consensus sequences of oomycetes and COS7 cells. Such incompatibility has been observed for transcriptional promoter and terminator regions between oomycetes and SV40 viruses, plants, plant viruses and higher fungi by Judelson *et al* (1992). This work indicated that oomycete promoters could support little or no expression of a marker gene in higher fungi, although transformation of lettuce with the marker gene was fused to either plant or oomycete promoters gave similar

results. Expression from oomycete promoters in animal cells was not tested, although the SV40 early promoter was shown to be ineffective when used to express genes in oomycetes (Judelson *et al.*, 1992).

Without obtaining precise sequence information for each of the major transcripts observed, it is impossible to determine their specific identities. However, it is possible to state that the larger transcript observed in transformed COS7 cells likely arises from the *nad-gdh* strand of the antisense gene pair, using a promoter located within the antisense gene pair region. It also seems likely that the smaller transcript arises from the complementary (*hsc70*) strand of the DNA duplex, although it is possible that the second transcript finds its origins on the *nad-gdh* strand from promoter $EP_{gdh}3$ and terminating at $ET_{gdh}2$.

2. Characterization of *P. ultimum* NAD-GDH

The NAD-GDHs of *A. klebsiana* and *P. debaryanum* have been extensively characterized (Stevenson, 1974), and the *nad-gdh* gene of *A. klebsiana* cloned, sequenced and studied (Yang, 1991). In this latter study, a putative *hsc70* gene was identified on the DNA strand complementary to *nad-gdh*, forming what has been termed an antisense gene pair (LéJohn *et al.*, 1994a and 1994b). Polymerase chain reaction analysis using primers designed to generate a 0.9 kb fragment in *A. klebsiana* comprising a portion of the antisense gene pair and the internal region of *nad-gdh* immediately upstream on the *nad-gdh* strand identified the potential presence of a similar antisense gene pair in *P. ultimum* (LéJohn, unpublished data). It was therefore of advantage to characterize some aspects of the NAD-GDH of *P. ultimum* in order to determine its (dis)similarity with that of *A. klebsiana* in conjunction with the future identification of any antisense gene pair arrangement which may involve the *P. ultimum nad-gdh*.

2.I. Enzyme Stability

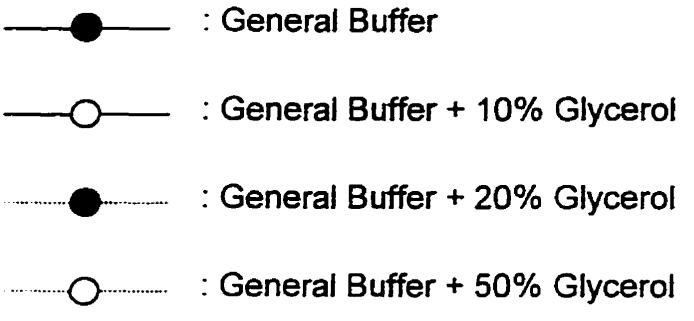
NAD-GDH stability over time was analyzed at several temperatures in order to determine enzyme storage conditions conducive to the use of crude cell extracts with a high level of NAD-GDH specific activity over the longest possible period of time. Glycerol, previously demonstrated to be an effective stabilizing agent for the storage of NAD-GDH from *A. klebsiana* (Yang, 1991), was chosen as a potential stabilizing agent for NAD-GDH in crude cell extracts of *P. ultimum*. In the presence of glycerol concentrations of 20%(v/v) and higher, NAD-GDH specific activity was decreased by less than 15% after 16 minutes incubation at 37°C, with no significant levels of NAD-GDH specific activity lost in the presence of 50%(v/v) glycerol over the same time (Figure 16). Similarly, less than 5% of initial NAD-GDH activity was lost after 24 hours of storage at 4°C in 50%(v/v) glycerol (Figure 17). Long term stabilization of enzyme activity at -20°C was also enhanced by glycerol (Figure 18), but even in the presence of 50%(v/v) glycerol, prolonged storage at -20°C is insufficient to prevent a drastic loss of NAD-GDH activity.

2.II. pH Effects

The effect of pH upon the activity of NAD-GDH from *P. ultimum* for both the oxidative deamination and reductive amination reactions was studied, with pH optima established at approximately 8.8 and 7.2, respectively (Figure 19). The increase in NAD-GDH specific activities from extreme pHs to pH

Figure 16: Retention of NAD-GDH activity over time at 37°C

NAD-GDH from mycelia grown overnight was induced with 15 mM L-glutamate during starvation for 4 hours as described (Methods and Materials *1.II.a. Short Term Culture* and *3.IV.b. Induction During Starvation*, respectively). Crude enzyme was extracted (Methods and Materials *3.V. Protein Extraction*) using general buffer with several different concentrations of glycerol, and samples were aliquoted and incubated at 37°C. Enzyme activity for the reductive amination of α-ketoglutarate to L-glutamate was assayed (Methods and Materials *3.VI. NAD-GDH Activity Assay*), with activities at each time compared with initial activities for each buffer to give percent enzyme activity remaining.



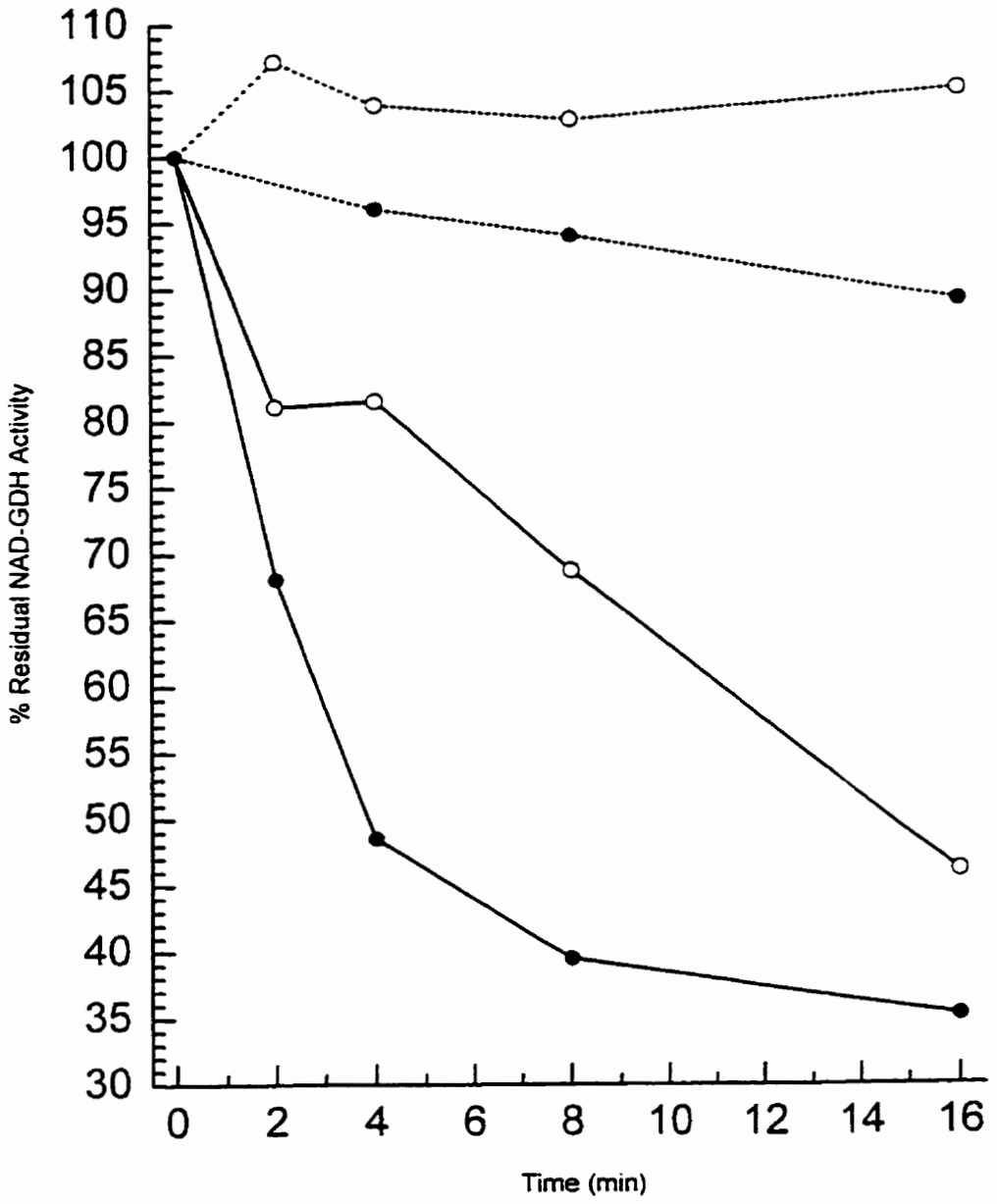
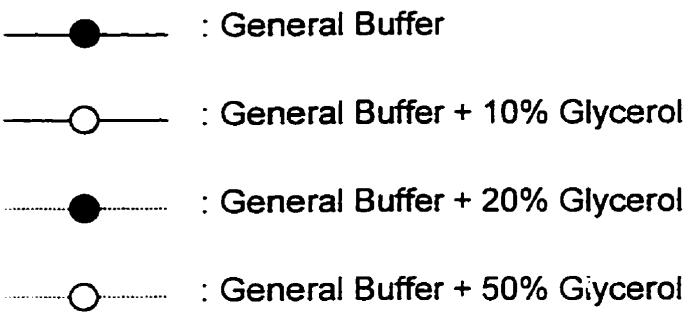

110
105
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
% Residual NAD-GDH Activity
0
2
4
6
8
10
12
14
16
Time (min)

Figure 17: Retention of NAD-GDH activity over time at 4°C

NAD-GDH from mycelia grown overnight was induced with 15 mM L-glutamate during starvation for 4 hours as described (Methods and Materials *1.II.a. Short Term Culture* and *3.IV.b. Induction During Starvation*, respectively). Crude enzyme was extracted (Methods and Materials *3.V. Protein Extraction*) using general buffer with several different concentrations of glycerol, and samples were aliquoted and stored at 4°C. Enzyme activity for the reductive amination of α-ketoglutarate to L-glutamate was assayed (Methods and Materials *3.VI. NAD-GDH Activity Assay*), with activities at each time compared with initial activities for each buffer to give percent enzyme activity remaining.



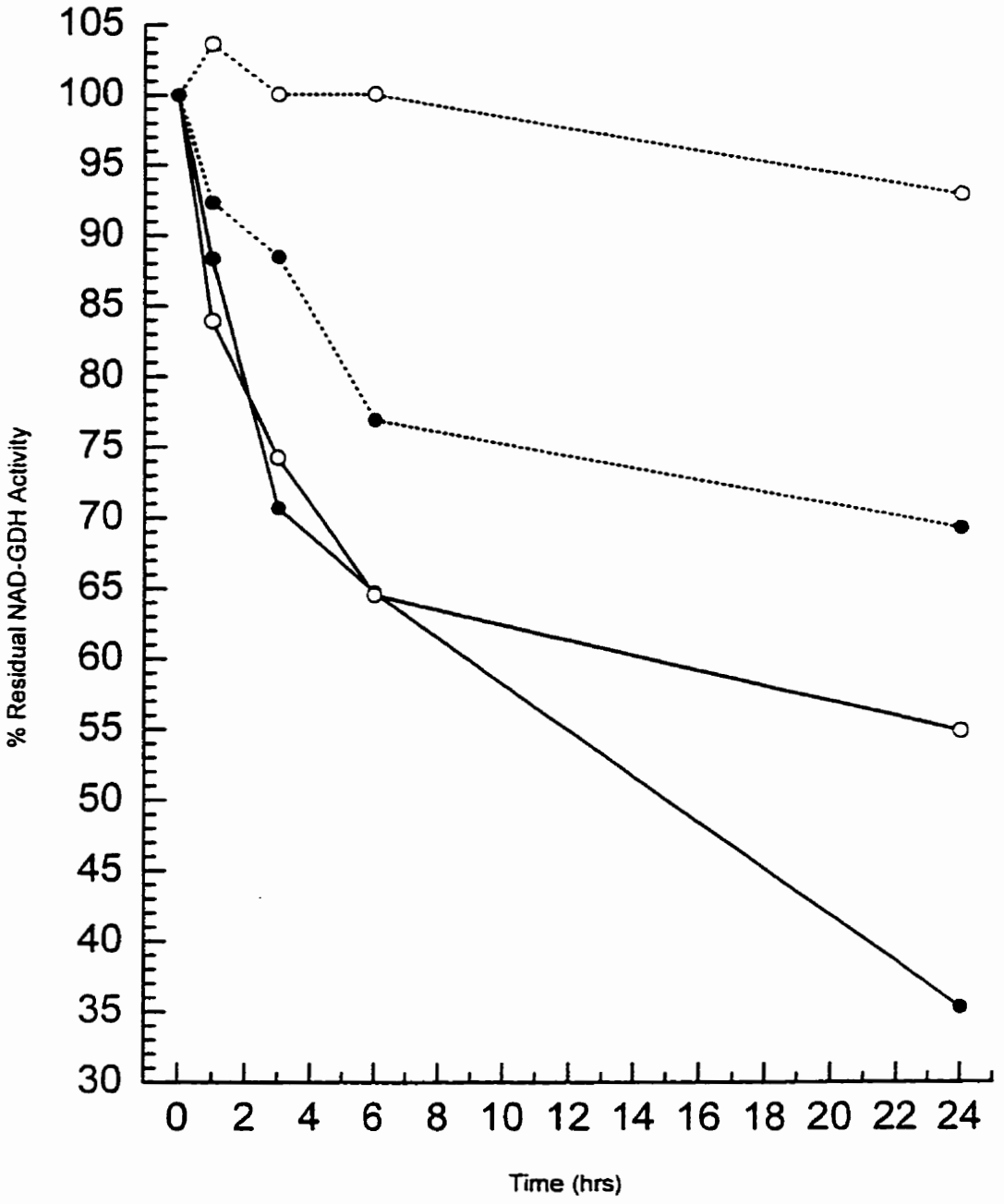
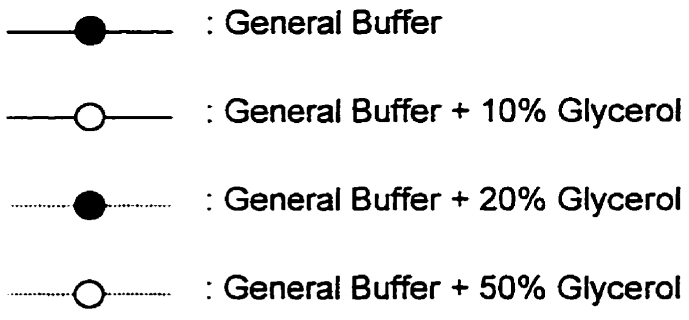

105
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
% Residual NAD-GDH Activity
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (hrs)

Figure 18: Retention of NAD-GDH activity over time at -20°C

NAD-GDH of mycelia grown overnight was induced with 15 mM L-glutamate during starvation for 4 hours as described (Methods and Materials *1.II.a. Short Term Culture* and *3.IV.b. Induction During Starvation*, respectively). Crude enzyme was extracted (Methods and Materials *3.V. Protein Extraction*) using general buffer with several different concentrations of glycerol, and samples were aliquoted and stored at -20°C. Samples were not thawed more than once. Enzyme activity for the reductive amination of α-ketoglutarate to L-glutamate was assayed (Methods and Materials *3.VI. NAD-GDH Activity Assay*), with activities at each time compared with initial activities upon extraction for each buffer to give percent enzyme activity remaining.



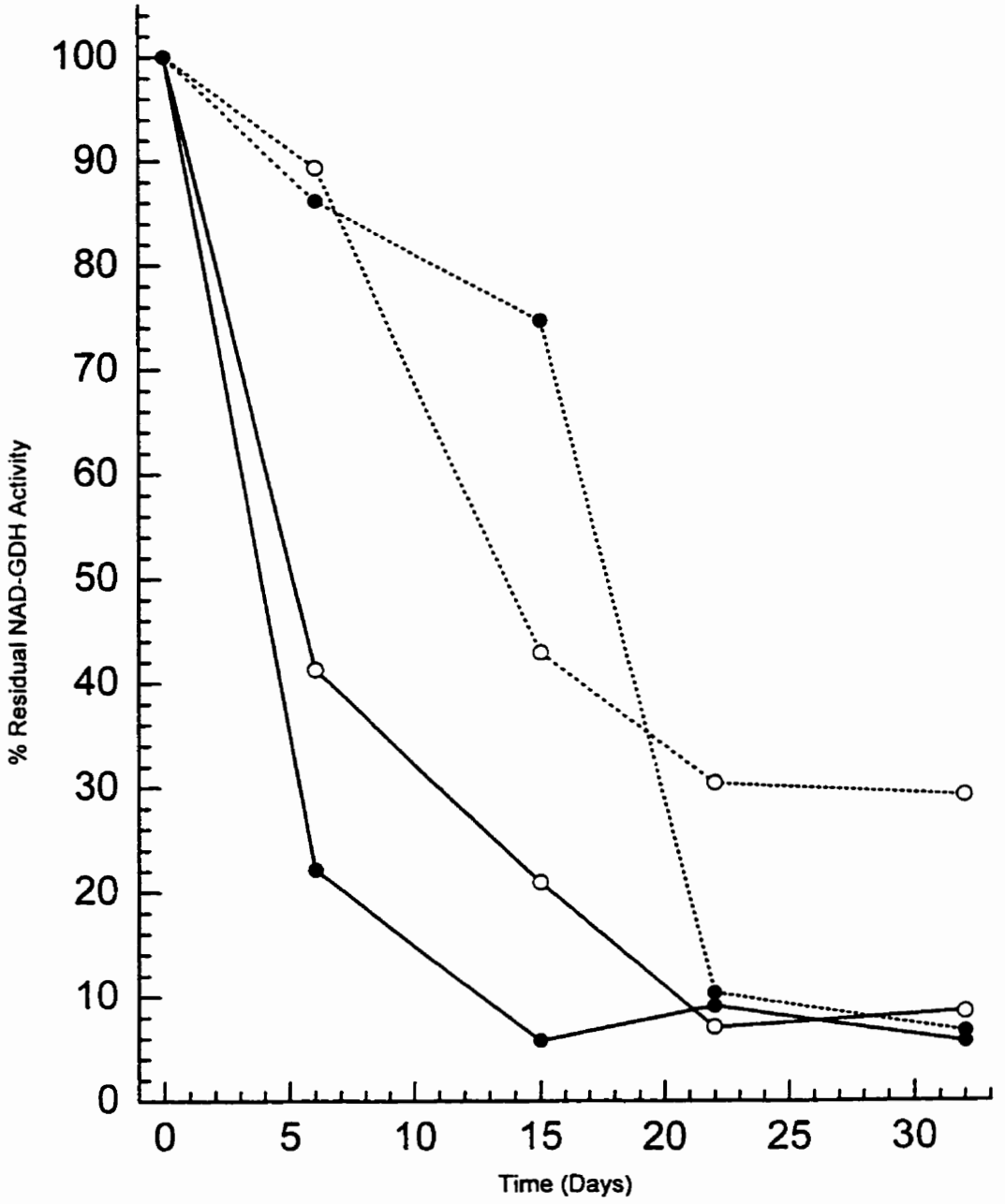
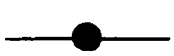
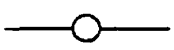
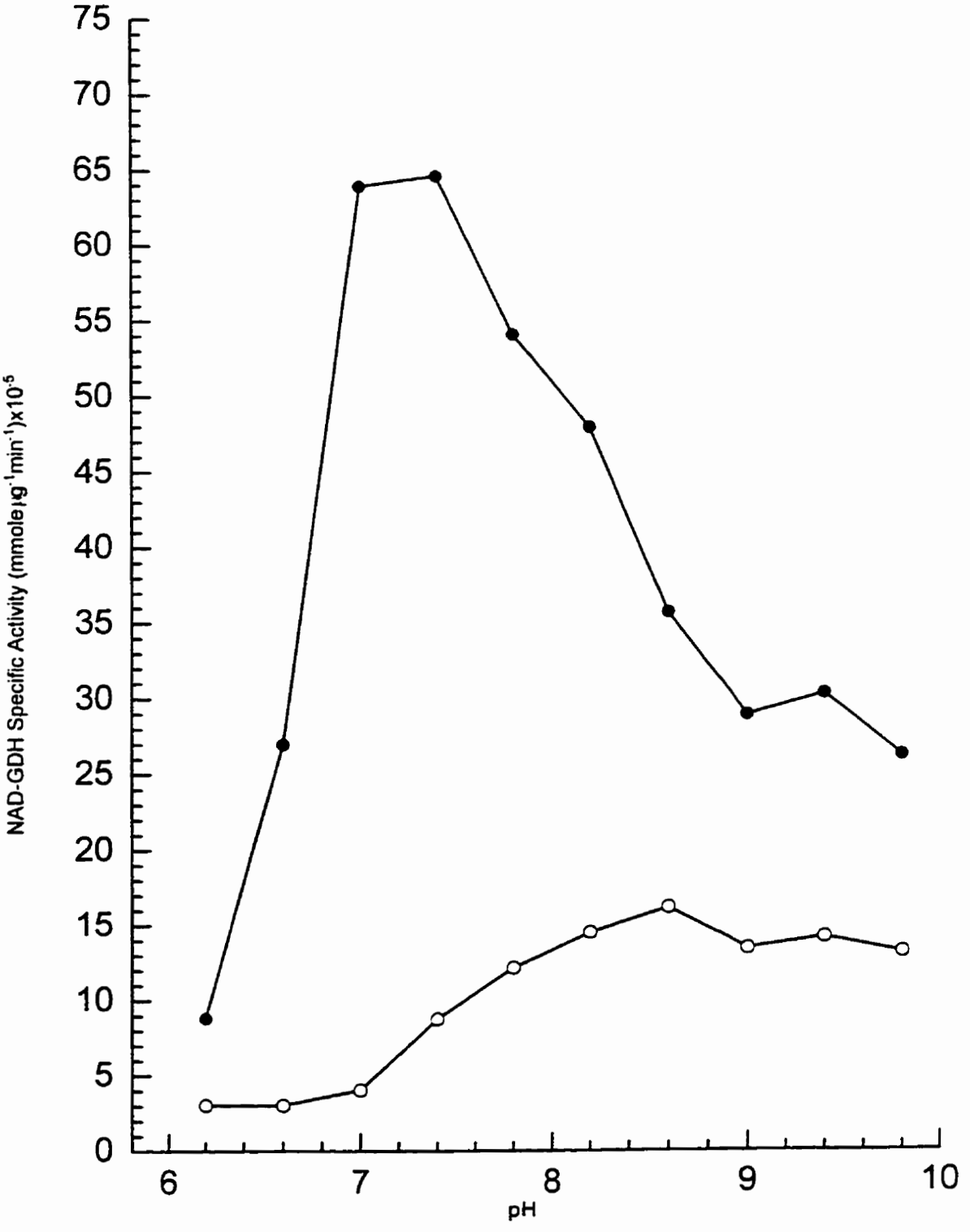

% Residual NAD-GDH Activity
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
Time (Days)

Figure 19: Effect of pH upon NAD-GDH activity

Crude protein obtained (Methods and Materials *3.I. Protein Extraction*) from mycelia grown overnight and induced during starvation for 4 hours with 15 mM L-glutamate as described (Methods and Materials *1.II.a. Short Term Culture* and *3.IV.b. Induction During Starvation*, respectively) was assayed (Methods and Materials *3.VI. NAD-GDH Activity Assay*) at several pHs between pH 6 and 10 (Materials and Methods *3.II. pH Optima Determination*) for both the oxidative deamination and reductive amination reactions to identify the pH optima of NAD-GDH for each reaction. Protein levels were quantified as described (Methods and Materials *3.VII. Protein Quantification*).


: Reductive Amination (αKG → L-Glu)


: Oxidative Deamination (L-Glu → αKG


NAD-GDH Specific Activity (mmole/µg-1min-1)x10-5
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
6
7
8
9
10
pH

optima were substantial, with greater than 8-fold and 5-fold increases in activity for the reductive and oxidative reactions, respectively.

2.III. $NADP^+$ Effects

P. ultimum NAD-GDH has been reported to be activeable by micromolar levels of $NADP^+$ at pH 8.0 (LéJohn, 1975), and has been demonstrated here to be activeable by $NADP^+$ in the reductive amination and oxidative deamination reactions at their respective pH optima (Figure 20). Activation of NAD-GDH activity was 7-fold greater for the amination of α-ketoglutarate to L-glutamate than for the reverse oxidative reaction. Only micromolar concentrations of $NADP^+$ were necessary to activate NAD-GDH in both cases, as has previously been reported in *A. klebsiana* and *P. debaryanum* (Stevenson, 1974). The cooperative binding of $NADP^+$ to more than one allosteric site was indicated by the biphasic nature of the log plot of *(Ve-Vo)/(Vmax-Ve)* versus log [$NADP^+$] for the reductive amination reaction, but was less distinct for the oxidative deamination reaction, which may be biphasic or monophasic (Figure 19) (Dixon and Webb, 1979; Segel, 1975; LéJohn *et al.*, 1970). This depiction of the data obtained also indicates that the Hill constant for each reaction (equal to the slope of the steeper of the two component lines of a biphasic plot, or of the only line in a monophasic plot) is less than one (0.76 μM^{-1} for the reductive amination reaction, 0.7 μM^{-1} for the oxidative deamination reaction), which identifies $NADP^+$ binding as

Figure 20: Activation of NAD-GDH by $NADP^+$

Mycelia grown overnight (Methods and Materials *1.II.a. Short Term Culture*) and induced with 15 mM L-glutamate for 4 hours during starvation (Methods and Materials *3.IV.b. Induction During Starvation*) were assayed for both the oxidative deamination and reductive amination reactions (Methods and Materials *3.VI. NAD-GDH Activity Assay*) in the presence of increasing concentrations of $NADP^+$. Protein concentration was determined as described (Methods and Materials *3.VII. Protein Quantification*). Data showing both the increase of specific activity with $NADP^+$ concentration (main) and fold activation (inset A) are presented. Estimation of the number(s) of allosteric sites can be made from the number of slope(s) observed by plotting $\log(V_e-V_o)/(V_{max}-V_e)$ against log[NADP+], wherein V_o is enzyme velocity in the absence of activator and V_e is velocity at the activator concentration tested, as presented in inset B.

—●— : Reductive Amination (αKG →L-Glu)

—○— : Oxidative Deamination (L-Glu →αKG

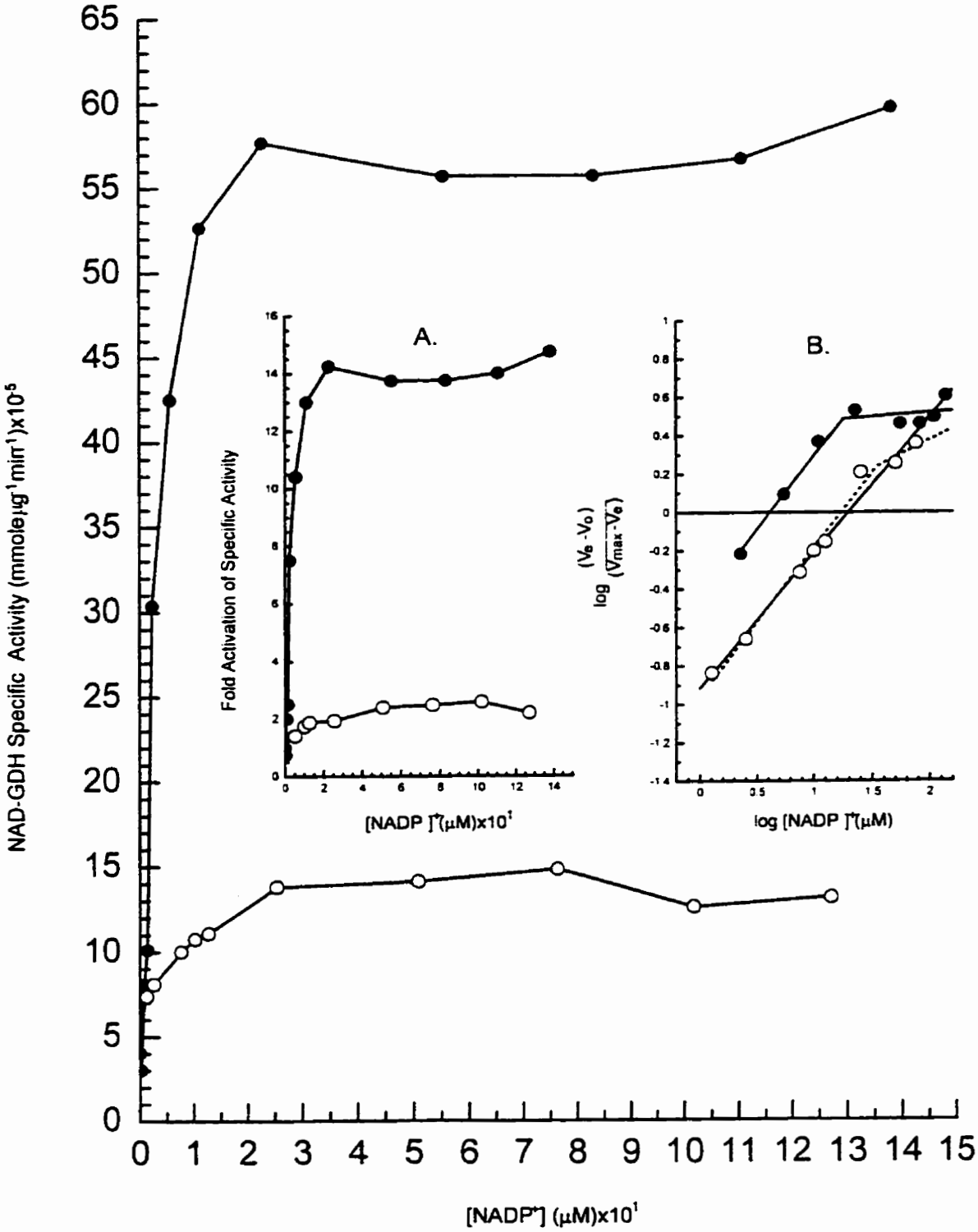
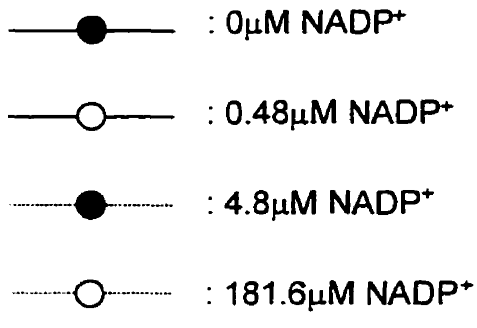

NAD-GDH Specific Activity (mmole$_{\mu g}^{-1}$min^{-1})x10^{-5}
[NADP$^+$] (μM)x10^1
A.
Fold Activation of Specific Activity
[NADP$^+$](μM)x10^1
B.
$\log \frac{(V_s - V_o)}{(V_{max} - V_s)}$
log [NADP$^+$](μM)

Figure 21: Kinetic analysis of the reductive amination of α-ketoglutarate to L-glutamate by NAD-GDH

Amination of α-ketoglutarate to L-glutamate using crude protein extracts of NAD-GDH obtained from mycelia grown and induced with L-glutamate as described for Figure 19. NAD-GDH assays (Materials and Methods *3. VI. NAD-GDH Activity Assay*) were done with different concentrations of $NADP^+$ as activator. Protein concentration was determined as described (Materials and Methods *3.VII. Protein Quantification*). Data obtained for each [$NADP^+$] were plotted as a linear regression on a Lineweaver-Burke double reciprocal plot (main) and as a curve displaying Michaelis-Menten kinetics (inset).



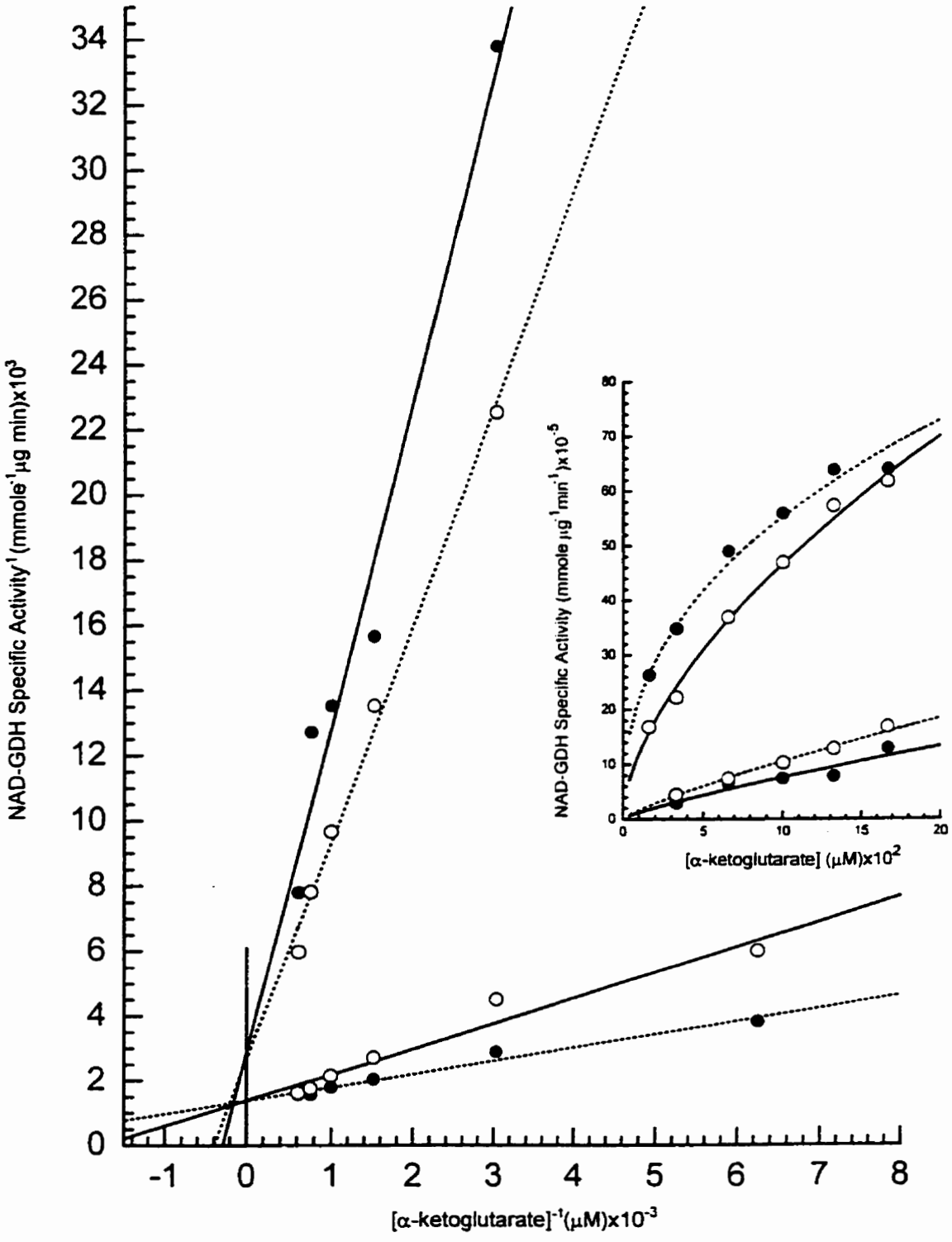
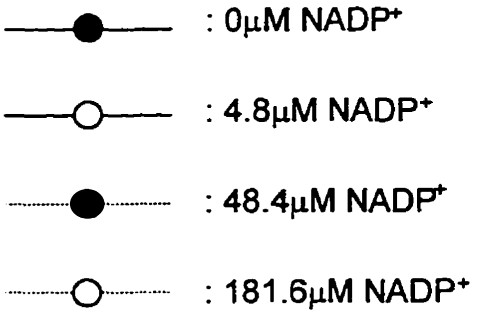
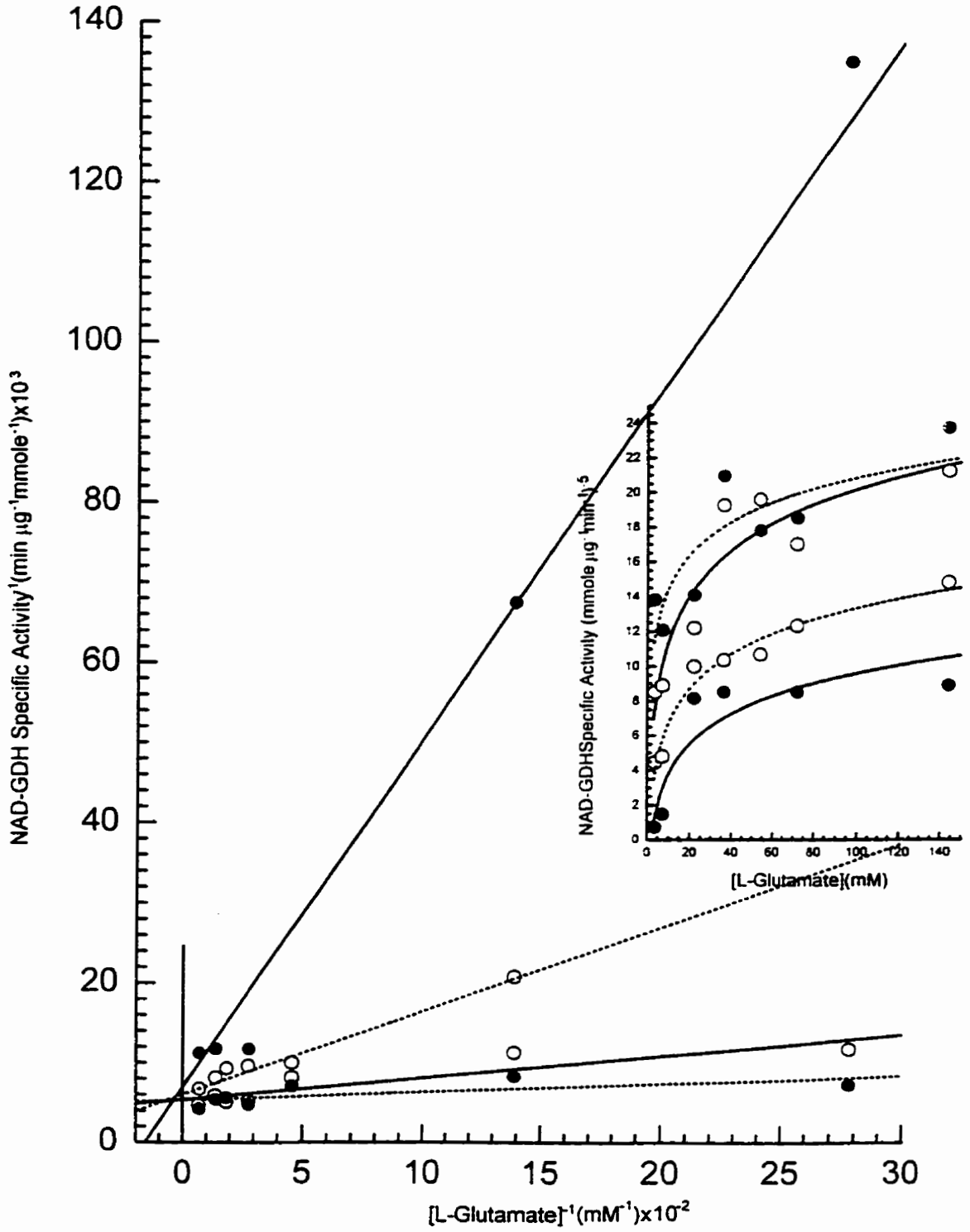

NAD-GDH Specific Activity^{-1}(mmole^{-1}µg min)x10^{3}
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
-1
0
1
2
3
4
5
6
7
8
[α-ketoglutarate]$^{-1}$(µM)x10^{-3}
NAD-GDH Specific Activity (mmole µg^{-1}min^{-1})x10^{-5}
0
5
10
15
20
[α-ketoglutarate] (µM)x10^{2}

Figure 22: Kinetic analysis of the oxidative deamination of L-glutamate to α-ketoglutarate by NAD-GDH

Deamination of L-glutamate to α-ketoglutarate by crude protein extracts of NAD-GDH obtained from mycelia grown and induced with L-glutamate as described for Figure 19. NAD-GDH assays (Materials and Methods *3. VI. NAD-GDH Activity Assay*) were done with different concentrations of $NADP^+$ as activator. Protein concentration was determined as described (Materials and Methods *3.VII. Protein Quantification*). Data obtained for each [$NADP^+$] were plotted as a linear regression on a Lineweaver-Burke double reciprocal plot (main) and as a curve displaying Michaelis-Menten kinetics (inset).




NAD-GDH Specific Activity^{-1}(min μg^{-1}mmole^{-1})x10^{3}
[L-Glutamate]$^{-1}$(mM^{-1})x10^{-2}
NAD-GDHSpecific Activity (mmole μg^{-1}min^{-1})x10^{-5}
[L-Glutamate](mM)

being negatively cooperative. In other words, the binding of one molecule of $NADP^+$ by the NAD-GDH of *P. ultimum* will inhibit the binding of a second molecule of $NADP^+$.

Due to the exhibition of residual NAD-GDH activity by crude proteins in the absence of $NADP^+$, it would appear that $NADP^+$ is acting as an activator of the NAD-GDH of *P. ultimum* rather than as a cofactor. Confirmation of this was achieved by enzyme velocity analysis using double-reciprocal plots (Dixon and Webb, 1979) with different concentrations of substrate (α-ketoglutarate or L-glutamate) for several different $NADP^+$ concentrations (Figures 21 and 22). These experiments also indicate that $NADP^+$ increases substrate binding affinity, as reflected by the 30-fold decrease of $K_{m(L\text{-}Glu)}$ and the less than 7-fold decrease in $K_{m(\alpha\text{-}KG)}$ as $[NADP^+]$ increases, with $K_{m(L\text{-}Glu)}$ greater than $K_{m(\alpha\text{-}KG)}$ by a factor of 3 to 20 fold in all cases. In contrast, there is less of an impact upon V_{max}, with $V_{max(L\text{-}Glu)}$ increasing by 1.3 fold while $V_{max(\alpha\text{-}KG)}$ doubles within the range of $NADP^+$ concentrations tested (Table 7).

2.IV. Induction of NAD-GDH Activity with L-Glutamate

2.IV.a. <u>Concentration Effects</u> L-glutamate was demonstrated to induce *P. ultimum* NAD-GDH activity in the reductive amination reaction both in isolation during starvation and as a supplement to growth medium of overnight cultures, with increases in NAD-GDH activity of up to 7 fold or 4.5

Table 7: V_{max} and K_m values of NAD-GDH

NAD-GDH V_{max} and K_m values for both the oxidative deamination and reductive amination reactions were derived from the y-intercepts and slopes of the appropriate double-reciprocal plots (Figures 21 and 22), as described by Stryer (1988).

nd: not done

[$NADP^+$] (μM)	Oxidative Deamination		Reductive Amination	
	$V_{max(L\text{-}Glu)}x10^{-4}$ (mmole $\mu g^{-1}min^{-1}$)	$K_{m(L\text{-}Glu)}$ (mM)	$V_{max(\alpha\text{-}KG)}x10^{-4}$ (mmole $\mu g^{-1}min^{-1}$)	$K_{m(\alpha\text{-}KG)}$ (mM)
0	1.4	62.1	3.5	3.51
0.484	nd	nd	3.7	2.93
4.84	1.7	17.3	7.2	2.49
48.4	1.9	5.0	nd	nd
181.6	1.9	1.9	7.2	0.56

Figure 23: Effect of L-glutamate concentration upon NAD-GDH induction

Mycelia grown overnight (Methods and Materials *1.II.a. Short Term Culture*) and induced with various concentrations of L-glutamate during and without starvation (Methods and Materials *3.IV.b. Induction During Starvation* and *3.IV.a. Induction Without Starvation*, respectively) were harvested and proteins prepared, assayed for the reductive amination reaction and quantified as described (Methods and Materials *3.V. Protein Extraction*, *3.VI. NAD-GDH Activity Assay* and *3.VII. Protein Quantification* respectively). Specific activities obtained for each L-glutamate concentration are given for L-glutamate concentrations depicted as both mM (main) and g/L (inset) for cells induced without starvation.

—●— : Induced, Growth Medium

—○— : Induced, Starvation Medium

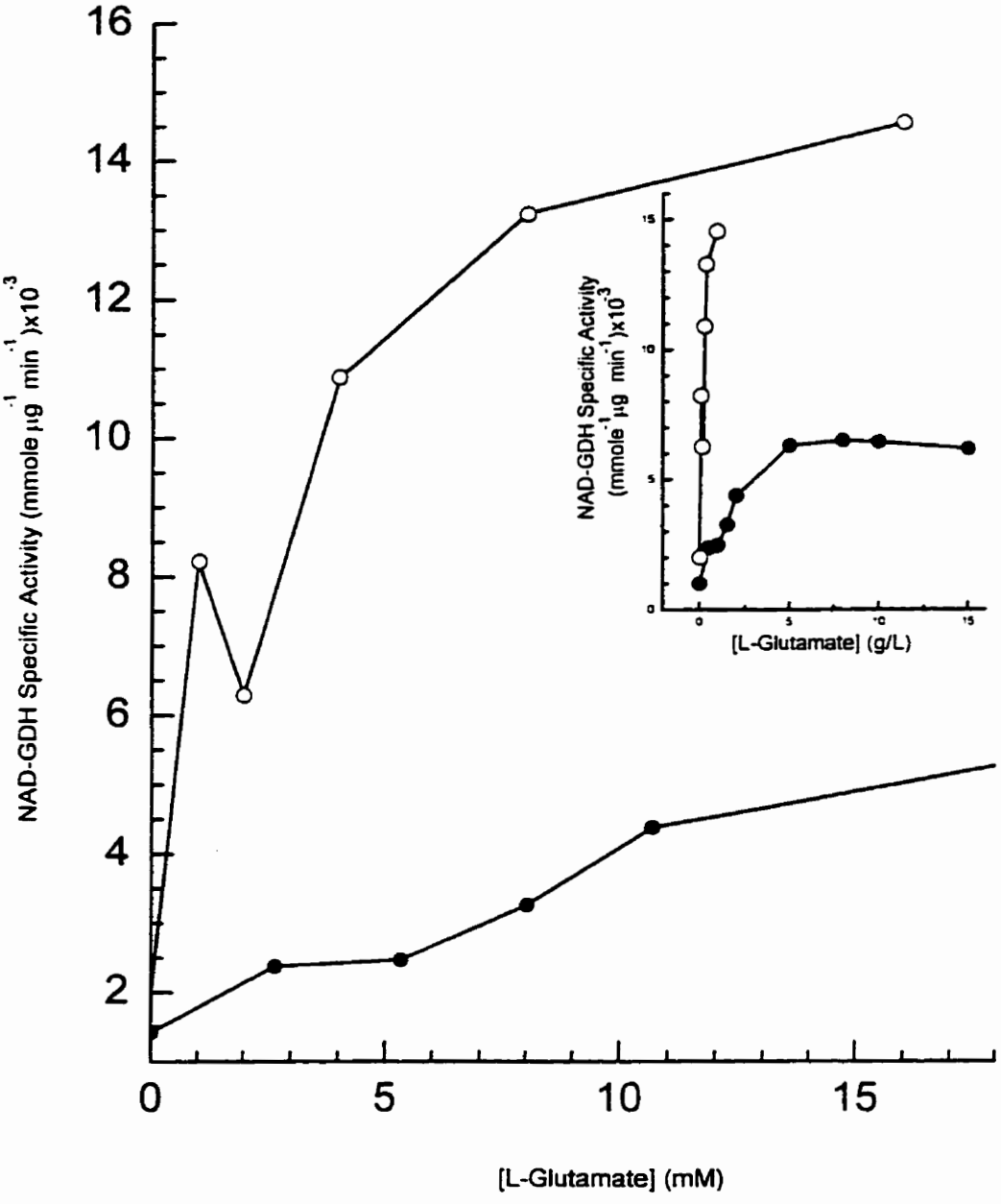
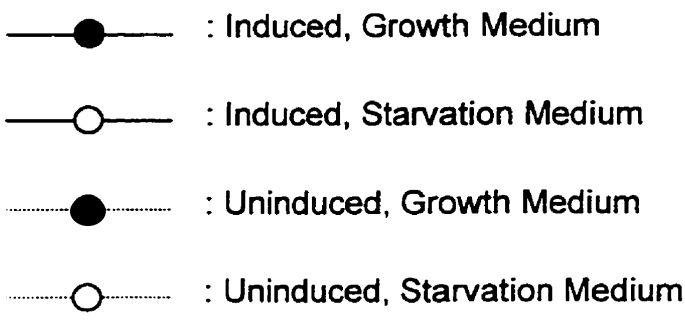
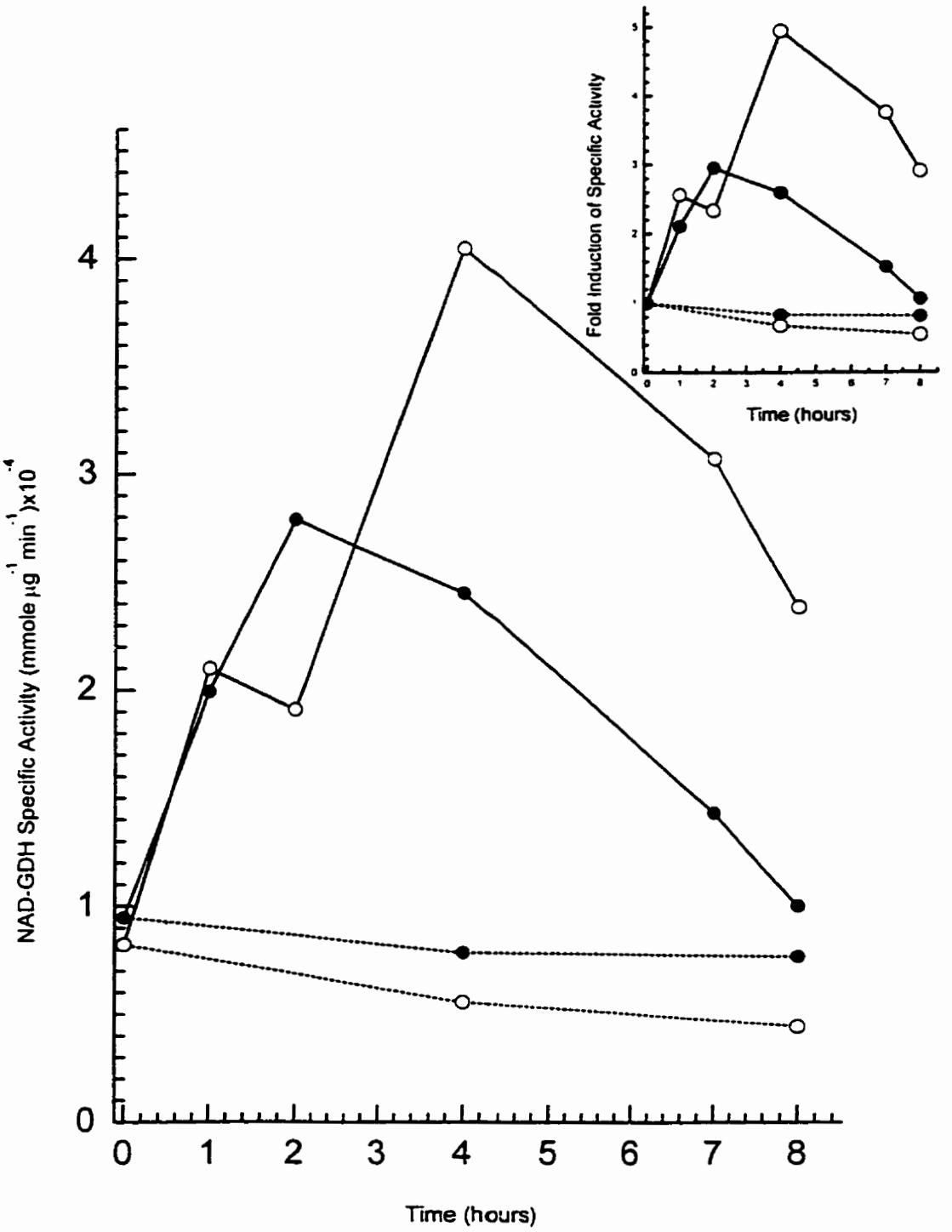

NAD-GDH Specific Activity (mmole µg min)x10
16
14
12
10
8
6
4
2
0
5
10
15
[L-Glutamate] (mM)
NAD-GDH Specific Activity
(mmole µg min)x10
[L-Glutamate] (g/L)

Figure 24: Induction of NAD-GDH over time in starved and unstarved cells.

Mycelia were grown and induced as described (Methods and Materials *1.II.a. Short Term Culture, 3.IV.a. Induction Without Starvation, 3.IV.b. Induction During Starvation*), using 15 mM L-glutamate or 15 g/L L-glutamate as inducing agent for starved and unstarved cells, respectively, for 0, 1, 2, 4 and 8 hours. Uninduced controls were cultured under identical conditions but without inducing agent for 4 and 8 hours. Crude proteins were extracted, assayed and quantified (Methods and Materials *3.V. Protein Extraction, 3.VI. NAD-GDH Activity Assay* and *3.VII. Protein Quantification* respectively) and specific activity (main) and fold induction (inset) plotted against induction time.




NAD-GDH Specific Activity (mmole μg⁻¹ min⁻¹)x10⁻⁴
0
1
2
3
4
0 1 2 3 4 5 6 7 8
Time (hours)
Fold Induction of Specific Activity
Time (hours)

fold for starved and unstarved cells, respectively (Figure 23). A concentration of 5g/L L-glutamate (approximately 27 mM) was found to maximize NAD-GDH induction in unstarved cells, with a concentration of 15 mM L-glutamate in starved cells giving a level of induction approaching the maximum possible.

2.IV.b. Time Effects NAD-GDH induction was shown to be dependent upon the time of exposure to as well as upon the concentration of L-glutamate (Figure 24). Induction of NAD-GDH specific activity in the reductive amination reaction of 5 fold after four hours of induction in cells starved during induction with 15 mM L-glutamate, and of 3 fold after two hours of induction with 15g/L L-glutamate in unstarved cells, were observed. A net decrease of NAD-GDH activity was also observed in uninduced cells grown in starvation medium or in GY medium as negative controls, with the decrease being most marked in cells grown under starvation conditions.

2.V. Discussion of NAD-GDH Characterization

2.V.a. Inherent instability of NAD-GDH is countered by high glycerol concentrations The NAD-GDH of *P. ultimum* was demonstrated to be inherently unstable, and as is the case for the NAD-GDH of *A. klebsiana*, is stabilized by the presence of glycerol in high concentrations to some extent. Higher concentrations of glycerol consistently preserved higher levels of NAD-GDH activity at all temperatures, with almost no detectable loss of

activity over the times studied at 37°C (Figure 16) and 4°C (Figure 17) with 50%(v/v) glycerol. However, it is apparent that *P. ultimum* NAD-GDH cannot be maintained at -20°C for more than one week (Figure 18), which is significantly less than reported for the NAD-GDH of *A. klebsiana* (Yang, 1991). Loss of *P. ultimum* NAD-GDH activity in crude cell extracts incubated at 37°C and 4°C was also greater at concentrations of less than 50% (v/v) glycerol than in corresponding experiments conducted with crude cell extracts from *A. klebsiana*, with the effect becoming more marked as glycerol concentrations are decreased (Figures 16 and 17).

2.V.b. The NAD-GDH of *P. ultimum* has distinct pH optima for the oxidative and reductive reactions it catalyzes This study has demonstrated that the NAD-GDH of *P. ultimum*, at saturating substrate and activator concentrations, has distinct pH activation curves and pH optima for the oxidative deamination of L-glutamate to α–ketoglutarate and the reverse reductive amination reaction (Figure 19). Sigmoid-like pH activation curves with an indicated activity minima at acidic pH, similar to that demonstrated for the oxidative NAD-GDH catalyzed reaction, have been taken as an indication of an active site which must be deprotonated at one or more locations, with deprotonated complexes able to react while protonated ones cannot (Cornish-Bowden and Wharton, 1988; Bender *et al.*, 1962). Reduced activity at acidic pH is due to the inability of amino acid residues in the active site to

be easily deprotonated, and activity maxima correspond to a state where almost all of the active sites are deprotonated. In the case of the *P. ultimum* NAD-GDH, unless different active sites are used for the oxidative and reductive reactions, it is more likely that the oxidative reaction's pH activation curve is really bell-shaped, with a broad pH optimum. Similar work with *P. debaryanum* NAD-GDH pH activation indicates that testing of the oxidative deamination reaction may not have extended to high enough pHs to determine if the oxidative deamination reaction of NAD-GDH followed a sigmoid or bell-shaped activation curve.

Enzyme affinity and not velocity can also be affected by the alteration of pH, but this is unlikely to be the cause of differences in enzyme activity in these experiments, due to the saturating concentrations of both substrate and activator used in enzyme assays. It is also possible that the lack of enzyme activity at extreme pHs is due to a loss of enzyme stability rather than decreasing enzyme efficiency (Dixon and Webb, 1979). This can be discounted for the *P. ultimum* NAD-GDH since the acidic pHs tested are not very extreme and the alkaline ones exhibit a stability (in the case of the oxidative deamination reaction) rather than a loss of enzyme activity.

The broad alkaline pH optimum observed for the oxidative deamination reaction is significantly different from that of the reductive amination of α-ketoglutarate to L-glutamate, which has a definite bell-shaped pH activation

curve with an optimum of pH 7.2. This indicates the presence of two or more groups in the active site of the enzyme, some of which must be protonated and others deprotonated in order to catalyze the conversion of α-ketoglutarate to L-glutamate (Cornish-Bowden and Wharton, 1988), with the pH at which these are best in balance being the pH optimum. Such bell-shaped pH activation curves characterize both reactions of *A. klebsiana* NAD-GDH and the reductive reaction of *P. debaryanum.*

The presence of distinct pH optima for both the reductive and oxidative reactions of NAD-GDH reported here for *P. ultimum* is also characteristic of *P. debaryanum*, although the pH optima of *P. debaryanum* are more alkaline than those of *P. ultimum* (Stevenson, 1974). The characteristic of a more alkaline pH optimum for the oxidation of L-glutamate to α-ketoglutarate than that of the reverse reductive reaction established in *P. debaryanum* (Stevenson, 1974) was also demonstrated in *P. ultimum*, and is also typical of many other GDHs (diPrisco and Garofano, 1974; Strecker, 1953). This is in contrast to the shared pH optima of the NAD-GDH catalyzed reactions observed in *A. klebsiana*, with a pH optimum of 8.0 in *A. klebsiana* for both reactions (Stevenson, 1974), which is intermediate to the two pH optima of *P. ultimum.* One feature of the *A. klebsiana* NAD-GDH shared with that of *P. ultimum* is an apparent secondary activity peak at pH 9.0 for *A. klebsiana* and at pH 9.4 for both reactions of NAD-GDH from *P. ultimum.* Such an

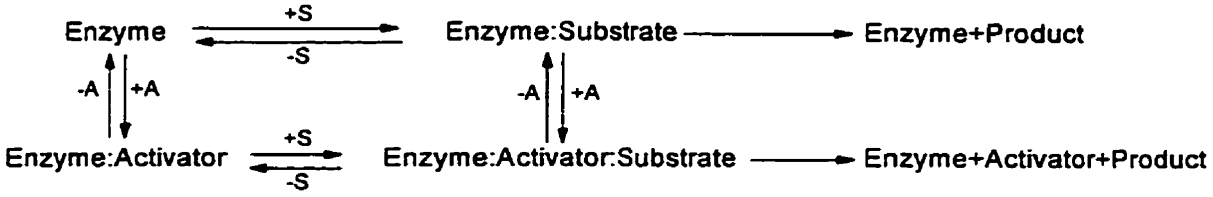
activity peak was not identified in the range of pHs tested for *P. debaryanum*. The substantial variation in activity with pH (up to 8 fold) is of a degree similar to that observed in *P. debaryanum*, although in *P. debaryanum* the greater activity change is seen for the oxidative deamination of L-glutamate rather than the reductive amination of α-ketoglutarate, as has been shown in this study for *P. ultimum*.

2.V.c. <u>NADP$^+$ is an activator of NAD-GDH</u> Both NAD-GDH catalyzed reactions were shown to be activated by micromolar concentrations of $NADP^+$, as has been previously demonstrated for other oomycetes (Yang and LéJohn, 1994; LéJohn, 1975; Stevenson, 1974). NAD-GDH activity levels for the reductive amination reaction were increased 14-fold in the presence of $NADP^+$ over those observed in the absence of exogenous $NADP^+$. This is significantly higher than the increases in NAD-GDH activity previously reported for *P. debaryanum* (5-fold) (Stevenson, 1974) and *A. klebsiana* (4-fold) (Yang and LéJohn, 1994; Stevenson, 1974). $NADP^+$ increased the rate of the oxidative deamination reaction by over 2-fold (Figure 20). This variation in activation may not be directly comparable, however, since inhibitor and activator effects in *P. debaryanum* and *A. klebsiana* have been shown to vary with pH (LéJohn and Stevenson, 1971; LéJohn *et al.*, 1969b; LéJohn and Jackson, 1968), and this could also be the case in *P. ultimum*.

Analysis of these same data on a Hill plot for cooperative binding ($\log[(V_e-V_o)/(V_{max}-V_e)]$ plotted against $\log[NADP^+]$) (Dixon and Webb, 1979; Segel, 1975) gave a biphasic plot for NAD-GDH in the reductive amination reaction direction, but was less distinct in the oxidative deamination direction (Figure 20). Similar results in several oomycetes, including *P. debaryanum*, have been previously described for GTP and acyl-CoA derivatives as activators of NAD-GDH (LéJohn *et al.*, 1970). The multi-phasic nature of such plots is indicative of a presumptive cooperativity of binding by a number of allosteric sites corresponding to the number of distinct slopes observed, in these cases one or two. These slopes also indicated that this cooperativity would be negative.

The presence of residual NAD-GDH activity levels in the absence of exogenous $NADP^+$ suggests that $NADP^+$ is not a cofactor but rather an activator, as is the case *P. debaryanum* (Stevenson, 1974). However, the possible presence of $NADP^+$ in the crude cell extracts could not be discounted, as it could be responsible for the residual NAD-GDH activity detected in the absence of exogenous $NADP^+$. Study of the relationship between NAD-GDH activity and substrate concentration at several $NADP^+$ concentrations gave double reciprocal plots typical of an enzyme in the presence of increasing amounts of an activator for both the oxidative and reductive reactions catalyzed by NAD-GDH (Figures 21 and 22), which

interacts with the enzyme in a manner analogous to that of a partial inhibitor (Dixon and Webb, 1979) as shown:



Derivation of V_{max} and K_m for both reactions of NAD-GDH was also accomplished from these double reciprocal plots for each $NADP^+$ concentration tested (Table 7). These indicate that, although $V_{max(\alpha\text{-}KG)}$ at pH 7.2 is never more than 4 fold greater than $V_{max(L\text{-}Glu)}$ at pH 8.6, substrate binding affinity as measured by $K_{m(\alpha\text{-}KG)}$ and $K_{m(L\text{-}Glu)}$ is lowered by a factor of 3 to 20 fold with increasing $NADP^+$ concentration for the conversion of α-ketoglutarate to L-glutamate at pH 7.2 than for the reverse reaction at pH 8.6, respectively. However, no interpretations of these data for *in vivo* enzyme activity and substrate preference can be made, as the internal substrate and activator concentrations are likely not at the saturating levels used in these experiments and internal pH of *P. ultimum* mycelia are unknown.

2.V.d. <u>NAD-GDH induction by L-glutamate is concentration and time dependent</u> Attempts to induce high levels of NAD-GDH in *P. ultimum* with L-glutamate were conducted by varying both L-glutamate concentration (Figure 23) and the time mycelia were incubated with L- glutamate (induction

time) (Figure 24). The relationship between these parameters and medium content, using cells either starved for both carbon and nitrogen sources other than L-glutamate or in complex growth (GY) medium were also examined. Under starvation conditions, *P. ultimum* NAD-GDH levels are highest after approximately 4 hours of enzyme induction, and approach their highest levels in the presence of L-glutamate concentrations greater than 15 mM. Under these circumstances, NAD-GDH specific activity increases up to 5 fold over uninduced levels. In contrast, cells induced under non-starvation conditions in the presence of GY medium (containing 0.5% w/v glucose and 0.05% w/v yeast extract) are most highly induced by two hours incubation with 15g/L L-glutamate (27 mM), with NAD-GDH specific activity increasing only 3 fold. The occurrence of maximum induction levels earlier in unstarved cells than starved cells may be due to the preferential use of glucose as a carbon source over L-glutamate, leading to the production of glucose catabolites which have been shown to repress NAD-GDH induction in *A. klebsiana* and *P. debaryanum* (Stevenson, 1974), but only after these catabolites reach a certain critical concentration. Such concentrations appear to be reached between two and four hours of induction, at which point NAD-GDH induction levels will decrease drastically. NAD-GDH induction during starvation also decreases after four hours, and is likely due to increased enzyme turnover as demands for amino acids other than L-glutamate exceed

the starving cell's supply. The slight decrease in NAD-GDH activity in the control cell cultures may be indicative of a region of balance between some basal level of *nad-gdh* transcription/translation and NAD-GDH degradation. Regardless of media content, NAD-GDH specific activity levels decreased slightly over the time of incubation in control cultures free of L-glutamate.

The analogous induction of NAD-GDH by L-glutamine in *A. klebsiana* reported by Braithwaite (1987) was hypothesized to be due to either a direct increase in gene expression or by the post-transcriptional modification of mRNAs (specifically methylation) to enhance the translational efficiency of these molecules. Another possibility is that there is an intracellular NAD-GDH pool which is only released for use by the cell when needed. Immunodetection of NAD-GDH in crude *A. klebsiana* protein extracts with anti-GDH antibodies (LéJohn *et al.*, 1994a; Yang and LéJohn, 1994; Yang, 1991) indicated that NAD-GDH levels increased in response to the presence of increasing inducer concentrations. A concurrent increase in levels of a 3.4 kb mRNA predicted to be the NAD-GDH message was detected by Northern blotting (LéJohn *et al.*, 1994a), indicating that the induction of NAD-GDH activity is a result of increased transcription and translation. Experiments necessary to confirm any of these hypotheses were not conducted in the work with *P. ultimum* reported here.

3. Characterization of an *hsp70* gene in *Pythium ultimum*

Characterization of the organization of the 17 kb genomic clone from which the sequence of *nad-gdh* in *A. klebsiana* was obtained, particularly the regions in the 5' upstream direction of *nad-gdh* (LéJohn, unpublished data), was conducted in conjunction with similar investigations of analogous regions in the *P. ultimum* genome organization obtained by screening a *P. ultimum* genomic library. Sequence information derived from this work indicated the presence of nearly identical ORFs and associated 5' regions in *A. klebsiana* and *P. ultimum* which had characteristics of *hsp70* genes. The study presented here describes a brief analysis of the response of *P. ultimum* to heat stress, as well as the PCR amplification and sequencing of a previously unsequenced part of the *P. ultimum* open reading frame (ORF) suspected to encode a *hsp70* gene in the region of the genomic clone directly bordering the λEMBL3 vector DNA.

3.I. Effects of Heat Stress on *P. ultimum*

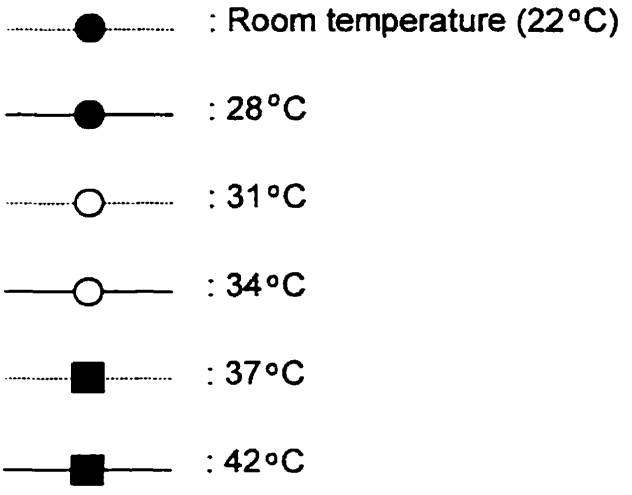
3.I.a. Effects upon growth rate and morphology

The growth rate of *P.ultimum* mycelia on potato-dextrose agar plates over 44 hours was found to peak at 28°C (1.35 mm hour^{-1}), with significant growth at room temperature (1.3 mm hour^{-1}) and 31°C (1.1 mm hour^{-1}), but with no significant growth observed at 34°C or higher temperatures (maximum of 0.04 mm hour^{-1} at 37°C) (Figure 25). These findings correspond to previously reported optimum and maximum growth temperatures for this species (van der Plaats-Niterink, 1981). Microscopic analysis (Figure 26) of *P. ultimum* grown at room temperature for 12 hours prior to being subjected to various growth temperatures for a further 24 hours revealed distinct morphological variations with increasing temperature, including the increasing presence of irregular swellings in individual hyphae which resemble sporangia up to a temperature of 34°C. No mycelial growth was observed at 42°C, with microscopic analysis indicating deformed mycelia which appear to have undergone plasmolysis. Incubation of *P. ultimum* for either 24 or 48 hours at 34°C, 37°C or 42°C were all found to be lethal, with no mycelial growth occurring after placement of these cultures at room temperature for one week.

3.I.b. Effects upon protein synthesis Examination of new protein synthesis during short-term temperature stress by SDS-PAGE identified only two protein bands exhibiting temperature dependent expression patterns one of

Figure 25: Effect of temperature upon mycelial growth of *P. ultimum*

Standardized *P. ultimum* inocula on agar plugs were used as a starting culture for potato-dextrose petri plates (150 mm diameter) which were grown at various temperatures as described (Methods and Materials *4.1. Morphological analysis of* P. ultimum, above). At least four measurements were taken for each plate at each time point, with the average extent of mycelial growth from the inoculation point being presented as cumulative growth. Growth rates at different temperatures (approximating room temperature at 22°C) are also shown (inset).



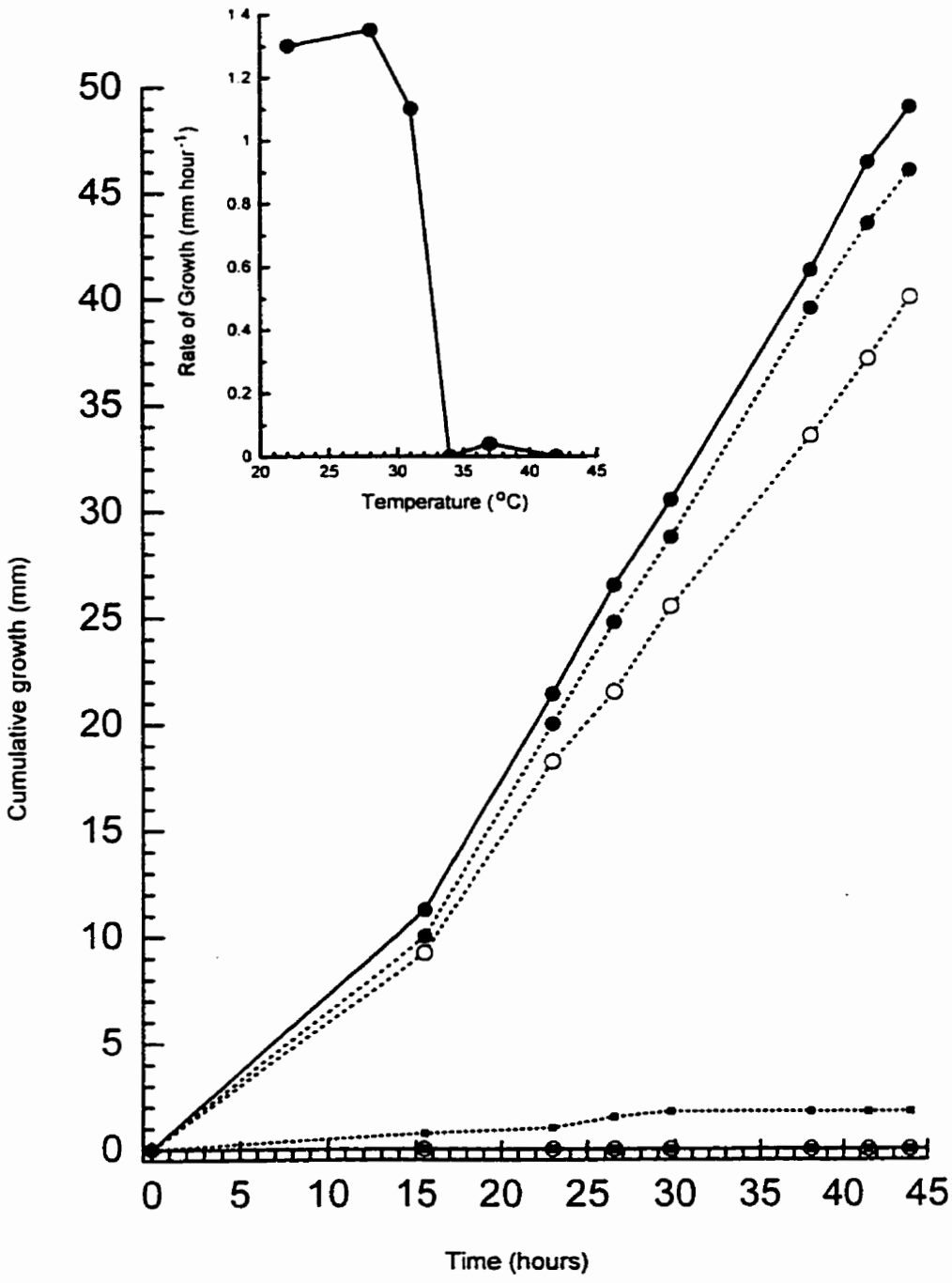

Cumulative growth (mm)
Time (hours)
Rate of Growth (mm hour-1)
Temperature (°C)

Figure 26: Effects of temperature upon hyphal morphology of *P. ultimum*

P. ultimum were cultured at several temperatures for 24 hours prior to microscopic photography (50x magnification) as described (Methods and Materials *4.1. Morphological analysis of* P. ultimum). The composite image presented here includes cultures incubated at A. room temperature (approximately 22°C), B. 28°C, C.31°C , D. 34°C , E. 37°C and F. 42°C. The scale in μm is given at bottom right.

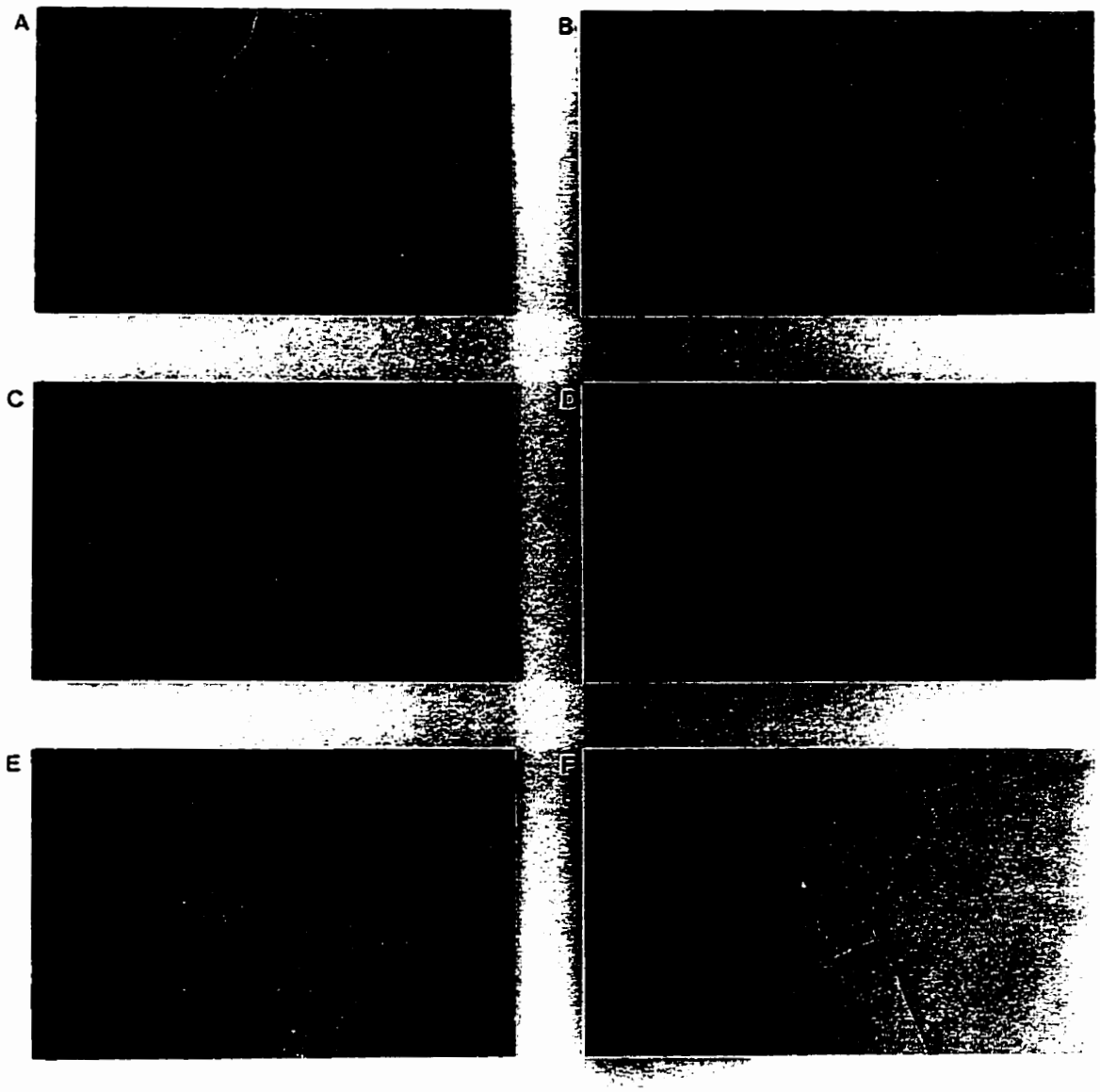
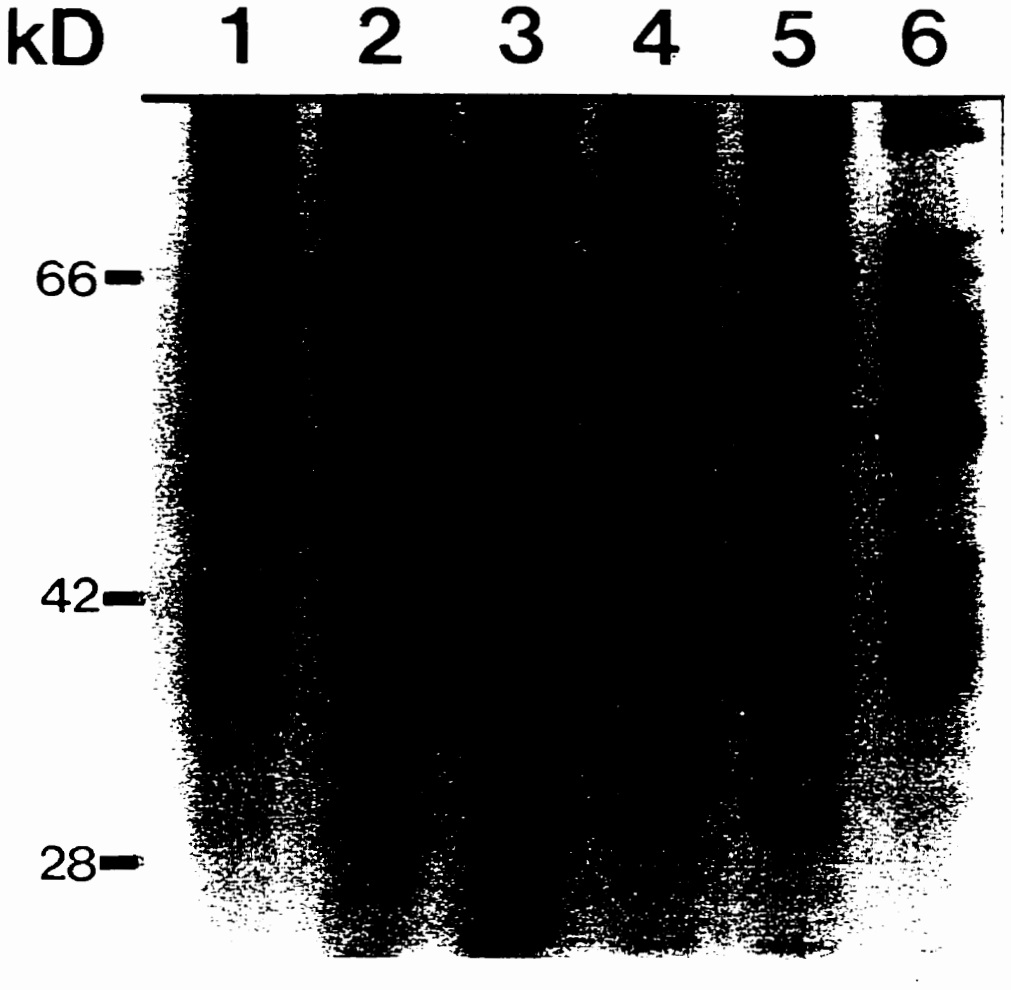

A
B
C
D
E
F
200 µm

Figure 27: Autoradiogram of differential protein synthesis in *P. ultimum* under heat stress conditions

P. ultimum was grown overnight in GY medium at room temperature was heat stressed for 30 minutes in the presence of ^{35}S-labeled methionine and cysteine as described (Methods and Materials *3.V.a. Protein radiolabeling*, above). Proteins were extracted and electrophoresed using SDS-PAGE (Methods and Materials *3.V. Protein extraction* and *3.V.b. SDS polyacrylamide gel electrophoresis (SDS-PAGE)*, above). Heat stress temperatures for each sample were as follows: Lane 1, room temperature (approximately 22°C); Lane 2, 28°C; Lane 3, 31°C,; Lane 4, 34°C; Lane 5, 37°C; Lane 6, 42°C. 300000 cpm were loaded in each lane. Both the 70kD (<) and 55kD (*) protein bands indicated in Lane 3 are also present in Lanes 4 through 6, and seem to be generated only at heat stress temperatures. Protein standards in kD are given on the left.


kD
1
2
3
4
5
6
66
42
28

approximately 55kD and another of 70kD whose production is induced by increasing temperature (Figure 27) over a relatively short period of exposure to the stress-inducing temperature. The pattern of heat stress related protein production tends to indicate that temperatures greater than 28°C induce a heat stress response in *P. ultimum*, which corresponds to the effects of temperature upon mycelial growth and morphology noted above. These results must be qualified, however, since proteins with uncharacteristically high or low methionine and/or cysteine contents will incorporate greater or lesser amounts of radiolabel than the norm, giving an inaccurate perception of the relative quantities of these proteins compared to others in the same sample. Also, multiple proteins of similar size may not be completely distinguishable with one-dimensional SDS-PAGE. This does not detract from the observations noted in these experiments, as the demonstration of the synthesis of proteins unique to the heat-stress condition is of interest.

3.II. Sequencing Template Preparation

Restriction endonuclease mapping of the *A. klebsiana* genomic library clone used to characterize the *nad-gdh* gene and associated antisense gene pair (LéJohn *et al.*, 1994a and 1994b; Yang, 1991) (Figure 28) allowed the preparation of several recombinant molecules containing different regions of the genomic clone for sequence analysis. The fragment closest to the λ–EMBL3 long arm in the *A. klebsiana* genomic clone is a 2.57 kb EcoRI

Figure 28: Restriction endonuclease digestion map of an *A. klebsiana* genomic library clone containing an antisense gene pair

Restriction endonuclease mapping, conducted by B. Yang (1991) and confirmed by DNA sequencing (LéJohn, unpublished data), of a genomic library clone used to sequence members of an antisense gene pair in *A. klebsiana* (LéJohn *et al.*, 1994a and 1994b). Fragment sizes in kb are as indicated.

Endonuclease Sites:
X: XbaI
E: EcoRI
M: MboI
S: SalI

nad-gdh:hsc70 antisense gene pair
λshort
λlong
M
E
X
E
X
X
SE
SE
M
?
2.35
0.45
4.53
2.77
1.6
0.1
2.47
0.1
1.5

restriction endonuclease fragment identified by the laboratory of LéJohn. Independent screening of a *P. ultimum* genomic library with the *A. klebsiana* antisense gene pair identified several positive clones, which were subsequently SalI restriction endonuclease digested and shotgun subcloned into pBluescript (LéJohn, unpublished data). One of these subclones contained a 2.57 kb SalI restriction endonuclease fragment suspected to be analogous to the 2.57 kb EcoRI restriction endonuclease fragment of *A. klebsiana*, and was used for subsequent analysis.

Since the insert DNA for the genomic library was prepared by partial MboI restriction endonuclease digestion, the terminus of the insert region of the genomic clone closest to the 2.57 kb SalI restriction endonuclease fragment (1.4 kb distant) must be an MboI restriction endonuclease site. Unfortunately, excision of the 1.4 kb intervening fragment by double digestion with MboI restriction endonuclease and SalI restriction endonuclease was not feasible, as the 1.4 kb intervening fragment could contain one or more MboI restriction endonuclease sites remaining uncut during library construction. Therefore, it was decided to amplify this region by PCR from genomic DNA. Primers external to the 1.4 kb region (primers 2.9prB and LarmEMBL3) as well as internal primers (primers HSPextL and HSPextR) based upon the already elucidated *A. klebsiana* sequence for the 2.57 kb EcoRI restriction endonuclease fragment and 1.4 kb intervening fragment were used,

generating PCR products of 1.4 and 0.7 kb from *P. ultimum* genomic library clone DNA (Figure 29). Both were subsequently used as sequencing templates.

3.III. Sequence Analysis

3.III.a. Characterization of the nucleotide sequence of the 2.57 kb SalI restriction endonuclease fragment and 1.4 kb PCR product Subcloning into pBluescript and sequencing of the 2.57 kb SalI restriction endonuclease fragment derived from the genomic library clone for *P. ultimum* (Heidi Wood, 1997) demonstrated that this region is almost completely identical to a corresponding 2.57 kb EcoRI restriction endonuclease fragment obtained from the independently identified 17 kb *A. klebsiana* genomic library clone originally used to isolate the *nad-gdh* gene (LéJohn *et al.*, 1994a and 1994b; Yang, 1991). Only three base pair differences with the *A. klebsiana* sequence were observed (Figure 31). Two of these changes (5'-C→A and A→C-3') are adjacent to and occur upstream of a large putative ORF, within a region bearing characteristic 5' regulatory signals (Table 8). The third change (C→T) is a silent mutation located within the aforementioned putative ORF. The ORF extends for approximately 355 bp within the 2.57 kb EcoRI restriction endonuclease clone to the end of this fragment, adjacent to the starting position of the 1.4 kb intervening fragment PCR product.

Several regulatory sequences immediately 5' of the predicted ORF were

Figure 29: PCR amplification of the terminal 1.4 kb fragment of a *P. ultimum* genomic library clone

PCR amplification with primers given in **A.** of the 1.4 kb intervening fragment adjacent to both the 2.57 kb SalI restriction endonuclease fragment and the λEMBL3 vector arm of a genomic library clone from *P. ultimum* (Methods and Materials *4.II.a Production of hsp70 DNA Template for Sequencing*) produced 1.4 kb and 0.7 kb PCR amplification fragments. These products correspond to the regions given in **B.** Successful amplification was confirmed by agarose gel electrophoresis (Methods and Materials *2.I.a. Insert and Vector Preparation*) and ethidium bromide staining (inset). Contents of marked lanes in the inset are noted below. Molecular sizes are indicated in kb.

Lane 1: 1 μg 1.4 kb PCR product

Lane 2: 1 μg 0.7 kb PCR product

A.

Designation	Primer Name	Primer Sequence (5' to 3')
a	2 9PrB	GATAATAAGCCTCAAATCAG
b	HSPextL	TGACAACCGCCTTGTGAATTA
c	HSPextR	TCCAGTCAGTGTATTGATAGTA
d	LarmEMBL3	GATCTGGGTCGACGGATC

B.

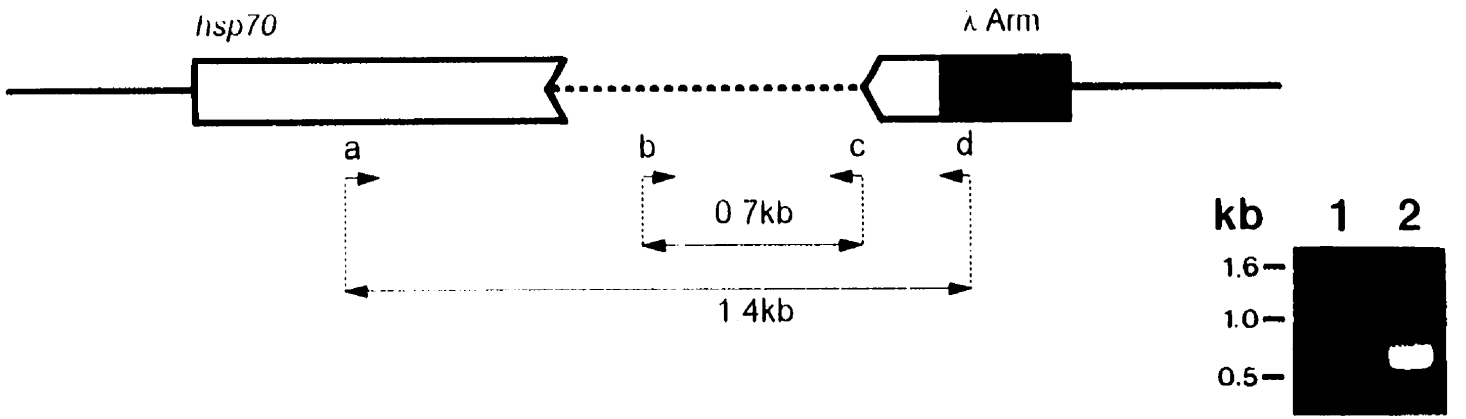


Figure 30: Sequencing strategy for the *P. ultimum* 2.57 kb SalI restriction fragment and 1.4 kb PCR product

Template and primer preparation for sequencing and sequencing itself were conducted as described (Methods and Materials *4.I Production of hsp70 DNA Template for Sequencing* and *4.II. DNA Sequencing*). Primer locations and sequencing direction are indicated by labeled arrows (solid for 2.57 kb SalI restriction endonuclease fragment sequencing, dotted for 1.4 kb PCR fragment). Individual sequences obtained from these primers are indicated by labeled boxes, with overlaps between sequences are indicated by vertical lines connecting the sequences involved. Primer sequences are as follows:

EVB:	5'-TTTGAGGCCACATTCATCTC-3'
1030:	5'-ATCAAGATTGACTTCG-3'
1006:	5'-ATATACACCAGTCAGTGCTG-3'
EVA:	5'-GCTTATCACTTGATTGTTC-3'
2.9PrB:	5'-GATAATAAGCCTCAAATCAG-3'
Pudb1:	5'-GAACTATTGATGTTTCTATTGT-3'
HSPextL:	5'-TGACAACCGCCTTGTGAATTA-3'
Pudb4:	5'-GCTTCTATTGTAACACGAGCTC-3'
HSPextR:	5'-TCCAGTCAGTGTATTGATAGTA-3'
LarmEMBL3:	5'-GATCTGGGTCGACGGATC-3'
T3:	5'-AATTAACCCTCACTAAAGGG-3'
T7:	5'-GTAATACGACTCACTATAGGGC-3'

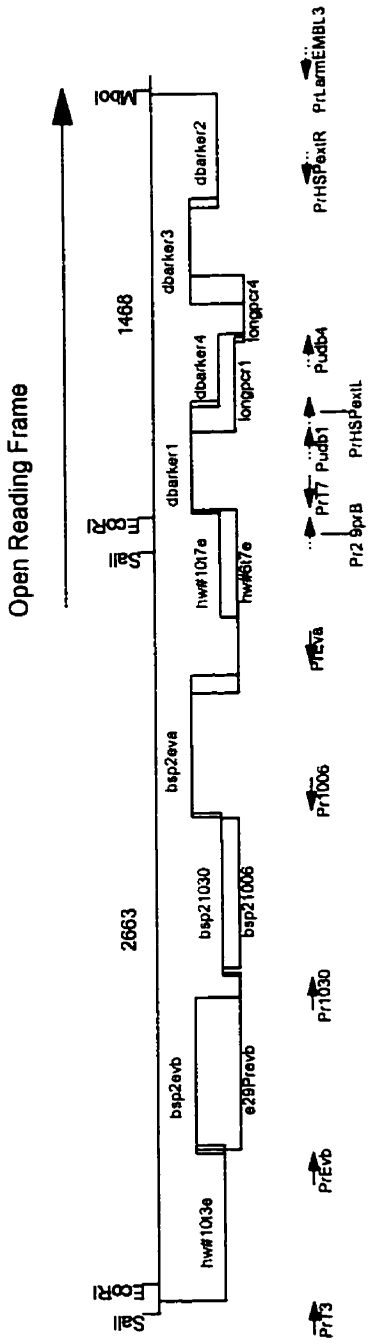
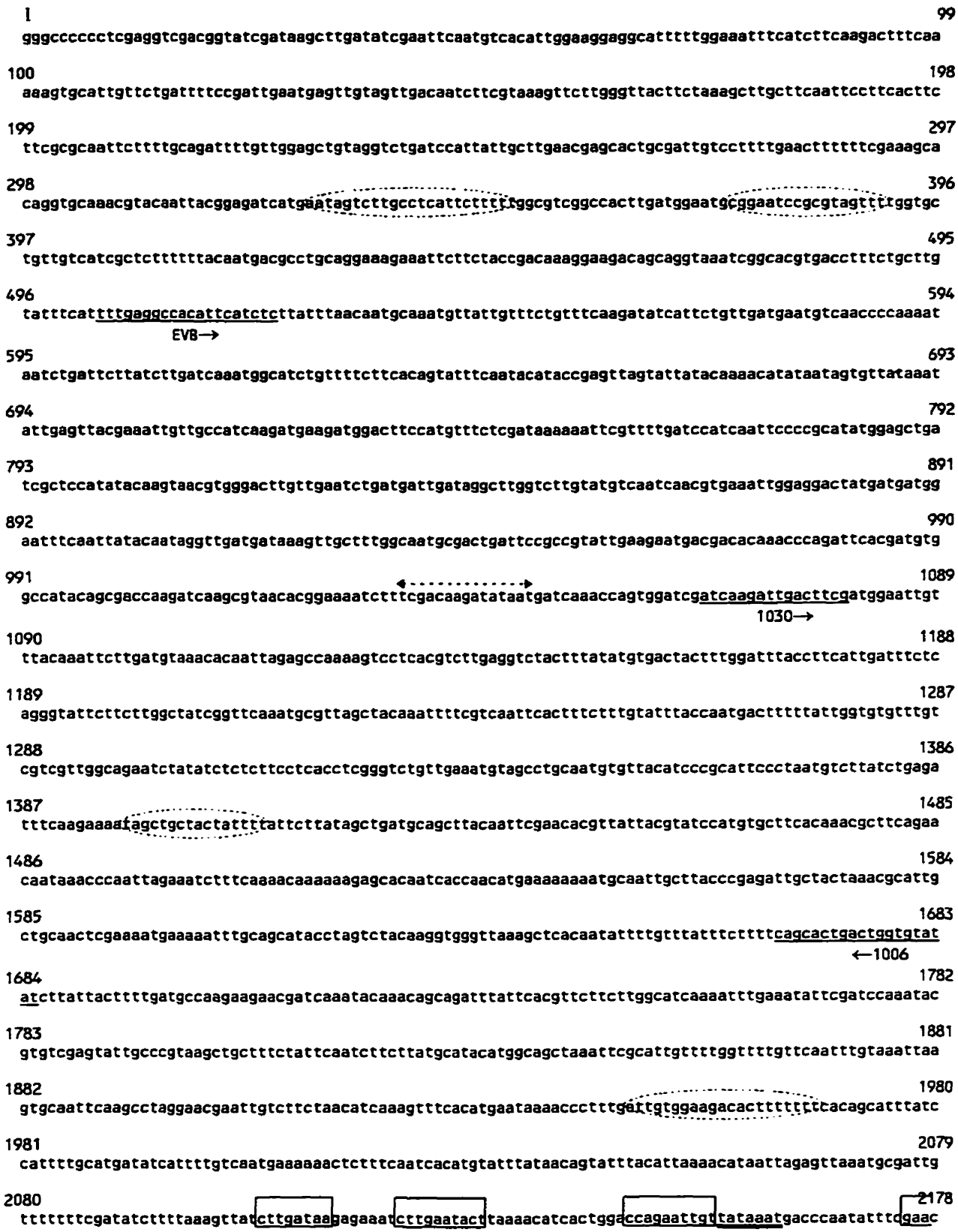
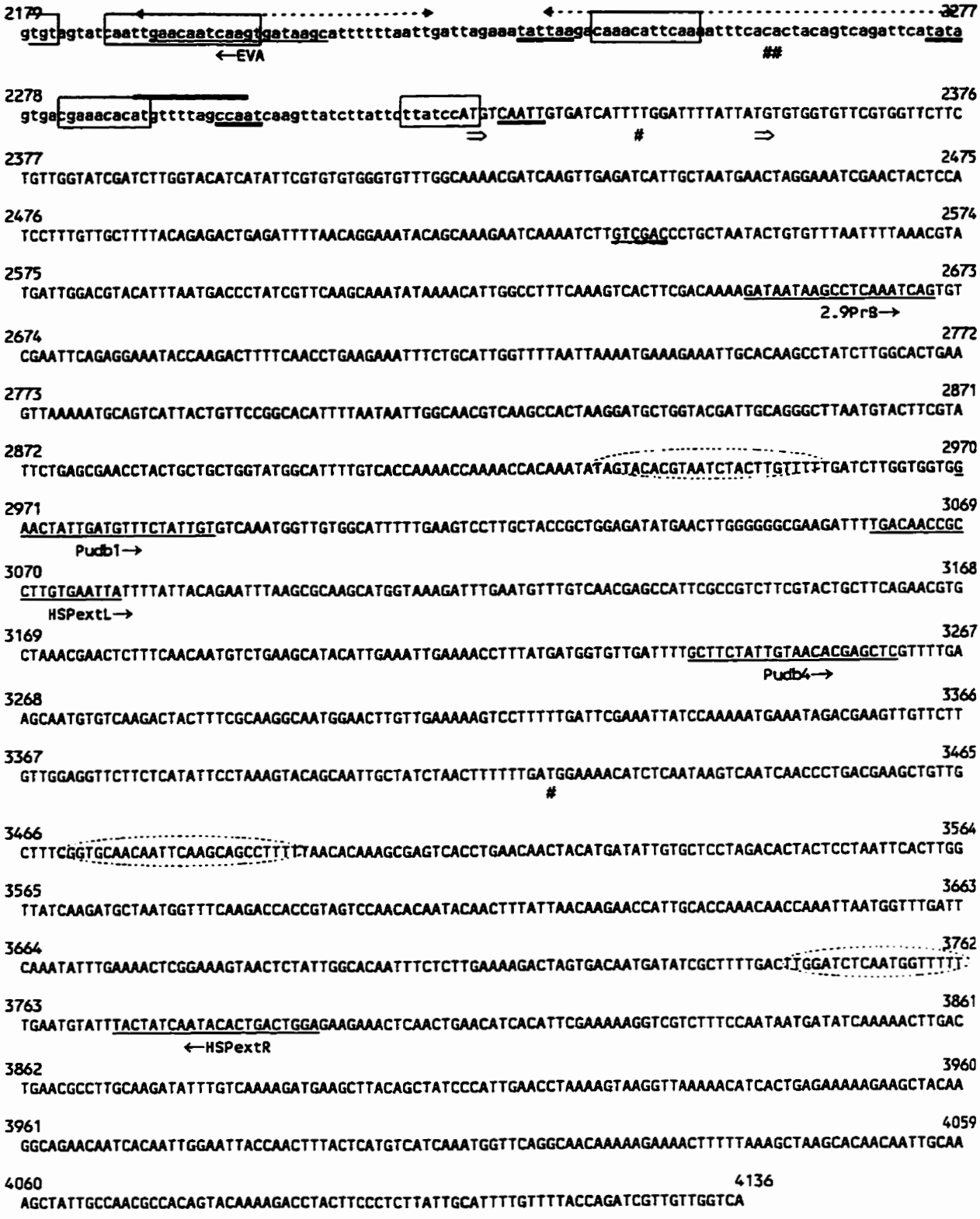

Open Reading Frame
MboI
1468
dbarker3
dbarker2
dbarker4
longpcr1
longpcr4
dbarker1
EcoRI
SalI
hwf#1017e
hwf#807e
bsp2evb
bsp2evb
2663
bsp21030
bsp21006
e28Prevb
hwf#1013e
EcoRI
SalI
Pr1006
Pr1030
PrEVb
Pr73
PrEVa
Pr77
Pr2
PudB1
PudB4
SprB
PrHSPexIL
PrHSPexIR
PrLamEMBL3

Figure 31: Combined DNA sequence of the 2.57 kb SalI restriction endonuclease fragment and the 1.4 kb PCR product from *P. ultimum*, containing a putative *hsp70* gene

The DNA sequence is given 5'→3', with only the sequence of the strand encoding a putative *hsp70* gene given. The end furthest from the λEMBL3 vector is 5'-most. Indicated are a long ORF (capitals); sequencing/PCR primer locations within the sequenced area (underlined, with identity and direction indicated); CCAAT and TATAAT 5' regulatory signals (doubly underlined); potential prokaryotic promoters (dotted, double arrowed overscores); potential prokaryotic ρ-independent terminators (dotted ellipses); heat shock elements (boxed); start codons (double-lined arrows); CTF/NF1 binding site (thick overscore); differences with the putative *hsp70* sequence in *A. klebsiana* (#); and the SalI restriction endonuclease site linking the 2.57 kb SalI restriction endonuclease fragment with the 1.4 kb PCR product (dotted underline).


1
99
gggccccccctcgaggtcgacggtatcgataagcttgatatcgaattcaatgtcacattggaaggaggcatttttggaaatttcatcttcaagactttcaa
100
198
aaagtgcattgttctgattttccgattgaatgagttgtagttgacaatcttcgtaaagttcttgggttacttctaaagcttgcttcaattccttcacttc
199
297
ttcgcgcaattcttttgcagattttgttggagctgtaggtctgatccattattgcttgaacgagcactgcgattgtccttttgaacttttttcgaaagca
298
396
caggtgcaaacgtacaattacggagatcatgaatagtcttgcctcattctttttggcgtcggccacttgatggaatgcggaatccgcgtagttttggtgc
397
495
tgttgtcatcgctctttttttacaatgacgcctgcaggaaagaaattcttctaccgacaaaggaagacagcaggtaaatcggcacgtgacctttctgcttg
496
594
tatttcattttgaggccacattcatctcttatttaacaatgcaaatgttattgtttctgtttcaagatatcattctgttgatgaatgtcaaccccaaaat
EVB→
595
693
aatctgattcttatcttgatcaaatggcatctgttttcttcacagtatttcaatacataccgagttagtattatacaaaacatataatagtgttataaat
694
792
attgagttacgaaattgttgccatcaagatgaagatggacttccatgtttctcgataaaaaattcgttttgatccatcaattccccgcatatggagctga
793
891
tcgctccatatacaagtaacgtgggacttgttgaatctgatgattgataggcttggtcttgtatgtcaatcaacgtgaaattggaggactatgatgatgg
892
990
aatttcaattatacaataggttgatgataaagttgctttggcaatgcgactgattccgccgtattgaagaatgacgacacaaacccagattcacgatgtg
991
1089
gccatacagcgaccaagatcaagcgtaacacggaaaatctttcgacaagatataatgatcaaaccagtggatcgatcaagattgacttcgatggaattgt
1030→
1090
1188
ttacaaattcttgatgtaaacacaattagagccaaaagtcctcacgtcttgaggtctactttatatgtgactactttggatttaccttcattgatttctc
1189
1287
agggtattcttcttggctatcggttcaaatgcgttagctacaaattttcgtcaattcactttctttgtatttaccaatgacttttttattggtgtgtttgt
1288
1386
cgtcgttggcagaatctatatctctcttcctcacctcgggtctgttgaaatgtagcctgcaatgtgttacatcccgcattccctaatgtcttatctgaga
1387
1485
tttcaagaaaatagctgctactatttttattcttatagctgatgcagcttacaattcgaacacgttattacgtatccatgtgcttcacaaacgcttcagaa
1486
1584
caataaacccaattagaaatctttcaaaacaaaaaagagcacaatcaccaacatgaaaaaaaatgcaattgcttacccgagattgctactaaacgcattg
1585
1683
ctgcaactcgaaaatgaaaaatttgcagcatacctagtctacaaggtgggttaaagctcacaatattttgtttatttcttttcagcactgactggtgtat
←1006
1684
1782
atcttattacttttgatgccaagaagaacgatcaaatacaaacagcagatttattcacgttcttcttggcatcaaaatttgaaatattcgatccaaatac
1783
1881
gtgtcgagtattgcccgtaagctgctttctattcaatcttcttatgcatacatggcagctaaattcgcattgttttggttttgttcaatttgtaaattaa
1882
1980
gtgcaattcaagcctaggaacgaattgtcttctaacatcaaagtttcacatgaataaaaccctttgattgtggaagacacttttttttcacagcatttatc
1981
2079
cattttgcatgatatcattttgtcaatgaaaaaactctttcaatcacatgtatttataacagtatttacattaaaacataattagagttaaatgcgattg
2080
2178
ttttttttcgatatctttaaagttatcttgataagagaaatcttgaatactaaaacatcactggaccagaattgttataaatgacccaatatttcgaac


←EVA
##
#
⇒
2.9PrB→
Pudb1→
HSPextL→
Pudb4→
#
←HSPextR

identified, including sequences with strong homologies to eukaryotic transcriptional regulatory motifs, particularly TATAA, CCAAT, and CTF/NF1 binding regions (Figure 31 and Table 8) (Lewin, 1990; Bienz, 1986). Sequences similar to those described for heat shock elements (nucleotide sequences recognized by heat shock transcription factor (HSF)) associated with transcription of heat shock genes (Lewin, 1990; Amin *et al.*, 1988; Bienz and Pelham; 1987) were also identified, as were putative cryptic prokaryotic transcriptional start and stop signals (Figure 32)

The nucleotide sequence obtained for the 1.4 kb and 0.7 kb PCR products was almost perfectly identical to that of the corresponding region of the *A. klebsiana* genome. The ORF identified in the 2.57 kb SalI restriction endonuclease fragment of *P. ultimum* extends through the entirety of the 1.4 kb PCR product to the λ- EMBL3 vector arm (Figures 30 and 31). Within this region, there is a single base transversion of a guanine in *A. klebsiana* to thymine in *P. ultimum*, resulting in the alteration of an amino acid residue from glutamate (*A. klebsiana*) to aspartate (*P. ultimum*) within the putative translation product encoded by this sequence. The 1.4 kb PCR product terminates before reaching any stop codon and/or 3' regulatory region which may be associated with the ORF.

3.III.b. Analysis of a suspected *P. ultimum hsp70* ORF Upon elucidation of the amino acid sequence of the ORF observed in the DNA sequence

Table 8: Regulatory sequences of the putative *P. ultimum hsp70* gene

Nucleotide sequences resembling regulatory consensus sequences of eukaryotic genes are listed according to type and location. Location is indicated consistent with the nucleotide numbering given in Figure 31, and is also given with regard to the start of the open reading frame (nucleotide 2348 in Figure 30), with negative values upstream (5'-wards) and positive values downstream (3'-wards) on the coding strand.

Regulator Type	Consensus Sequence	Sequence	Location	
			2.57 kb Sall Start	ORF Start
TATA Box	TATAAT	TATA	2273	-51
		TATTAA	2230	-94
		TATAAAT	2153	-171
CAAT Box	GGCCAATCT	GTCAATT	2329	+5
		GCCAATC	2297	-27
HSE	CnnGAAnnTC CnnG	CTTATCCATG	2317	-7
		CGAAACACATG	2281	-43
		CAATTGAACAATCAAG	2196	-128
		CGAACGTGTAG	2182	-142
		CCAGAATTG	2143	-181
		CTTGAATAC	2118	-206
		CTTGATAA	2103	-221
CTF/NF1	TGG(A/C)n_5G CCAA	TGTTTTAGCCAA	2290	-34

described above, the encoded putative translation product was screened against sequences deposited in the GenBank database from the American National Institutes of Health (NIH) using a non-redundant protein search with the BLAST alignment algorithm (<<*http://www.ncbi.nlm.nih.gov/BLAST/*>>) (Altschul *et al.*, 1990). This indicated that a hypothetical protein encoded by the *P. ultimum* ORF would be highly homologous to a number of *hsp70* genes from a variety of sources (Table 9), including the oomycetes *Achlya klebsiana* and *Bremia lactucae*. The amino acid sequence most similar to that of the putative *P. ultimum hsp70* is the *A. klebsiana* heat shock cognate 70 gene (*hsc70*) which forms an antisense gene pair with *nad-gdh* (almost 65% amino acid identity and over 80% similarity). Alignment of the primary amino acid sequences of the translation products of the *P. ultimum hsp70* and the *A. klebsiana hsc70* reveals a dramatic decrease in sequence identity and similarity as the C-terminal end of the amino acid sequence is approached. Long stretches of amino acid identity were observed in the N-terminal half of alignment of the two sequences, as were amino acid motifs characteristic of heat shock proteins (heat shock signals 1 and 2) (LéJohn *et al.*, 1994a; Bairoch, 1991; Chapelle *et al.*, 1986) (Figure 32). Peptidase sites bordering the second start methionine could indicate the synthesis of a propeptide sequence from the putative *hsp70*, which would be post-translationally modified in order to become active. Comparison of the nucleic

Table 9: *P. ultimum* translated ORF BLAST search results

Screening of GenBank conducted using the BLAST search system (Altschul *et al.*, 1990) identified several heat shock 70 proteins similar to the translation product of the *P. ultimum* ORF spanning the 2.57 kb SalI restriction endonuclease fragment and the 1.4 kb PCR product. The twelve most similar HSP70s are listed. Values for both % identity and % similarity were calculated for the overlap regions only. Amino acids are defined here as being similar if they have the same hydropathic classification as previously outlined (Table 1). *P(N)* represents the probability of the indicated overlaps being coincidental.

Accession Number	Organism	Total Overlap (Amino Acids)	% Identity	% Similarity	*P(N)*
U02504	*A. klebsiana*	508	64.96	81.69	2.6×10^{-210}
M27825	*B. lactucae*	417	60.43	76.98	9.5×10^{-191}
AF005993	*T. aestivum*	515	56.50	76.12	1.9×10^{-183}
AF034618	*S. oleracea*	516	56.40	75.97	1.9×10^{-183}
L41253	*L. esculentum*	515	55.73	76.50	6.9×10^{-183}
X13301	*P. hybrida*	515	56.31	75.53	3.3×10^{-182}
AF025951	*D. discoideum*	499	58.12	77.76	7.5×10^{-182}
X67711	*O. sativa*	515	55.53	75.92	5.7×10^{-181}
Z80223	*C. elegans*	526	55.89	75.86	6.8×10^{-181}
X74604	*A. thaliana*	513	55.56	76.02	7.8×10^{-181}
U35064	*O. tschawytshca*	318	53.46	75.47	2.2×10^{-180}
L26336	*H. sapiens*	521	56.24	76.20	1.6×10^{-179}

Figure 32: Alignment of the *A. klebsiana* HSC70 protein and predicted *P. ultimum hsp70* translation product amino acid sequences

Amino acid sequences were obtained from GenBank (*A. klebsiana* HSC70) or by translating known DNA sequences (*P. ultimum hsp70* translation product), and are given with the N-terminus as residue number 1. Alignment was done using a combination of the BLAST search algorithm (Altschul *et al.*, 1990) and GeneRunner™ 3.0 (©Hastings Software). Amino acid identity (*) and similarity (·) between the sequences are both indicated. The amino acid sequence disparity between the *P. ultimum* (aspartate) and *A. klebsiana* (glutamate) sequences is also indicated (#). The heat shock signals 1 (Chapelle *et al.*, 1986) and 2 (LéJohn *et al.*, 1994a; Bairoch, 1991) are indicated within double boxes. Peptidase sites as described in the text near the second start methionine residue in the *P. ultimum* HSP70 are indicated with downward pointing arrows.

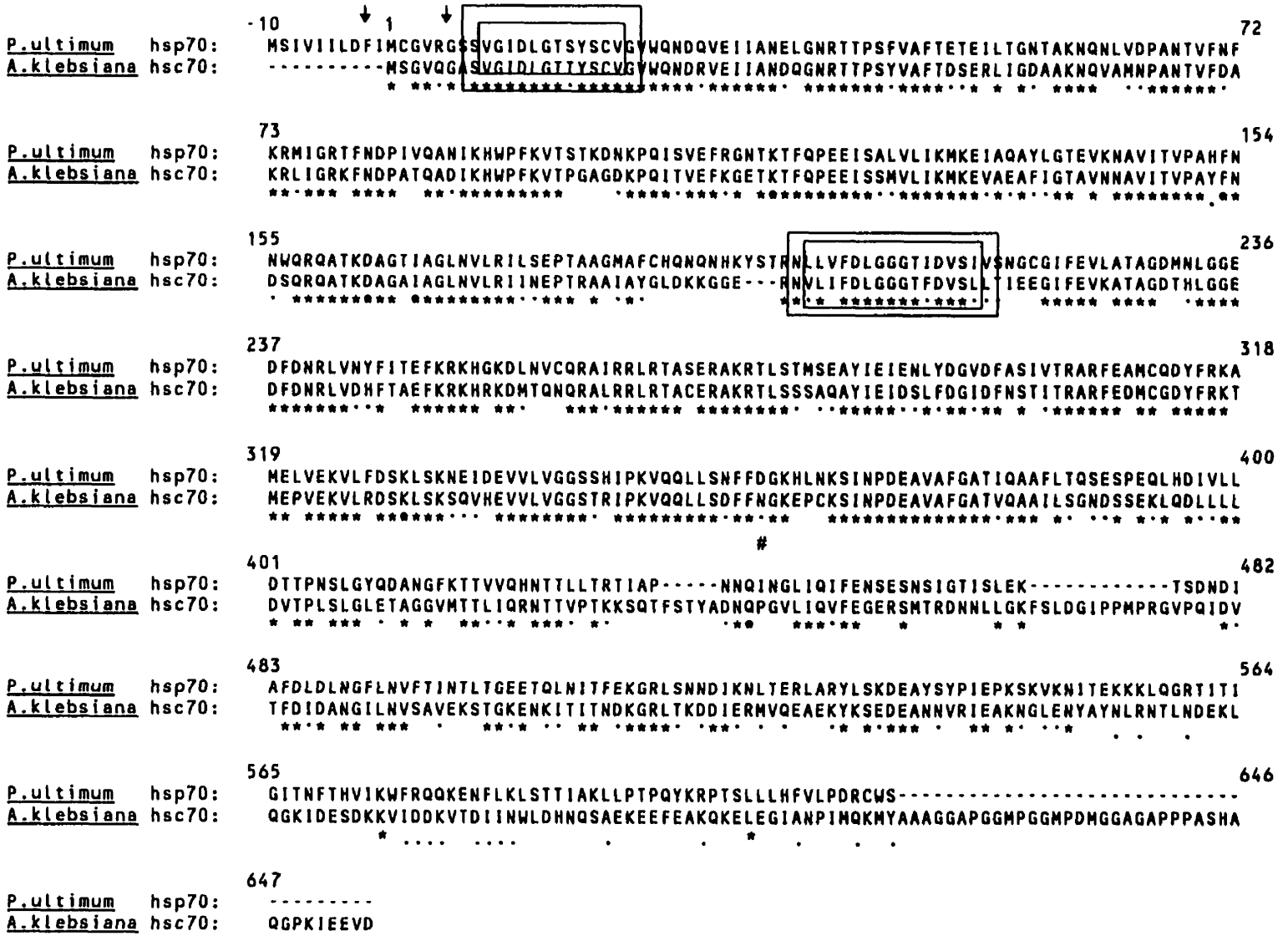
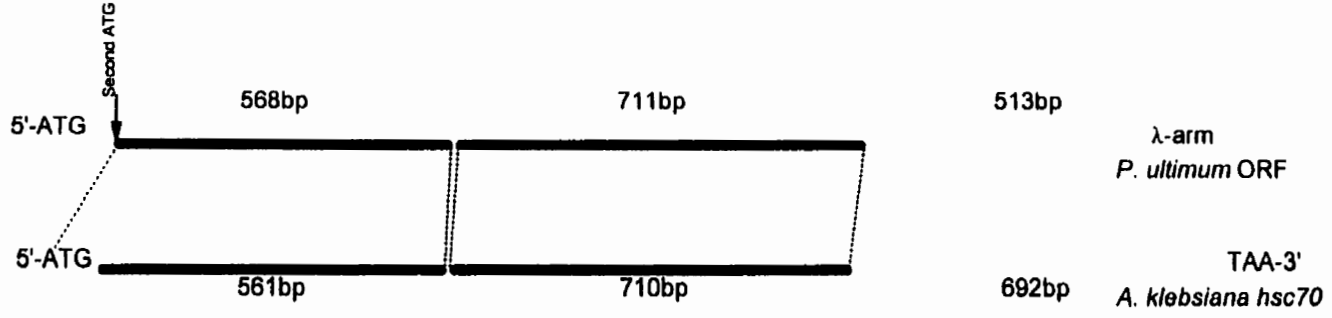

-10
1
72
P.ultimum hsp70: MSIVIILDFIMCGVRGSSVGIDLGTSYSCVGVWQNDQVEIIANELGNRTTPSFVAFTETEILTGNTAKNQNLVDPANTVFNF
A.klebsiana hsc70: ----------MSGVQGASVGIDLGTTYSCVGVWQNDRVEIIANDQGNRTTPSYVAFTDSERLIGDAAKNQVAMNPANTVFDA
73
154
P.ultimum hsp70: KRMIGRTFNDPIVQANIKHWPFKVTSTKDNKPQISVEFRGNTKTFQPEEISALVLIKMKEIAQAYLGTEVKNAVITVPAHFN
A.klebsiana hsc70: KRLIGRKFNDPATQADIKHWPFKVTPGAGDKPQITVEFKGETKTFQPEEISSMVLIKMKEVAEAFIGTAVNNAVITVPAYFN
155
236
P.ultimum hsp70: NWQRQATKDAGTIAGLNVLRILSEPTAAGMAFCHQNQNHKYSTRNLLVFDLGGGTIDVSIVSNGCGIFEVLATAGDMNLGGE
A.klebsiana hsc70: DSQRQATKDAGAIAGLNVLRIINEPTRAAIAYGLDKKGGE---RNVLIFDLGGGTFDVSLLTIEEGIFEVKATAGDTHLGGE
237
318
P.ultimum hsp70: DFDNRLVNYFITEFKRKHGKDLNVCQRAIRRLRTASERAKRTLSTMSEAYIEIENLYDGVDFASIVTRARFEAMCQDYFRKA
A.klebsiana hsc70: DFDNRLVDHFTAEFKRKHRKDMTQNQRALRRLRTACERAKRTLSSSAQAYIEIDSLFDGIDFNSTITRARFEDMCGDYFRKT
319
400
P.ultimum hsp70: MELVEKVLFDSKLSKNEIDEVVLVGGSSHIPKVQQLLSNFFDGKHLNKSINPDEAVAFGATIQAAFLTQSESPEQLHDIVLL
A.klebsiana hsc70: MEPVEKVLRDSKLSKSQVHEVVLVGGSTRIPKVQQLLSDFFNGKEPCKSINPDEAVAFGATVQAAILSGNDSSEKLQDLLLL
#
401
482
P.ultimum hsp70: DTTPNSLGYQDANGFKTTVVQHNTTLLTRTIAP-----NNQINGLIQIFENSESNSIGTISLEK-----------TSDNDI
A.klebsiana hsc70: DVTPLSLGLETAGGVMTTLIQRNTTVPTKKSQTFSTYADNQPGVLIQVFEGERSMTRDNNLLGKFSLDGIPPMPRGVPQIDV
483
564
P.ultimum hsp70: AFDLDLNGFLNVFTINTLTGEETQLNITFEKGRLSNNDIKNLTERLARYLSKDEAYSYPIEPKSKVKNITEKKKLQGRTITI
A.klebsiana hsc70: TFDIDANGILNVSAVEKSTGKENKITITNDKGRLTKDDIERMVQEAEKYKSEDEANNVRIEAKNGLENYAYNLRNTLNDEKL
565
646
P.ultimum hsp70: GITNFTHVIKWFRQQKENFLKLSTTIAKLLPTPQYKRPTSLLLHFVLPDRCWS----------
A.klebsiana hsc70: QGKIDESDKKVIDDKVTDIINWLDHNQSAEKEEFEAKQKELEGIANPIMQKMYAAAGGAPGGMPGGMPDMGGAGAPPPASHA
647
P.ultimum hsp70: ---------
A.klebsiana hsc70: QGPKIEEVD

Figure 33: Areas of sequence similarity between the *A. klebsiana hsc70* gene and the suspected *P. ultimum hsp70* gene.

Regions of homology at the nucleic acid sequence level illustrated using a schematic representation of the sequenced portion of the putative *P. ultimum hsp70* gene. Homologous regions are connected by dotted lines. Only the ORF, and not the 5' regulatory region, was examined.


Second ATG
5'-ATG
568bp
711bp
513bp
λ-arm
P. ultimum ORF
5'-ATG
561bp
710bp
692bp
TAA-3'
A. klebsiana hsc70

acid sequences of the putative *P. ultimum hsp70* and the *A. klebsiana hsc70* genes indicate a 70.4% sequence identity in two blocks representing 69.6% of the sequenced region of the putative *P. ultimum hsp70* ORF (1273 bases) (Figure 33).

3.IV. Discussion of *P.ultimum* Stress Response and ORF Sequence Data

3.IV.a. *P. ultimum* growth and morphology are affected by temperatures in excess of 28°C The effects of temperature upon growth, morphology and protein production of *P. ultimum* (Figures 25, 26 and 27) indicate an optimum growth temperature of between approximately 22°C (room temperature) and 28°C, with substantial growth at temperatures of up to 31°C, although this is accompanied by some abnormal morphological features. Beyond this temperature, cell growth is either halted entirely or severely retarded, with cell death observed after 24 hours at 34°C and higher. These data imply that a heat stress response likely occurs when *P. ultimum* is grown at greater than a threshold temperature of between 28°C and 34°C. The production of sporangia-like bodies may also be an indicator of such a response, as cellular differentiation has been linked to stress protein synthesis in some organisms (Heikkila, 1993; Zimmerman *et al.*, 1983). The 70kD protein produced in response to heat stress is of the range of molecular weights from 68 to 74kD (Nagao *et al.*, 1990) typical of the HSP70 stress response

proteins, while the 55kD protein also produced in response to heat stress has an apparent molecular size similar to that of a mitochondrial heat shock cognate protein HSP58 of *Tetrahymena* (Lindquist and Craig, 1988; McMullin and Hallberg, 1988). The production of proteins of similar molecular sizes (70kD and 56kD, respectively) has previously been observed during nutritional stress in *A. klebsiana* both with and without supplementation of the medium with L-glutamine (Braithwaite, 1987). It is not known if the 70kD and 55kD protein bands of *P. ultimum* correspond to single proteins or more than one protein with similar electrophoretic mobilities.

3.IV.b. <u>A transcribable ORF is present</u> Sequencing of the 1.4 kb PCR product of *P. ultimum* in this study in conjunction with previous analysis of the adjacent 2.57 kb SalI restriction endonuclease fragment identified a region of over 1800 bp forming a single, uninterrupted ORF (Figure 31) almost identical in sequence to a similar ORF and 5' regulatory regions found in *A. klebsiana* (LéJohn, unpublished data). The region immediately 5' of the ORF is replete with typical eukaryotic transcriptional regulatory signals, including TATA- and CCAAT-like sequences. This region extends over more than 100 bp upstream of the first of two ATG start codons (Table 7). In addition to these eukaryotic consensus sequences, putative cryptic prokaryotic transcriptional start and stop signals were also identified (Figure 31). These regions may have significance if this *P. ultimum* ORF is to be

transcribed and expressed *in vitro* in a bacterial host, as was attempted with the *A. klebsiana* antisense gene pair in *E. coli*. Of the four base pair differences between the *A. klebsiana* and *P. ultimum* identified, only one found within the PCR amplified region causes an alteration to the suspected translation product. This is a conservative mutation converting a glutamate residue (*A. klebsiana*) to aspartate (*P. ultimum*), and should not have much of an effect upon the protein product. Since this base pair variance occurs in the 1.4 kb fragment obtained by PCR amplification, it is possible that this mutation is an experimental artifact in either the *P. ultimum* or the *A. klebsiana* sequence given the relatively low fidelity of the Taq polymerase used during the PCR process which has an error rate of $5x10^{-5}$ bases (Watson *et al.*, 1992). In a similar manner, the error rate of the sequencing procedure used of $2.5x10^{-4}$ bases may also be a factor in the observed sequence differences. Such possibilities also apply for all the other differences between the *A. klebsiana* and *P. ultimum* sequences observed.

A second, silent mutation was also identified between two potential start codons of the ORF (Figure 31). This region has no corresponding sequence in the *A. klebsiana* antisense gene pair *hsc70*, and it is not known which start codon would be used as the start site for translation. It is interesting to note that hydrophobic regions similar to the ten N-terminal amino acids encoded by the *P. ultimum* ORF show similarity to propeptide sequences necessary

for protein transport through the membrane of the endoplasmic reticulum (Verner and Schatz, 1988). Of further interest is the identification of several peptidase sites near the second start methionine (amino acid 11), including chymotrypsin (amino acid 9) and the peptidases endoproteinase arg-C, trypsin and carboxypeptidase B (all at amino acid 15). Further investigation of these characteristics was not conducted.

The final two nucleotide sequence variations identified between the *A. klebsiana* and *P. ultimum* ORFs are found adjacent to one another in the region immediately 5' of the first start ATG of the ORF, but appear to have no effect upon the 5' regulatory consensus sequences identified (Table 7). The almost identical nature of these sequences in *A. klebsiana* and *P. ultimum*, including not only the ORF but also the 5' regulatory sequences observed and untranscribed regions, indicates that the conservation of this locus is likely vital for cell survival, and is therefore likely transcribed and translated. It is important to note at this point that the almost perfect identity between the *P. ultimum* sequence reported here and the corresponding sequence in *A. klebsiana* were initially obtained from genomic library clones identified independently several years apart. Therefore, the sequence information given here is almost certainly not from cross contamination of clones or resequencing of the same clone by accident.

3.IV.c. <u>The *P. ultimum* ORF may encode a putative *hsp70* gene</u> If the ORF identified in *P. ultimum* is assumed to be transcribed and translated, the type of protein product which could be generated by this nucleotide sequence must be determined. Based upon the sequence data obtained, it is apparent that the ORF, if transcribed and translated, has the potential to produce a heat shock 70 protein (HSP70). Evidence in support of this includes: the presence of heat shock element transcriptional regulators 5' of the ORF (Table 7 and Figure 31); the presence of amino acid arrangements typical of HSP70 proteins (Figure 32); significant amino acid and nucleic acid sequence homology with other heat shock 70 proteins and genes (Table 8 and Figures 32 and 33); and a pattern of amino acid homology corresponding to the predicted functional domains of members of the HSP70 protein family (Figure 32).

Amino acid and nucleotide sequences characteristic of HSP70s were identified through examination of the *P. ultimum* ORF. At the protein level, amino acid sequences characteristic of HSP70 proteins, termed heat shock signals 1 and 2, were identified (LéJohn, 1994a; Bairoch, 1991; Chappell *et al.*, 1986). These amino acid motifs consist of either 11 amino acids with a hydrophobic-hydrophilic-hydrophobic profile or 13 amino acids of hydrophobic character, respectively. These sequences are maintained in the

heat shock 70 proteins of a wide variety of organisms with a high level of conservation.

The 5' untranscribed region upstream of the ORF contains several heat shock element (HSE) sequences, which are transcriptional regulatory elements characteristic of *hsp70* genes (Amin *et al.*, 1988). The HSE has a consensus sequence consisting of one to three directly adjacent 14 bp elements (Bienz and Pelham, 1987) (Table 7), which identify regions of 27 bp to which transcriptional factors specific for the heat shock response (HSFs) will bind. This interaction can result in either the transcription of otherwise untranscribed genes, or the increased transcription in organisms where HSFs are necessary for a basal level of transcription at all times (Deshaies *et al.*, 1988). These sequences can be located anywhere within 400 bp 5' of the start ATG codon of the *hsp70* gene (Bienz and Pelham, 1987). In the case of the *P. ultimum* ORF, several sequences similar to the HSE consensus sequence exist in the 5' untranslated region within 230 bp of the first start codon (Table 7 and Figure 31).

The similarities between heat shock protein 70 amino acid sequences deposited in GenBank and the *P. ultimum* ORF translation product also point towards the *P. ultimum* ORF encoding an *hsp70*-like protein. At the amino acid sequence level, a selection of twelve HSP70s share at least 55% identity and 75% similarity with the *P. ultimum* ORF translation product, over

an average sequence overlap of about 490 amino acids (80% of the 610 amino acids encoded by the *P. ultimum* ORF sequenced to date) (Table 8). Alignment of nucleic acid sequences of the *P. ultimum* ORF and the *A. klebsiana hsc70* indicates a 70% identity between the two sequences over 70% of the sequenced portion of the *P. ultimum* ORF, with conserved regions closer to the 5' end of the ORF than the 3' end (Figure 33). This follows a pattern predicted by the hypothesized functional domains of the HSP70s. Members of this protein family are believed to consist of two general domains, an N-terminal ATPase domain and a C-terminal target binding domain (Gething and Sambrook, 1992). As different HSP70s will interact with different target proteins, it follows that there should be a large degree of variability between the C-termini of HSP70s, associated with a tendency towards sequence conservation of the N-termini. Comparison of the amino acid sequences of the putative *P. ultimum* HSP70 with the *A. klebsiana* HSC70 indicates a high degree of identity and similarity between the two proteins at the N-terminus and over the first two thirds of their overlap. This homology is rapidly lost over an area of less than one hundred amino acids until the two protein products are completely dissimilar at their C-terminal ends (Figure 32). This trend is reflected by the high degree of nucleic acid sequence identity between the corresponding genes of these

CONCLUSIONS

1. The Antisense Gene Pair of *A. klebsiana*

While transcript production from both recombinant molecules containing the antisense gene pair of *Achlya klebsiana* occurred in prokaryotic and eukaryotic hosts, these products are not suspected to encode functional HSC70 or NAD-GDH proteins. The transcripts generated may instead arise from regions within the antisense gene pair resembling consensus sequences for prokaryotic and eukaryotic transcriptional promoter and terminator regions. This observation must be qualified by the fact that the resemblance of a DNA sequence to a transcriptional regulatory consensus sequence is not necessarily a prerequisite for nor an indication of biological function. This is especially true for oomycetes, whose transcriptional promoter regions have shown incompatibility with those of many other organisms (Judelson *et al.*, 1992). Similarly the degree of incompatibility of post-transcriptional modification machinery such as spliceosomes between oomycetes and other eukaryotes has not been determined.

Of the hypothetical transcripts and translation products which could arise from the combinations of cryptic consensus sequences identified, several transcripts would be of a predicted size similar to those transcripts experimentally observed. Given the provisions mentioned above, it would appear that the multiple transcripts produced in both prokaryotic and eukaryotic hosts is due to the concurrent but not necessarily simultaneous transcription of overlapping transcripts from the antisense gene pair region using either the same (prokaryotic) or different (eukaryotic) DNA strand as template. None of the hypothetical prokaryotic transcripts can generate a protein product greater than 9 amino acids in length which corresponds to the coding region of either member of the *A. klebsiana* antisense gene pair. The same is true for all eukaryotic transcripts save 1L, 1M, 2L, 2M and 2N, which may or may not produce a protein corresponding to the *hsc70* ORF. These observations question the potential capacity of the antisense gene pair to be expressed *in vitro*.

2. The NAD-GDH of *P. ultimum*

The characterization of the *Pythium ultimum* NAD-GDH in this thesis demonstrated a number of shared characteristics with the NAD-GDHs of *Achlya klebsiana* and *Pythium debaryanum*. An inherent instability mitigated by storage in high concentrations of glycerol has also been demonstrated for the *A. klebsiana* NAD-GDH (Yang, 1991). The use of $NADP^+$ as an activator

by *P. ultimum* NAD-GDH is characteristic of oomycetes NAD-GDHs in general (LéJohn, 1975). $NADP^+$ showed a more marked effect upon enzyme velocity in the reductive amination reaction, while substrate affinity was more greatly affected for the oxidative deamination reaction. Similar results have been reported for the NAD-GDHs of *A. klebsiana* and *P. debaryanum* (Stevenson, 1974). The effect of $NADP^+$ upon NAD-GDH kinetics also indicated the presence of two allosteric sites involved in the reductive amination reaction, and one or two for the reverse reaction. This could be due to modulation of the NAD-GDH mediated conversion of α-ketoglutarate to L-glutamate by NH_4^+, which is not only one of the substrates for this reaction but also an inhibitor of NAD-GDH in *P. debaryanum* and *A.klebsiana* (Stevenson, 1974). The presence of two distinct pH optima for the reductive amination and oxidative deamination reactions is not a characteristic shared with the NAD-GDH of *A. klebsiana*, although it is a feature of the NAD-GDH of *P. debaryanum*. The induction of NAD-GDH by L-glutamate is characteristic of several oomycetes, including *P. debaryanum* (LéJohn and Stevenson, 1970), with L-glutamine acting as an inducer of *A. klebsiana* NAD-GDH (Yang and LéJohn, 1994; Yang, 1991; Braithwaite, 1987). Levels of induction were also observed to be significantly lower in cells kept in complex growth medium in comparison to those purged of carbon and nitrogen sources prior to L-glutamate induction.

This basic characterization of the NAD-GDH of *P. ultimum* indicates that it shares several characteristics of oomycete NAD-GDHs in general, and of the NAD-GDHs of *A. klebsiana* and *P. debaryanum* in particular. However, there are minor differences between the *P. ultimum* and *A. klebsiana* NAD-GDHs which do not exist between the enzymes of *P. ultimum* and *P. debaryanum*.

3. The *hsp70* of *P. ultimum*

Analysis of the effect of temperature upon the growth and morphological characteristics of *P. ultimum* indicated that the span of temperatures from 28°C to 31°C likely include a stress response to heat, and also correspond to the highest temperatures tested which are not lethal after 24 hours exposure. Fluorographic analysis of proteins pulse-labeled with ^{35}S-methionine and cysteine during short duration heat stress confirmed the production of protein bands unique to heat stressed cells of 55 and 70kD at these heat stress inducing temperatures. These protein bands may or may not correspond to single proteins, and may or may not correspond to similar proteins previously shown to be produced by *A. klebsiana* in response to nutritional stress (Braithwaite, 1987).

The incomplete sequence data of the suspected *hsp70* gene identified an ORF and 5' region replete with several types of eukaryotic transcription initiation signals, including those specific for heat shock proteins. The amino acid sequence encoded by this ORF showed a high degree of homology with

HSP70s from a variety of sources, and contained the highly conserved peptide motifs heat shock signals 1 and 2. Unfortunately, the 3' end of this suspected gene was not contained within the sequenced region, and therefore cannot be part of the genomic clone used as a template for the PCR amplification to obtain sequencing template.

4. Summary and Prospectus

The results of the investigations presented in this thesis indicate that multiple transcripts arise from recombinant molecules containing the antisense gene pair of *A. klebsiana* in both prokaryotic and eukaryotic hosts. This potential, in association with evidence suggesting the existence of an antisense gene pair like arrangement in *P. ultimum* (LéJohn, unpublished data), led to the investigation of the NAD-GDH of this organism to determine if it shared any biochemical similarities with the NAD-GDH of *A. klebsiana.* The NAD-GDH of *P. ultimum* was shown to be typical of the oomycete NAD-GDHs (including inherent enzyme instability during storage, action of $NADP^+$ as an activator, the presence of more than one allosteric site) with some characteristics such as pH optima and induction by L-glutamate rather than L-glutamine which differentiate it from the NAD-GDH of *A. klebsiana.* The identification of a suspected *P. ultimum hsp70* gene almost identical to a counterpart sequence in *A. klebsiana* associated with the genomic locus of the antisense gene pair was also been reported here, and further increased

suspicions that *P. ultimum* has a sequence arrangement like the *A. klebsiana* antisense gene pair. More evidence to support this contention arose from the sequence data obtained for an exon X like region PCR amplified from *P. ultimum* genomic DNA (*Appendix A*, below), which indicated that this PCR product is almost identical to the region of the *A. klebsiana nad-gdh* gene which overlaps the *hsc70* of the antisense gene pair. Briefly then, this thesis presents evidence that *P. ultimum* and *A. klebsiana* possess biochemically similar but non-identical NAD-GDHs which, in *A. klebsiana*, is encoded by a previously identified member of an antisense gene pair (LéJohn *et al.*, 1994a and 1994) which can generate multiple transcripts *in vitro*, and resides in a locus which has regions with nucleotide sequences almost perfectly identical to sequences identified in *P. ultimum*.

The research presented in this thesis provide as a useful starting point for further investigation of the antisense gene pair of *A. klebsiana*. The presence of prokaryotic and eukaryotic promoter and terminator consensus sequences within the *nad-gdh* gene may preclude the use of cDNAs of the antisense gene pair for *in vitro* expression of either the *A. klebsiana nad-gdh* or *hsc70*. However, studies of the interaction of the two proteins of the antisense gene pair will be primarily concerned with the region of gene overlap, which has been shown here to entirely lack transcriptional regulatory consensus sequences on either DNA strand. It may therefore be possible to express

these regions by themselves *in vitro* to study their ability to interact with one another. In a similar fashion, the *in vitro* expression of the *hsp70* sequenced here would allow HSP70 characterization and generation of anti-HSP70 antibodies. Anti-HSP70 antibodies could then be used to help determine the stress conditions under which the *P. ultimum hsp70* is predominantly expressed, HSP70 purification by affinity chromatography for more detailed structural analysis and investigation of the prevalence and distribution of this HSP70 amongst other oomycetes. This research area could be conducted as a supplement to the probing of genomic DNA and cDNA libraries of other oomycetes for the *P. ultimum hsp70* gene. Unfortunately, the presence of cryptic prokaryotic transcriptional promoter and terminator consensus sequences within the *hsp70* sequence may make HSP70 expression *in vitro* difficult, but this is by no means certain.

In the shorter term the sequencing of the 3' end of the putative *hsp70* to give the complete *hsp70* sequence can be accomplished. This can be best conducted through the screening of either genomic and/or cDNA libraries from *P. ultimum* and *A. klebsiana*, using the extreme 3' end of the region of the *hsp70* already sequenced as a probe. This region is the most suitable segment of the *hsp70* to use as a probe since it should be dissimilar from other *hsp70*s and should therefore reduce any hybridization to clones representing non-target *hsp70* genes. Sequence information for the 3' end of

the putative *hsp70* will allow a more accurate appraisal of the potential for this ORF to be transcribed and translated.

The nearly identical nature of the sequenced regions of the putative *hsp70* genes from *P. ultimum* and *A. klebsiana*, when viewed in conjunction with the PCR products previously generated from *P. ultimum* corresponding to the antisense gene pair of *A. klebsiana* (LéJohn, unpublished data), make it tempting to hypothesize that a similar antisense gene exists in *P. ultimum*. Identification of the *nad-gdh* gene of *P. ultimum* would allow the hypothesis of a *P. ultimum nad-gdh:hsc70* antisense gene pair to be confirmed or refuted. In addition to this, sequence information derived for a *P. ultimum nad-gdh* would permit a comparison between the amino acid primary structures of the *P. ultimum* and *A. klebsiana* NAD-GDHs, which may help indicate regions of (dis)similarity which could be associated with the differences and similarities of kinetic characteristics demonstrated in this thesis.

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R: Paper in Russian.

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A. Sequencing of a *Pythium ultimum* Genomic Region Similar to *Achlya klebsiana*'s Antisense Gene Pair

Evidence that *P. ultimum* possesses an antisense gene pair similar to that of *A. klebsiana* was obtained through two different PCR amplifications. One consisted of the amplification of a 0.95 kb region corresponding to intron 9 as well as adjacent regions of exons IX and X from *P. ultimum* (LéJohn, unpublished data). The second was the PCR amplification of a fragment of approximately 1.8 kb using *P. ultimum* genomic DNA as template and primers complementary to the end sequences of the *A. klebsiana nad-gdh* exon X. A control amplification was done using *Achlya* genomic DNA as template, which also yielded a 1.8 kb amplification product.

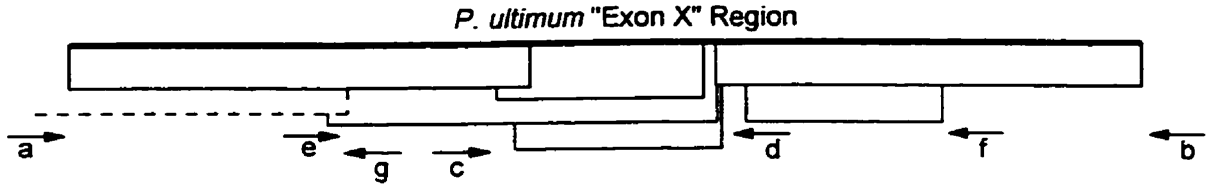
A.I. Results and Discussion

A.I.a. <u>Amplification of an "Exon X" sequence from *P. ultimum*</u> PCR amplification and sequencing of a 1.8 kb PCR product generated from *P. ultimum* genomic DNA which partly overlaps a previously sequenced 0.95 kb PCR product (LéJohn, unpublished data) identified a large degree of sequence identity between it and the *A. klebsiana nad-gdh* exon X (Figure A-

Figure A-1: Sequencing strategy and primers used for *P. Ultimum* Exon X PCR amplification and sequencing

A 1.8 kb PCR amplification product for DNA sequencing was generated from a genomic DNA template using primers 5'ExonXPst and 3'ExonXPst (Methods and Materials *2.IV.c. Polymerase chain reaction*, above). DNA sequencing was conducted as described (Methods and Materials *4.II.b. DNA Sequencing*, above). Some sequence data was obtained through the direct sequencing of the PCR product (primers Pu5'ExonX, Pu3'ExonX and Pr3'Ex) or by the sequencing of PCR product ligated into pBAD-TOPO (Invitrogen) according to the maufacturer's instructions (primers Pyt5'ExonX, Pyt3'ExonX and Pyt5'Ex-X-R) (Appendix B, below) or pBluescript (fragment 0.95 kb PCR, dashed lines (LéJohn, unpublished data)) (Methods and Materials *2.I. Recombinant Molecule Construction*, above). The sequencing strategy for the 1.8 kb PCR product is given (**A.**) with labeled arrows indicating primer locations and boxes outlining the extent of reliable sequence data from each primer. Also presented are the oligonucleotide primers used (**B.**) for both the initial PCR amplification and DNA sequencing of this region.

A.



B.

Designation	Primer Name	Primer Sequence (5' to 3')
a	5'ExonXPst	GACTGCAGTTCCTTTTGCTTGGCTTCGAA
b	3'ExonXPst	AGCTGCAGTCAAATGAAAGCTGACGTCAA
c	Pyt5'Ex-X	TGGACTGTGGCACCCAAAGCCACAG
d	Pyt3'ExR-X	TTCGATGTGTCGCTTTTGACCATTG
e	Pyt5'Ex-X-R	GTCATCGAACGCTCACCTTCAAAC
f	Pyt3'ExR-X-2	GTTCATTGGTACTGCTGTTTACAAC
g	T7	GTAATACGACTCACTATAGGGC

2). Overall, these sequences have a greater than 99% nucleic acid sequence identity. Of the non-identical nucleotides, three were unconfirmed. This exceedingly high level of identity yields 98% and 99% amino acid identity for the predicted HSC70 and NAD-GDH proteins, respectively (Figures A-3 and A-4). Of the ten non-identical amino acids in the HSC70 sequence, three were not deciphered due to the presence of unconfirmed nucleotides in their codons, but must encode hydrophobic amino acids, and therefore will either represent conservative amino acid changes or no change at all. None of the unidentified bases have an effect upon the predicted NAD-GDH protein.

All of the confirmed discrepancies with the *A. klebsiana* sequence were transversions (three T→A, two C→G and one G→C) (Figure A-4). These are expressed in the suspected NAD-GDH protein as three conservative amino acid changes and one non-conservative change, while the *hsp70* strand encodes four conservative and two non-conservative amino acid changes. All unconfirmed nucleotides are in codons which will not be altered on the *nad-gdh* strand, and must encode hydrophobic amino acids on the *hsc70* strand. This bias in favour of nucleotide changes which encode conservative amino acid alteration provides may reflect an evolutionary pressure to minimize alterations to the encoded protein, which is what would be expected if the *P. ultimum* "exon X" is part of a functional gene.

Figure A-2: *P. ultimum* PCR product and *A. klebsiana nad-gdh* exon X nucleotide sequence alignment.

Analysis of DNA sequence data and nucleic acid alignments was conducted using the computer program GeneRunner™ 3.0 (©Hastings Software). The DNA sequence is given 5'→3', with only the sequence of the strand encoding the *A. klebsiana nad-gdh* gene of a previously described antisense gene pair (LéJohn *et al.*, 1994c) (top line) and the corresponding strand of the *P. ultimum* PCR product (bottom line) given. Indicated are nucleotide sequence discrepancies (*), primer locations for DNA sequencing within the sequenced area (underlined, with identity and direction indicated); and unconfirmed bases in the *P. ultimum* PCR product (?).


1
110
A. klebsiana exon X: TTCCTTTTGC TTGGCTTCGA ATTCTTCCTT TTCAGCGGAT TGGTTGTGGT CGAGCCAGTT AATGATGTCG GTGACCTTGT CGTCAATGAC CTTCTTGTCG CTTTCATCGA
P. ultimum "exon X": TTCCTTTTGC TTGGCTTCGA ATTCTTCCTT TTCAGCGGAT TGGTTGTGGT CGAGCCAGTT AATGATGTCG GTGACCTTGT CGTCAATGAC CTTCTTGTCG CTTTCATCGA
111
220
A. klebsiana exon X: TCTTGCCTTG GAGCTTTTCG TCGTTGAGGG TGTTGCGGAG GTTGTAGGCG TAGTTTTCAA GACCGTTCTT GGCTTCAATG CGGACGTTGT TGGCTTCATC TTCCGACTTG
P. ultimum "exon X": TCTTGCCTTG GAGCTTTTCG TCGTTGAGGG TGTTGCGGAG GTTGTAGGCG TAGTTTTCAA GACCGTTCTT GGCTTCAATG CGGAGCTTGT TGGCTTCATC TTCCGACTTG
**
221
330
A. klebsiana exon X: TACTTTTCAG CTTCTTGCAC CATGCGTTCA ATATCATCCT TGGTGAGGCG ACCCTTGTCG TTGGTAATGG TAATCTTGTT TTCTTTACCA GTCGACTTTT CGACGGCAGA
P. ultimum "exon X": TACTTTTCAG CTTCTTGCAC CATGCGTTCA ATATCATCCT TGGTGAGGCG ACCCTTGTCG TTGGTAATGG TAATCTTGTT TTCTTTACCA GTCGACTTTT CGACGGCAGA
331
440
A. klebsiana exon X: CACGTTCAAG ATACCGTTGG CATCAATGTC GAAGGTGACA TCAATTTGTG GAACACCACG AGGCATTGGA GGAATACCAT CGAGGGAGAA CTTCCCGAGC AAGTTGTTGT
P. ultimum "exon X": CACGTTCAAG ATACCGTTGG CATCAAGTTC GAAGGTGACA TCAATTTGTG GAACACCACG AGGCATTGGA GGAATACCAT CGAGGGAGAA CTTGCCGAGC AAGTTGTTGT
•
441
550
A. klebsiana exon X: CACGGGTCAT CGAACGCTCA CCTTCAAACA CTTGAATCAA CACACCAGGT TGGTTGTCAG CGTAGGTGGA GAAAGTTTGC GACTTCTTGG TTGGCACAGT AGTGTTACGT
P. ultimum "exon X": CACGGGTCAT CGAACGCTCA CCTTCAAACA CTTGAATCAA CACACCAGGT TGGTTGTCAG CGTAGGTGGA GAAAGTTTGC GACTTCTTGG TTGGCACAGT AGTGTTACGT
Pyt5'Ex-X-2
551
660
A. klebsiana exon X: TGGATGAGGG TAGTCATGAC ACCACCAGCA GTTTCCAAAC CAAGAGAGAG AGGAGTGACA TCAAGAAGCA ACAAGTCTTG GAGCTTTTCA GACGAGTCGT TACCGCTCAA
P. ultimum "exon X": TGGATGAGGG TAGTCATGAC ACCACCAGCA GTTTCCAAAC CAAGAGAGAG AGGAGTGACA TCAAGAAGCA ACAAGTCTTG GAGCTTTTCA GACGAGTCGT TACCGCTCAA
661
770
A. klebsiana exon X: AATGGCAGCT TGGACTGTGG CACCGAAAGC AACAGCTTCA TCAGGGTTGA TCGACTTGCA TGGTTCCTTG CCGTTGAAGA AGTCCGAAAG CAATTGTTGG ACCTTTGGGA
P. ultimum "exon X": AATGGCAGCT TGGACTGTGG CACCGAAAGC AACAGCTTCA TCAGGGTTGA TCGACTTGCA TGGTTCCTTG CCGTTGAAGA AGTCCGAAAG CAATTGTTGG ACCTTTGGGA
Pyt5'Ex-X
771
880
A. klebsiana exon X: TACGGGTGGA ACCACCGACA AGGACGACTT CATGGACTTG GCTCTTGGAG AGCTTCGAGT CACGGAGGAC CTTTTCAACA GGTTCCATAG TCTTGCGGAA GTAGTCACCG
P. ultimum "exon X": TACGGGTGGA ACCACCGACA AGGACGACTT CATGGACTTG GCTCTTGGAG AGCTTCGAGT CACGGAGGAC CTTTTCAACA GGTTCCATAG TCTTGCGGAA GTAGTCACCG
881
990
A. klebsiana exon X: CACATGTCTT CGAAACGGGC ACGGGTGATG GTGGAGTTGA AATCAATACC ATCGAAGAGC GAGTCGATTT CAATGTAAGC TTGGGCCGAA GAAGAAAGAG TACGCTTGGC
P. ultimum "exon X": CACATGTCTT CGAAACGGGC ACGGGTGATG GTGGAGTTGA AATCAATACC ATCGAAGAGC GAGTCGATTT CAATGTAAGC TTGGGCCGAA GAAGAAAGAG TACGCTTGGC
991
1100
A. klebsiana exon X: ACGTTCACAA GCGGTACGAA GACGGCGAAG GGCACGTTGG TTTTGGGTCA TATCCTTGCG GTGCTTGCGC TTGAATTCAG CGGTAAAGTG GTCGACGAGG CGGTTATCGA
P. ultimum "exon X": ACGTTCACAA GCGGTACGAA GACGGCGAAG GGCACGTTGG TTTTGGGTCA TATCCTTGCG GTGCTTGCGC TTGAATTCAG CGGTAAAGTG GTCGACGAGG CGGTTATCGA
1101
1210
A. klebsiana exon X: AATCTTCACC ACCCAAGTGG GTATCACCAG CAGTAGCCTT GACTTCGAAG ATACCTTCTT CAATGGTCAA AAGCGACACA TCGAAGGTAC CACCACCAAG ATCGAAAATG
P. ultimum "exon X": AATCTTCACC ACCCAAGTGG GTATCACCAG CAGTAGCCTT GACTTCGAAG ATACCTTCTT CAATGGTCAA AAGCGACACA TCGAAGGTAC CACCACCAAG ATCGAAAATG
Pyt3'ExR-X

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                    1211                                                                                                1320
A. klebsiana exon X: AGAACATTGC GTTCACCACC CTTCTTGTCA AGACCGTAGG CAATGGCGGC ACGAGTAGGT TCGTTAATGA TACGAAGGAC GTTAAGACCA GCAATGGCAC CAGCATCCTT
P. ultimum "exon X": AGAACATTGC GTTCACCACC CTTCTTGTCA AGACCGTAGG CAATGGCGGC ACGAGTAGGT TCGTTAATGA TACGAAGGAC GTTAAGACCA GCAATGGCAC CAGCATCCTT

                    1321                                                                                                1430
A. klebsiana exon X: GGTAGCTTGA CGTTGCGAGT CGTTGAAATA AGCTGGGACG GTAATGACAG CGTTGTTAAC AGCAGTACCA ATGAAGGCTT CAGCAACTTC CTTCATCTTG ATCAAAACCA
P. ultimum "exon X": GGTAGCTTGA CGTTGCGAGT CGTTGAAATA AGCTGGGACG GTAATGANAG CGTTGTAAAC AGCAGTACCA ATGAAGGCTT CAGCAACTTC CTTCATCTTG ATCAAAACCA
                                                                      ?         * Pyt3'ExR-X-2
                    1431                                                                                                1540
A. klebsiana exon X: TCGAGGAAAT TTCTTCAGGT TGGAAAGTCT TAGTTTCACC CTTGAATTCG ACGGTGATTT GTGGCTTGTC ACCAGCACCA GGGGTAACCT TGAATGGCCA GTGCTTAATA
P. ultimum "exon X": TCGAGGAAAT TTCTTCAGGT TGGAAAGTCT TAGTTTCACC CTTGAATTCG ACGGTGATTT GTGGCTTGTC ACCAGCACCA GGGGTAACCT TGAATGGCCA GTGCTTAATA

                    1541                                                                                                1650
A. klebsiana exon X: TCGGCTTGAG TAGCTGGGTC GTTGAATTTA CGACCGATCA AACGCTTAGC ATCGAACACA GTGTTGGCAG GGTTCATGGC AACTTGGTTC TTAGCGGCAT CACCAATAAG
P. ultimum "exon X": TCGGCTTGAG AAGCTGGGTC GTTGAATTTA CGACCGATCA AACGCTNAGC ATCGAACACA GTGTTGGCAG GGTTCATGGC AACTTGGTTC TNAGCGGCAT CACCAATAAG
                          *                                       ?                                                   ?
                    1651                                                                                                1760
A. klebsiana exon X: ACGTTCGCTG TCAGTGAAAG CAACGTACGA TGGGGTGGTA CGGTTACCTT GATCGTTGGC AATAATTTCA ACACGATCGT TTTGCCAGAC ACCGACACAC GAATAAGTCG
P. ultimum "exon X": ACGTTCGCTG TCAGTGAAAG CAACGTACGA TGGGGTGGTA CGGTAACCTT GATCGTTGGC AATAATTTCA ACACGATCGT TTTGCCAGAC ACCGACACAC GAATAAGTCG
                                                          *

                    1761                                                                                          1863
A. klebsiana exon X: TACCGAGATC GATACCGACG GAAGCTCCTT GGACACCAGA CATGCTTGTA GCAAATTCAA AAATGAAGTG GACTCTCCAC CACACTTGAC GTCAGCTTTC ATT
P. ultimum "exon X": TACCGAGATC GATACCGACG GAAGCTCCTT GGACACCAGA CATGCTTGTA GCAAATTCAA AAATGAAGTG GACTCTCCAC CACAC----- ---------- ---
```

Figure A-3: Amino acid sequence alignment of the *hsc70* strand translation products of the *A. klebsiana* antisense gene pair *hsc70* and corresponding region of the of the *P. ultimum* PCR product

Protein alignments were conducted using the BLAST search algorithm (Altschul *et al.*, 1990). Amino acid 1 of both sequences is at the N-terminus. The *A. klebsiana* HSC70 (LéJohn *et al.*, 1994b) amino acid sequence is given on the top line, that of the translation product of the *P. ultimum* PCR product on the bottom line. Amino acid identity (*) and similarity (·) between the sequences are both indicated. The presence of undeciphered amino acids in the *P. ultimum* sequence are also indicated (?).

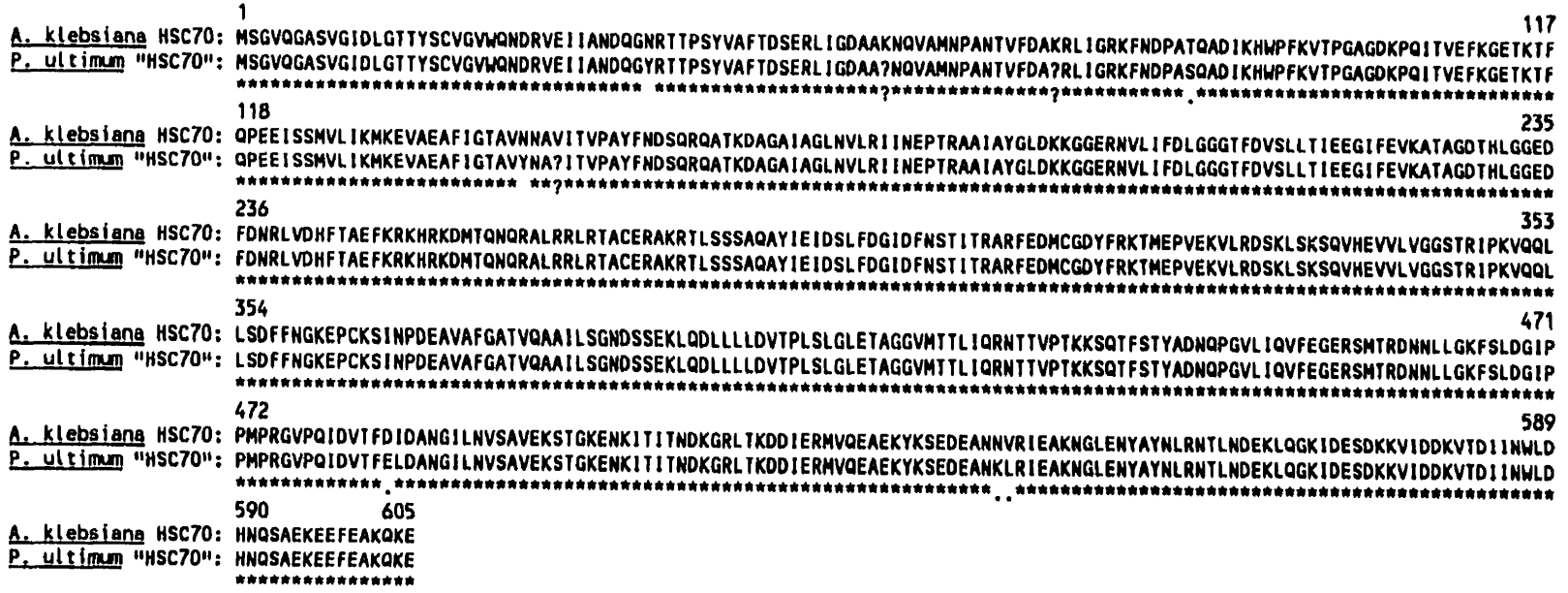
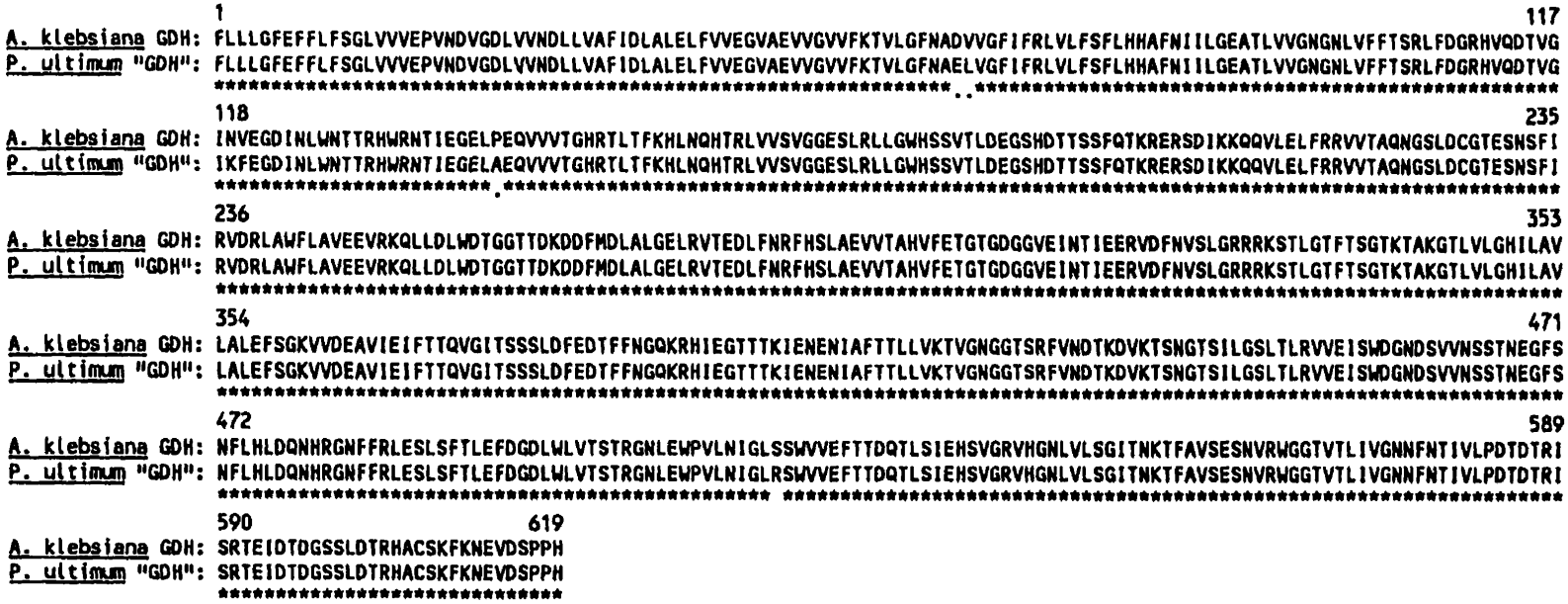

1
117
A. klebsiana HSC70: MSGVQGASVGIDLGTTYSCVGVWQNDRVEIIANDQGNRTTPSYVAFTDSERLIGDAAKNQVAMNPANTVFDAKRLIGRKFNDPATQADIKHWPFKVTPGAGDKPQITVEFKGETKTF
P. ultimum "HSC70": MSGVQGASVGIDLGTTYSCVGVWQNDRVEIIANDQGYRTTPSYVAFTDSERLIGDAA?NQVAMNPANTVFDA?RLIGRKFNDPASQADIKHWPFKVTPGAGDKPQITVEFKGETKTF
118
235
A. klebsiana HSC70: QPEEISSMVLIKMKEVAEAFIGTAVNNAVITVPAYFNDSQRQATKDAGAIAGLNVLRIINEPTRAAIAYGLDKKGGERNVLIFDLGGGTFDVSLLTIEEGIFEVKATAGDTHLGGED
P. ultimum "HSC70": QPEEISSMVLIKMKEVAEAFIGTAVYNA?ITVPAYFNDSQRQATKDAGAIAGLNVLRIINEPTRAAIAYGLDKKGGERNVLIFDLGGGTFDVSLLTIEEGIFEVKATAGDTHLGGED
236
353
A. klebsiana HSC70: FDNRLVDHFTAEFKRKHRKDMTQNQRALRRLRTACERAKRTLSSSAQAYIEIDSLFDGIDFNSTITRARFEDMCGDYFRKTMEPVEKVLRDSKLSKSQVHEVVLVGGSTRIPKVQQL
P. ultimum "HSC70": FDNRLVDHFTAEFKRKHRKDMTQNQRALRRLRTACERAKRTLSSSAQAYIEIDSLFDGIDFNSTITRARFEDMCGDYFRKTMEPVEKVLRDSKLSKSQVHEVVLVGGSTRIPKVQQL
354
471
A. klebsiana HSC70: LSDFFNGKEPCKSINPDEAVAFGATVQAAILSGNDSSEKLQDLLLLDVTPLSLGLETAGGVMTTLIQRNTTVPTKKSQTFSTYADNQPGVLIQVFEGERSMTRDNNLLGKFSLDGIP
P. ultimum "HSC70": LSDFFNGKEPCKSINPDEAVAFGATVQAAILSGNDSSEKLQDLLLLDVTPLSLGLETAGGVMTTLIQRNTTVPTKKSQTFSTYADNQPGVLIQVFEGERSMTRDNNLLGKFSLDGIP
472
589
A. klebsiana HSC70: PMPRGVPQIDVTFDIDANGILNVSAVEKSTGKENKITITNDKGRLTKDDIERMVQEAEKYKSEDEANNVRIEAKNGLENYAYNLRNTLNDEKLQGKIDESDKKVIDDKVTDIINWLD
P. ultimum "HSC70": PMPRGVPQIDVTFELDANGILNVSAVEKSTGKENKITITNDKGRLTKDDIERMVQEAEKYKSEDEANKLRIEAKNGLENYAYNLRNTLNDEKLQGKIDESDKKVIDDKVTDIINWLD
590
605
A. klebsiana HSC70: HNQSAEKEEFEAKQKE
P. ultimum "HSC70": HNQSAEKEEFEAKQKE

Figure A-4: Amino acid sequence alignment of the *nad-gdh* strand translation products of the *A. klebsiana* antisense gene pair *nad-gdh* and corresponding region of the of the *P. ultimum* PCR product

Protein alignments were conducted using the BLAST search algorithm (Altschul *et al.*, 1990). Amino acid 1 of both sequences is at the N-terminus. The *A. klebsiana* NAD-GDH (LéJohn *et al.*, 1994c) amino acid sequence is given on the top line, that of the translation product of the *P. ultimum* PCR product on the bottom line. Amino acid identity (*) and similarity (·) between the sequences are both indicated.


1 117
A. klebsiana GDH: FLLLGFEFFLFSGLVVVEPVNDVGDLVVNDLLVAFIDLALELFVVEGVAEVVGVVFKTVLGFNADVVGFIFRLVLFSFLHHAFNIILGEATLVVGNGNLVFFTSRLFDGRHVQDTVG
P. ultimum "GDH": FLLLGFEFFLFSGLVVVEPVNDVGDLVVNDLLVAFIDLALELFVVEGVAEVVGVVFKTVLGFNAELVGFIFRLVLFSFLHHAFNIILGEATLVVGNGNLVFFTSRLFDGRHVQDTVG
118 235
A. klebsiana GDH: INVEGDINLWNTTRHWRNTIEGELPEQVVVTGHRTLTFKHLNQHTRLVVSVGGESLRLLGWHSSVTLDEGSHDTTSSFQTKRERSDIKKQQVLELFRRVVTAQNGSLDCGTESNSFI
P. ultimum "GDH": IKFEGDINLWNTTRHWRNTIEGELAEQVVVTGHRTLTFKHLNQHTRLVVSVGGESLRLLGWHSSVTLDEGSHDTTSSFQTKRERSDIKKQQVLELFRRVVTAQNGSLDCGTESNSFI
236 353
A. klebsiana GDH: RVDRLAWFLAVEEVRKQLLDLWDTGGTTDKDDFMDLALGELRVTEDLFNRFHSLAEVVTAHVFETGTGDGGVEINTIEERVDFNVSLGRRRKSTLGTFTSGTKTAKGTLVLGHILAV
P. ultimum "GDH": RVDRLAWFLAVEEVRKQLLDLWDTGGTTDKDDFMDLALGELRVTEDLFNRFHSLAEVVTAHVFETGTGDGGVEINTIEERVDFNVSLGRRRKSTLGTFTSGTKTAKGTLVLGHILAV
354 471
A. klebsiana GDH: LALEFSGKVVDEAVIEIFTTQVGITSSSLDFEDTFFNGQKRHIEGTTTKIENENIAFTTLLVKTVGNGGTSRFVNDTKDVKTSNGTSILGSLTLRVVEISWDGNDSVVNSSTNEGFS
P. ultimum "GDH": LALEFSGKVVDEAVIEIFTTQVGITSSSLDFEDTFFNGQKRHIEGTTTKIENENIAFTTLLVKTVGNGGTSRFVNDTKDVKTSNGTSILGSLTLRVVEISWDGNDSVVNSSTNEGFS
472 589
A. klebsiana GDH: NFLHLDQNHRGNFFRLESLSFTLEFDGDLWLVTSTRGNLEWPVLNIGLSSWVVEFTTDQTLSIEHSVGRVHGNLVLSGITNKTFAVSESNVRWGGTVTLIVGNNFNTIVLPDTDTRI
P. ultimum "GDH": NFLHLDQNHRGNFFRLESLSFTLEFDGDLWLVTSTRGNLEWPVLNIGLRSWVVEFTTDQTLSIEHSVGRVHGNLVLSGITNKTFAVSESNVRWGGTVTLIVGNNFNTIVLPDTDTRI
590 619
A. klebsiana GDH: SRTEIDTDGSSLDTRHACSKFKNEVDSPPH
P. ultimum "GDH": SRTEIDTDGSSLDTRHACSKFKNEVDSPPH

A.I.b. Discussion of the *P. ultimum* "Exon X" sequence information The sequence information described here for the *P. ultimum* 1.8 kb PCR fragment and part of a previously sequenced overlapping 0.95 kb PCR product (LéJohn, unpublished data) indicates that a gene arrangement exists in *P. ultimum* which is almost identical to the region of the antisense gene pair of *A. klebsiana* which contains the overlapping segments of the *nad-gdh* and *hsc70* ORFs. Those discrepancies which were observed between the sequences of these two species tend to encode conservative amino acid alterations in the predicted protein products, and may be due to errors introduced either during PCR amplification or the DNA sequencing of the *P. ultimum* PCR fragment. This work carries with it the implication that the antisense gene pair of *A. klebsiana* may not be unique, but may be a DNA arrangement shared with the genomes of other oomycetes.

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B. Attempts to Amplify the *hsp70* mRNA for Sequence Analysis

Attempts to extend the suspected *hsp70* gene sequence information to its 3' terminus and concurrently demonstrate the active transcriptional nature of this gene were made through reverse transcriptase PCR (RT-PCR) amplification of *P. ultimum* total RNA. A sequence specific to the suspected *P. ultimum hsp70* and oligo-dT were used as primers for this amplification.

B.I. Results and Discussion

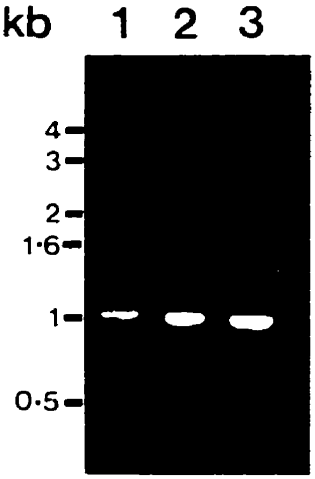
B.I.a. <u>Amplification and nucleotide sequencing of distinct products during RT-PCR</u> Distinct RT-PCR products were generated from total RNA derived from cells cultured at several temperatures in starvation medium supplemented with L-glutamate or heat stressed at 31°C in GY growth medium. PCR fragments of similar sizes were also generated by PCR amplification with *P. ultimum* genomic DNA as template and Pudb4 as the sole primer present (Figure B-1). Southern blotting of the RT-PCR products and hybridization to part of the suspected *P. ultimum hsp70* gene resulted in strong

Figure B-1: Genomic origin of and comparison between two RT-PCR products with *P. ultimum hsp70*

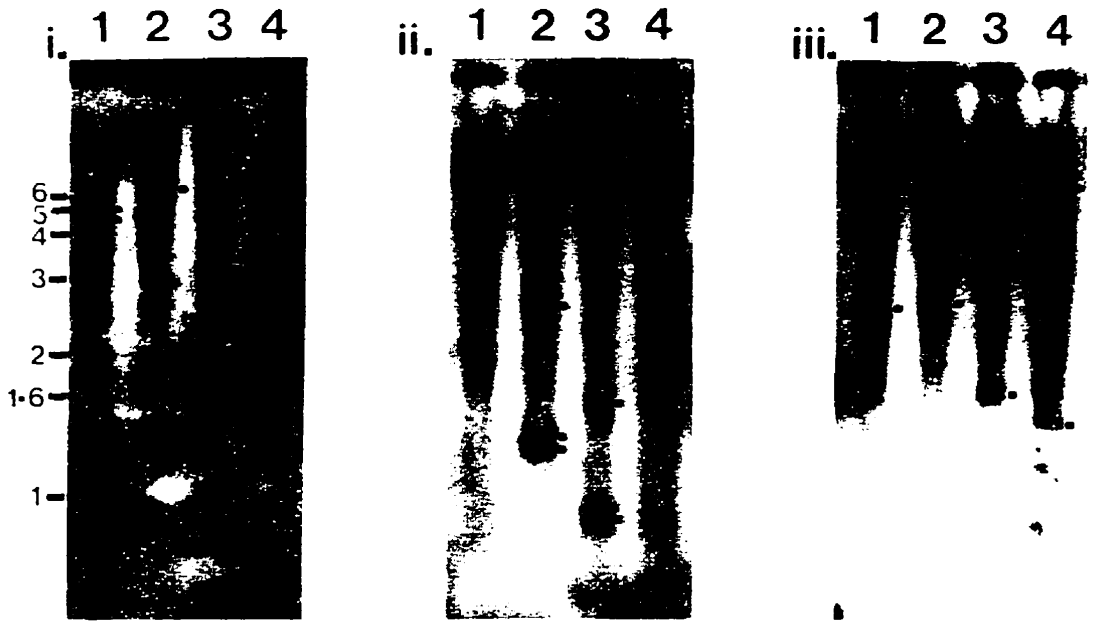
Production of PCR products from genomic DNA using only Pudb4 as primer similar to RT-PCR products observed (Figures B-1 and B-4) was conducted as described (Figure B-2, below). Plasmid constructs of the RT-PCR products were used to probe restriction endonuclease digested *P. ultimum* genomic DNA to determine if they and the suspected *hsp70* arose from similar or distinct regions of the *P. ultimum* genome (Methods and Materials *2.I.d. Insert orientation determination*, *2.IV.d. Probe radiolabeling* and *2.IV.e. Probe hybridization and autoradiography*, above) with Southern blotting conducted using 0.5 M NaOH as transfer buffer overnight at room temperature. Recombinants were generated using 50 ng RT-PCR product ligated into the pBAD-TOPO vector (Invitrogen) and immediately transformed into commercially available competent cells (One-Shot™ Competent Cells, Invitrogen) as per the manufacturer's instructions. Transformants were picked after overnight incubation at 37°C on LB plates supplemented with 100 μg/mL ampicillin. Successful transformation was verified by PCR (Methods and Materials *2.IV.c. Polymerase chain reaction*, above) using the same primers as for the initial RT-PCR reactions. **A.** Agarose gel electrophoresis of RT-PCR products and PCR products using different

primers and templates. Lane 1, RT-PCR (primers Pudb4 and oligodT) of RNA extracted from cells heat stressed at 28°C in starvation medium supplemented with 15 mM L-glutamate; Lane 2, same as Lane 1, but with heat stress conducted at 31°C; Lane 3, PCR of *P. ultimum* genomic DNA using only primer Pudb4. **B.** Southern blot analysis of identical agarose gels probed with: i, *hsp70* 0.7 kb PCR product; ii, pBADRTPCR-1; iii, pBADRTPCR-2. Lanes in each comprise *P. ultimum* genomic DNA digested the following restriction endonucleases: Lane 1, BamHI; Lane 2, EcoRI; Lane 3, HindIII; Lane 4, SalI. Bands observed are identified with solid boxes. Molecular sizes are given for all figures in kb to the left.

A.



B.



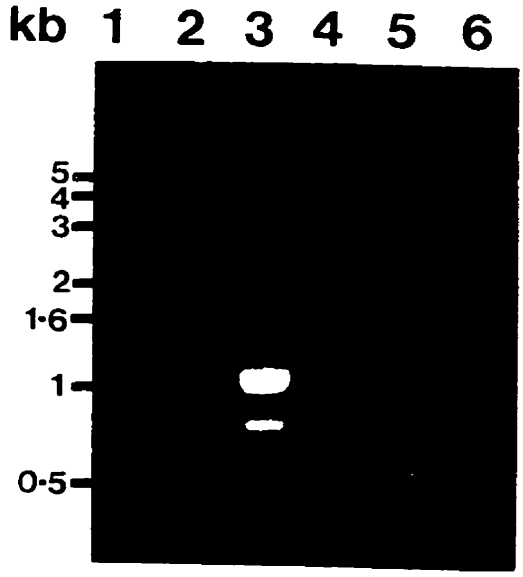
labeling in several lanes to 1.0 kb and 0.75 kb RT-PCR fragments (Figures B-2, B-3 and B-4). The RT-PCR products for the RNA samples from mycelia heat stressed at 31°C in GY medium were shotgun cloned into pBAD-TOPO (Invitrogen) to generating two distinct clone types containing inserts corresponding to the 1.0 kb or 0.75 kb RT-PCR products (pBADRTPCR-1 and pBADRTPCR-2, respectively). Use of these to probe restriction endonuclease digested *P. ultimum* genomic DNA demonstrated that pBADRTPCR-1 and pBADRTPCR-2 contain inserts distinct from both one another and from the suspected *P. ultimum hsp70* (Figure B-1). The complete sequence of the 1.0 kb RT-PCR fragment subcloned into pM13 (Figure B-5) identified several ORFs with amino acid sequence similarity to several known proteins (Table B-1). None of corresponded to the suspected *hsp70* or a heat stress protein of any type, and no ORF spanning the entire 1.0 kb as would be expected for an RT-PCR product arising from an mRNA was observed.

B.I.b. Discussion of RT-PCR amplification data Initial RT-PCR analysis identified a product produced by mycelia exposed to heat and nutritional stress conditions (Figures B-2 and B-4). The greater intensity of ethidium bromide staining under UV light of products obtained from mycelia grown at 28°C and 31°C in starvation medium supplemented with 15 mM L-glutamate is indicative of a larger population of template molecules in these samples,

Figure B-2: RT-PCR and PCR reamplification of total RNAs derived from heat-stressed cells

Five hundred ng total RNA was recovered (Methods and Materials *2.III. Total RNA Extraction*, above) from overnight cultures of *P. ultimum* which were subsequently incubated for one hour at the same temperatures used for *P. ultimum* morphological analysis (Methods and Materials *4.I. Morphological Analysis of* P. ultimum, above) was used as template for 50 μL RT-PCR reactions, using the Titan™ One Tube RT-PCR kit (Boehringer-Mannheim). Five μL of each RT-PCR reaction was used as template for conventional 100 μL Taq polymerase PCR reactions (Methods and Materials *2.IV.c. Polymerase chain reaction*, above). Ten μL aliquots of each reamplification reaction were electrophoresed 5 hours at 50 V on a 1.5% agarose gel prior to (Methods and Materials *2.I.a. Insert and vector preparation*, above) to identify products and for Southern blotting overnight at room temperature with 0.5M NaOH as transfer buffer. Membranes were pre-hybridized, hybridized to probe, washed and autoradiographed as described (Methods and Materials *2.IV.e. Probe hybridization and autoradiography*, above). The 0.7 kb PCR product obtained from the suspected *P. ultimum hsp70* gene (Results and Discussion *3.II. Sequencing template preparation*, above) was used as probe. Both the gel prior to Southern blotting (top) and

after autoradiography (bottom) are shown. Lanes correspond to the heat stress temperatures used on the cells from which the RNA templates were derived as follows: Lane 1, room temperature (approximately 22°C); Lane 2, 28°C; Lane 3, 31°C; Lane 4, 34°C; Lane 5, 37°C; Lane 6, 42°C. Standard sizes are given to the left in kb.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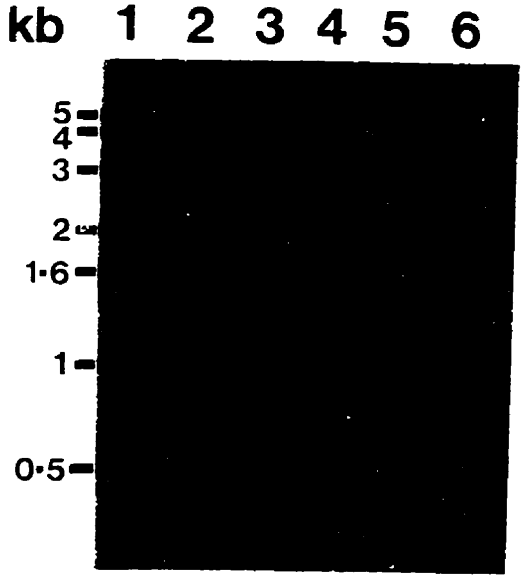
1
0·5


kb 1 2 3 4 5 6
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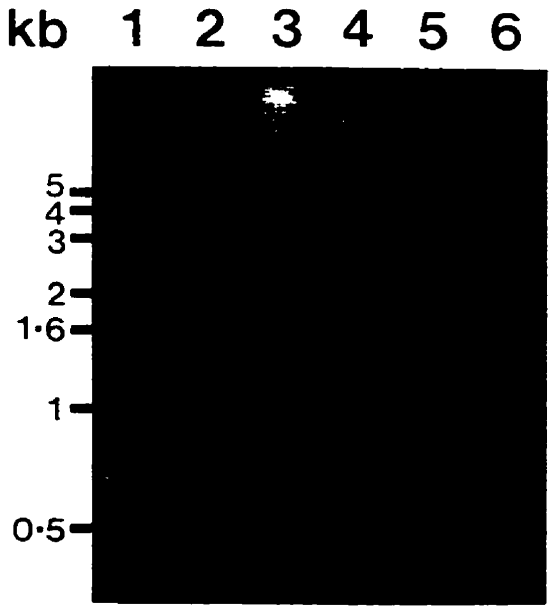
4
3
2
1·6
1
0·5

Figure B-3: RT-PCR and PCR reamplification of total RNAs derived from heat and nutritionally stressed cells

RT-PCR and Southern blotting were conducted as described (Figure B-1), but with cells incubated at heat stress temperatures for 1 hour in 20 mL starvation medium. Both the gel prior to Southern blotting (top) and after autoradiography (bottom) are shown. Lanes correspond to the heat stress temperatures used on the cells from which the RNA templates were derived as follows: Lane 1, room temperature (approximately 22°C); Lane 2, 28°C; Lane 3, 31°C; Lane 4, 34°C; Lane 5, 37°C; Lane 6, 42°C. Standard sizes are given to the left in kb.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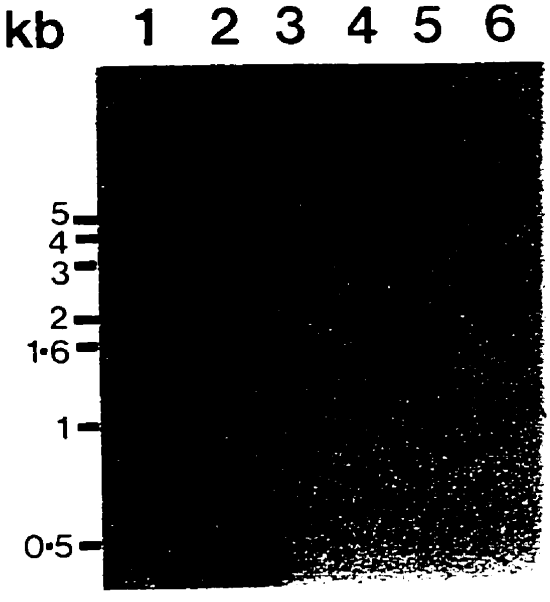
kb
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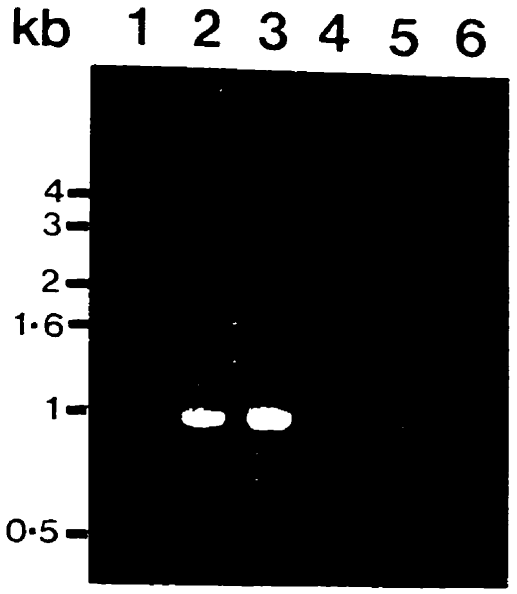
Figure B-4: RT-PCR and PCR reamplification of total RNAs derived from heat and nutritionally stressed cells in medium supplemented with 15 mM L-glutamate

RT-PCR and Southern blotting were conducted as described (Figure B-1), but with cells incubated at heat stress temperatures for 1 hour in 20 mL starvation medium supplemented with 15 mM L-glutamate. Both the gel prior to Southern blotting (top) and after autoradiography (bottom) are shown. Lanes correspond to the heat stress temperatures used on the cells from which the RNA templates were derived as follows: Lane 1, room temperature (approximately 22°C); Lane 2, 28°C; Lane 3, 31°C; Lane 4, 34°C; Lane 5, 37°C; Lane 6, 42°C. Standard sizes are given to the left in kb.


kb
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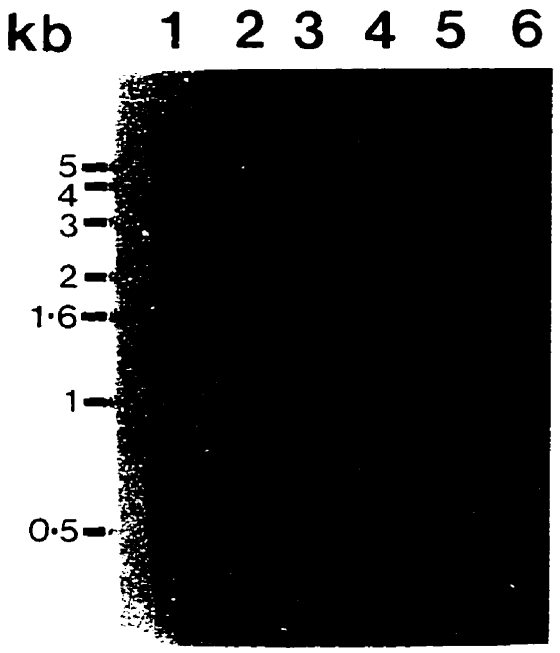
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2
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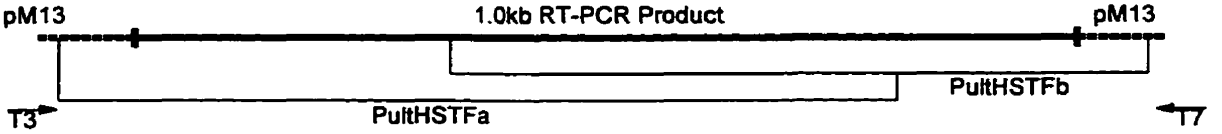
1
0·5

Figure B-5: Complete nucleic acid sequence of the *P. ultimum* 1.0 kb RT-PCR product

The 1.0kb RT-PCR product was purified after agarose gel electrophoresis by the freeze squeeze method (Methods and Materials *2.IV.c. Polymerase chain reaction*, above) and made blunt-ended by incubation with DNA polymerase large fragment as for random primer labeling (Methods and Materials *2.IV.d. Probe radiolabeling*, above). The blunt-ended fragment was ligated into the EcoRV site of dephosphorylated pM13 and transformed into *E. coli* XL-1 Blue (Methods and Materials *2.I.b. Vector dephosphorylation, ligation and bacterial transformation*, above). After confirmation of the presence of insert by PCR as described (Figure B-1, above), one positive clone was selected for plasmid purification and DNA sequencing using commercial T3 and T7 primers as described (Methods and Materials *2.I.c. Plasmid amplification and recovery* and *4.II.b. DNA sequencing*, above). The sequencing strategy with labeled arrows indicating primer locations and boxes outlining the extent of reliable sequence data from each primer (**A.**) and complete nucleic acid sequence of the 1 kb RT-PCR product (**B.**) are presented. Sequences for the primers used in sequencing, are the same as for Figure 30, above. The nucleotide sequence is that of the T3 primed strand. ORFs encoding theoretical peptides with some homology to known

proteins are indicated by solid (T3 primed strand) or dotted (T7 primed strand) arrows, with arrows indicating the N-terminus to C-terminus orientation of the peptide products. The Pudb4 primer binding site is boxed.

A.



B.

1 100
TCTGGAAGCACAAGCGACCAGCGAAAGACACGTTGACGATGTCAAGTGCGCGCAAGTCAACTCGCTGCTCAAGATCAACCTCCGGAAACGCTACTTGTTT

101 200
AAATGGTGAAAAAGGGCGCCACGTACTTATTGAAGCGTCTCGTGCGAACGTGAAGCAAACCGCTGCTACTTGTGATCATTTATATCCTATTCTGTTACTC
ORF 1
ORF 2

201 300
TAAGTTCGTTCACCAGCCAAATGCAATGCTAACACGACTTAACCAGCGATATTTATAGTTCTATCAATGCGTCAAGGTTACGCCTTCGCTTCTTCTGTGT
ORF 3

301 400
AAAATGGACCCAATGACCGCACGAGAGCCAATGCAGCTCGTCGGTGATGCGCAACTCGCGACGCCATGTTTTTTGAAGCCCAGGTAGATCTTTCGAAGAA

401 500
ACCTGGCGTCCCACCTGTATCCAAATGTACTGTATGTGTCTCGATGAAAGCGTCTTGACTTTTCTCGTAAGATGGCACTCGACATGCTGTCTCGAGTATG

501 600
AACGAAGCTGATGAGAAGCAAACCCCTGACAATGCAAAGCACAACCCACTCAAACAAGCCACTTCTGGAATTTCCCGTTTTTCCTTTCTACGAAGTACAG

601 700
ACTTGAGGTCTTCCGGGTTTAATCATCTATCTGGGGGCCATTTTTCACGTGCTGCTCCTTCTTTTTGAAAGAGATCATTTACTTTTTCGCGTTCCCATGG
ORF 4
ORF 5

701 800
ACATCTCCGAACTCGACGCGATGAGCGAGTTTATTGATATTGTCGATATCGATAGCATCGATAGCAGCGACTTGGACGAAGACGGTGACATGGTGATGCC

801 900
AGATTCCTTTGGCGGGATGATTCGTTCCCATGAGGACGGGGTGCAAATCCTGTGTGACCAATGTGCCGACGATCCACCAAACCAAGACGAACAAGTCGAC

901 934
GATGCCCGCGCCAGAGCTCGTGTTACAATAGAAG
ORF 6

Table B-1: *P. ultimum* 1.0 kb RT-PCR product ORF BLAST search results

Screening of GenBank conducted using the BLAST search system (Altschul *et al.*, 1990) identified several proteins similar to the theoretical translation products ORFs identified in the 1 kb RT-PCR product. Sizes of these ORFs are given as are the sizes of regions overlapping segments of homologous proteins. Values for both % identity and % similarity were calculated for the overlapping regions only. Amino acids are defined here as being similar if they have the same hydropathic classification as previously outlined (Table 1).

ORF	ORF Size (Amino Acids)	Homologous Protein	Total Overlap (Amino Acids)	Identity/Similarity (%)
1	66	SapB (*Campylobacter fetus*)	55	27/54
		Secreted protein (*Neisseria meningitidis*)	56	33/56
		X gene product (*D. melanogaster*)	49	30/44
2	35	C09D8.1 (*Caenorhabditis elegans*)	30	36/62
3	73	Mkr4 Zinc Finger Protein (Murine)	25	44/56
4	84	Tsx Outer Membrane Protein (*E. coli*)	24	45/65
5	56	AOF1001 (*S. cerevisiae*)	32	53/62
		YOL155c (*S.cerevisiae*)	32	53/62
6	33	ATP dependent RNA helicase (*B. subtilis*)	23	52/73

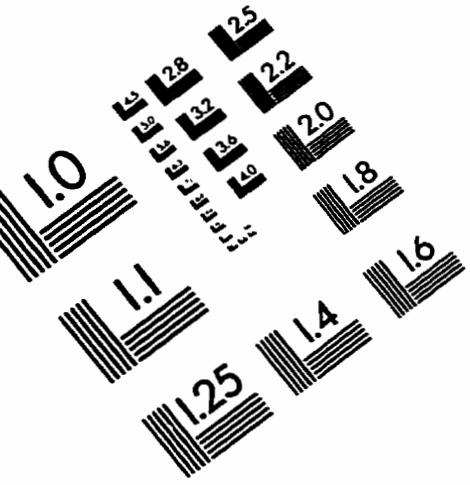
as would be expected for the amplification of a stress protein mRNA.

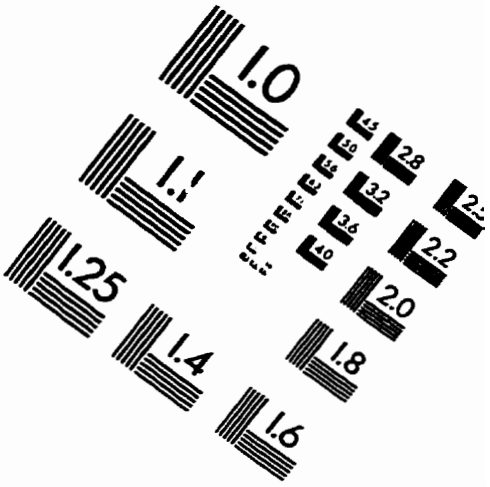
The fact that Pudb4 by itself was sufficient to prime PCR amplification of a 1.0 kb fragment from genomic *P. ultimum* DNA (Figure B-1) brought the source of the template and the identity of the RT-PCR products observed into question. Subsequent analysis of hybridization patterns for two distinct RT-PCR clones to restriction endonuclease digested *P. ultimum* genomic DNA indicated that the RT-PCR products were not only different from one another, but were also distinct from the suspected *P. ultimum hsp70* (Figure B-1). This contradicted the Southern blot analysis of the RT-PCR products already mentioned (Figures B-2 and B-4) which clearly indicated hybridization between the putative *hsp70* and the RT-PCR products.

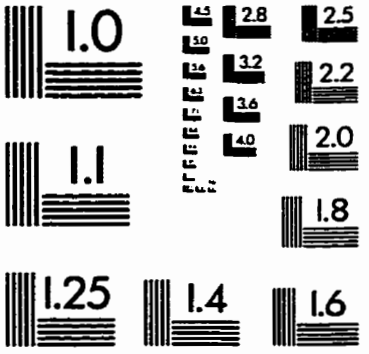
The hybridization patterns of pBADRTPCR-1 and pBADRTPCR-2 to restriction endonuclease digested *P. ultimum* genomic DNA and the capacity of Pudb4 as sole primer to produce 1.0 kb PCR products from *P. ultimum* genomic DNA indicate that the RT-PCR products obtained may be artifacts arising from DNA contamination of the total RNA samples. Sequencing of this region confirmed this template does not correspond to the suspected *P. ultimum hsp70* (Figures B-5), and demonstrated the presence of several short ORFs which is inconsistent with a mRNA template (Figure B-5 and Table B-1). In all, 14 ORFs were identified, six of which have some degree of similarity at the amino acid sequence level with previously

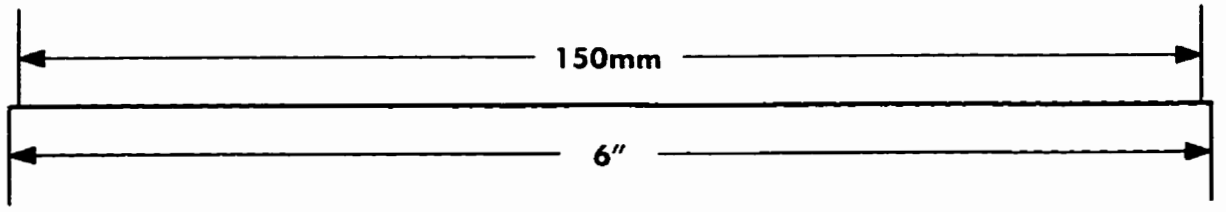
characterized proteins (Table B-1). Of these, only three ORFs have homology with previously characterized proteins extending over at least 70% of the ORF product protein. Sequence information obtained for the 1.0 kb RT-PCR product also indicated that Pudb4 only primed the PCR amplification from one end of the product molecule (Figure B-5), and may indicate the presence of a contaminating primer.

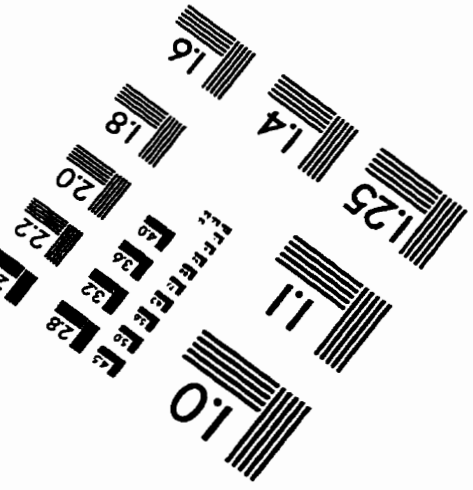
IMAGE EVALUATION
TEST TARGET (QA-3)

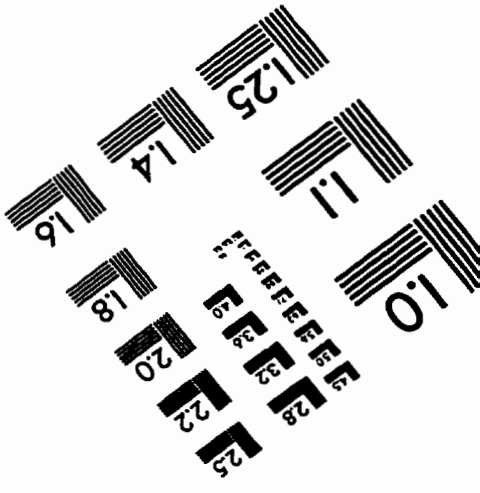












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