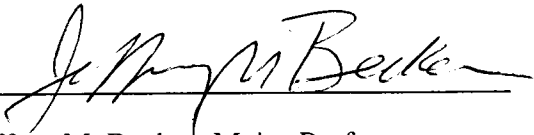
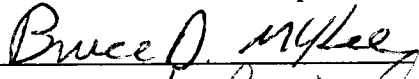


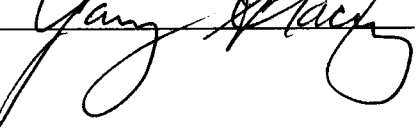
To the Graduate Council:

I am submitting herewith a dissertation written by Mark Andrew Lubkowitz entitled "Oligopeptide Transport: Cloning and Characterization of a New Gene Family." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.


Jeffrey M. Becker, Major Professor

We have read this dissertation
and recommend its acceptance:





Accepted for the Council:


Associate Vice Chancellor and
Dean of the Graduate School

**OLIGOPEPTIDE TRANSPORT : CLONING AND
CHARACTERIZATION OF A NEW GENE
FAMILY**

**A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville**

Mark Lubkowitz

August 1997

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Science is often perceived as a field of individualism where a person can make a discovery. As I look back upon my experiences I find that nothing is further from the truth, and I am now convinced that no one makes a discovery on their own. Bench work science is perhaps one of the most social and interactive professions imaginable. With that said, I would like to acknowledge “the team” that ultimately made this work possible.

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I will always remember and cherish the friendships which developed during my time in the trenches of the Becker lab. I have enjoyed the long days and nights in the lab and in the brew pub with all of you and I wish everyone the best in the future. I would like to acknowledge two individual in particular from the Becker lab, David Barnes and Melinda Hauser. Through the course of my graduate studies I developed a very dynamic and what I hope was a synergistic relationship with these two remarkable individuals.

My development certainly would not have been as complete without you two.

Finally, I would like to thank my wife, Ginger Gay, for her love, patience, support, and the seemingly endless number of sacrifices she endured over the years.

ABSTRACT

Peptide transport is a widely observed phenomenon defined as the translocation of peptides 2-6 residues in length across the plasma membrane in an energy-dependent manner. Internalized peptides are rapidly hydrolyzed by peptidases, and the resulting amino acids are used for protein synthesis or alternatively as a source of nitrogen or carbon. Physiological evidence suggested that the pathogenic fungus *Candida albicans* has at least two different peptide transporters: a di-/tripeptide transporter named CaPtr2p and an oligopeptide transporter system which accommodates peptides of 3-5 residues. The purpose of this study was to 1) clone and characterize the gene(s) responsible for oligopeptide transporter in *C. albicans* and 2) explore the role of oligopeptide transport in virulence.

Part II of this dissertation describes how the oligopeptide transport gene *OPT1* was cloned from *C. albicans*. Using growth conditions under which *Saccharomyces cerevisiae* strain PB1X-9B does not transport tetra- and pentapeptides, we were able to identify *OPT1* as a gene that allowed PB1X-9B to utilize tetra- and pentapeptides as a sole source of an auxotrophic supplement. *OPT1* bestowed oligopeptide transport activity to PB1X-9B as measured by sensitivity to toxic oligopeptides and the ability to accumulate a radiolabeled tetrapeptide.

Part III of this dissertation details how we identified the *OPT1* homolog *isp4* from *S. pombe* and how subsequent characterization of a deletion strain revealed that *isp4* encodes an oligopeptide transporter. Furthermore, based upon the four criteria of protein length, function, topology, and conserved functional domains, we proposed that *OPT1* from *C. albicans*, *isp4* from *S. pombe*, and *YPR194C* and *YJL212C* from *S. cerevisiae*

comprise the first identified members of a novel family of transport proteins.

Part IV of this dissertation summarizes the construction and characterization of several *OPT1* disruptant strains. Analyses of the disruptants revealed that Opt1p is a high-affinity/low-capacity transporter and that another oligopeptide transport system exists in *C. albicans*. The role of *OPT1* in one murine model of systemic candidiasis was explored and the possible roles for *OPT1* in virulence are discussed.

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PART 1
GENERAL INTRODUCTION

CHAPTER I

INTRODUCTION

Peptide transport is a widely observed phenomenon defined as the translocation of peptides 2-6 residues in length across the plasma membrane in an energy-dependent and saturable manner. Internalized peptides are rapidly hydrolyzed by peptidases, and the resulting amino acids are used for protein synthesis or alternatively as a source of nitrogen or carbon (Reviewed by Becker and Naider, 1995; Payne and Smith, 1994). In addition to acquiring nutrients, peptide transport systems have also been shown to participate in other biological functions such as quorum sensing (Swift *et al.*, 1996), cell wall recycling (Payne and Smith, 1994), and in aiding organisms to evade the host immune response (Parra-Lopez *et al.*, 1993) as will be explained in greater detail below. For the purposes of this dissertation peptide transporters will be divided by substrate length into two categories: those that transport di-/tripeptides (the di-/tripeptide transporters) and those that transport peptides 3-6 residues in length (the oligopeptide transporters). Prior to my work, two evolutionarily distinct families of peptide transporters had been identified: the ATP Binding Cassette (ABC) Superfamily and the Peptide Transporter (PTR) Family (Steiner *et al.*, 1995). I have now further lengthened the list of peptide transport families by identifying and characterizing an evolutionarily distinct group of proteins called the OPT Family (Part 3; Lubkowitz *et al.*, 1997). This dissertation will be presented in four parts consisting of a review of the literature (Part 1), the cloning and characterizing of the oligopeptide transporter *OPT1* from *Candida albicans* (Part 2), the identification of an *OPT1* homolog and the establishing of a new family of transport proteins (Part 4), and the affect of *OPT1* deletion on virulence in a systemic murine model of candidiasis (Part 4).

CHAPTER II

TRANSPORTER FAMILIES

The ABC Family

The ABC (ATP Binding Cassette) proteins are a large group of membrane-bound importing or exporting proteins that use ATP hydrolysis to energize the translocation of their substrates. They have been identified in prokaryotes, lower eukaryotes, higher eukaryotes, and archaeobacteria. Their substrates are as diverse as the organisms from which they were identified and range from small ions to large macromolecules (reviewed by Higgins, 1992). The ABC proteins have been topographically analyzed and several conserved structural features have been identified. Perhaps the most striking feature is the symmetry exhibited by these twelve transmembrane domain proteins. The twelve membrane spanning segments exhibit sequence symmetry around a center axis, thereby giving the appearance of a 2x6 configuration. Furthermore, each six transmembrane domain is associated with an intracellular domain responsible for binding ATP. The 2x6 symmetry as well as the associated ATP binding domains are thought to have arisen as a result of a past gene duplication event. In prokaryotes each of the four domains is encoded by a separate Open Reading Frame (ORF), while in eukaryotes all four domains are encoded by the same ORF. In addition to topographical similarities, several highly conserved regions have also been found at the primary amino acid level leading to the identification of two signature motifs. The most prominent of these motifs is the ATP binding sites denoted the Walker A or B motif. In addition to the Walker motifs, a signature motif of 5 amino acids located once in the protein upstream of the Walker B motif has been identified. Although highly conserved, the functional relevance of this motif has not been established.

The PTR Family

The PTR (Peptide Transport) Family is a newly established group of transport proteins that has been identified in both prokaryotes and eukaryotes and contains approximately 12 members. In the relatively short amount of time since its discovery in 1994 several common features have been identified. Unlike the ABC Family, the PTR proteins use the proton motive force to energize the translocation of their substrates. Kinetic analyses performed in a *Xenopus* oocyte with a PTR transporter from rabbit and human intestine has demonstrated that one proton is symported with every peptide (Fei *et al.*, 1994). The only member of the PTR Family to be analyzed topographically *in vivo* is the di-/tripeptide transporter Dpt1p from *Lactococcus lactis* (Hagting *et al.*, 1997). Results from these studies, in congruence with the hydropathy analysis, indicated that Dpt1p contains twelve transmembrane spanning regions. Even though Dpt1p is the only PTR member to be analyzed topographically *in vivo*, the nearly superimposable hydropathy plots of the PTR proteins has led our laboratory to hypothesize that the other members of this family also contain twelve transmembrane domains. In addition to nearly identical hydropathy plots, two highly conserved regions have been identified from the primary amino acid sequences. The most prominent of these regions is found in the fifth transmembrane domain, is absolutely conserved across all species, and has been termed the FING Motif (Steiner *et al.*, 1995). Preliminary data involving site directed mutagenesis of the FING motif suggests that this motif is required for transporting peptides (J. Wiles, unpublished observation). Whether its mode of action is structural, peptide translocation, or proton translocation has not been determined.

CHAPTER III

PEPTIDE TRANSPORT IN PROKARYOTES

Introduction

Most peptide transport research has been carried out in prokaryotes with studies being performed in *Eschericia coli*, *Salmonella typhimurium*, *Lactococcus lactis*, *Bacillus subtilis*, *Streptomyces coelicolor*, and *Streptococcus pneuoniae* (Payne and Smith, 1994; Hagting *et al.*, 1994, Koide and Hoche, 1994; Parra-Lopez *et al.*, 1993; Nodwell *et al.*, 1996; Cundell *et al.*, 1995). Multiple peptide transporter systems have been identified in all of the organisms thus far examined with the exception of *Streptomyces coelicolor*. The single peptide transport system in *Streptomyces coelicolor* was identified through a screen designed to detect genes involved in signaling filamentous differentiation (Nodwell *et al.*, 1996). Peptide transport, per se, has not been examined in this organism, and hence the lack of observed multiplicity may be due simply to an insufficient amount of research.

The first peptide transporters cloned from prokaryotes were all ABC proteins and consisted of the di-/tripeptide transporter *dpp*, the tripeptide transporter *tpp*, and the oligopeptide transporter *opp* from *E. coli* and *S. typhimurium* (reviewed by Payne and Smith, 1994). The proposed role of these seemingly redundant systems was originally for acquiring nutrients. The later observation that the *opp* system could accommodate peptides containing *D* stereoisomers led to the proposal that this system was responsible for recycling cell peptides. In addition to providing peptides as a source of nutrients in prokaryotes, peptide transporters have now been shown to provide resistance to defensins (Parra-Lopez *et al.*, 1993), mediate adhesion (Cundell *et al.*, 1996), and transduce signal peptides (Solomon *et al.*, 1996). The only non-ABC peptide transporter studied in detail

to date is Dptp of *L. lactis* (Hagting *et al.*, 1994) although data from the *E. coli* sequencing initiative has led to the identification of a putative PTR family member.

Peptide transporters play a role in prokaryotic cell to cell signaling

The use of peptides as signal molecules has been widely observed. The paradigm model of signaling by peptides consists of a signal peptide binding to a membrane bound receptor thereby leading to the induction of the appropriate response. More recently, several investigators have shown that in some organisms peptides are actually internalized via a peptide transporter before inducing the appropriate response. Such a mechanism was demonstrated for the induction of sporulation and competence in *B. subtilis* (Solomon *et al.*, 1996). Although single celled organisms, prokaryotes exhibit an array of population coordinated behaviors some of which are density dependent. The phenomenon of density dependent behavior has recently been termed "quorum sensing" and includes such behaviors as competency, sporulation, fruiting body formation, virulence, antibiotic production, and bioluminescence (Swift *et al.*, 1996)). A wide variety of quorum sensing signal molecules have been identified including acyl homoserine lactones, amino acids, and small peptides.

Perhaps the best understood system of quorum sensing that involves an imported signal peptide is sporulation and competency in *Bacillus subtilis*. Current studies suggest that in *B. subtilis* competency is regulated through two peptides whose concentrations increase with population density. The first peptide discovered was ComX which consists of 9 or 10 amino acids with a modified tryptophan (Magnuson *et al.*, 1994). ComX binds to ComP, a membrane bound receptor that is the histidine protein kinase of a two component regulatory pathway. The activated ComP undergoes autophosphorylation after which a phosphate is transferred to the response regulator and transcription factor ComA.

The phosphorylated ComA then activates the competency genes by initiating the transcription of *srfA*.

In addition to ComA, the pentapeptide Competency and Sporulation Factor (CSF), ERGMT, is also required for the acquisition of density dependent competency (Solomon *et al.*, 1996). CSF is translated as a 40 amino acid peptide prior to being processed and secreted as the pentapeptide ERGMT. The ABC type oligopeptide transporter SpoOK then imports the CSF into the cell where it inhibits RapC, a phosphatase responsible for the dephosphorylation of ComA-P (Solomon *et al.*, 1996). Together these two peptides coordinate density dependent competency by either activating a two component regulatory cascade (ComX) or by repressing the phosphatase responsible for the deactivation of ComA.

In addition to importing CSF leading to the deactivation of RapC, two observations led Solomon *et al.* (1996) to propose that the oligopeptide transporter SpoOK plays an additional role in competency, possibly through interactions with other phosphatases or kinases. First, SpoOK mutants, as well as SpoOK and RapC double mutants, are deficient in competency. Because the target and mode of action of CSF is the inactivation of RapC and because CSF enters the cell through SpoOK, one would predict that a mutation in SpoOK could be bypassed by a mutation in RapC. Second, a mutant in CSF production still exhibits a competency level above a SpoOK mutant. These two observations imply that SpoOK, in addition to regulating RapC, interacts with other regulatory processes involved in acquiring competency. Although the direct role of the oligopeptide transporter SpoOK is still unknown, what is clear is that peptide transport systems are not just simply for foraging nutrients.

Peptide transporters affect virulence

Many global regulators of virulence programs have been identified but none have been found to be a peptide or peptide based. However, peptide transporters have been shown to affect virulence in *Salmonella typhimurium* by mediating resistance to host antimicrobial peptides (Parra-Lopez *et al.*, 1993; Groisman *et al.*, 1992). Furthermore, two different peptide transport systems were shown to affect adherence to endothelial and lung cells in *Streptococcus pneumoniae*.

Intracellular parasites, such as *S. typhimurium*, must be able to overcome the many challenges presented by the hostile host environment in order to colonize their target niche. One of these challenges is the ability to resist pore-forming antimicrobial peptides. These pore forming peptides have been widely observed in nature and consist of small (23-39 amino acids) cationic peptides which are predicted to form amphipathic alpha helical pores in the membrane (reviewed by Boman, 1991). The antimicrobial properties of some of these compounds have been extensively documented, such as the magainins from *Xenopus*, the cecropins from moth hemolymph, and the defensins from phagocytes.

In order to determine the nature of antimicrobial peptide resistance in *S. typhimurium*, Groisman *et al.* (1992) screened a *S. typhimurium* mutant library for hypersensitivity to the peptides protamine and melittin. Several mutants were identified that exhibited cross resistance and mapped to the same locus. Further characterization of these mutants demonstrated that they were also hypersensitive to neutrophil granular extracts but still sensitive to defensins. Furthermore, Groisman *et al.* (1992) demonstrated that these mutants were avirulent in a murine model of infection, even at an inoculum size 1000 fold larger than the LD₅₀ of the wildtype. Cloning of the mutated locus revealed that it encoded an ABC type oligopeptide transporter named Sensitivity-to-Antimicrobial-Peptides. Two possible mechanisms for this mediated resistance have been proposed. The oligopeptide transporter of *Salmonella* discovered by Groisman *et al.*

(1992) could be responsible for transporting the pore-forming peptides into the cell and away from the plasma membrane where they would be subsequently degraded by intracellular proteases. Alternatively, the antimicrobial peptides could bind to the transporter itself gene product which in turn could activate a resistance determinant. However, no peptide transport system has ever been shown to accommodate a peptide of this size. Given the widespread observance of antimicrobial peptides, it seems likely that other prokaryotes may also have evolved a similar mechanisms to evade toxicity through a peptide transport system.

CHAPTER IV

PEPTIDE TRANSPORT IN EUKARYOTES

Saccharomyces cerevisiae

Di- and tripeptide transport has been extensively studied in *S. cerevisiae* while oligopeptide transport activity has only been reported in a limited number of strains (reviewed by Becker and Naider, 1995). The lack of observed oligopeptide transport activity in *S. cerevisiae* may not necessarily be reflective of the true extent of oligopeptide transport in this organism simply because of the limited number of growth conditions and substrates tested previously. In an attempt to understand the molecular mechanisms governing di- and tripeptide transport in *S. cerevisiae*, Island *et al.* (1991) screened a mutagenized population for strains that were resistant to toxic dipeptides. Subsequent complementation analyses of the peptide transport deficient strains indicated that at least three genes, denoted *PTR1/UBR1*, *PTR2*, and *PTR3*, are responsible for di-/tripeptide transport activity in *S. cerevisiae*. The *PTR2* gene was found to encode the actual transporter (Perry *et al.*, 1994) and was the first identified member of the PTR Family (Steiner *et al.*, 1995) while the protein products of *PTR1/UBR1* and *PTR3* were found to regulate *PTR2* activity at the mRNA level (Alagramam *et al.*, 1995, Barnes *et al.*, 1997). Regulation of *PTR2* appears to be very complicated as it falls under the control of the nitrogen catabolite repression system. Rich nitrogen sources, such as ammonium sulfate, repress peptide transport activity while poor nitrogen sources do not. In addition to being regulated by nitrogen source, *PTR2* is also induced by micromolar amounts of certain amino acids (Island *et al.*, 1987).

Bartel *et al.* (1990) originally identified *UBR1* in a screen designed to identify bypass suppressors of the N-end rule of ubiquitin mediated protein degradation. The N-end rule states that the stability of a protein is, at least partially, determined by the N-

terminal amino acid. In a wildtype cell β -galactosidase engineered to contain arginine as the N-terminal residue (Arg- β -Gal) has a half life of 2 minutes as a result of being marked for degradation by the ubiquitin proteolytic degradation system and therefore forms a white colony when grown on a medium containing the chromophoric substrate X-Gal. Bartel *et al.*, (1990) generated a class of mutants that exhibited a decrease in the ability to degrade Arg- β -Gal as measured by blue colony formation when grown on a medium containing X-Gal. Using this color based selection scheme Bartel *et al.* (1990) was able to clone *UBR1* by screening for clones that restored Arg- β -Gal instability as indicated by growth as a white colony. This study led to the proposal that Ubr1p is one of the recognition components of the ubiquitin system. *UBR1* was later recloned as *PTR1* by functional complementation of a peptide transport deficient mutant and hence renamed *PTR1/UBR1* (Alagramam *et al.*, 1995).

Combining the results of these two studies it is apparent that a *ptr1/ubr1* deletion strain is characterized by two seemingly unrelated phenotypes: peptide transport deficiency and Arg- β -Gal stability. The observation by Alagramam *et al* (1995) that *PTR2* is not transcribed in a *ptr1* mutant led to the proposal of two possible models. The first model states that Ptr1p/Ubr1p activates *PTR2* transcription by mediating ubiquitination and hence the degradation of a *PTR2* repressor. Recently, Varshavsky (1996) proposed that this repressor is encoded by *CUP9*, a gene whose protein product was previously shown to be a repressor of the copper transporter. However, this assertion appeared in a review and the evidence suggesting that Cup9p is the target of Ptr1p/Ubr1p has not been formally presented. The second model proposes that Ptr1p/Ubr1p is a bifunctional protein mediating two separate phenomenon: transcriptional regulation of *PTR2* and ubiquitination. Computer aided analyses of Ptr1p have identified several features common to DNA binding proteins such as an acidic tail and a putative leucine zipper. In an attempt to determine if Ptr1p/Ubr1p is a *PTR2* transcriptional

activator, a truncated Ptr1p that lacked the 15 C-terminal residues was expressed in a *ptr1/ubr1* deletion strain. Anderson (unpublished observation) observed that the truncated Ptr1p could not functionally complement a *ptr1* deletion strain and did not increase the stability of Arg-β-Gal when expressed in a *ubr1* deletion strain. Although this experiment was not able to conclude whether Ptr1p is a transcriptional activator, it did demonstrate the importance of the acidic tail in both phenotypes. Regardless of the model, the environmental factors and protein signals triggering Ptr1p/Ubr1p mediated derepression (model I) or transcriptional activation (model II) of *PTR2* have yet to be identified.

The remaining gene implicated in di- and tripeptide transport activity in *S. cerevisiae* is *PTR3* and was recently cloned by Barnes *et al.* (1997). Barnes *et al.* (1997) has noted that *ptr3* deletion strains show a decrease in *PTR2* mRNA levels. Furthermore, the proportional decrease in mRNA levels between a *PTR3* wildtype strain and a *ptr3* deletion strain appears to be independent of nitrogen source when the strains are grown in the presence of amino acid inducers. The recent finding that Ptr3p affects the steady state mRNA levels of *PTR2* as well as the amino acid permeases *BAP1* and *GAP1* in a nitrogen independent manner has led to the hypothesis that Ptr3p mediates the amino acid inducibility of *PTR2* through an undetermined mechanism.

Only three complementation groups involved in di-/tripeptide transport were originally identified by Island *et al.* (1991). However, the mutant screen only identified those strains which were peptide transport deficient and not those mutants which would show an increase in peptide transport. Given the multiple environmental factors which affect di- and tripeptide transport activity it seems likely that other regulatory proteins will be identified in the future. Furthermore, studies have not focused on those genes that may mediate the uptake of peptides larger than tripeptides in *S. cerevisiae*.

Candida albicans

Peptide transport in *C. albicans* consists of at least two separate systems: one for di- and tripeptides and another for oligopeptides. Three primary observations revealed the existence of at least two distinct peptide transport systems. The first observation was that mutants resistant to the toxic peptide analogs bacilysin, polyoxin, and nikkomycin Z (all demonstrated substrates of the di/tripeptide transport system) were able to transport tetra- and pentapeptides at wildtype levels. Conversely, mutants resistant to various toxic tetrapeptides were able to transport dipeptides at wildtype levels (Payne and Shallow, 1985; Milewski *et al.*, 1988; McCarthy *et al.*, 1985). Secondly, peptide uptake experiments with radiolabeled compounds and chromophoric substrates demonstrated that dipeptides did not compete with tetra- and pentapeptides for entry into the cell (Milewski *et al.*, 1988; McCarthy *et al.*, 1985; Yadan *et al.*, 1984). Conversely, tetra- and pentapeptides did not compete with labeled dipeptides for entry into the cell. Thirdly, regulation studies demonstrated that sensitivity to toxic dipeptides in strain CBS-562 was influenced by nitrogen source and micromolar amounts of amino acids (Basrai *et al.*, 1992) while sensitivity to toxic tetra- and pentapeptides was not. Sensitivity to toxic tripeptides was characterized by an intermediate response to nitrogen source and amino acid influence with respect to di- and tetra-/pentapeptides. The conclusion from these studies is that *C. albicans* possesses at least two peptide transport systems, one for di- and tripeptides and another for oligopeptides. The size limitation of the oligopeptide transporter appears to vary slightly from strain to strain but, in general, peptides 3-5 residues in length are transported (for review see Becker and Naider, 1995; Payne and Smith, 1994). While the above studies provide strong physiological evidence for the presence of two peptide transport systems in *C. albicans*, final confirmation awaits genetic analysis.

Di- and tripeptide transport activity in *S. cerevisiae* and *C. albicans* share some common features. First, both *S. cerevisiae* and *C. albicans* have a high affinity for hydrophobic or alanine containing di- and tripeptides and a low affinity for peptides containing basic or acidic residues (Payne *et al.*, 1991; Milewski *et al.*, 1988; McCarthy *et al.*, 1985). Second, neither organism will transport dipeptides containing D-amino acids, although *C. albicans* will transport oligopeptides containing D-residues in a position dependent manner (Shallow *et al.*, 1991). Finally, in both organisms di-/tripeptide transport activity is regulated by nitrogen source and is inducible by micromolar amounts of amino acids (Basrai *et al.*, 1992; Island *et al.*, 1987). Interestingly, there is very little overlap with respect to which amino acids act as inducers. Although peptide transport seems to be regulated by the same environmental factors in these two yeasts the mechanism of regulation appears to be very different. Induction of di/tripeptide transport in *C. albicans* appears to operate independently of *de novo* protein synthesis as supported by uptake experiments in the presence of cycloheximide (Payne *et al.*, 1991). Cultures of *C. albicans* induced for peptide transport in the presence of cycloheximide exhibited wildtype peptide transport activity. Interestingly, an identical experiment performed in *S. cerevisiae* led to the complete loss of peptide transport activity (Island *et al.*, 1991), implying that in *S. cerevisiae* peptide transport is regulated primarily at the transcriptional level whereas in *C. albicans* regulation appears to occur at the protein level (Payne *et al.*, 1991). This observation has been further substantiated after Perry *et al.*, (1994) found that *PTR2* transcription was inducible by leucine and regulated by nitrogen source. A di-/tripeptide transporter has been cloned from *C. albicans* and *S. cerevisiae*, named *CaPTR2* and *PTR2*, respectively Basrai *et al.*, 1995; Perry *et al.*, 1994). *S. cerevisiae ptr2* deletion strains have thus far been found to be peptide transport deficient in all conditions tested. This observation suggests *PTR2* encodes the only di-

/tripeptide transporter in *S. cerevisiae*. However, whether di/tripeptide activity can be solely attributed to *CaPTR2* in *C. albicans* awaits further analysis.

Prior to this study, very little was known about the oligopeptide transport system(s) in *C. albicans*. Only one study has been reported in which the regulation of the oligopeptide transport system was examined. Basrai *et al.* (1992) showed that in strain CBS-562 sensitivity to toxic tetra- and pentapeptides was not influenced by nitrogen source or inducible by amino acids. However, I will present data in Part 2 and Part 4 which indicates that in strain SC5314 oligopeptide transport activity is regulated by nitrogen source

Peptide transport in Plants

Peptide transporters have been shown to play a large role in the germination of the cereal grains barley, wheat, rice, and maize (Higgins and Payne, 1978; Sopanen *et al.*, 1978; Salmenkallio and Sopanen, 1989; Payne and Walker-Smith, 1987). During germination, the proteins in the endosperm are hydrolyzed by various proteases and the resulting amino acids and small peptides are translocated by transporters in the scutella to the embryo where they can serve as a source of amino acids. Payne and Walker-Smith (1987) identified two putative peptide transporters by thiolaffinity labeling in the scutellum of germinating barley. The two proteins were predominantly found 15 hours after imbibition and coincided with the appearance of peptide transport activity. Pulse-chase experiments confirmed that synthesis of these proteins occurred primarily 15-20 hours after imbibition. However, high levels of peptide transport activity were detectable for three days post-imbibition. The authors concluded that the peptide transporters were synthesized 15-20 hours post-imbibition but remained active in the scutellum for up to three days due to slow turnover.

Two PTR type peptide transporters, *AtPTR2-A* and *AtPTR2-b*, have been cloned from *Arabidopsis thaliana* (Steiner *et al.*, 1994; Song *et al.*, 1996). Northern blot analyses of total mRNA from *A. thaliana* failed to detect *PTR2-B* message. However, *AtPTR2-B* message was detected through RT-PCR in RNA isolated from roots. Steiner *et al.* (1994) found that when germinated on a medium containing toxic peptides, seeds would form roots 1-3 mm long before the toxic peptide inhibited further root elongation. Furthermore, *At PTR2-B* message could not be detected after two days of germination. These observations are consistent with the observations by Payne and Walker-Smith (1987) that led to the proposal that peptide transporters are developmentally regulated and are most highly synthesized during a defined period after imbibition. The sensitivity exhibited by the seedlings as well as the lack of detectable mRNA at day 2 is also suggestive of long lived proteins and is therefore in congruence with the findings of Payne and Walker-Smith (1987).

Conclusion

In this literature review I have highlighted two aspects of peptide transport: the diverse array of biological functions in which peptide transport is a participant and various regulatory schemes. Furthermore, I have briefly reviewed the two families, ABC and PTR, under which all peptide transporters previously identified have fallen. Keeping with this theme, I will now discuss how our laboratory recently cloned a novel oligopeptide transporter gene from *C. albicans* and *S. pombe*. Furthermore, we will make the argument that these proteins are not members of the ABC or PTR Families but rather constitute the first members of a new family. Finally, I will present data that suggests that oligopeptide transport activity affects virulence levels in *C. albicans*. Possible mechanisms connecting virulence and peptide transport will be discussed.

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PART 2
CLONING AND CHARACTERIZATION OF AN
OLIGOPEPTIDE TRANSPORT GENE FROM
CANDIDA ALBICANS*

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ABSTRACT

A *Candida albicans* oligopeptide transport gene, *OPT1*, was cloned from a *C. albicans* genomic library through heterologous expression in the *Saccharomyces cerevisiae* di-/tripeptide transport mutant PB1X-9B. When transformed with a plasmid harboring *OPT1*, *S. cerevisiae* PB1X-9B, which did not express tetra-/pentapeptide transport activity under the conditions used, was conferred with an oligopeptide transport phenotype as indicated by growth on the tetrapeptide Lysyl-Leucyl-Leucyl-Glycine, sensitivity to toxic tetra- and pentapeptides, and an increase in the initial uptake rate of the radiolabeled tetrapeptide Lysyl-Leucyl-Glycyl-[³H]Leucine. The level of oligopeptide transport was found to be influenced in the heterologous host by the source of nitrogen used for growth. The entire 3.8 kb fragment containing the oligopeptide transport activity was sequenced and an open reading frame of 2349 nucleotides containing a 58 nucleotide intron was identified. The deduced protein product of 783 amino acid residues contained twelve hydrophobic regions suggestive of a membrane transport protein. Sequence comparisons revealed that similar proteins are encoded by genes from *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* and that *OPT1* is not a member of the ABC or PTR membrane transport families.

CHAPTER I

INTRODUCTION

Peptide transport, a phenomenon defined as the translocation of peptides across the plasma membrane in an energy-dependent manner, has been well documented in bacteria, plants, fungi, and mammals (for reviews see Becker & Naider, 1995; Payne and Smith, 1994). Upon internalization, peptides are quickly hydrolyzed into their amino acid components to serve as sources of amino acids or nitrogen. In addition to acquiring nutrients from the environment, peptide transport has been shown to play a role in recycling cell wall peptides and in transducing signals for group behaviors such as sporulation and competency in *B. subtilis* and chemotaxis in *E. coli*. Recently it has been proposed that in *Salmonella typhimurium* peptide transporters aid the bacteria in evading the host immune response by transporting membrane disrupting peptides away from the plasma membrane (Parra-Lopez *et al.*, 1993). Similarly, in *Streptococcus pneumoniae* the peptide transporters encoded by *plpA* and the *amiA* loci play a role in virulence by modulating adherence to epithelial and endothelial cells (Cundel *et al.*, 1995).

Our lab has recently identified a family of di-/tripeptide transporters named the PTR (Peptide TRansport) Family. This family is characterized by several conserved motifs, has twelve putative transmembrane domains, and is driven by the proton motive force. Members of the PTR family have been identified in a broad variety of eukaryotes and one prokaryote as well (Steiner *et al.*, 1995). Well characterized members of the PTR family are the di- and tripeptide transporters from *S. cerevisiae* (*ScPTR2* , Perry *et al.*, 1994) and from *C. albicans* (*CaPTR2* , Basrai *et al.*, 1995). Both *CaPTR2* and *ScPTR2* have been shown to be regulated by nitrogen source and inducible by micromolar amounts of amino acids; their encoded proteins have broad substrate specificities with a

preference for peptides containing hydrophobic residues (Basrai *et al.*, 1992; Island *et al.*, 1987). Prior to the establishment of the PTR family, all peptide transporters cloned were from prokaryotes and were members of the ATP Binding Cassette (ABC) Superfamily (Higgins, 1992). Recently, transporters from the PTR family have been identified in the prokaryote *Lactococcus lactis* (Hagting *et al.*, 1995). However, in eukaryotes all peptide transporters thus far identified are members of the PTR family.

In addition to the di-/tripeptide transporter (*CaPTR2*) in *C. albicans*, three observations indicated the existence of another distinct peptide transport system. The first observation was that mutants resistant to the toxic peptide analogs bacilysin, polyoxin, and nikkomycin Z (all demonstrated substrates of the di-/tripeptide transport system) were able to transport tetra- and pentapeptides at wild type levels, and, conversely, mutants resistant to various toxic tetrapeptides were able to transport dipeptides at wildtype levels (Payne and Shallow, 1985; Milewski *et al.*, 1988; McCarthy *et al.*, 1985). Secondly, peptide uptake experiments with radiolabeled compounds and chromophoric substrates demonstrated that dipeptides did not compete with tetra- and pentapeptides for entry into the cell, and *vice versa* tetra- and pentapeptides did not compete with labeled dipeptides (Milewski *et al.*, 1988; McCarthy *et al.*, 1985; Yadan *et al.*, 1984).. Thirdly, sensitivity to toxic di- and tripeptides was influenced by nitrogen source and micromolar amounts of amino acids while sensitivity to toxic tetra- and pentapeptides was not regulated by similar means (Basrai *et al.*, 1992). The conclusion from these studies was that *C. albicans* possessed two peptide transport systems: one for di- and tripeptides and another for oligopeptides. The size limitation of the oligopeptide transporter appeared to vary slightly from strain to strain, but in general tetra- and pentapeptides were transported well and tripeptides were transported to a lesser extent (for review see Becker and Naider, 1995). While the above studies provided strong physiological evidence for the presence of two peptide transport systems in *C. albicans*, final confirmation required genetic

analysis. In an attempt to further define the molecular basis of peptide transport, we report here the cloning of a novel oligopeptide transporter from *C. albicans* that does not code for an ABC- or PTR-type transporter.

CHAPTER II

MATERIALS AND METHODS

Strains, Vectors and Media

The strains used in this study were *S. cerevisiae* PB1X-9B (*MATa ura3-52 leu2-3,112 lys1-1 his4-38 ptr2-2*) (Perry *et al.*, 1994) and *C. albicans* SC5314 (Fonzi and Irwin, 1993).

C. albicans and *S. cerevisiae* cells were maintained on YEPD medium (2% dextrose, 1% Yeast Extract, 2% Peptone, and 1.5% agar). The minimal medium used for most studies was made by adding 10 ml of 10X filter sterilized YNB (Yeast Nitrogen Base, Difco) with ammonium sulfate and without amino acids to 90 ml of sterile water containing 2 g glucose and auxotrophic supplements (Sherman *et al.*, 1986). For those experiments where proline was used as a sole nitrogen source YNB without amino acids and without ammonium sulfate was supplemented with 0.1% proline. The mutant strain *S. cerevisiae* PB1X-9B was grown in Synthetic Complete Medium (SC) which consisted of minimal medium with histidine, uracil, lysine and leucine. *S. cerevisiae* PB1X-9B transformed with pRS202 based plasmids was grown on SC lacking uracil (SC-Ura).

The *C. albicans* library used for cloning *OPT1* was kindly provided by Gerry Fink (Liu *et al.*, 1994). The library was created by partially digesting *C. albicans* strain 1006 genomic DNA (Goshorn and Sherer, 1989) with *Sau3A* and cloning the resulting fragments (>4 kb) into the *Sall* site of pRS202, a *URA3/2* μ based plasmid (Christianson *et al.*, 1992).

Peptide medium consisted of minimal medium supplemented with auxotrophic requirements minus the amino acid leucine plus 100 μ M of one of the following peptides: Lysyl-Leucine (KL), Lysyl-Leucyl-Glycine (KLG), Lysyl-Leucyl-Leucyl-Glycine

(KLLG), Lysyl-Leucyl-Glycyl-Leucine (KLGL), or Lysyl-Leucyl-Leucyl-Leucyl-Glycine (KLLLG). Abbreviations for toxic peptides and amino acids used in this study are as follows: Ethionine (Eth), Alanyl-Ethionine (AEth), Leucyl-Ethionine (LEth), Lysyl-Leucyl-Ethionine (KLEth), Lysyl-Leucyl-Leucyl-Ethionine (KLLEth), Lysyl-Leucyl-Alanyl-Ethionine (KLAEth), and Lysyl-Leucyl-Leucyl-Leucyl-Ethionine (KLLLEth). All amino acids were in the *L* configuration.

Enzymes, chemicals and reagents

Restriction endonucleases, T4 DNA ligase, T4 DNA polymerase, and alkaline phosphatase were purchased from New England BioLabs or Promega and were used according to the manufacturers specifications.

Synthesis of Radioactive Lys-Leu-Gly-[³H]Leu

KLGL was prepared by conventional automated solid phase peptide synthesis on an Applied Biosystems Model 433A synthesizer. Peptide was cleaved from the resin with trifluoroacetic acid (TFA) and purified using a C₁₈ reversed phase column (19 x 300 mm) to >99% homogeneity with a 5 to 20% linear gradient of acetonitrile in water over 60 minutes. The product was verified using mass spectrometry [(M⁺+1)=430.2; calculated=429.6].

Tritiated KLGL (Lys-Leu-Gly-[³H]Leu) was prepared by solution phase peptide synthesis as follows. BocLys(Boc)-Leu-Gly-OH (5.2 mg; 10 μmol) was dissolved in 108 μl of a 0.092 μmol/μl solution of N-hydroxysuccinimide (10 μmol) in dry dioxane. Dicyclohexylcarbodiimide (10 μmol in 57 μl) in dry dioxane was added and the reaction mixture was stirred for one hour at ambient temperature. Leu (0.65 mg, 5 μmol), dissolved in 1 ml of water, was added to radioactive leucine (American Radiolabeled Chemicals, Inc., St. Louis, MO; Specific Activity 60 Ci/mmol; Concentration 1mCi/ml in

2% ethanol). This solution was evaporated to dryness, redissolved in 250 µl of water/dioxane (4:1) containing N-methyl morpholine (50 µmol) and the solution containing the activated tripeptide was added. The resulting reaction mixture was stirred for 6 hours at ambient temperature, 5.5 ml of TFA was then added, and after 5 minutes the reaction mixture was evaporated to dryness. The residue was redissolved in 500 µl of water, injected onto a Waters µBondapak C₁₈ column (7.8 x 300 mm) and eluted isocratically using 5% acetonitrile in water, containing 0.025% TFA. Product eluting at the KLGL position was collected, evaporated, redissolved in water (200 µl) and analyzed by high pressure liquid chromatography (HPLC) and on silica thin layers using a Butanol:Acetic acid:water (4:1:5) mobile phase. TLC plates were exposed to film overnight at -80 °C and developed to show one radioactive spot with the mobility of the desired tetrapeptide. KLG-[³H]L was >97% pure according to HPLC. Specific radioactivity was 90 mCi/mmol. Peptide was diluted with nonradioactive KLGL as required.

DNA Manipulations

Small scale plasmid DNA preparations from *E. coli* transformants were performed as described in Sambrook *et al.* (1989). Plasmid DNA from *S. cerevisiae* transformants was isolated as described previously (Ward, 1990). Whole cell DNA from *C. albicans* was obtained by the procedure described by Ausubel *et al.* (1990).

Yeast transformations were done using the procedure described by Gietz *et al.* (1991) and plates were incubated at 30 °C for 4 days or longer.

For Southern analyses whole cell DNA was digested with restriction enzymes and electrophoresed on 1.0% agarose gels. Southern blotting was done as described in Sambrook *et al.* (1989). Hybridization was performed at 60° C for twelve hours in a Hybritube (Gibco BRL) followed by two washes of 1X SSC, 0.1% SDS at 42° C and

two washes of 0.1X SSC and 0.1% SDS at 60° C. The probe used for Southern blots was generated via PCR using the primers LC2 (5' GCATGGATTGTTTCCTGACTGG 3') and FT2 (5' CCAATACCAAACAAATGAGGC). The product was 408 bp in length and its position within the *OPT1* ORF is depicted in Figure 1. The Southern blot displayed in Figure 2 was processed using the program Adobe Photoshop.

For plasmid curing experiments *S. cerevisiae* transformants were grown nonselectively in YEPD broth for about 40 generations. Cells were then plated on YEPD plates to obtain isolated colonies which were picked, washed with water, resuspended at 5×10^6 cells/ml in sterile water, and spotted onto the appropriate peptide medium.

The nucleotide sequence of the 3.8 kb insert in plasmid pOPT1 was generated through automated cycle sequencing using an ABI 373A Automated sequencer (Smith *et al.*, 1986). The insert of pOPT1 was digested with either *TaqI* or *Sau3A*, subcloned into M13, and ssDNA isolated as a template for sequencing from randomly chosen plaques. The sequenced fragments were assembled using the software DNASTAR and the remaining gaps were filled using properly placed primers. Primers were purchased from Bioserve Biotechnologies. Final assembly was performed using Autoassembler from ABI.

Growth and transport assays

Growth assays to determine the phenotype of the cells were done as described by Island *et al.* (1991). Briefly, 3 μ l of culture from a suspension of 5×10^6 cells/ml were spotted to the surface of the medium and plates were incubated at 30 °C for 4-7 days.

Uptake of KLG-[³H]L was determined using a protocol for uptake of dipeptides as described by Basrai *et al.* (1995) with a few modifications. *S. cerevisiae* cultures were grown overnight to exponential phase in SC-Ura medium. Cells were harvested by centrifugation, and resuspended in 2% glucose at a cell density of 2×10^8 cells/ml. Two

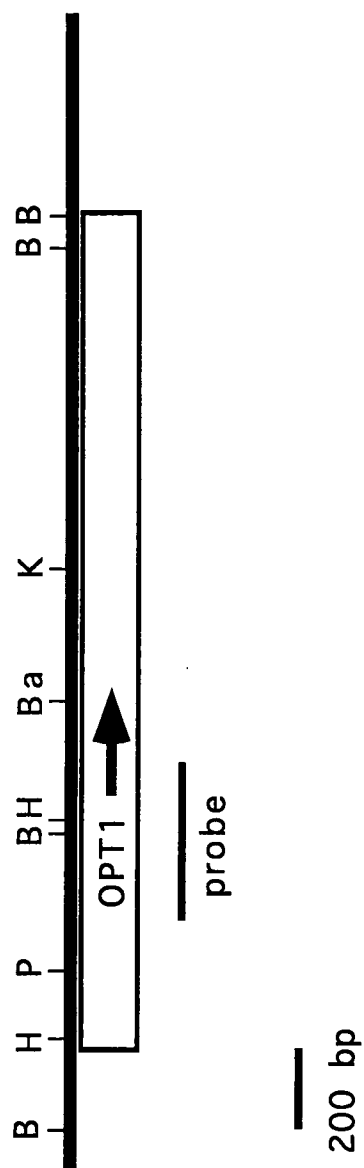


Figure 1. A partial restriction map of the 3.8 kb fragment from pOPT1. The location and orientation of the open reading frame are indicated as well as the location of the probe used in Southern blots. Abbreviation of restriction sites are as follows: B-*BstXI*; H-*HincII*; P-*PvuII*; Ba-*BamHI*; and K-*KpnI*.

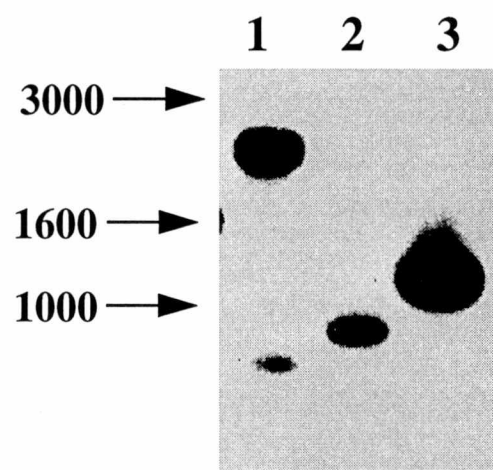


Figure 2. Southern blot. Analysis of hybridization of a probe of *OPT1* to genomic DNA isolated from *C. albicans* SC5314 was performed as described in Materials and Methods. Lanes: 1, *Hinc*II digest; 2, *Pvu*II/*Bam*HI digest; 3, *Pvu*II/*Kpn*I digest. Size markers are in bp.

hundred and fifty microliters of cell suspension were added to an equal volume of an uptake assay reaction mixture and incubated at 30°C. The final concentrations of the components in the uptake assay solution were: glucose (2%, w/v), 10mM sodium citrate/potassium phosphate buffer (pH 5.0), and KLG-[³H]L (150 µM; 8.5 mCi/mmol). Competition experiments were done in the presence of either 1.5 mM L, KL, KLG, KLLG, or KLLL. At various time points, 90 µl portions were removed and filtered through a membrane. The yeast cells retained on the filter were washed twice with ice-cold distilled water, once with room temperature distilled water, and the residual radioactivity was measured by liquid scintillation. There was no peptide adsorption to the cell surface or sticking to filters since at 0° C the counts were at background level. The uptake results, calculated on the basis of 50% counting efficiency (determined using L-[³H]lysine as a standard, and the specific activity of the peptide), were expressed as nmol of peptide uptake per minute per mg cell dry weight.

Sensitivity assays

Sensitivity to ethionine, a toxic methionine analog, and ethionine-containing peptides was determined by the method described by Island *et al.* (1987). Cells were grown overnight to exponential phase of growth in SC, washed, and resuspended at 5×10^6 cells /ml in sterile water. One ml of this cell suspension was added to 3 ml molten Noble agar (0.8% final concentration) and overlayed on 20 ml of the same medium used to prepare the inoculum. A disk (6 mm diameter, Difco) was placed on the plate and 0.38 µmoles of the compounds to be tested were applied to the disks. Zones of inhibition were measured after 24-48 hr incubation at 30 °C. Each test comprised at least three independent assays and the results represented in the Tables are means of the values obtained. Maximum variation between the zones of inhibition measured for each test were ≤3 mm. A value of 7 mm for the diameter of zone of inhibition represents a minimal

growth inhibition value as the disk diameter was 6 mm. Photodocumentation of sensitivity assays was done with a Umax Scanner and processed through Adobe Photoshop.

Nucleotide sequence accession number

The nucleotide sequence reported in this paper has been entered in the GenBank data base and assigned accession number U60973.

CHAPTER III

RESULTS

Cloning of an Oligopeptide Transporter

We have recently cloned di- and tripeptide transporters of *Candida albicans* (*CaPTR2*) (Basrai *et al.*, 1995) and *Arabidopsis thaliana* (*AtPTR2-A* and *AtPTR2-B*) (Steiner *et al.*, 1994; Song, *et al.*, 1996) through heterologous expression in *Saccharomyces cerevisiae*. Unlike *C. albicans*, *S. cerevisiae* has been found to transport only a limited number of tetra- and pentapeptides under a limited number of growth conditions (reviewed by Becker and Naider, 1995). Therefore, as our primary screen, we transformed the *S. cerevisiae* di-/tripeptide transport mutant PB1X-9B with a high copy number *C. albicans* genomic library and screened for the ability of *S. cerevisiae* to grow on a normally non-utilized tetrapeptide as a sole source of auxotrophic supplements.

A pRS202 based *C. albicans* genomic library was transformed into *S. cerevisiae* PB1X-9B and 32,000 *URA3*⁺ transformants were obtained. Transformants were pooled into 6 groups of approximately 5,200 transformants each and subsequently plated onto a medium containing 50 μ M Lys-Leu-Leu-Gly (KLLG) as the sole source of leucine and lysine as well as ammonium sulfate as a nitrogen source. A double auxotrophic selection was employed to preclude the possibility of cloning the *C. albicans* *LEU2* or *LYS1* homologs. *S. cerevisiae* PB1X-9B can not utilize the tetrapeptide KLLG as a sole source of lysine or leucine when grown on a medium containing a rich nitrogen source such as ammonium sulfate (unpublished observation). Oligopeptide transport (OPT) positive colonies appeared after 5-7 days of incubation at 30 C.

Curing of the plasmid by growth in nonselective conditions as well as shuttling the plasmid through *E. coli* and back into *S. cerevisiae* PB1X-9B demonstrated that the OPT activity was plasmid borne. Subsequently two different plasmids, denoted pOPT1

and pOPT24 containing inserts of 3.8 and 4.3 kb respectively, were recovered from a representative sample of OPT⁺ colonies. Initial restriction mapping demonstrated that the smaller of the two plasmids pOPT1 overlapped entirely with the larger plasmid pOPT24. Therefore, the plasmid pOPT1 (Fig. 1) was used in all subsequent experiments.

Southern Blot

Southern blot analysis was done to ensure that *OPT1* was derived from *C. albicans* genomic DNA and to determine if there were other homologous genes. Genomic DNA was isolated from *C. albicans* SC5314 and digested with the restriction enzymes *HincII*, *BamHI/PvuII*, and *PvuII/KpnI*. The resulting fragments were separated on a 1% agarose gel and Southern blotting performed as described in materials and methods. The *PvuII/KpnI* and *PvuII/BamHI* digests were each predicted to yield one band while the *HincII* digest was predicted to yield two bands. As seen in Figure 2, each digest produced their predicted bands; 617 bp and a band of >2700 bp for *HincII* (Lane 1), 790 bp for *PvuII/BamHI* (Lane 2), 1163 bp for *PvuII/KpnI* (Lane 3).

Nucleotide and Deduced Amino Acid Sequence of *OPT1*

Sequence analysis revealed the presence of two ORFs, separated by a type II intron, and encoding a hydrophobic protein of 783 amino acids with an apparent MW of 88 kD and a pI of 7.1 (Fig. 3). The first ORF contained 1626 nucleotides while the second ORF contained 723 nucleotides excluding the stop codon. The intron separating the two ORFs was 58 nucleotides in length and contained the highly conserved 5' splice site (GCATGT), 3' splice site (TAG), and branch point (TACTAAC) (Rymond and Rosbash, 1992). The two ORFs and intron constitute the gene *OPT1*. The size and hydrophobic nature of the predicted protein product of *OPT1* are suggestive of a

Figure 3. Nucleotide and predicted amino acid sequences of *OPT1*. The predicted amino acids are italicized and numbered to the left of the figure while nucleotides are numbered to the right. The 5' and 3' splice sites as well as the conserved branch point of the intron are boxed. The codon CUG (CTG in the DNA) codes for serine not leucine in *C. albicans* (Omaha *et al.*, 1993).

GATCATGTGGCTAAAAATTCATATGGCTTTATGCTGTGGCTTCAGCTCTACTGATAATTGATATCTTTATT -410

CCTATACATAAATATTAAAGCCACTTGATTATTGCTCATAGGGCCAAAAACAAAAAGATGCAGAACCATCTAAAGTTTGGTTTGGCTTTGCTATT -312

TTGTGCTAGTGAGATTAAATAGTTATCTTTTCATGACAAAAATCCTTTAGACTACTTTTATTCCATTGGTTGGTTTACGATTATCAATCGTCAT -214

AGTTCAATTTTGTAAAAATTTATCTTTTCAATCCCAACCTTTTAAATAGTCAGTAATTTCTCTCATAGGAATTTCAAGTTTCCACTTTTGTGCTTT -116

CCTTCTATTTTCTTTTATAAGTTTACTGTTTCGTGAAATATTATTCATTGTATTATTTTACTAAGTCAACCACATTGATTCATTCTTAACACT -18

TATTATAAGTACTTACT -1

1 ATG GAC AAA ATA AGG GCA GTA ATT AGT GGA GGT GAG AAA CCT CCC GTT GAC ACT GAC AAC GAT CAC AAC ACA GAC 75

M D K I R A V I S G G E K P P V D T D N D H N T D

26 TTT GAG GCT GAC AGA AAA ATG CCA GAT TTG GAT ATT GTA GTT TCC AAA TCA CAA GAA TTT GAC CCA CTC ACC TCC 150

F E A D R K M P D L D I V V S K S Q E F D P V T S

51 CAC TTG GTT AAT GAT ATT ATG GAA GAT GAA TAT GCT GCT CAT GTT GAA GAT GAT TCT CCT TAT CCA GAA GTT 225

H L V N D I M E D E Y A A V H V E D D S P Y P E V

76 AGA GCA GCT GTT CCT TCT ACT GAC GAC CCA ACT TTA CCT CAA AAT ACC ATT AGA GCC TGG GTT ATT GGT TTG ATA 300

R A A V P S T D D P T L P Q N T I R A W V I G L I

101 TTG ACT AGC GTT GGT TGT GGT ATG AAT ATG TTG TTG AGT TTC CAT AGT CCC TCA TTT GCT ATC ACC ACT TTT GTC 375

L T T V G C G M N M L F S F H S P S F A I T T F V

126 ACA TCC ATT TTG GCT TGG CCA ATT GGG AAC TTT TGG GCA TGG ATT GTT CCT GAC TGG AAG ATT TTT GGT GCT TCG 450

T S I L A W P I G N F W A W I V P D W K I F G A S

151 TTA AAT CCA GGT CCA TTC AAC GTT AAA GAA CAT ACT ATC ACT ATT ATG GCC AAC GTT TCT TTT GGT ATT GGT 525

L N P G P F N V K E H T I I T I M A N V S G F G T G

176 GCC GCA TAT GCC ACA GAT ATT TTG CTT GCA CAA AAT ATG TTT TAT AAA TCA AAT TTT GGT TGG GGG GAT CCA GAA GTT 600

A Y A T D I L L A Q N M F Y K S N F G W G Y N L

201 TTA CTT ATC TGG AGT ACC CAA TGT ATT GGG TTT GCT TTC GGA GGT GTT ATG AGA AGA TTT GTT GTT GAC AGT CCA 675

L L I W S T Q C I G F A F G G V M R R F V T G S P

226 GGG GCC ATC TGG CCC CTG AAT TTG GTC ACC GCA ACA TTC TTG ACT AAT ATG CAC ATT AAC GAA AAC CAC ACT GCT 750

G A I W P S N L V T A T F L T N M H I N E N H T A

251 AAT GGC TGG AAA ATT TCT CGT CTT GCA TTT TTC GTG ATC GTG TTT GTT GCC TCA TTT GTT TGG TAT TGG TTC CCA 825

N G W K I S R L A F F V I V F V A S F V W Y W F P

276 GGT TAT ATT TTC CAG GCT TTA TCG TAT TTT TCT TGG ATC ACC TGG ATT AAA CCA AAC AAT GTC ATT ATC AAC CAA 900

G Y I F Q A L S Y F S W I T W I K P N N V I I N Q

301 GTT TTC GGT TCT TCA TCT GGS TTA GGT ATG ATT CCT AAC AAC ATT GCC TTG GAC TGG AAC CAA ATT GCA GGG TAT 975

V T F G S S S G L G M I P N N I A L D W N Q I A G Y

326 ATT GGG TCT CCA TTG ATT CCA CCA GCT AGT GTT ATT GCT ACA ATT TTT GGA TCC ATT GTG CTT ATT TTC TGG ATT 1050

I G S P L I P P A S V I A T I F G S I V L I F W I

351 GTT GTG CCA GCT ATT CAC TAT TCC AAC ACT TGG TAC TCC CAA TAC TTG CCA ATC TCA TCT ACT GGA TCG TTT GAT 1125

V V P A I H Y S N T W Y S Q Y L P I S S T G F D

376 AGG TTC CAA CAA ACT TAT AAT GTG TCA AAA ATT ATC GAC CAT AAA ACT TTA TCA TTC AAT GAA GCG GAA TAC AAA 1200

R F Q Q T Y N V S K I I D H K T L S F N E A E Y K

401 AAG TAT ATT TTC CAG GCT TTA TCC ACC ACC TTT GCT ATT TCC TAT GGG CTA TCA TTT GCC TCC ATT TTA GCC ACT 1275

K Y S P L F L S T T F A I S Y G L S F A S I L A T T

426 ATA ACA CAC ACC ATT TGC TTC CAT GGA CGT GAC CTT ATC GCA TCG TTG AAG GCC AAA GAA AAA CCA GAT GTT CAC 1350

I T H T I C F H G R D L I A S L K A K E K P D V H

451 AAT AGA TTA ATG AAA GCA TAC AAA CCA GTG CCT GAA TGG TGG TAC CTA GTT GTC TTC TTG GTC TTT TTC GGT ATG 1425

N R L M K A Y K P V P A S V I A T I F W Y L V V F L V F T T G M

476 TCC ATA GCC ACC GTA CGT GCT TGG CCT ACT GAA ATG CCA GTA TGG GGG TTA GTT TTT GCT CTT ATC ACT GCT ACT 1500

S I A T V R A W P T E M P V W G L V F A L I I A I

501 ATA TTT TTA TTA CCC GTT GCT ATC ATT TAT GCA AAA ACG AAT ATT GCT GTT GGT TTA AAC GTT GTA ACC GAG TTC 1575

I F L L P V A I I Y A K T N I A V G L N V V T E F

526 ATC GTG GGC TAC GTA CTA GGT GGA CGT CCC CTA TGT ATG ATT TTG TTG TTT AAG GCAATG ATTAGAATTTCGACATATAATCA 1656

I V G Y V L G G R P L C M M L F K

543 GTCAGTT TACTAAC CTAATTTTGAA TAG ACC TTC GGA TAC ATC ACT AAT AAC CAA GCT GTT ACT TTT GTG CAG GAT ATG 1735

T F G Y I T N N Q A V T F V Q D H

560 AAA CTT GGG CAC TAC ATG AAA ATA GAT CCG CGC ACT TTG TTT TGG GCG CAG TTT GCT GCT ACC ATA TGG GGA TCG 1810

K L G H Y M K I D P R T L F W A Q F A A T I W G S

585 TTA GTT CAG ATC GCA GTT TTG GAG TGG GCC TAT GGT GCA ATC GAC AAT TTG TGT GCT GCT GAC CAA AAA AAT CAT 1885

L V D Q I A V L E W A Y G A I D N L C A A D Q K N H

610 TAC ACA TGT CCA AAC GGT AAA GTT TTC TTA AAT GCT TCG ATC ATT TGG GGT GTC ATT GGA CCC CAA CGT CAA TTC 1960

Y T C P N G K V F F N A S I I W G V I G P Q R Q F

635 TCA CTT GGG CAG ATT TAT TAT GGG TTA CTT TTC TTT ATC ATT GGT GCT GTG ACC CCT GTG ATC AAT TGG TTG 2035

S H G Q I Y Y G L L F F F I I G A V T P V I N W L

660 ATC TTG AAA AAA TGG CCA AAC TCT CCA GTC AAG TAT TTG CAT TGG CCA GTG TTC TTT TCT GGG CAA GGG TAT ATT 2110

I L K K W P N S P V K Y L H W P V F F S G T G Y I

685 CCT CCA GCC ACT CCA TAT AAC TAT ACC TCC TAC TGT GCT GTG GGT TTG TTC TTT GGA TGG TGG ATT AAA AAG AAG 2185

P A T P Y N Y T S Y C A V G L F F G W I K K K

710 TGG TTC CAC TGG TGG TCT AAA TAC AAC TAT TCC TTG TCT GCG GGC TTG GAT ATT GGT TTG GCA TGG TCG TCG TTG 2260

W F H W S K Y N Y S L S A G L D I G L A W G S L

735 ATC ATT TTC TTG TGC TTG AGT TTA ACA AAC ACC GAC TTC CCA TCG TGG TGG GGA AAC GAT GTG ATC AAC ACC ACT 2335

I I F L C L S L T N T D F P A S W W G N D V I N T T

760 CTC GAC ACT CAG GTT GTC ACC AAT ATC AGA CAC ATA TTG AAA GAG GGA GAG GCA TTT GGG CCA TCT TCC TGG TAA 2410

L D T Q V V T N I R H I L K E G E A F G P S S W

GCTGACGAAGAACACACACACACATTATGCTTCTTATGCTGCTGCTTTTATGATAGTGAACGTGTTTTTATAAGAATGTAATTTAATTTATGTT 2509

ATATATATGCCAAATATTTTACTGCCATTTTATATTTCTTCTGCCACTAAAAATGATAGGAGGTTTGTATACTGGGTGCTTGTGTTTACACGCGGG 2608

CTCTTTTATTTATGATTGTAACAGTCTCTAAGGAAGTTACGAACCTATAGGTGAGTCTAAAAATGAAAGGGAGTGAGGTTCTCTTATATCTCTTT 2707

TTGGCAAGTAAATGTGCTGCTTTGATATATTAGAAAGCAATCCATTAATAGATGAAATATATTTGATGATGAAAGAGTATGGTTGTTCAAAAT 2806

GAAAGATCAATATAAAATTCGAGAGAGAACGTGATGTTTATAGAGTAAAAAATTGAGCTGATAACTTCGCAACCAATTCGAACAAGCATAGTTTGCA 2905

AATATGAATACATCTAGAAAAAGTGAATCTATGAGGAATATGCAGGATATTCATGATCTCTTAGCAAAATATTAAGGTTCAATCGGTTTGTGAT 3004

GGTTGCAAAATTTATCATTCGCGGTGTAAGTACACCAAGAGTTAGACACCTACATGATCTTTTGTGTTTTCATCTTTTGTATTTCCATATAAAAGT 3103

TCTCCCGTTTCTCTTCTCTTTCTTCTGAAAAATGAACAATATAGAAATCTTTTGTGGTGAATCACTGATTCGCTAACCTTCGTTCTTGAATTTAT 3202

ATTCTATTAATTTGTTGATCATCGCTGCTTCTATTTCCATCTCTCTGTTTCAAAATCAAAATATAAAAGTTCAAGAGATGATCAATAGTGACAATTT 3301

TCAGCTCGTGTAACCTGTAAGTGAAGTAAATACAGACTCTGCAGATACACATGCTGAAAAATTAATGACAAAGGTATGTTGAACATGATC 3392

membrane bound protein with at least twelve putative transmembrane domains of 20-24 amino acid residues.

A search of the database using the BLAST algorithm (Altschul *et al.*, 1990) identified two ORFs from *S. cerevisiae* and one ORF from *S. pombe* as having significant homology. The ORFs YJL212C and YPR194C from *S. cerevisiae* were identified during the genome sequencing project and were not assigned any function. The remaining ORF, *ISP4* from *S. pombe*, was identified as a gene of unknown function that was up-regulated as a result of inducing meiosis through nitrogen starvation (Sato *et al.*, 1994). However, whether this induction was meiosis specific or due simply to nitrogen starvation was not determined. The predicted protein products of the putative homologs were aligned (Fig. 4) using the PileUp program (Feng and Doolittle, 1987) from the Genetics Computer Group (GCG) software (Devereux *et al.*, 1984) and percent identity and similarity calculated using the GCG program Bestfit. The protein Isp4p from *S. pombe* exhibited the best homology with 48% identity and 70% similarity. The two proteins from *S. cerevisiae* exhibited lower homology with 40% identity and 63% similarity for YJL212C and 34% identity and 59% similarity for YPR194C.

The PTR family of peptide transporters is characterized by the signature motif FYXXINXGSLs (Steiner, *et al.*, 1995) whereas the ABC transporters are characterized by the ATP binding Walker motifs (Higgins, 1992). The predicted protein product of *OPT1* did not contain the PTR signature motif or the ABC Walker motif. Furthermore, a comparison of *OPT1* with the PTR di-/tripeptide transporter *CaPTR2* using the GCG program Bestfit revealed only 18% identity between the two transporters. These data indicated that *OPT1* is not a member of the PTR or ABC families of membrane transporters.

Figure 4. Comparison of oligopeptide transporters. A) The Proteins Opt1p, Isp4p, Yjl212cp , and Ypr194cp were aligned using the program PileUp. Conserved residues are in capitals and denoted as consensus while nonconserved residues are in lower case. The amino acids in each respective protein are numbered to the right.

Peptides as Growth Substrates

To determine the size constraints of peptide utilization mediated by pOPT1 we tested the ability of *S. cerevisiae* PB1X-9B (a mutant in the di-/tripeptide transporter) harboring either pRS202 (the parent vector) or pOPT1 (pRS202 containing the 3.8 kb insert with the *OPT1* gene) to grow on KL, KLG, KLLG, and KLLLG as a sole source of leucine. Previously it has been shown that di- /tripeptide transport activity in *C. albicans* and *S. cerevisiae* is regulated by nitrogen; rich nitrogen sources such as ammonium sulfate repress, while poor nitrogen sources such as proline derepress transport. Therefore, we simultaneously determined the effect of nitrogen source on oligopeptide transport activity by supplying either ammonium sulfate or proline as the nitrogen source. When grown on a medium containing ammonium sulfate, PB1X-9B(pOPT1) was only able to utilize the peptide KLLG as a source of leucine whereas PB1X-9B(pRS202) did not utilize any of the peptides tested. When grown on a medium containing 0.1% proline, PB1X-9B(pOPT1) was also able to utilize the tetrapeptide KLLG as a sole source of leucine, although the growth was much more robust than the growth exhibited on the ammonium sulfate medium. No growth was observed on KL, KLG and KLLLG for PB1X-9B(pOPT1) or PB1X-9B(pRS202).

Sensitivity of *S. cerevisiae* Transformants to Toxic Peptides

S. cerevisiae PB1X-9B is sensitive to the toxic amino acid ethionine (Eth) but is resistant to ethionine containing di-, tri-, tetra-, and pentapeptides. We utilized disk sensitivity assays to determine if cells transformed with pOPT1 were sensitive to toxic peptides and whether this sensitivity was dependent upon nitrogen source. In those conditions where ammonium sulfate was used as a nitrogen source, no zone of growth inhibition was seen for the transformed strain in the presence of AEth, LEth, or KEth, whereas a 33 mm zone of inhibition was seen for ethionine alone (Table 1). A small and

Table 1 : Sensitivity of *S. cerevisiae* PB1X-9B transformants to various ethionine-containing peptides

<i>S. cerevisiae</i> Transformants [†] :	Nitrogen source	Zone of inhibition (mm) *					
		Eth	AEth	KLEth	KLLEth	KLAEth	KLLAEth
pRS202	(NH ₄) ₂ SO ₄	33	none	none	none	none	none
pOPT1	(NH ₄) ₂ SO ₄	33	none	none	diffuse [§]	diffuse [§]	diffuse [§]
pRS202	proline	33	none	none	none	none	none
pOPT1	proline	35	none	none	32	34	32 [‡]

* A total of 0.38 μ moles was spotted on a 6 mm disk placed on a lawn of cells. "None" indicates no growth inhibition. Each test comprised three or more independent assays and the results represented in the Table are means of the values obtained. Maximum variation between the zones of inhibition for each test was ≤ 3 mm.

[†] Transformants tested harbored either the parent vector (pRS202) or the parent containing vector containing the 3.8 kb fragment (pOPT1).

[§] A small and diffuse halo with no distinct zone of growth inhibition was observed.

[‡] The halo observed was hazy.

diffuse zone of growth inhibition (about 11-15 mm) was seen for KLEth, KLAeth, and KLLAeth. When 0.1% proline was used as a nitrogen source, a zone of complete growth inhibition was seen for the toxic peptides KLEth, KLAeth, and KLLAeth for PB1X-9B(pOPT1) but not for PB1X-9B(pRS202) (Fig. 5; Table 1). Neither strain exhibited sensitivity to the toxic dipeptide or tripeptide Aeth and KLEth when proline was used as a nitrogen source.

Transport of Lys-Leu-Gly-[³H]Leu in *S. cerevisiae* Transformants

To determine if *S. cerevisiae* transformants harboring pOPT1 could accumulate a radiolabeled tetrapeptide, uptake assays were performed with the radiolabeled substrate KLG-[³H]L with cells grown to log phase in SC-Ura with either ammonium sulfate or 0.1% proline as a nitrogen source. PB1X-9B(pOPT1) grown in SC with ammonium sulfate exhibited a significant uptake rate compared to no uptake by PB1X-9B(pRS202) (Fig. 6a). Furthermore, PB1X-9B(pOPT1) demonstrated a higher initial rate of uptake when compared to *C. albicans* SC5314 grown in the same medium. This higher initial rate can be explained by overexpression due to high copy number or alternatively by the lack of requisite regulatory elements which may be absent in the heterologous host. All three strains had a higher rate of initial uptake when grown in SC-Ura with 0.1% proline as a nitrogen source (Fig. 6b). PB1X-9B(pRS202) did accumulate the tetrapeptide KLGL under these conditions but apparently not to a large enough extent to support growth on KLLG or to exhibit sensitivity to KLEth or KLAeth (Table 1; Fig. 5).

To more rigorously determine the size constraints of the oligopeptide transporter, the accumulation of KLG-[³H]L was measured in the presence of 10-fold molar excess of the competitors L, KL, KLG, KLLG, and KLLLG. SC-Ura with ammonium sulfate was chosen as the growth medium because under these growth conditions PB1X-9B(pOPT1)

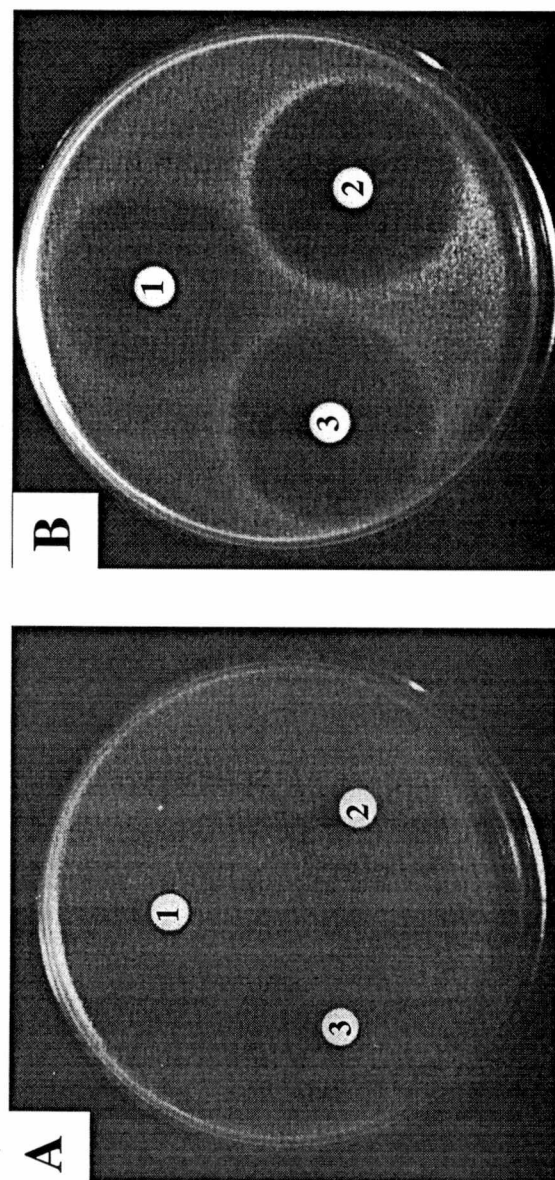


Figure 5. Toxic peptide inhibition assay. Sensitivity to the ethionine-containing peptides KLLAEth (1), KLAETH (2), and KLEth (3) on a 0.1% proline medium was determined as described in Materials and Methods. A. PB1X-9B(pRS202); B. PB1X-9B(pOPT1).

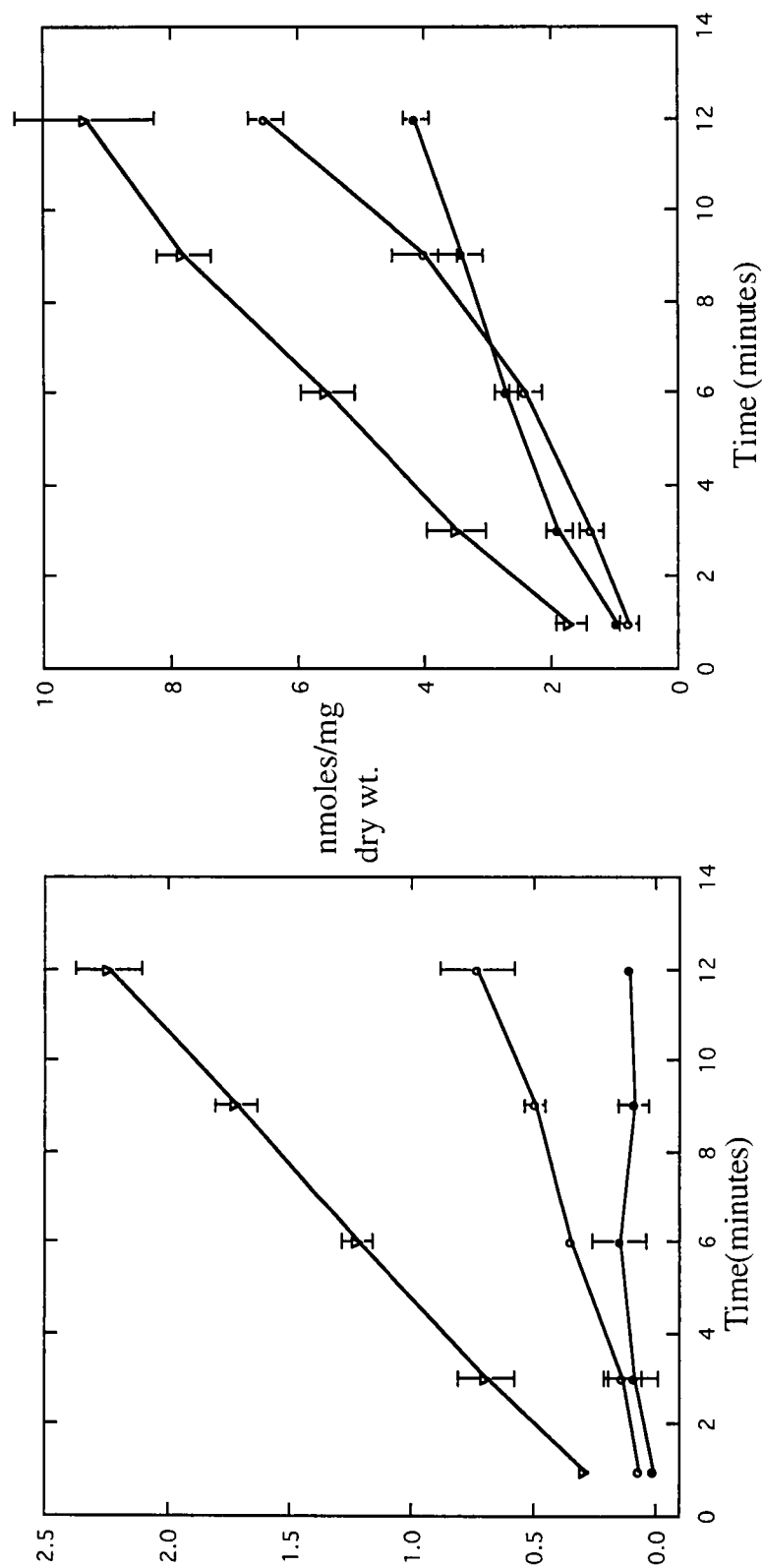


Figure 6. Peptide transport assay. Accumulation of KLG-[³H]L was measured over a twelve minute time course as described in Materials and Methods. Strains *S. cerevisiae* PB1X-9B harboring pRS202 (●) or pOPT1 (▽) and *C. albicans* SC5314 (o) were grown in SC-Ura media with either ammonium sulfate (Figure 6a) or 0.1% Proline (Figure 6b) as a nitrogen source.

accumulated KLG-[³H]L whereas PB1X-9B(pRS202) did not (Figure 6a). As seen in Figure 7, L and KL do not compete with the uptake of KLG-[³H]L whereas competition was seen with KLLG and KLLLG. The tripeptide KLG exhibited decreased competition in comparison to KLLG or KLLLG (Fig. 7) possibly due to a lower affinity, although this low level of KLG uptake is below the threshold to support full growth when used as an auxotrophic supplement. Uptake rates were calculated from a bestfit of the slope for each set of data. The uptake rate of KLG-[³H]L in the presence of no competitor, L, or KL was 0.24, 0.25, and 0.26 nm/min/mg of dry weight, respectively. When KLG was used as a competitor the uptake rate was 0.12 nmoles/min/mg of dry weight which was approximately 50% of the no-competitor rate. The uptake rate approximated zero when KLLG and KLLLG were used as competitors.

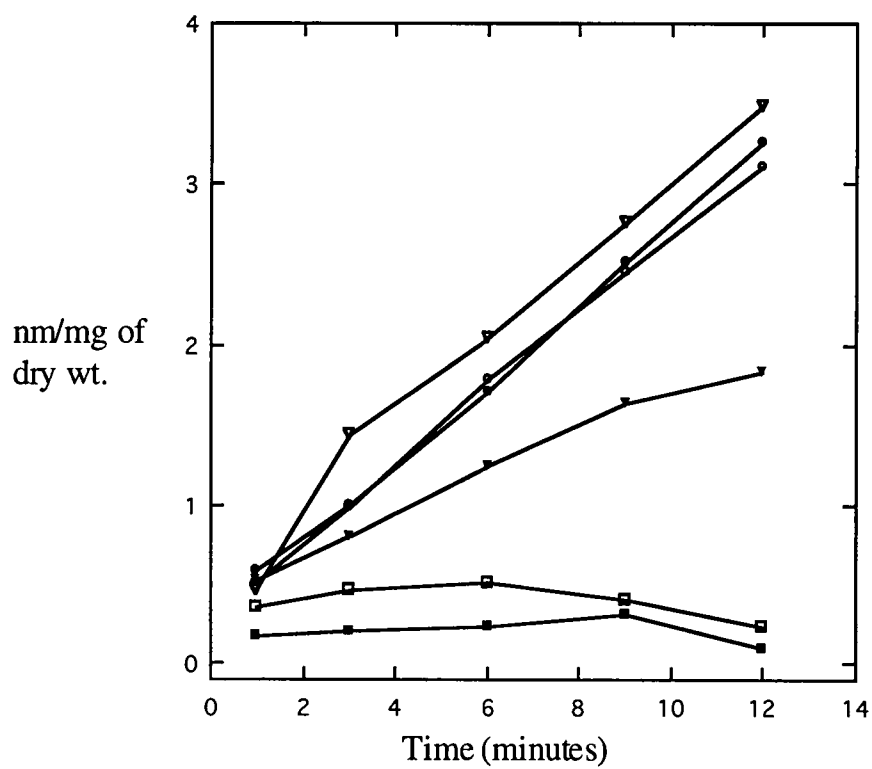


Figure 7. Peptide transport competition experiment. Accumulation of KLG-[³H]L (○) was measured in the presence of a 10 fold molar excess of the competitors L (●), KL(▽), KLG (▼), KLLG (◻), or KLLL (■) over a twelve minute time course.

CHAPTER IV

DISCUSSION

Three lines of evidence support the cloning of an oligopeptide transport gene from *C. albicans*. First, the plasmid pOPT1 conferred the ability to utilize the peptide KLLG to satisfy the leucine auxotrophic requirement of *S. cerevisiae* PB1X-9B when grown on a medium with a rich or poor nitrogen source. Secondly, the *S. cerevisiae* strain PB1X-9B was not sensitive to the toxic peptides KLL⁺EtH, KLA⁺EtH, or KLLA⁺EtH when grown on a minimal medium with 0.1% proline as a nitrogen source but was sensitive when transformed with the plasmid pOPT1 (Fig. 5; Table 1). Similarly, a very faint zone of growth inhibition was seen for KLL⁺EtH, KLA⁺EtH, and KLLA⁺EtH when PB1X-9B(pOPT1) but not PB1X-9B(pRS202) was grown in a medium containing ammonium sulfate. Finally, PB1X-9B(pRS202) had an initial uptake rate of zero for the radiolabelled substrate KLG-[³H]L when grown on a media with ammonium sulfate whereas PB1X-9B(pOPT1) had a dramatically higher initial uptake rate (Fig. 6a). When the growth media contained proline as a nitrogen source, the initial uptake rate was 2.5 times higher for PB1X-9B(pOPT1) than for PB1X-9B(pRS202) (Fig. 6b). Furthermore, uptake was competed by KLLG and KLLL⁺G and to a lesser degree KLG (Figure 7). The fact that leucine did not compete with KLG-[³H]L for uptake excluded the possibility that *OPT1* coded for a secreted protease. Therefore, we propose that we have cloned an oligopeptide transporter from *C. albicans* capable of transporting tetra- and pentapeptides and to a lesser extent tripeptides.

The predicted protein product of *OPT1* did not show any significant homology to any members of the ABC superfamily or PTR family of transporters. Furthermore, a search of the Prosite (Bairoch, 1992) and Motifs (Devereux *et al.*, 1984) databases for protein motifs did not reveal any previously identified functional domains common to

transport proteins with the exception of potential glycosylation sites. However, the twelve putative transmembrane domains separated by hydrophilic regions as well as the expression of transport activity in a heterologous host are suggestive of an integral membrane transporter. Because three ORFs of significant homology as well as several Expressed Sequence Tags (data not shown) were identified, the possibility exists that *OPT1* constitutes the first identified member of a new family of transporters. We are currently testing this hypothesis in our laboratory by testing these ORFs for oligopeptide transport activity.

Opt1p is able to accommodate peptides of 3-5 residues; peptides of larger than five were not tested. As demonstrated by growth assays, halo assays, and competition experiments tetrapeptides were most readily transported by Opt1p. On the other hand, pentapeptides did enter the cell as demonstrated by sensitivity to KLLAEth and supported by the competition between KLLLG and KLG-[^3H]L. However, KLLLG was not able to support growth when used as a source of leucine possibly due to the inability of cellular peptidases to release leucine from this peptide. Similarly, KLG was able to compete slightly for entry into the cell with KLG-[^3H]L, but KLG did not support growth and KLEth was not toxic. Because the number of possible tri- and pentapeptides is large and our sample size small, we can not conclude that Opt1p has a lower affinity for tri- and pentapeptides than for tetrapeptides.

Sequence analysis revealed the presence of a 58 nucleotide intron located within the 3' half of *OPT1*. The 5' splice site, 3' splice site, and branch point are identical to previously reported type II introns within fungi (Rymond and Rosbash, 1992). It is interesting to note that the di-/tripeptide transporter *CaPTR2* also contains a small type II intron that is located within the 3' half of the gene. It has been suggested that introns play a regulatory role. However, a comparison of the two introns did not reveal any apparent

consensus sequences that might be suggestive of a common regulatory element or of a common ancestry.

To date only one study has been published addressing the regulation of oligopeptide transport activity in *C. albicans*. Basrai *et al.*, (1992) concluded that sensitivity to toxic oxalysine-containing tetra- and pentapeptides was not influenced by nitrogen source or by the presence of amino acid inducers. However, our findings suggest that when expressed in *S. cerevisiae*, *OPT1* is regulated by nitrogen source. The discrepancy in results may be explained by differences in the levels of regulation or substrate specificity between the two different strains used in the studies, or alternatively by superimposition of a *S. cerevisiae* regulatory mechanism on the *CaOPT1* gene expressed heterologously.

A search of the database using the BLAST algorithm identified three putative homologs of *OPT1*. The *ISP4* gene from *S. pombe* exhibited the highest homology and was identified by Sato *et al.* (1994) through a subtractive hybridization experiment using RNA isolated from nitrogen starved and non-nitrogen starved cells. In *S. pombe*, nitrogen starvation induces meiosis and therefore this nitrogen-starvation/meiosis-inducing screen identified genes that were either induced during meiosis or regulated by the nitrogen catabolite repression system. Based upon the high homology between *OPT1* and *ISP4* and the established role of nitrogen regulation in many peptide transport systems, we hypothesize that *ISP4* encodes an oligopeptide transporter that is regulated by nitrogen source. The remaining two putative homologs were from *S. cerevisiae* and were identified during the genome sequencing project. Interestingly, few favorable conditions have been identified for oligopeptide transport activity in *S. cerevisiae*. As seen in Figure 6, PB1X-9B(pRS202) when grown in a medium containing proline exhibited an initial uptake rate of KLG-[³H]L that was comparable to the initial uptake rate of PB1X-9B(pOPT1) when grown in a medium with ammonium sulfate. However, under these conditions PB1X-9B(pRS202) exhibited no sensitivity to the toxic

tetrapeptides KLL^{Eth} and KLA^{Eth} and was not able to utilize the tetrapeptide KLLG as a sole source of leucine, whereas PB1X-9B(pOPT1) did grow on KLLG and exhibited slight sensitivity to the toxic peptides KLL^{Eth} and KLA^{Eth}. This discrepancy in results could be explained by an uptake rate exhibited by PB1X-9B(pRS202) that may not necessarily be reflective of total peptide accumulation over the prolonged incubation times necessary for growth and sensitivity assays. We are currently trying to determine if these two putative *S. cerevisiae* *OPT1* homologs are responsible for this low level oligopeptide transport activity.

In summary, we report the cloning of a second peptide transport gene from *C. albicans* through heterologous expression in *S. cerevisiae*. The existence of multiple peptide transport systems has been documented extensively in both prokaryotes and eukaryotes. The redundancy of this phenomenon underscores its importance. With the identification of two peptide transport genes in *C. albicans*, *CaPTR2* and now *OPT1*, the role of peptide transport in virulence, induction of secreted acid proteinases, and cell wall recycling can now be explored in greater detail.

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PART 3
SCHIZOSACCHAROMYCES POMBE ISP4
ENCODES A TRANSPORTER REPRESENTING
A NOVEL FAMILY OF OLIGOPEPTIDE
TRANSPORTERS*

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ABSTRACT

We have recently cloned an oligopeptide transport gene from *Candida albicans* denoted *OPT1*. This gene showed significant sequence similarity to three ORFs with no previously established function: *isp4* from *Schizosaccharomyces pombe* and YJL212C and YPR194C from *Saccharomyces cerevisiae*, identified during the genome project. The *S. pombe* gene *isp4* was originally identified by Sato *et al.* as a gene that was up-regulated through nitrogen starvation induction of meiosis (S. Sato, H. Suzuki, U. Widgastuti, Y. Hotta, and S. Tabata. Curr. Genet. 26:31-37, 1994). However, an *isp4Δ* strain exhibited a wildtype phenotype with respect to sexual differentiation. We have found that the same *isp4Δ* strain is deficient in tetrapeptide transport activity as measured by its resistance to toxic tetrapeptides, by its inability to accumulate a radiolabeled tetrapeptide, and by the inability to utilize tetrapeptides as a sole source of an amino acid to satisfy an auxotrophic requirement. Sequence analyses as well as physiological evidence has led us to propose that the proteins encoded by *isp4* and the genes identified from *S. cerevisiae* and *C. albicans* comprise a new group of transporters specific for small oligopeptides, which we have named the OPT Family.

CHAPTER I

INTRODUCTION

Peptide transport is a widely observed phenomenon exemplified by the translocation of peptides 2-6 residues in length across the plasma membrane in an energy-dependent manner (Becker and Naider, 1995; Payne and Smith, 1994). Internalized peptides are quickly hydrolyzed by peptidases and the resulting amino acids are used for protein synthesis or alternatively as a source of nitrogen or carbon. In addition to acquiring nutrients, peptide transport systems have also been shown to participate in other biological functions such as sensing of population density (Koide and Hoche, 1994; Rudner *et al.*, 1991) and in aiding organisms to evade the host immune response (Parra-Lopez *et al.*, 1993). To date, two evolutionarily distinct families of peptide transporters have been identified: the ATP Binding Cassette (ABC) Superfamily (Higgins, 1992) and the Peptide Transporter (PTR) Family (Steiner *et al.*, 1995).

The ABC Superfamily of transporters has been extensively studied and members have been identified in prokaryotes and eukaryotes. The ABC proteins are characterized by twelve transmembrane domains which can be subdivided into a 2 X 6 configuration based upon symmetry. Each six membrane spanning segment is associated with one highly conserved ATP binding site termed the Walker motif (Higgins, 1992), and it is presumed that hydrolysis of ATP at this site provides the energy necessary to translocate the appropriate substrate. A broad category of substrates have been reported for ABC transporters ranging from ions, to large macromolecules, to low molecular weight nutrients. No importing ABC peptide transporters have been identified in eukaryotes. ABC peptide transporters mediating import have been identified in *Lactococcus lactis* (Kunji *et al.*, 1995), as well as several other prokaryotes (Higgins, 1992) such as

Eschericia coli, *Bacillus subtilis*, *Salmonella typhimurium*, and *Streptomyces coelicolor* (Higgins, 1992; Nodwell *et al.*, 1996).

The PTR family is a newly identified group of transport proteins (Steiner *et al.*, 1995). Currently, this family has eight members and, with the exception of the *Arabidopsis thaliana* nitrate transporter *CHLI* (Tsay *et al.*, 1993), all have been shown to be involved in the import of peptides. Members of this family are characterized by several conserved motifs including a highly conserved signature motif. PTR transporters are also predicted to have twelve transmembrane domains, but unlike ABC proteins, translocation of their substrate is energized by the proton-motive force. In eukaryotes, PTR transporters have been cloned from human (Liang *et al.*, 1995), rabbit (Fei *et al.*, 1994), *Arabidopsis thaliana* (Steiner *et al.*, 1994; Song *et al.*, 1996), *Candida albicans* (Basrai *et al.*, 1995), and *Saccharomyces cerevisiae* (Perry *et al.*, 1994). The only PTR transporter identified in prokaryotes is *DtpT* from *Lactococcus lactis* (Hagting *et al.*, 1994) although a putative member of the PTR family has been revealed in the *E. coli* sequencing project.

In fungi, peptide transport is best characterized in *S. cerevisiae* and *C. albicans* from which several peptide transporters and their regulatory elements have been cloned. We have recently described the cloning and characterization of a novel oligopeptide transporter from *C. albicans* denoted *OPT1*. We report here the identification of a related peptide transporter from *Schizosaccharomyces pombe* that exhibits high homology to the oligopeptide transporter from *C. albicans*. Furthermore, we report the identification of a new family of peptide transporters that are not structurally related to the ABC or PTR transporter families.

CHAPTER II

MATERIALS AND METHODS

Strains, Vectors and Media

The strains used in this study were *S. pombe* JY800 (h^{90} *leu1 ura4-D18 ade6-M216*) and SS004 (h^{90} *leu1 ura4-D18 ade6-M216 isp4::ura4⁺*) kindly provided by Dr. Shusei Sato of the Kazusa DNA Research Institute (Sato *et al.*, 1994).

The plasmid pART1 was kindly provided by Dr. Stevan Marcus and has been described previously (McLeod *et al.*, 1987). The plasmid pOIP4 consisted of the *C. albicans* oligopeptide transporter *OPT1* under control of the *ADH* promoter. The plasmid pOIP4 was created by amplifying *OPT1* via PCR and cloning the resulting product into the *Pst*I site of pART1. The primers used in the PCR reaction were HET1 5'-TTTCAGCATGTGTATTCTGCAG-3' and HET2 5'-CTAAGTCAACCACTGCAGATTCCATTCC-3'.

S. pombe cells were maintained on YEPD medium (2% dextrose, 1% Yeast Extract, 2% Peptone, and 1.5% agar). The minimal medium (MM) used for most studies was made by adding 10 ml of 10X filter-sterilized YNB (6.7 g/100 ml; Yeast Nitrogen Base, Difco) with ammonium sulfate and without amino acids to 90 ml of sterile water containing 2 g glucose and auxotrophic supplements (adenine, 20 µg/ml; leucine, 30 µg/ml; and uracil, 20 µg/ml). For those experiments where proline was used as a sole nitrogen source, YNB without amino acids and without ammonium sulfate was supplemented with 0.1% proline.

Peptide media consisted of MM minus the amino acid leucine plus 100 mM of one of the following peptides: Lysyl-Leucine (KL), Lysyl-Leucyl-Glycine (KLG), Lysyl-Leucyl-Leucyl-Glycine (KLLG), Lysyl-Leucyl-Glycyl-Leucine (KLGL), or Lysyl-Leucyl-Leucyl-Leucyl-Glycine (KLLLG). Abbreviations for toxic amino acids and

peptides used in this study are as follows: Ethionine (Eth), Oxalysine (O), Oxalysyl-Glycine (OG), Oxalysyl-Leucyl-Glycine (OLG), Oxalysyl-Leucyl-Leucyl-Glycine (OLLG), Oxalysyl-Leucyl-Leucyl-Leucyl-Glycine (OLLLG), Alanyl-Ethionine (AEth), Leucyl-Ethionine (LEth), Lysyl-Leucyl-Ethionine (KLEth), Lysyl-Leucyl-Leucyl-Ethionine (KLLEth), Lysyl-Leucyl-Alanyl-Ethionine (KLAEth), and Lysyl-Leucyl-Leucyl-Leucyl-Ethionine (KLLLEth). All amino acids were in the *L* configuration. All peptides were purchased from Sigma corp. (St. Louis, MO) or synthesized by methods described previously (Basrai *et al.*, 1995; Island *et al.*, 1987; Perry *et al.*, 1994).

Note on gene and protein nomenclature: we have used the conventional nomenclature for genes and proteins specific for each organism mentioned. For example, the wildtype *C. albicans* gene *OPT1* encodes the protein Opt1p while in *S. pombe* the gene *isp4* encodes Isp4.

Synthesis of Radioactive Lys-Leu-Gly-[³H]Leu

KLGL was prepared by conventional automated solid phase peptide synthesis on an Applied Biosystems Model 433A synthesizer. Peptide was cleaved from the resin with trifluoroacetic acid (TFA) and purified using a C18 reversed phase column (19 x 300 mm) to >99% homogeneity with a 5 to 20% linear gradient of acetonitrile in water over 60 minutes. The product was verified using mass spectrometry [(M⁺+1)=430.2; calculated=429.6].

Tritiated KLGL (Lys-Leu-Gly-[³H]Leu) was prepared by solution phase peptide synthesis as follows. BocLys(Boc)-Leu-Gly-OH (5.2 mg; 10 μmol) was dissolved in 108 μl of a 0.092 μmol/μl solution of N-hydroxysuccinimide (10 μmol) in dry dioxane. Dicyclohexylcarbodiimide (10 μmol in 57 μl) in dry dioxane was added and the reaction mixture was stirred for one hour at ambient temperature. Leu (0.65 mg, 5 μmol), dissolved in 1 ml of water, was added to radioactive leucine (American Radiolabeled

Chemicals, Inc., St. Louis, MO; 60 Ci/mmol; 1mCi/ml in 2% ethanol). This solution was evaporated to dryness, redissolved in 250 μ l of water/dioxane (4:1) containing N-methyl morpholine (50 μ mol) and the solution containing the activated tripeptide was added. The resulting reaction mixture was stirred for 6 hours at ambient temperature, 5.5 ml of TFA was then added, and after 5 minutes the reaction mixture was evaporated to dryness. The residue was redissolved in 500 μ l of water, injected onto a Waters μ Bondapak C18 column (7.8 x 300 mm) and eluted isocratically using 5% acetonitrile in water, containing 0.025% TFA. Product eluting at the KLGL position was collected, evaporated, redissolved in water (200 μ l) and analyzed by high pressure liquid chromatography (HPLC) and on silica thin layers using a Butanol:Acetic acid:water (4:1:5) mobile phase. TLC plates were exposed to film overnight at -80 C and developed to show one radioactive spot with the mobility of the desired tetrapeptide. KLG-[³H]L was >97% pure according to HPLC. Specific radioactivity was 90 mCi/mmol. Peptide was diluted with nonradioactive KLGL as required.

Growth and transport assays

For growth assays, cells were grown in liquid culture to log phase, washed once with water, and resuspended at final concentration of 1×10^6 cells/ml in water. Three microliters of the cell suspension or subsequent dilution were spotted to the surface of the medium and plates were incubated at 30 °C for 3-4 days.

Uptake of KLG-[³H]L was determined using a protocol for uptake of dipeptides as described by Basrai *et al.* (1995) for *C. albicans* with a few modifications. *S. pombe* cultures were grown overnight to $1-2 \times 10^6$ cells/ml in MM. Cells were harvested by centrifugation, and resuspended in 2% glucose at a cell density of 2×10^8 cells/ml. Two hundred and fifty microliters of cell suspension were added to an equal volume of an uptake assay reaction mixture and incubated at 30°C. The final concentrations of the

components in the uptake assay solution were: glucose (2%, w/v), 10mM sodium citrate/potassium phosphate buffer (pH 5.0), and KLG-[³H]L (150 μM; 8.5 mCi/mmol). At various time points, 90 μl portions were removed and filtered through a membrane. The yeast cells retained on the filter were washed three times with ice-cold distilled water and the residual radioactivity retained on the cells on the filter was measured by liquid scintillation. There was no peptide adsorption to the cell surface or sticking to filters since at 0° C the counts were at background level. The uptake results were expressed as nmoles of peptide uptake per minute per mg cell dry weight and are an average of four independent assays.

Competition experiments were performed in an identical manner as uptake assays except that the reaction mixture contained a 10-fold molar excess of the competitor (KLLLG, KLLG, KLG, KL, or L; final concentration 1.5 mM). Each competition experiment was repeated twice with similar results.

Sensitivity assays

Sensitivity to ethionine (a toxic methionine analog), oxalysine (a toxic lysine analog), ethionine-containing peptides, and oxalysine-containing peptides was determined by the method described by Island *et al.* (Island *et al.*, 1987). Cells were grown overnight to exponential phase of growth in MM, washed, and resuspended at 5×10^6 cells/ml in sterile water. One ml of this cell suspension was added to 3 ml molten Noble agar (0.8% final concentration) and overlayed on 20 ml of the same medium used to prepare the inoculum. A disk (6 mm diameter, Difco) was placed on the plate and the compounds to be tested were applied to the disks. A total of 0.38 μmoles of ethionine or ethionine-containing peptides was spotted while 0.25 μmoles of oxalysine or oxalysine-containing peptides was spotted. Zones of inhibition were measured after 24-48 hr incubation at 30 °C. Each test comprised at least three independent assays and the results

represented in the Tables are means of the values obtained. Maximum variation between the zones of inhibition measured for each test were ≤ 2 mm. A value of 7 mm for the diameter of zone of inhibition represents a minimal growth inhibition value as the disk diameter was 6 mm. Photodocumentation of sensitivity assays was done with a Umax Scanner and processed through Adobe Photoshop.

Nucleotide accession numbers

The Genbank/EMBL accession numbers for the ORFs analyzed during this study are as follows: *C. albicans* *OPT1*, accession number U60973; *S. pombe* *isp4*, accession number 729859; *S. cerevisiae* ORF YJL212C, accession number Z49487; and *S. cerevisiae* ORF YPR194C, accession number U25841.

CHAPTER III

RESULTS

***isp4* of *S. pombe* shows high homology to an oligopeptide transporter from *Candida albicans*.**

We have recently cloned an oligopeptide transport gene (*OPT1*) from *Candida albicans* through heterologous expression in *Saccharomyces cerevisiae* (Lubkowitz *et al.*, 1997). A search of the Genetics Computer Group (GCG) database (Devereux *et al.*, 1984) identified three open reading frames (ORF) of unknown function that exhibited significant sequence similarity to *OPT1*. The *S. pombe* gene *isp4*, a nitrogen starvation-induced gene of previously unknown function (Sato *et al.*, 1994), showed the highest resemblance with 70% similarity and 48% identity. We therefore tested whether *isp4* encoded an oligopeptide transporter by various physiological measurements.

Peptides as Growth Substrates.

To determine if *isp4* encoded an oligopeptide transporter we assayed the *isp4*⁺ strain JY800 and the *isp4Δ* strain SS004 for the ability to utilize leucine-containing peptides in order to satisfy their leucine auxotrophic requirement. Because previous studies had indicated nitrogen as an important controlling condition of peptide transport in *S. cerevisiae* (Island *et al.*, 1987; Island *et al.*, 1991), we simultaneously tested the effect of nitrogen source by providing in the medium either a rich nitrogen source (ammonium sulfate) or a poor nitrogen source (proline). The *isp4*⁺ strain JY800 was able to utilize the peptides KL, KLG, KLLG, and KLGL as a source of leucine when grown on a medium containing proline while the *isp4Δ* strain SS004 was only able to utilize the peptides KL and KLG (Fig.1). Neither strain was able to grow on the pentapeptide KLLLG. When grown on a medium containing ammonium sulfate, both strains were only able to grow

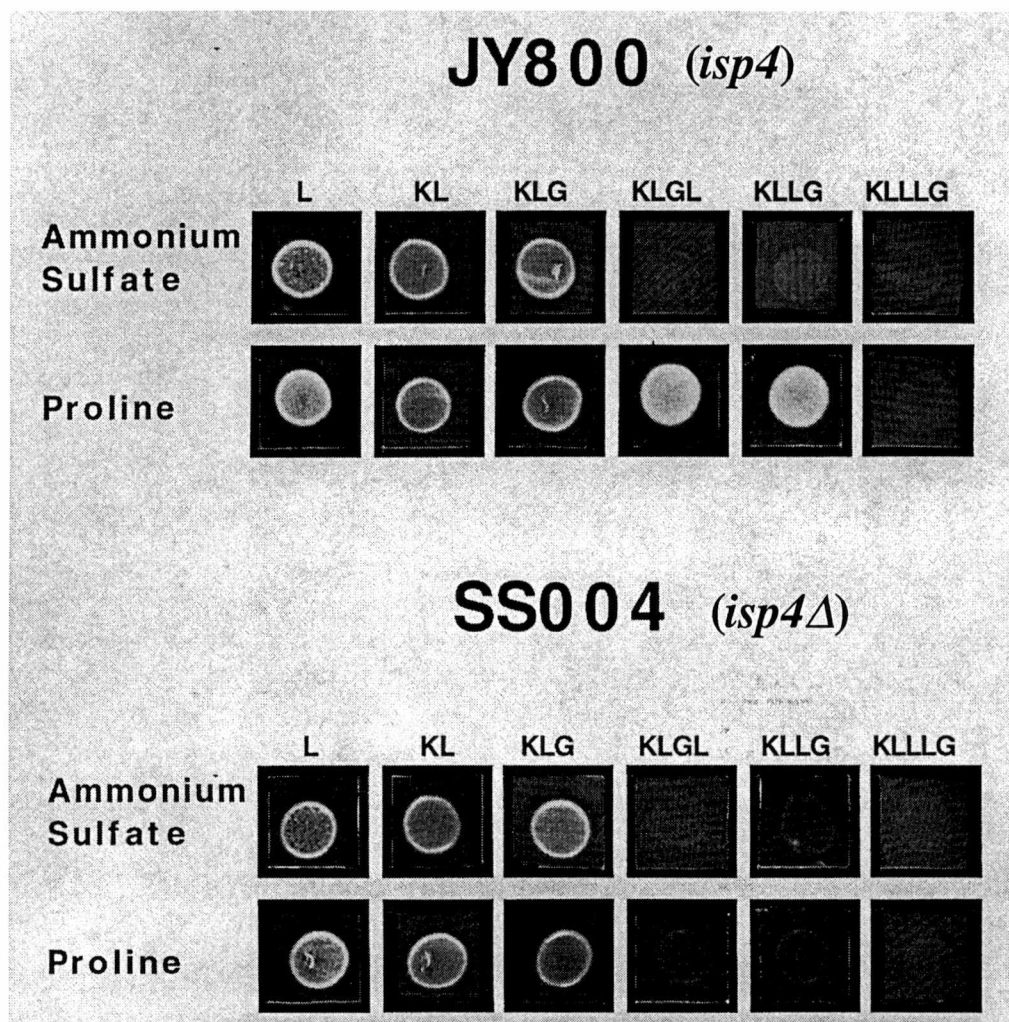


Figure 1. Growth assay of *S. pombe* strains. Strain JY800 and SS004 were tested for their ability to utilize leucine and leucine-containing peptides as an auxotrophic supplement on media containing either ammonium sulfate or proline as a nitrogen source.

and KLG. In general growth on di- and tripeptides was equivalent in both strains. The possibility of *isp4* encoding a peptidase specific for tetrapeptides only is highly unlikely and further precluded by the direct transport competition experiments presented below.

Sensitivity of JY800 and SS004 to toxic peptides.

We measured the sensitivity of JY800 and SS004, in a medium containing either ammonium sulfate or 0.1% proline as a nitrogen source, to various toxic peptides through halo assays to determine if *isp4* conferred sensitivity (Table 1). When grown on the medium containing 0.1% proline, strain JY800 was sensitive to the ethionine-containing peptides AEth, KEth, KLEth, and KLAeth and to the oxalysine-containing peptides OG, OLG, and OLLG. On the other hand, SS004 exhibited sensitivity to the toxic di- and tripeptides AEth, KEth, OG, and OLG but not to the toxic tetrapeptides OLLG, KLEth, and KLAeth. Neither strain exhibited sensitivity to the toxic pentapeptides KLLAeth and OLLLG. An example of these halos are shown for KEth, KLAeth, and KLLAeth in strain JY800 and SS004 (Fig. 2). Although a 31 mm halo was measured for KLAeth in strain JY800, the zone of growth inhibition was not as distinct as the zone formed for KEth.

When ammonium sulfate was used as a nitrogen source, both strain JY800 and SS004 exhibited equal sensitivity to the ethionine-containing peptides AEth and KEth. Neither strain exhibited sensitivity to the toxic tetrapeptides KLEth and KLAeth or the toxic pentapeptide KLLAeth.

Accumulation of the radiolabeled peptide Lys-Leu-Gly-[³H]Leu.

The *isp4* deletion strain SS004 could not utilize tetrapeptides as a sole source of leucine whereas the *isp4*⁺ strain JY800 could utilize tetrapeptides when grown in a medium containing 0.1% proline as a sole nitrogen source (Fig. 1). Similarly, JY800

Table 1 : Sensitivity of *S. pombe* strains to various ethionine- or oxalysine-containing peptides ^a

<i>S. pombe</i> Strain ^b	Nitrogen Source	Zone of inhibition (mm) ^c					
		Eth ^d	AEth	KLEth	KLLEth	KLAEth	KLLAEth
JY800	0.1% proline	40	33	34	34 ^d	31	none
SS004	0.1% proline	40	33	32	none	none	none
JY800	(NH ₄) ₂ SO ₄	24	36	34	none	none	none
SS004	(NH ₄) ₂ SO ₄	23	36	35	none	none	none

Nitrogen Source		O ^d	OG	OLG	OLLG	OLLLG
JY800	0.1% proline	32	20	16	24	none
SS004	0.1% proline	30	20	17	none	none

^a Disk sensitivity assays were performed on a medium of SC without amino acids with 0.1% proline as a sole nitrogen source and supplemented with the appropriate auxotrophic requirements.

^b Strain Genotypes: *S. pombe* JY800 (*leu1 ura4-D18 ade6-M216 ISP4*)
S. pombe SS004 (*leu1 ura4-D18 ade6-M216 isp4::URA4*).

^c For those experiments that utilized ethionine (Eth) or ethionine-containing peptides, a total of 0.38 μmoles was spotted on a 6 mm disk placed on a lawn of cells. For those experiments that utilized oxalysine (O) or oxalysine-containing peptides, a total of

Table 1 (con't)

0.25 μ moles was spotted. "none" indicates no growth inhibition. Each test comprised three or more independent assays and the results represented in the Table are means of the values obtained. Maximum variation between the zones of inhibition for each test was ≤ 2 mm.

- d The outline of the zone of inhibition was not very sharp and a hazy zone of inhibition was observed.

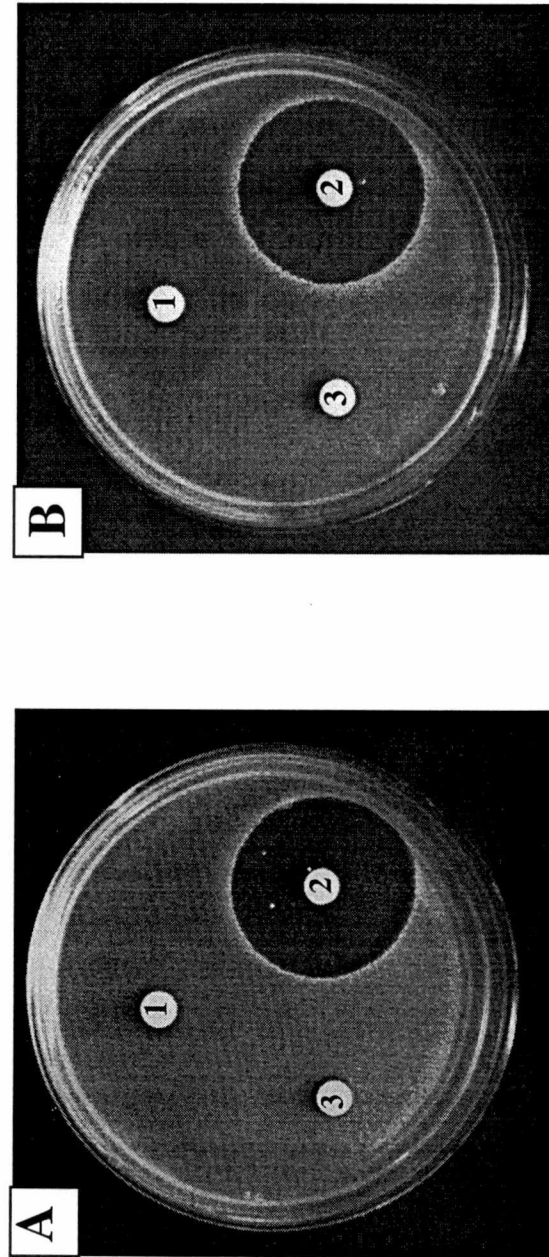


Figure 2. Toxic peptide inhibition assay of *S. pombe* strains. Sensitivity to the toxic peptide 1) KLAeth, 2) KLEth, and 3) KLLAEth on proline media was performed as described in Materials and Methods. Panel A: SS004, Panel B: JY800

was only resistant to a toxic pentapeptide while SS004 was resistant to toxic tetra- and pentapeptides (Fig. 2; Table 1) when grown in a 0.1% proline medium. We concluded that *isp4* was a tetrapeptide transporter that is down regulated by a rich nitrogen source such as ammonium sulfate. To further test this hypothesis, the accumulation of KLG-[³H]L by JY800 and SS004 was measured in a medium containing either ammonium sulfate or 0.1% proline as a nitrogen source. As seen in Figure 3, JY800, when grown in a proline medium, had an initial uptake rate of 4.3 nmoles/min/mg of dry wt while SS004 did not accumulate the peptide. When grown in a medium containing ammonium sulfate JY800 had an initial uptake rate of 0.7 nmoles/min/mg of dry wt while SS004 still did not accumulate the peptide.

We further tested the substrate length requirements of *isp4* by measuring the effect of a 10-fold molar excess of L, KL, KLG, KLLG, or KLLL on the accumulation of KLG-[³H]L. As seen in figure 4, KLLG and KLLL were good competitors while KLG exhibited only slight competition. On the other hand, the amino acid leucine and the dipeptide KL did not compete with the accumulation of KLG-[³H]L (data not shown).

Sequence comparisons.

In addition to the *C. albicans* gene *OPT1*, two open reading frames (ORFs) with significant similarity to *isp4* were identified by a BLAST search (Altshul *et al.*, 1990). The oligopeptide transporter *OPT1* from *C. albicans* had the highest similarity to *isp4* with 48% identity and 70% similarity, and it was the only ORF identified with a previously established function. The two remaining ORFs from *S. cerevisiae*, named YJL212C and YPR194C (Walsh and Barrell, 1996), identified during the *S. cerevisiae* genome sequencing project, exhibited 40% identity and 63% similarity and 34% identity and 59%, respectively, to *isp4*. The sequences were aligned using the GCG programs Pileup and Pretty and conserved residues identified (Fig. 5). In addition to these full

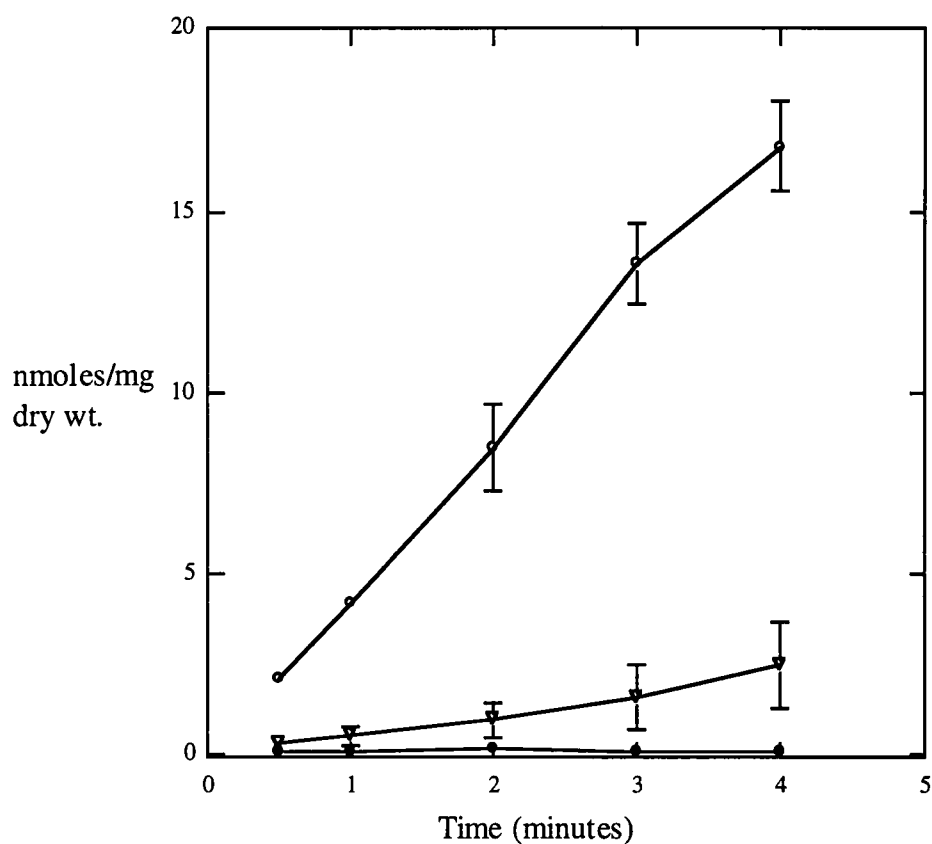


Figure 3. Peptide transport assay of *S. pombe* strains. Accumulation of KLG- $[^3\text{H}]\text{L}$ by JY800 grown in a 0.1% proline medium (o), JY800 grown in a ammonium sulfate medium (∇), and SS004 ($\bullet$) was measured over a four minute time course as described in Materials and Methods. The uptake rate for SS004 was the same for both growth media. Error bars represent 1 standard deviation away from the mean. For those points where no error bar appears the standard deviation is smaller than the diameter of the point.

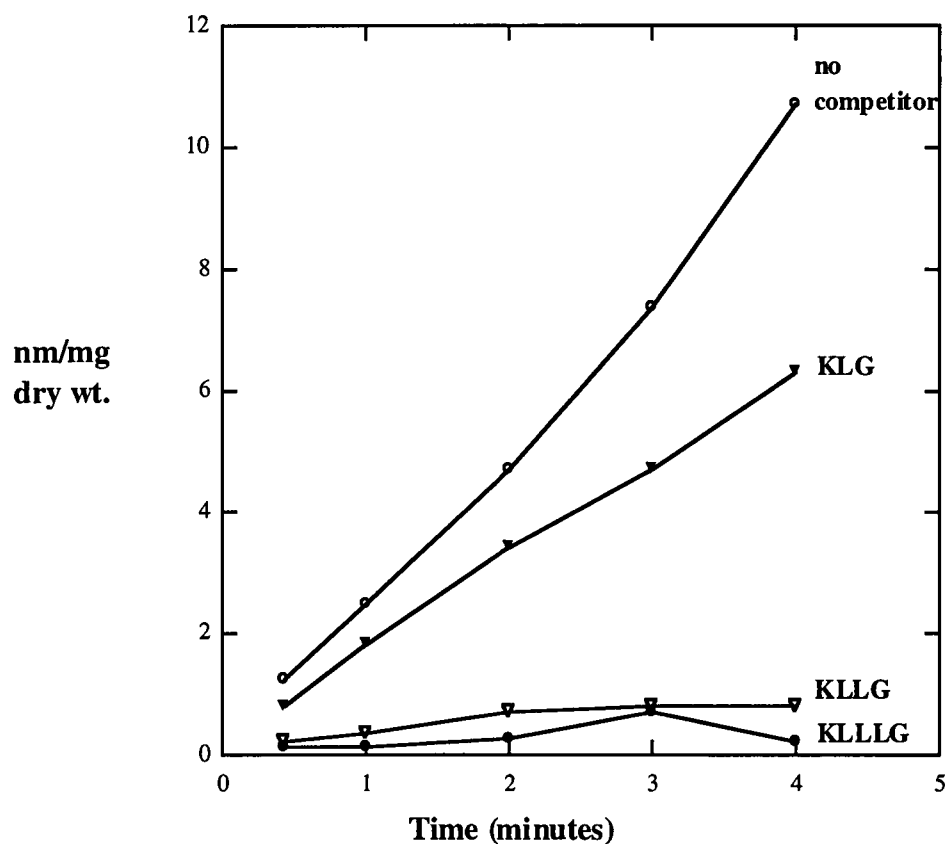


Figure 4. Competition experiment with *S. pombe* JY800. The ability of a 10-fold molar excess of KLG(▼), KLLG(▽), and KLLL'G(●) to competitively inhibit the accumulation of KLG-[³H]L was measured over a four minute time course as described in Materials and Methods. The accumulation of KLG-[³H]L(o) in the absence of competitors was also measured. The amino acid L and the dipeptide KL did not act as competitors (data not shown). The experiment was repeated twice with identical results.

Figure 5. Sequence comparison of Isp4 homologs. The proteins Isp4, Opt1p, Yjl212cp, and Ypr194cp were aligned using the GCG programs Pileup and Pretty. Areas of conservation are in capital while non conserved residues are in lower case. The putative Casein Kinase II sites located in the first hydrophilic stretch are underlined (residues 4 to 66 of Isp4) as well as the putative tyrosine kinase site located near the center of the proteins (residues 456 to 464 of Isp4). Conserved glycyl and prolyl residues occurring in hydrophobic regions are boxed.

79

length ORFs several Expressed Sequence Tags (ESTs) with significant similarity were identified (data not shown).

It has been suggested that identification of conserved sequences within a group of proteins can identify regions of functional and structural significance (Saier, 1994). Therefore, we searched the Pileup for large regions of conservation and/or conserved substitutions. All four proteins contained the sequence SPYXEV_RssVXX_pDDP(T/S) (Table 2; s-small residue, p-polar residue). A BLAST search did not identify any additional full length proteins in the database that contained this motif suggesting that this motif is unique to this group of proteins. The closest match was the neurolin transmembrane glycoprotein from zebra fish which contained the sequence SPKPEVQWSVNGTDDDET (Kanki *et al.*, 1994).

Hydropathy plots of Isp4, Opt1p, Yjl212p, and Ypr194cp were generated by the method of Kyte and Doolittle (Kyte and Doolittle, 1982) using the software DNASTar. Nearly identical and superimposable plots were generated for all four proteins (Fig. 6). The only deviation identified was in the *S. cerevisiae* protein Ypr194cp which contained approximately 60 more residues in the hydrophilic N-terminus. Using a minimum stretch of 20 hydrophobic residues, we identified 12-14 putative transmembrane domains in each protein. Transport proteins with 12 transmembrane domains have been extensively documented while transport proteins with 14 transmembrane domains are less common (Saier, 1994).

Functional Complementation of SS004 with *OPT1*

The oligopeptide transport phenotype associated with *isp4* and *OPT1* as well as the strong sequence similarity between the two genes led us to investigate whether *OPT1* could functionally complement oligopeptide transport in the *isp4* deletion strain SS004. The ORF of *OPT1* was placed under the control of the *S. pombe ADH* promoter as

Table 2. Comparison of signature motifs

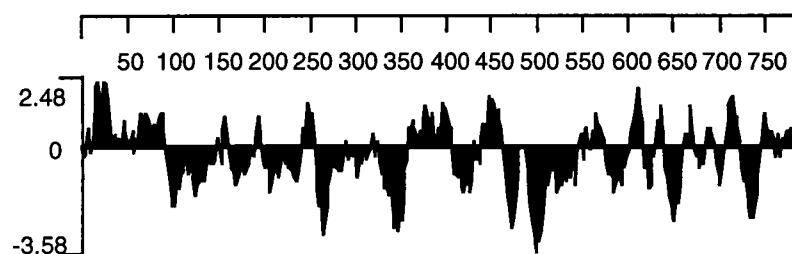
Protein ^a	Position ^b	Motif ^c
Isp4p	70	SPYPEVRAAVPPTDDPS
Opt1p	70	SPYPEVRAAVPSTDDPT
Scyj1212cp	85	SPYPEVRSAVSIEDDPT
Yscp9677p	144	SPYQEVRAVVDPEDDPT
consensus	---	SPYxEVRssVxxpDDP (S/T)
neurolin	431	SPKPEVQWSVNGTDDDET

^a A highly conserved region was identified and a consensus sequences was generated.

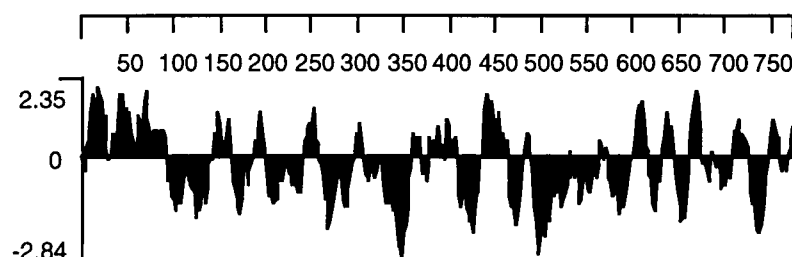
A BLAST search of the consensus sequence did not reveal any additional full length proteins with this motif. The closest match was a sequence from the neurolin transmembrane glycoprotein of zebrafish.

^b Sequence positions of the N-terminal residue of the motif within each protein are listed.

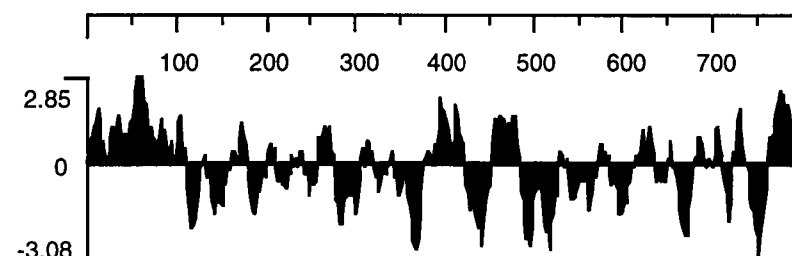
^c Conserved residues are in capitols. Abbreviations of consensus in lower case: x-any amino acid, s-small amino acid, p-polar amino acid.



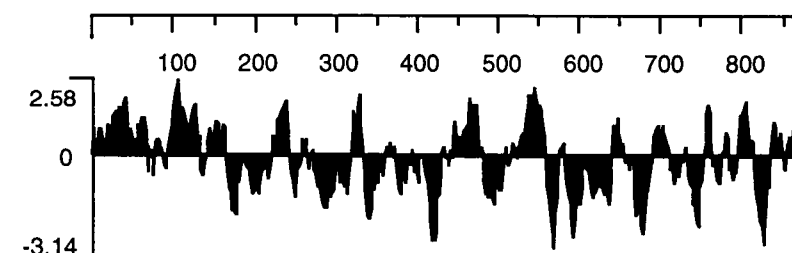
Opt1p



Isp4p



Yjl212cp



Ypr194cp

Figure 6. Hydropathy analysis of OPT family. Hydropathy plots of the proteins Isp4, Opt1p, Yjl212cp, and Ypr194cp were generated through the software DNASTar by the method of Kyte and Doolittle using a window of 13 residues. Hydrophobic residues are located below the center line while hydrophilic residues are represented above the line.

described in Material and Methods. The resulting plasmid pOIP4 was transformed into SS004 and oligopeptide transport activity measured by sensitivity to toxic peptides. Strain SS004 harboring pOIP4 exhibited the same phenotype as SS004 harboring only the parent plasmid pART1 when sensitivity to toxic peptides was measured indicating that heterologous complementation had not occurred.

CHAPTER IV

DISCUSSION

Four observations have led us to conclude that *isp4* encodes an oligopeptide transporter. First, the *isp4* deletion strain SS004 was not able to satisfy its leucine auxotrophic requirements with the tetrapeptides KLGL and KLLG whereas an the *isp4*⁺ strain JY800 could utilize these tetrapeptides (Fig. 1). Second, the strain SS004 was resistant to the toxic tetrapeptides OLLG, KLA⁺EtH, and KLL⁺EtH whereas strain JY800 was sensitive to these same peptides (Fig. 2; Table 1). Thirdly, JY800 exhibited an initial uptake rate of 4.3 nmoles/min/mg of dry weight for the peptide KLG-[³H]L whereas SS004 could not accumulate the radiolabeled substrate (Fig. 3). Finally, Isp4 showed high similarity to the *C. albicans* oligopeptide transporter Opt1p (Fig. 5). The possibility that *isp4* coded for an extracellular carboxypeptidase or endopeptidase was eliminated by the fact that neither leucine or a dipeptide competed with the uptake of KLG-[³H]L; hydrolysis of this tetrapeptide by such proteins would produce [³H]L or G-[³H]L whose uptake should have been inhibited by these competitors. If an aminopeptidase activity played a role, KLG-[³H]L would have first have been cleaved to LG-[³H]L which should have been efficiently competed for by KLG. Therefore, we conclude that Isp4 is not a protease.

The *isp4* gene was identified by Sato *et al.* (1994) through a subtractive hybridization screen designed to identify genes specifically induced during meiosis. In *S. pombe* meiosis can be induced through nitrogen starvation. Therefore, Sato *et al.* (1994) used mRNA isolated from nitrogen starved cells and mRNA from non-nitrogen starved cells for their subtractive hybridization screen. This strategy had the potential to yield two types of genes: those that were expressed during meiosis and those that were regulated through the nitrogen catabolite repression system (NCR). Indeed an ORF that showed

striking similarity to *GAP1*, the *S. cerevisiae* NCR-regulated General Amino Acid Permease (Jauniaux and Grenson, 1990), was identified as well as an ORF encoding a protein that showed striking similarity to *S. cerevisiae* Proteinase B (Moehle *et al.*, 1987), a serine proteinase responsible for protein degradation during sporogenesis. Sato *et al.* (1994) found that an *isp4* disruptant manifested a wildtype phenotype with respect to sporulation efficiency and spore viability. Furthermore, they demonstrated that *isp4* mRNA induction occurred prior to the onset of meiosis. No other phenotypes were tested in this previous study. In this report, we have demonstrated that *isp4* is required for tetrapeptide transport activity. Furthermore, tetrapeptide transport activity was repressed by the rich nitrogen source ammonium sulfate (Fig. 1 & 3, Table 1). Therefore, we propose that *isp4* encodes an oligopeptide transporter up-regulated due to nitrogen starvation. Whether this transport system plays a role in meiosis remains to be demonstrated.

Multiplicity of peptide transport systems has been extensively documented in both prokaryotes and eukaryotes. In *S. typhimurium* and *E. coli* genes encoding components of several distinct systems have been cloned (Payne and Smith, 1994). All of these prokaryotic systems consist of several components with one being a member of the ABC family. In *C. albicans* two peptide transporters have been cloned: *PTR2*, a di-/tripeptide transporter (Basrai *et al.*, 1995), and *OPT1*, an oligopeptide transporter (Lubkowitz *et al.*, 1997). It appears that *S. pombe* has at least two peptide transport systems, an oligopeptide transporter encoded by *isp4* and an unidentified system(s) responsible for di- and tripeptides. The strain SS004 was not able to transport tetrapeptides but was able to transport di- and tripeptides as demonstrated by growth on KL and KLG and by its sensitivity to the toxic peptides OL, OLG, AEth, and KLEth. Furthermore, the dipeptide KL did not compete for entry into the cell with the radiolabeled peptide KLG-[³H]L indicative of a different route of entry. The slight competition exhibited by KLG with

KLG-[³H]L demonstrated that there was substrate overlap between the system encoded by *isp4* and the other system(s) responsible for di- and tripeptide entry.

The *C. albicans* oligopeptide transporter *OPT1* is able to accommodate peptides of 3-5 residues with varying degrees of affinity (Lubkowitz *et al.*, 1997). *OPT1* has the highest affinity for tetrapeptides followed by pentapeptides and a much lower affinity for tripeptides. In the current study, we found that a deletion of *isp4* conferred a phenotype characterized by the inability to utilize the tetrapeptides KLLG and KLGL as growth substrates and the loss of sensitivity to the toxic tetrapeptides KLAeth, KLEeth, and OLLG. Competition experiments designed to more closely exam the substrate length requirements of *isp4* demonstrated that in addition to the tetrapeptide KLLG, the pentapeptide KLLLG and, to a lesser degree, the tripeptide KLG competed with a radiolabeled tetrapeptide (Fig. 4). Despite the observation that KLLLG was able to competitively inhibit the accumulation of KLG-[³H]L, KLLLG was not able to act as sole source of leucine. The inability of strain JY800 to utilize the pentapeptide KLLLG as a source of leucine could be explained by several possibilities. First, the competition exhibited by KLLLG over a four minute time course may not necessarily be indicative of the total peptide accumulation during the lengthy incubations times required for growth assays. Alternatively, the pentapeptide KLLLG may be a poor substrate for internal peptidases. The tripeptide KLG also competed with KLG-[³H]L but to a much lesser degree. We concluded that Isp4 has a lower affinity for tripeptides than for tetrapeptides, although only a limited number of peptides were tested as possible substrates for both Isp4 and Opt1p.

The *C. albicans* oligopeptide transporter *OPT1* was not able to functionally complement the *isp4* deletion in SS004. Despite this observation the nearly identical phenotypes and strong sequence similarity has led us to conclude that these proteins are homologs. The inability of *OPT1* to functionally complement the *isp4* deletion has

several potential explanations. First, the universal codon CUG is decoded as a serine in *C. albicans* (Ohama *et al.*, 1993). Therefore, the putative protein product may not be functional. Second, functionality of the *C. albicans* Opt1p may require accessory proteins which may be absent or be incompatible with Opt1p in *S. pombe*. Finally, the activity associated with *OPT1* and *isp4* appears to be highly regulated. Therefore, the overexpressing of *OPT1* by the *ADH* promoter may prevent the proper functionality or expression of this gene in the heterologous host. To our knowledge there has only been one report, involving complementation of temperature sensitive mutants, of successful expression of a *C. albicans* gene in *S. pombe* (Damagnez and Cottarel, 1996). Functional complementation of a gene knockout has not been reported.

We found that Isp4, Opt1p, Yjl212cp, and Ypr194cp not only shared large regions of homology but did not share significant sequence similarity with any other proteins in the database. Furthermore, rigorous comparisons with previously identified ABC and PTR transporters did not reveal any significant homology or regions of homology. We believe, therefore, that these proteins comprise a distinct group of evolutionarily related transporters. Saier (1994) has proposed that such computer aided assertions can be substantiated by meeting four criteria: 1) proteins must be of similar length, 2) proteins must have similar functions, 3) proteins must be topographically similar, and 4) functional domains must be positionally conserved. It is considered statistically improbable that all these characteristics would arise through convergent evolution.

As seen in Figure 5, all four proteins are approximately the same length with Ypr194cp having three short non-homologous regions, an approximately 60 residue stretch at the N-terminus as well as two shorter stretches of approximately 12-20 residues. A search of Isp4 and its three putative homologs with the GCG protein analysis software Motifs and Prosite (Bairoch, 1992) identified several common features. All

members contained 3-6 putative Casein Kinase II phosphorylation sites (Pinna, 1990) prior to the first large hydrophobic domain. Similarly a putative tyrosine kinase phosphorylation site (Patschinsky *et al.*, 1982) was identified that is located in a conserved region of all four proteins. Both casein kinase II and tyrosine kinases are able to phosphorylate membrane bound proteins.

In addition to the conserved sites identified by Prosite and Motifs, several conserved and unique motifs have been identified. The structural, regulatory, and mechanistic relevance of these motifs is not known. The largest of these motifs consisted of 10 conserved residues which we have termed the SPY motif. This sequence appears to be a signature motif located prior to the first hydrophobic domain (Table 2). A search of the database did not reveal any proteins of established function with this motif, although several ESTs were found.

Nearly identical hydropathy plots were generated for Isp4, Opt1p, Yjl212p, and Ypr194cp (Fig. 6). Presumably this identity is indicative of structural conservation. Twelve or fourteen putative transmembrane domains were identified from the plots. Both twelve and fourteen transmembrane domain transporters have been previously described although fourteen transmembrane domain transporters are much less common (Saier, 1994). The residues glycyl and prolyl in hydrophobic stretches are thought to have structural significance through the introduction of a "kink" in the putative transmembrane alpha helices (Deber *et al.*, 1990; Yaron and Naider, 1993). It has been suggested that the "kink" allows plasticity within the membrane bound portion of the protein and that this plasticity accommodates the structural flexibility required for the translocation of the substrate. Several glycyl and prolyl residues are conserved within hydrophobic regions of the OPT family and their positions are depicted in Figure 5.

All previously cloned peptide transporters have been members of the ABC (prokaryotes) or PTR (primarily eukaryotes) families. Members of the ABC family have

twelve transmembrane domains, contain two highly conserved ATP binding sites termed Walker Motifs A and B, and the signature motif LSGGQ (Higgins, 1992). On the other hand, the PTR transporters are characterized by twelve transmembrane domains and the conserved motif FYXXINXGSL (Steiner *et al.*, 1995). Neither signature motif was found in Isp4 or Opt1p although hydropathy analysis did predict at least twelve membrane spanning regions. Furthermore, a direct comparison of Isp4 with the ABC peptide transporter Opp from *B. subtilis* (Perego *et al.*, 1991) and the PTR protein ScPTR2 from *S. cerevisiae* (Perry *et al.*, 1994) using the GCG program Bestfit yielded only 15.3% and 19.8% identity, respectively. Therefore, based upon the high similarity between the four predicted proteins, the lack of homology with previously identified transport families, the nearly identical topology, and the oligopeptide transport activity associated with *OPT1* and *isp4*, we propose that these proteins constitute a newly identified family of transporters which we have called the OPT family.

In summary, we report the first identification of a peptide transporter from *S. pombe* and the discovery of a new family of transporters called the OPT family. OPT family proteins appear to have 12-14 transmembrane domains and contain the signature motif SPYXEVRRXXVXXXDDP(S/T). The energy source necessary for the translocation of the peptides, the relevance of the various motifs, and the function of the two *S. cerevisiae* homologs is currently under investigation in our laboratory.

Acknowledgments

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PART 4

CHARACTERIZATION OF *OPT1* DISRUPTANT STRAINS*

*We intend to submit this chapter for publication after the addition of several experiments as "Lubkowitz, M. A., Hauser, M., Naider, F., and J. M. Becker. (1997)."

CHAPTER I

INTRODUCTION

With the increase in AIDS, immunosuppressive therapy, and distribution of broad-spectrum antibiotics, disseminated fungal infections have become an increasing threat to humans in terms of economics as well as health. In particular, *C. albicans* has now become not only the most widespread of all fungal pathogens but also the third leading killer of persons with AIDS (Graybill, 1993). *C. albicans* is a normal constituent of the human gastrointestinal flora and is responsible primarily for topical infections such as vaginal candidiasis and oral thrush. However, the recent rise in immunocompromised individuals has led to the increase in the number of systemic infections caused by this organism (Reviewed by Odds, 1994)

As highlighted in Part I, peptide transporters have been shown to satisfy many biological functions. Specifically, in the pathogenic organism *S. typhimurium* peptide transporters have been shown to mediate resistance to anti-microbial peptides and in the pathogenic organism *S. pneumonia* to affect adherence (Cundell *et al.*, 1995). The observation that *C. albicans* produces several extracellular proteases that appear to be required for full virulence and that the global inducer(s) of the virulence program have not been identified, led us to hypothesize two possible mechanisms by which peptide transporters may be affecting virulence.

First, *C. albicans* secretes several extracellular proteases during colonization and infection of the host. Several of the genes encoding these Secreted Aspartyl Proteases (SAPs) have been cloned and been implicated as a virulence factor (Reviewed by Odds, 1994). SAP production can be stimulated *in vitro* by the addition of Bovine Serum Albumin to the growth medium (Lerner and Goldman, 1993). Because the degradation products of the SAPs are peptides, it is possible that these peptides are internalized by a

peptide transporter which then acts as an inducer of the SAP genes. In such a model, the peptide transporter would indirectly affect virulence by mediating the translocation of SAP inducers.

Second, the lack of an established inducer of the virulence program has led us to hypothesize that a host peptide or peptides could be the inducing signal. Such a peptide or peptides could be produced by the host and be freely available to the fungus. Alternatively, such a peptide or peptides could be produced as degradation products of SAP mediated proteolysis. Presumably these inducers would be internalized via a peptide transporter before eliciting the appropriate response. As discussed in Part 1, signal peptides internalized by transport systems have been observed in *B. subtilis* as in the example of the competency and sporulation peptide CSF. *B. subtilis* strains that are not able to transport CSF are characterized by a decrease in sporulation efficiency and competency. In an analogous manner, if *OPT1* does transport the inducer of a virulence program then one would predict that an *OPT1* deletion strain would be attenuated.

In addition to these hypotheses, the lack of observed mammalian homologs of *OPT1* in the Express Sequence Tag (EST) database raises the prospect of this transporter being exploited as a novel drug target if the transporter itself is required for virulence or survival in the host. Because *C. albicans* is a weak pathogen operating at the threshold of pathogenesis, any mutation that decreases the organisms ability to grow in the host should lead to attenuation. If such a scenario proves true, then *OPT1* itself becomes a potential drug target.

These three prospects warrant investigating the role of *OPT1* in systemic candidiasis. In an attempt to determine whether *OPT1* affects virulence, *OPT1* heterozygous and homozygous deletion strains were constructed, characterized, and tested for virulence in a murine model of systemic candidiasis.

CHAPTER II

MATERIALS AND METHODS

Strains, Media, and Vectors

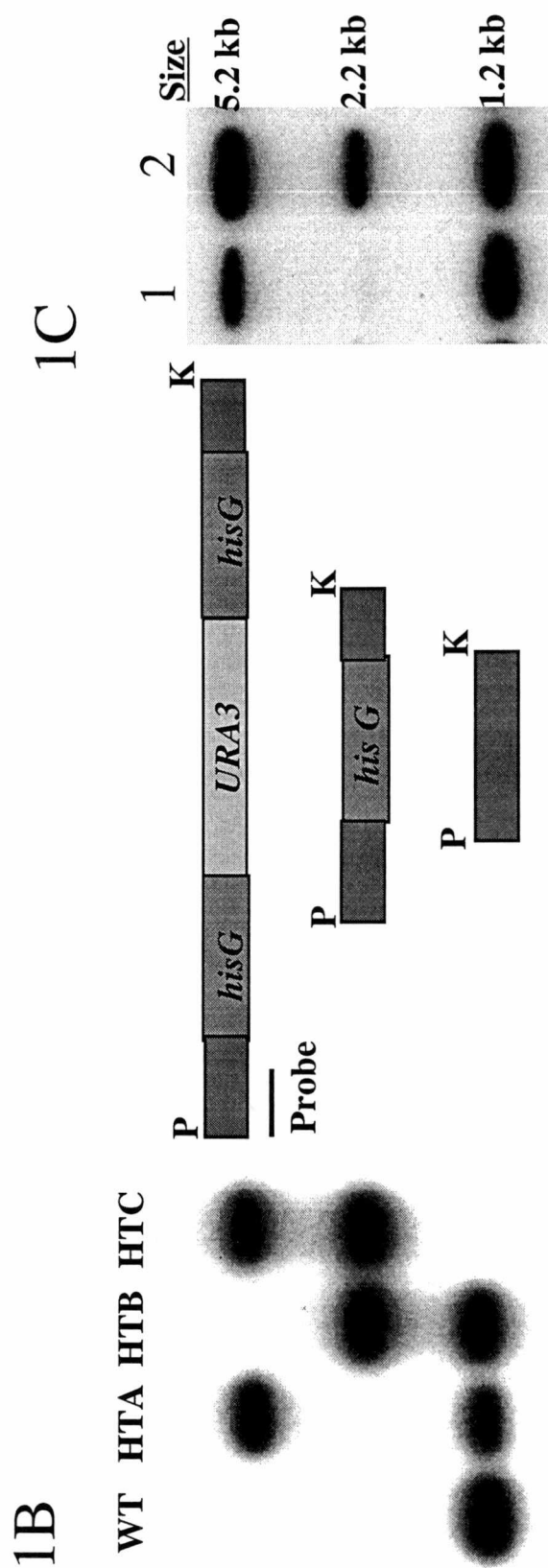
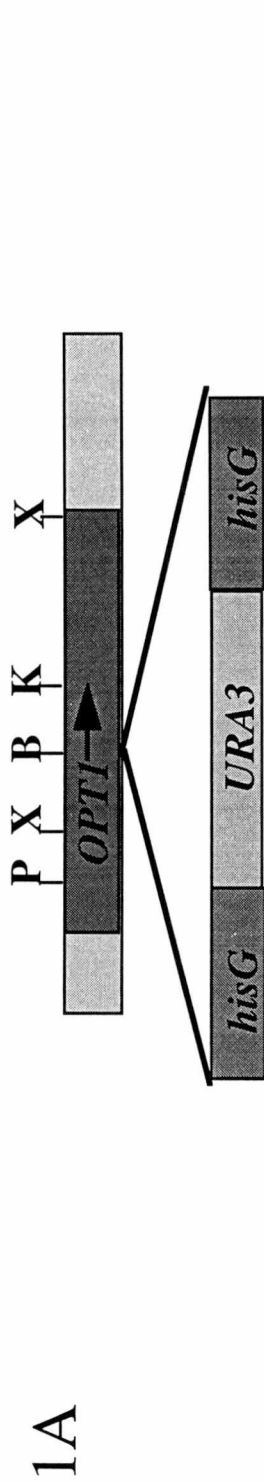
The media used and the plasmid pOP1 were described in Part 2, Materials and Methods. For *C. albicans* transformation procedures, transformants were plated onto MM with 1 M sorbitol as an osmotic stabilizer. *C. albicans* strain CAI4 (*ura3::imm434/ura3::imm434*) is a Ura⁻ derivative of the clinical isolate SC5314 and has been described previously (Fonzi and Irwin, 1993).

Construction of *OPT1* disruptants

Plasmid pOP2 was constructed by deleting the 45 bp *HindIII/NotI* fragment in the multiple cloning site of pOP1 (Lubkowitz *et al.*, 1997; Part 2). Plasmid pOP1 was digested with *HindIII/NotI*, recessed ends were filled in with Klenow, and the vector recircularized by blunt-end ligation. The resulting plasmid pOP2 contains a unique *BamHI* site located 1023 bp downstream of the start codon and 1384 bp upstream of the stop codon (Fig. 1A). A 4.045 kb *BamHI/BglII* fragment from plasmid pCUB6 (Fonzi and Irwin, 1993) containing the *hisG-URA3-hisG* fragment (Ura-Blaster) was cloned into the unique *BamHI* site of pOP2 to create pOP2Δ (Fig. 1A).

To obtain heterozygous *OPT1* disruptants 10 μg of plasmid pOP2Δ was digested with *BstXI*, ethanol precipitated, and resuspended in 10 μl of TE. Two microliters of the final suspension was then electroporated into CAI4 as described previously (Ausubel *et al.*, 1990) and transformants selected on MM containing 1 M sorbitol. Ura⁺ prototrophs were checked for URA-Blaster integration at the *OPT1* locus through Southern blot analyses. A total of 15 μg of genomic DNA from URA⁺ transformants was digested with *KpnI/PvuII*, electrophoresed in a 0.9% agarose gel, and Southern blot analysis performed

Figure 1. (A) Partial restriction map of the *OPT1* locus and Southern blots of *OPT1* disruptants. Restriction sites represented: P-*PvuII*, X-*BstXI*, B-*BamHI*, and K-*KpnI*. The disrupting construct was created by cloning the Ura-Blaster cassette into the unique *BamHI* site of plasmid pOP2 to create pOP2Δ. pOP2Δ was then digested with *BstXI* and subsequently electroporated into *C. albicans* CAI4. (B and C) Southern blot of *OPT1* disruptant strains. DNA was isolated from the designated strain and subjected to Southern blot analysis as described in Materials and Methods. The predicted products and sizes after digestion with *PvuII/KpnI* are represented for each sequential step of the disruption procedure. Figure 1B consists of the wildtype CAI4 and a representative of each predicted genotype: lane 1-CAI4 wildtype, lane 2-HTA6 heterozygous disruptant (*OPT1/opt1::Ura-Blaster*), lane 3 HTB14 heterozygous disruptant (*OPT1/opt1::hisG*), lane 4-HTC22 homozygous disruptant (*opt1::Ura-Blaster/opt1::hisG*). Figure 1C depicts the proposed gene duplication event that occurred after the transformation of HTB3 with the Ura-Blaster cassette. Subsequent analysis of twelve Ura⁺ transformants revealed two genotypes: lane 1-*OPT1/OPT1/opt1::Ura-Blaster* and lane 2-*OPT1/opt1::hisG/opt1::Ura-Blaster*. A faint band corresponding to 2.1 kb was detected in all lanes by overexposure of the autoradiogram.



as described (Lubkowitz *et al.*, 1997; Part 2). Putative heterozygous disruptants were designated by the letters HTA followed by a colony number. The probe used in all Southern blots is depicted in Figure 1 and was generated by PCR as described in Part 2.

The *URA3* marker was regenerated in the heterozygous disruptants by growing in non-selective conditions and then plating onto a medium containing 5-fluoro-orotic acid (5-FOA) (Boeke *et al.*, 1987). Briefly, an HTA strain was grown overnight under selective conditions in liquid medium. This culture was used to inoculate 3 ml of pre-warmed YEPD supplemented with 100 µg/ml uridine at a final density of 1×10^6 cells/ml and the cultures were then grown at 30 C. with shaking for 5-6 hours. Aliquots from this culture were plated onto MM supplemented with 100 µg/ml uridine and 100 µg/ml 5-FOA. Colonies resistant to 5-FOA appeared in 2-4 days. Regeneration of the *URA3* marker via interchromosomal recombination was distinguished from regeneration by intrachromosomal recombination by Southern blot analyses. No *URA*⁻ colonies were detected that arose by a mutation in the *URA3* gene. These strains were designated HTB followed by a colony number.

Several HTB strains were transformed as described above in order to disrupt the second allele of *OPT1*. To facilitate the identification of homozygous disruptants, approximately 100 *Ura*⁺ colonies were picked, suspended in sterile water, and spotted onto 0.1% proline medium containing either 50 µM OLLG or OLLLG. Spots were periodically examined between 18 and 48 hours of incubation and scored for growth. At the end of 48 hours all strains exhibited some level of growth. Those strains which exhibited the most resistance to the toxic peptide as measured by rapid growth were checked by Southern blot. Strains obtained during the second round of transformation were designated HTC plus a colony number.

Halo and Uptake Assays

Halo assays were performed as described in Part 2 and 3. Values reported for halo diameters were calculated by averaging the diameter of at least three different plates. Uptake assays were performed as described in Part 2 and 3. Values represented are an average of at least three different measurements.

Kinetic Analyses

Accumulation of KLG-[³H]L was measured as reported in Part 2 except that the final values reported have been adjusted to account for non-specific peptide adherence. The amount of non-specific peptide adherence was calculated by performing each experiment at 4 C. The final values were obtained by subtracting the amount accumulated at any given time point by the amount adhering at 4 C. An increase in non-specific adherence was observed as the concentration of KLG-[³H]L increased. However, the amount of non-specific peptide adherence did not increase in a time dependent manner. For kinetic analyses, the initial uptake rate was calculated by measuring the accumulation of KLG-[³H]L 20 seconds after the addition of the substrate. The value was converted to nmoles/min by multiplying by three and then adjusted to account for non-specific peptide adherence.

Murine Model of Systemic Candidiasis

The murine model of systemic candidiasis was performed as described earlier (Bulawa *et al.*, 1995). Briefly, four-week-old ICR male mice were injected in the tail vein with a 100 µl suspension containing 1×10^6 cells. Prior to injection, cells were grown to log phase in MM at 30 C, pelleted by centrifugation, washed once with sterile water, and resuspended at a concentration of 1×10^7 cells/ml in water. Cells were kept on ice prior to injection. Mice were checked twice daily for dead or moribund individuals. Moribund

animals were terminated by cervical dislocation and scored as dead on the day of termination. All animals were terminated by day 21. All experiments were performed within the guidelines established by the Institutional Animal Care and Usage Committee.

The spleen and both kidneys were removed from animals sacrificed on day 5 and day 10 and placed in a pre-weighed sterile Kaypack bag containing 1 ml of sterile water and the weight of each organ was determined. The organs were then crushed by hand in the bag, diluted in a 1:10 series, plated onto YEPD in an agar overlay, and incubated for 36 hours at 30 C. CFUs per g of tissue was calculated from the dilution that produced 30-300 colonies per plate.

CHAPTER III

RESULTS

Construction of *OPT1* heterozygous and homozygous disruptant strains

Previously we reported the cloning and characterization of the oligopeptide transport gene *OPT1* from *C. albicans* (Lubkowitz *et al.*, 1997; Part 2). To determine if *OPT1* is responsible for all of the oligopeptide transport activity observed in *C. albicans* and to determine if *OPT1* affects virulence, we constructed a heterozygous and homozygous disruptant of *OPT1* in strain CAI4 using the Ura-Blaster cassette (Fonzi and Irwin, 1993; see Materials and Methods for details on strain construction). The Ura-Blaster was cloned into the *Bam*HI site of pOP2 to create the disrupting plasmid pOP2 Δ (Fig. 1A). A *Bst*XI fragment from pOP2 Δ was then transformed into CAI4 and URA⁺ prototrophs obtained. Twelve transformants, designated HTA1-12, were selected and screened for integration of the disrupting cassette at the *OPT1* locus. All Southern blot analyses used genomic DNA digested with *Kpn*I and *Pvu*II as well as the probe depicted in Figure 1. As illustrated by strain HTA6 in Figure 1B, all twelve colonies chosen exhibited the *OPT1/opt1::hisG-URA3-hisG* genotype (data not shown) by producing the predicted 5.2 kb band for the disrupted locus and the 1.2 kb band for the native locus when analyzed by Southern blot. Disruption of the second allele required that the *URA3* marker be regenerated. Therefore, strain HTA6 was grown in a non-selective medium as described in Materials and Methods and subsequently was plated onto a medium containing 5-fluoro-orotic acid (5-FOA), a toxic substrate of the *URA3* gene product. Resistance to 5-FOA can be acquired by intrachromosomal recombination between the *hisG* sequences leading to the excision of the *URA3* gene and one copy of the *hisG* sequence, or, alternatively, 5-FOA resistance can occur through interchromosomal recombination leading to the regeneration of the wildtype locus. Twelve 5-FOA resistant

strains, designated HTB13-24, were analyzed by Southern blot and found to have an *OPT1/opt1::hisG* genotype as indicated by the disappearance of the 5.2 kb band corresponding to the disrupted allele and the emergence of a 2.3 kb band (Figure 1B, lane 3). None of the twelve strains examined underwent interchromosomal recombination resulting in an *OPT1/OPT1* genotype. To generate a homozygous disruptant, HTB13 and HTB14 were retransformed with the *Bst*XI fragment of pOP2Δ and URA⁺ prototrophs obtained. Forty prototrophs were subsequently spotted onto MM containing 50 μM OLLG and monitored for growth. Two strains designated HTC2 and HTC22, derived from HTB13 and HTB14, respectively, grew on this medium within 24 hours while all the remaining strains grew only after a prolonged incubation period. Southern blot analyses confirmed that HTC2 and HTC22 no longer harbored an intact copy of the *OPT1* ORF (Figure 1B, lane 4). However, HTC2 did not produce the predicted 5.2 kb band but rather a much larger band possibly due to tandem integration of the Ura-Blaster cassette (data not shown). Tandem integrations have been reported previously (Bulawa *et al.*, 1995). Over-exposure of autoradiograms revealed a faint band corresponding to 2.1 kb for all strains tested possibly indicating the presence of an *OPT1* homolog.

Gow *et al* (1994) unexpectedly reported triploidy at the *CHS2* locus after two rounds of transformation with a Ura-Blaster revealed a third copy of this gene. It was suggested that this third allele may have arisen by a gene duplication event as a result of repeated transformations and integrations at the same locus. During the construction of the *OPT1* disruptants, a gene duplication event seems to have occurred in one of the lineage's. The heterozygous disruptant HTA7 and its Ura⁻ progeny HTB3 have identical genotypes to HTA6 (*OPT1/opt1::hisG-URA3-hisG*) and HTB 13 (*OPT1/opt1::hisG*), respectively. However, two types of progeny were observed in the twelve Ura⁺ transformants analyzed after a second round of transformation of HTB3. Seven of the twelve transformants were found to have an *OPT1/opt1::hisG/opt1::hisG-URA3-hisG*

genotype as exhibited in Figure 1C, lane 2. The remaining five transformants had an *OPT1/opt1::hisG-URA3-hisG* genotype. However, the 1.2 kb band corresponding to the *OPT1* allele is much brighter than the band corresponding to the *opt1::hisG-URA3-hisG* allele (Figure 1C, Lane 1). This increase in brightness was not observed in lanes where three alleles were present suggesting that the genotype of these strains in actuality is *OPT1/OPT1/opt1::hisG-URA3-hisG*. Repeated attempts to disrupt the third allele failed to produce a homozygous disruptant.

A second set of disruptants was later generated to increase the number of strains analyzed. These strains were constructed in an analogous manner to HTA6 and HTC22. The heterozygous strains are denoted HTA14 and HTA15. The second round of transformation only produced one homozygous strain denoted HTC49. HTA14 is the parent strain of HTC49. The parental/sibling relationship of these *OPT1* disruption strains are shown in Figure 2.

Characterization of *OPT1* deletion strains

In Part 2 we demonstrated that oligopeptide transport activity in *C. albicans* is repressible by rich nitrogen sources and derepressible by poor nitrogen sources. For example, when grown in a medium containing 0.1% proline as a sole nitrogen source, *C. albicans* SC5314 was able to accumulate the radiolabeled peptide KLG-[³H]L with an initial uptake rate of 0.4 nmole/min/mg of dry wt. However, when grown in a medium containing ammonium sulfate (MM) as a sole nitrogen source SC5314 had an uptake rate of 0.06 nmoles/min/mg of dry wt for the same substrate (Lubkowitz *et al.*, 1997; Part 2). To determine if Opt1p was responsible for the nitrogen-dependent oligopeptide transport activity previously observed in SC5314, we measured the sensitivity of the *OPT1* wildtype strain CAF2-1 (a heterozygous *URA3* deletion strain and a derivative of SC5314), the heterozygous disruptants HTA6 and HTA14, and the homozygous

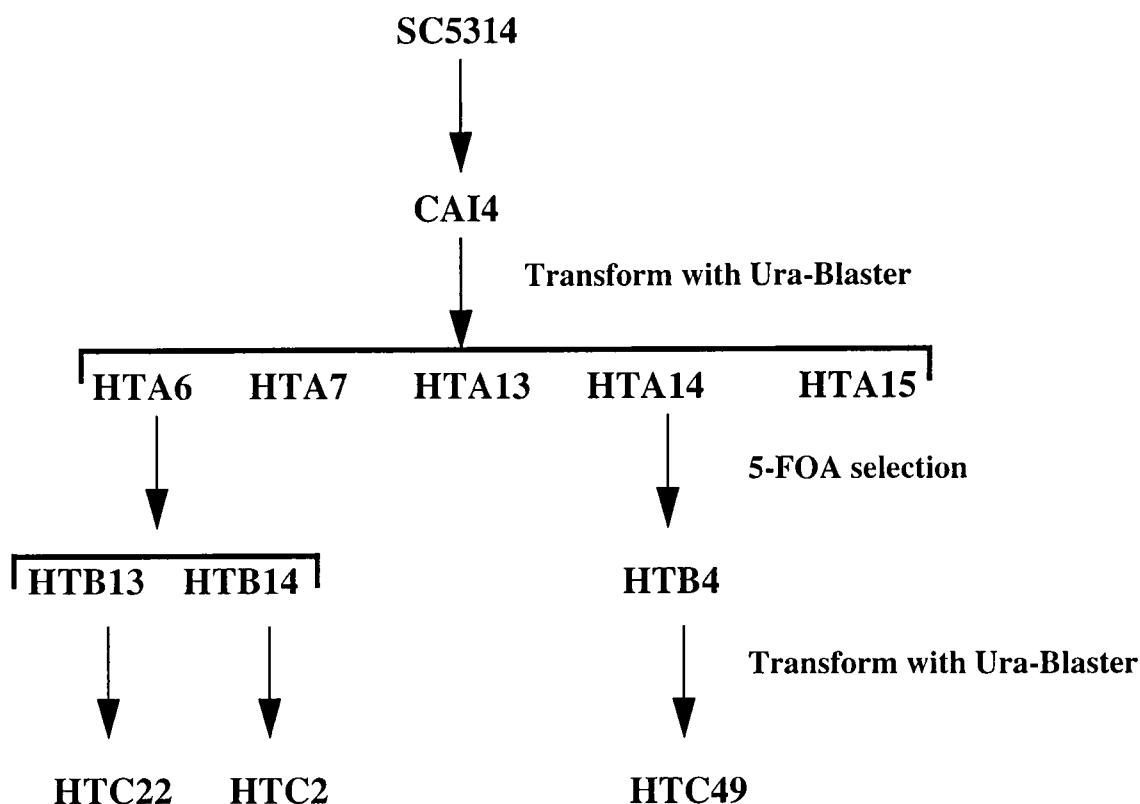


Figure 2. Pedigree of *OPT1* disruptant strains. Strains were constructed as described in Materials and Methods. Strain SC5314 is a clinical isolate and has been described previously (Fonzi and Irwin, 1993). Strain CAI4 is a *ura⁻* derivative of SC5314. The genotype of each strain with respect to the *OPT1* locus is as follows: SC5314-*OPT1/OPT1*, CAI4-*OPT1/OPT1*, HTA strains-*OPT1/opt1::hisG-URA3-hisG*, HTB strains-*OPT1/opt1::hisG*, HTC22 & 49-*opt1::hisG-URA3-hisG/opt1::hisG*, and HTC2-*opt1::hisG-URA3-hisG-hisG-URA3-hisG/opt1::hisG*.

disruptants HTC22 and HTC49 to the toxic oxalysine-containing peptides OG, OLG, OLLG, and OLLLG. Strain HTA6 is the parent strain of HTC22 while HTA14 is the parent strain of HTC49. When grown in a 0.1% proline medium, all five strains exhibited the same level of sensitivity to the toxic amino acid analog oxalysine and the toxic dipeptide OG (Table 1). However, a decreasing level of sensitivity was observed for the toxic peptides OLG, OLLG, and OLLLG in a copy number dependent manner. Furthermore, for several of the peptides tested two halos were observed: a distinct inner halo of complete growth inhibition surrounded by an outer halo of partial growth inhibition. When incubated for an additional 24 hours these halos became more discernible (Fig 3). Because the size and character of the halo changed with time, halo diameters were only compared within each individual experiment. Despite this variation, the trends observed between CAF2-1 and the heterozygous and homozygous disruptants was consistent and repeatable between experiments.

When measured after 18-24 hours of growth in 0.1% proline medium, the wildtype strain CAF2-1 formed a halo with unpronounced edges of 24-26 mm to the toxic tripeptide OLG. After prolonged incubation, the hazy edge formed into the outer ring depicted in Figure 3. The heterozygous knockouts HTA6 and HTA14 both formed a large hazy halo after 18-24 hours that was comparable in size to the halo formed by CAF2-1 (Table 1). However, the hazy edge had considerably more growth and a distinct inner halo was discernible at 24 hours. The homozygous knockouts HTC22 and HTC49 formed a very faint outer ring after overnight incubation and had a distinct inner ring of growth inhibition. When incubated for an additional 24 hours the outer ring became completely filled in. A similar pattern was observed for the tetrapeptide OLLG and the pentapeptide OLLLG. That is, after 24 hours, HTA6 and HTA14 had formed halos of comparable size to the halo formed by CAF2-1 but with edges of partial growth inhibition. In the homozygous knockouts, two halos were observed: an outer ring of

Table 1 : Sensitivity of *C. albicans* strains grown on 0.1% proline media to various oxalysine-containing peptides

<i>C. albicans</i> Strain:	Zone of inhibition (mm) ^a				
	O	OG	OLG	OLLG	OLLLG
CAF2-1	17	24	24	26	23
HTA6	17	25	15 (24) ^{b,d}	27 ^c	24(11) ^b
HTC22	18	26	14 (25) ^{b,d}	11	12
CAF2-1	19	25	26	27	26
HTA14	19	25	17 (27) ^{b,d}	28 ^c	27 ^b
HTC49	20	25	17	13(23)	25(25)

a A total of 0.25 μ moles was spotted on a 6 mm disk placed on a lawn of cells. "Faint" indicates a small (> 8 mm) zone with incomplete growth inhibition. Each test comprised three or more independent assays and the results represented in the Table are means of the values obtained. Maximum variation between the zones of inhibition for each test was $\leq$ 3 mm.

b Two halos were seen. The first halo was a clear zone of growth inhibition surrounded by a halo of partial growth inhibition. The diameter of the larger halo of partial growth inhibition is in parantheses.

c The outline of the zone of the inhibition was not very sharp and a hazy zone of inhibition was observed.

d The halo for the homozygous disruptant was more diffuse than the halo for the heterozygous disruptant.

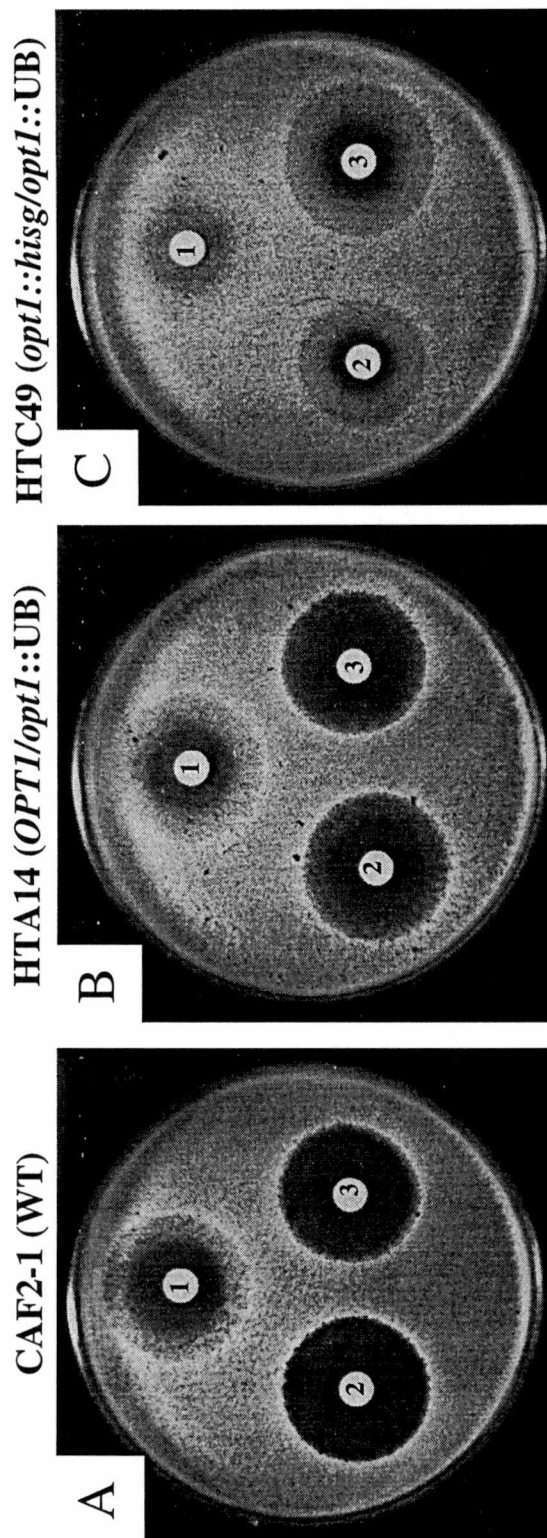


Figure 3. Toxic peptide inhibition assay with *OPT1* disruptant strains. Sensitivity to the oxalysine-containing peptides OLG (1), OLLG (2), and OLLLG (3) on 0.1% proline medium was determined as described in Materials and Methods. (a) CAF2-1; (b) HTA14; (c) HTC49.

partial growth inhibition and an inner ring of complete growth inhibition. The zones of complete growth inhibition as well as the zones of partial growth inhibition were more pronounced after 48 hours of incubation (Fig. 3). Furthermore, differences in halo sizes were not a function of growth rate for CAF2-1, HTA6, and HTC22 as these strains were found to have identical doubling times in both 0.1% proline media and MM (data not shown).

In medium containing ammonium sulfate as the primary nitrogen source, comparable halos, both in size and in character, were seen for CAF2-1, HTA6, HTA14, HTC22, and HTC49 for oxalysine and OG (Table 2). For the peptides OLG, OLLG, and OLLLG the halo size was smaller than the halo formed by the same strain when 0.1% proline was used as the nitrogen source. Halos of comparable size to CAF2-1 were seen for both lineages to OLG. However, with increased incubation time the edge of growth inhibition became less distinct in the heterozygous knockouts and even less distinct in the homozygous knockouts. The heterozygous disruptant HTA6 only exhibited slight sensitivity to OLLG and formed a halo comparable to that observed for HTC22. On the other hand, the heterozygous knockout HTA14 formed a halo that was intermediate to the levels of sensitivity exhibited by CAF2-1 and HTC49. This discrepancy between heterozygotes could be explained by allelic variation if opposite alleles are disrupted in each strain. All of the strains exhibited very little sensitivity to the pentapeptide OLLLG.

The accumulation of the radiolabeled substrate KLG-[³H]L was measured over a four minute time course with cells grown in either MM or 0.1% proline media. As seen in Figure 4A, when grown in proline medium the wildtype strain CAF2-1 accumulated the highest level of KLG-[³H]L followed by HTA6 and then HTC22. An identical pattern of accumulation was seen for the same strains when grown in MM although the total levels of accumulation were not as high (Fig. 4B).

Table 2 : Sensitivity of *C. albicans* strains grown on MM to various oxalysine-containing peptides

<i>C. albicans</i> Strain:	Zone of inhibition (mm) ^a				
	O	OG	OLG	OLLG	OLLLG
CAF2-1	14	22	14	22	faint
HTA6	14	20	12 ^d	9	faint
HTC22	11	19	12 ^d	9	faint
CAF2-1	15	25	17	26	11 ^c
HTA14	15	24	18 ^d	12(24) ^b	11 ^c
HTC49	16	25	17 ^d	faint	faint

a A total of 0.25 μ moles was spotted on a 6 mm disk placed on a lawn of cells. "Faint" indicates a small (> 8 mm) zone with incomplete growth inhibition. Each test comprised three or more independent assays and the results represented in the Table are means of the values obtained. Maximum variation between the zones of inhibition for each test was $\leq$ 3 mm.

b Two halos were seen. The first halo was a clear zone of growth inhibition surrounded by a halo of partial growth inhibition. The diameter of the halo of partial growth inhibition is in parantheses.

c The outline of the zone of the inhibition was not very sharp and a hazy zone of inhibition was observed.

d The halo for the homozygous disruptant was more difuse than the halo for the heterozygous disruptant.

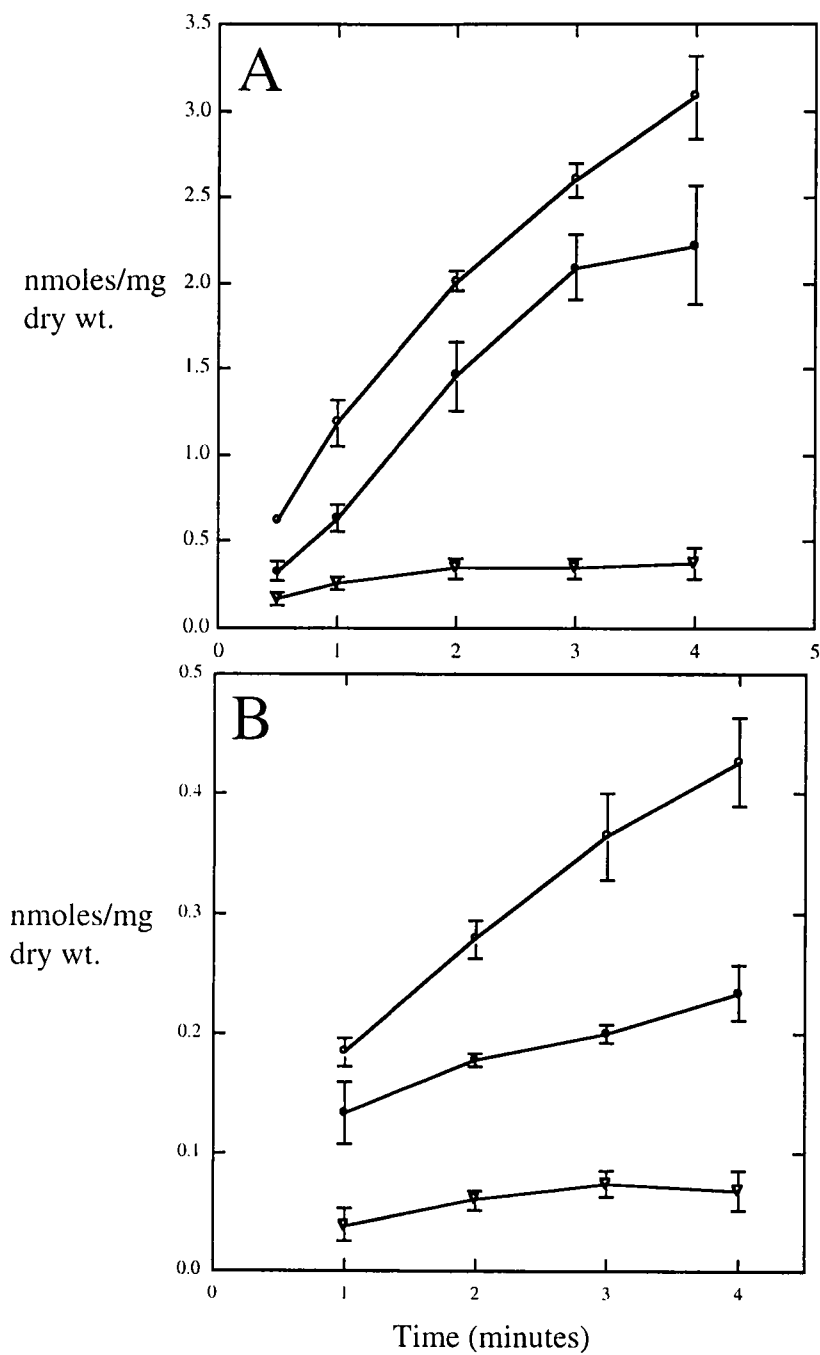


Figure 4. Peptide transport assay with *OPT1* disruptants. Accumulation of KLG-[³H]L (150 μ M) was measured over a four minute time course as described in Materials and Methods. CAF2-1 (o), HTA6(•), and HTC49 (∇) grown in either 0.1% proline medium (a) or MM (b). Final values were adjusted for non-specific peptide adherence as detailed in Materials and Methods.

The formation of a halo by HTC22 and HTC49 over a prolonged incubation suggests that the peptides OLG, OLLG, and OLLLG are entering the cell through an alternative route. To determine if another oligopeptide transport system exists in *C. albicans*, the wildtype strain CAF2-1 and the homozygous knockouts HTC22 and HTC49 were analyzed kinetically.

Kinetic Analyses of CAF2-1, HTC22, and HTC49

Because of the large halos exhibited by HTC22 and HTC49 in 0.1% proline medium to toxic tetra and pentapeptides and the very low initial uptake rates measured previously in MM, 0.1% proline was chosen as the nitrogen source for the growth medium of the kinetic experiments. The accumulation of KLG-[³H]L at concentrations of 3, 150, and 200 μ M by CAF2-1 was measured over a time course at twenty second intervals. The initial uptake rate was found to be linear for the first 40 seconds (Fig. 5) and subsequently all initial uptake rates were measured 20 seconds after the addition of KLG-[³H]L. The initial uptake rate of KLG-[³H]L at concentrations of 3.1, 6.3, 12.5, 25, 50, 100, and 200 μ M was then determined for CAF2-1, HTC22, and HTC49 as described in Materials and Methods. The experiment was also performed with cells incubated at 4 C and these counts were used to adjust for the non-specific adherence of the peptide to the cells. The adjusted values were plotted in an Eadie-Hofstee format (V_o vs V_o/S) which weights the lower concentrations and amplifies any deviations away from linearity. Under this form of analysis two transport systems with different kinetic properties are predicted to yield a curvilinear plot that can be divided into two intersecting lines while a single transport system yields one line. As seen in Figure 6, CAF2-1 exhibits a plot characteristic of one transport system. HTC49 and HTC22 had initial uptake values at or near zero after being adjusted for background levels and hence were not plotted. Using the transformed data, an apparent K_m of 11.5 μ M and a V_{max} of 0.45

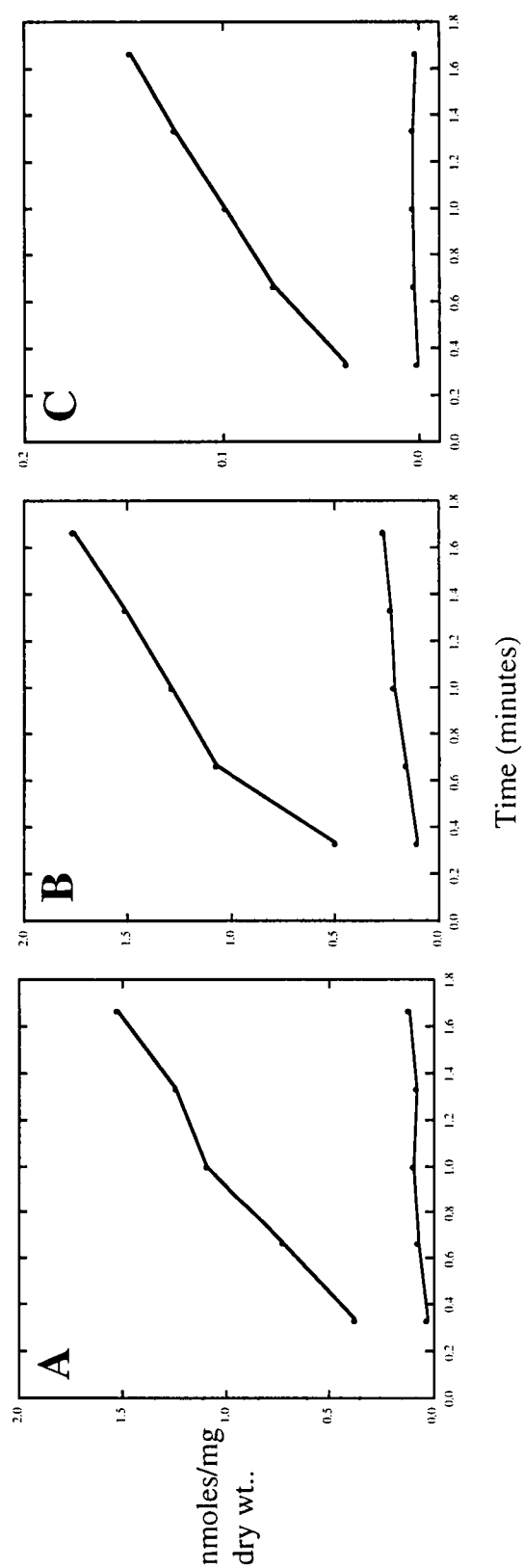


Figure 5. Peptide transport assay with *OPT1* disruptants. Accumulation of KLG-[³H]L at concentrations of 200 (a), 150 (b), and 3.1 (c) μ M was measured at 20 second intervals over a 100 second time course as described in Materials and Methods with CAF2-1 (●) and HTC49 (o) grown in 0.1% proline medium. The final values were adjusted for non-specific peptide adherence as detailed in Materials and Methods.

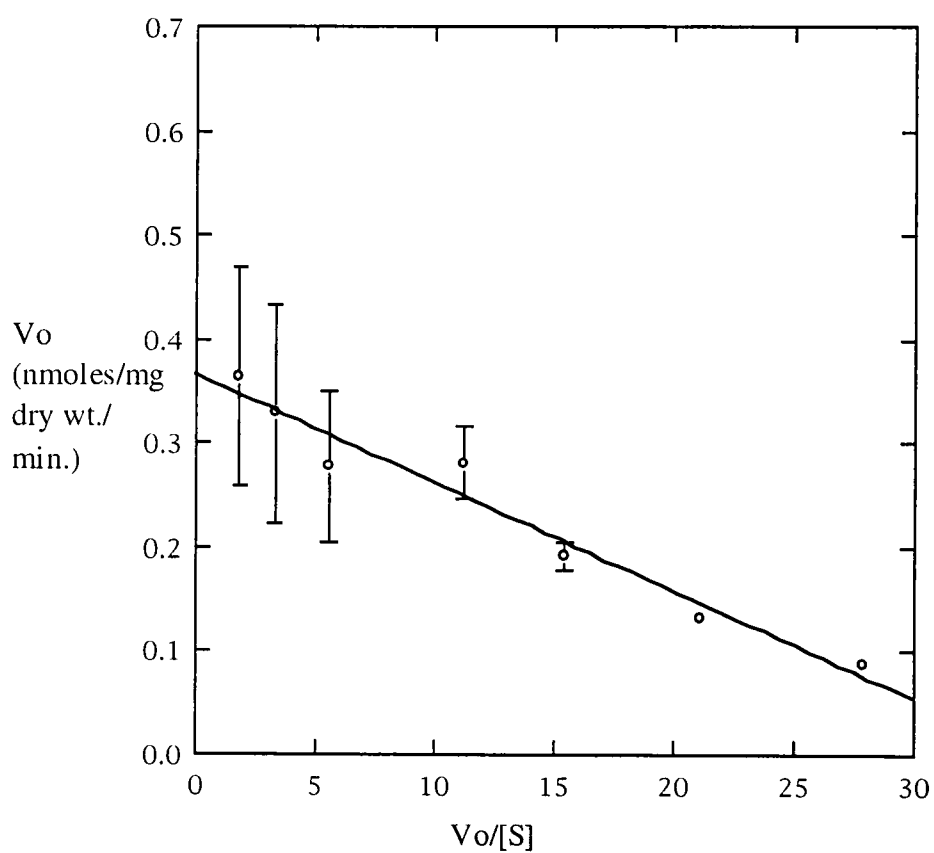


Figure 6. Eadie-Hofstee transformation of kinetic data. The initial uptake rate of KLG- ^3H L at concentrations of 3.1, 6.3, 12.5, 25, 50, 100, and 200 μM was measured for CAF2-1 grown in 0.1% proline medium. Final values were adjusted for non-specific peptide adherence as described in Materials and Methods.

nmoles min⁻¹ mg of dry wt.⁻¹ was calculated for strain CAF2-1 from two independent experiments repeated in triplicate. Because HTC49 and HTC22 did not have a measurable amount of peptide transport activity under the conditions tested we concluded that the kinetic properties observed in CAF2-1 are attributable to *OPT1*.

To determine whether CAF2-1 has the same affinity for OLLG and KLG-[³H]L the initial uptake rate of KLG-[³H]L at concentrations of 3.1, 6.3, 12.5, and 25 µM was measured in the presence of different fixed concentrations of OLLG (0, 5, 10, 25, and 50 µM). The background levels of peptide adherence were measured at 4 C for the entire range of KLG-[³H]L concentrations in the absence of competitor or in the presence of 50µM OLLG. The level of background counts was found to be independent of OLLG and these values were used to adjust the initial uptake rates for non-specific peptide adherence. The data obtained was transformed into a Lineweaver-Burke format (1/V_o vs 1/[S]) and the slope of each line calculated. The slope of each line was plotted against the concentration of OLLG to yield a Dixon plot (Fig. 7). The apparent K_i was then calculated from the x-intercept (-K_i) and found to be 13 µM.

Does *OPT1* affect virulence?

In order to determine whether *OPT1* affects virulence, CAF2-1, HTA6, and HTC22 were used in a murine model of systemic candidiasis as described in Materials and Methods. As seen in Figure 8, both the heterozygous and the homozygous strain exhibited decreased virulence when compared to the wildtype. Unexpectedly, the heterozygous disruptant HTA6 was slightly more attenuated than the homozygous knockout HTC22. To determine whether this phenotype was repeatable in other disruptants, strains HTA7, HTA14, HTA15, and HTC22 were subsequently tested for attenuation. For comparison purposes the day of 50% survival was calculated and tabulated. The heterozygotes HTA7, HTA14, and HTA15 exhibited near wildtype levels

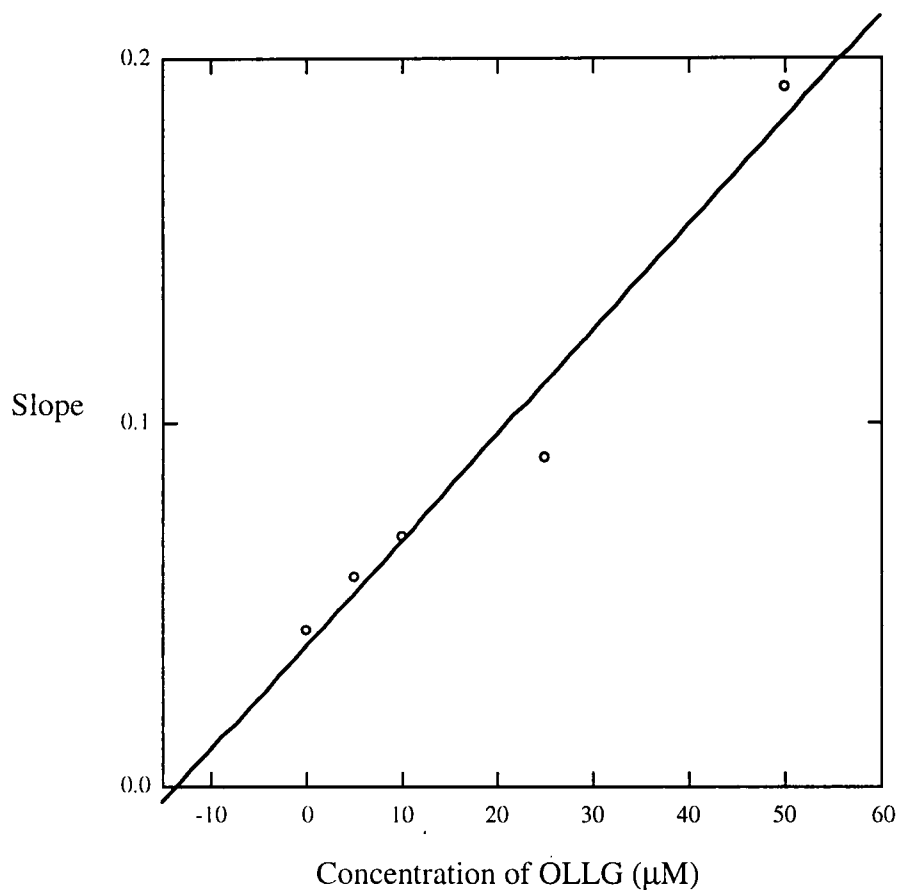


Figure 7. Dixon plot. The initial uptake rate of KLG- ^3H L at concentrations of 3.1, 6.3, 12.5, 25, and 50 μM was measured in the absence of a competitor or in the presence of 5, 10, 25, or 50 μM OLLG. The values obtained were then plotted in a Lineweaver-Burke format ($1/V_o$ vs $1/[S]$) and the slope of each line calculated. The slope of each line was plotted against the concentration of OLLG and the K_i calculated from the x-intercept.

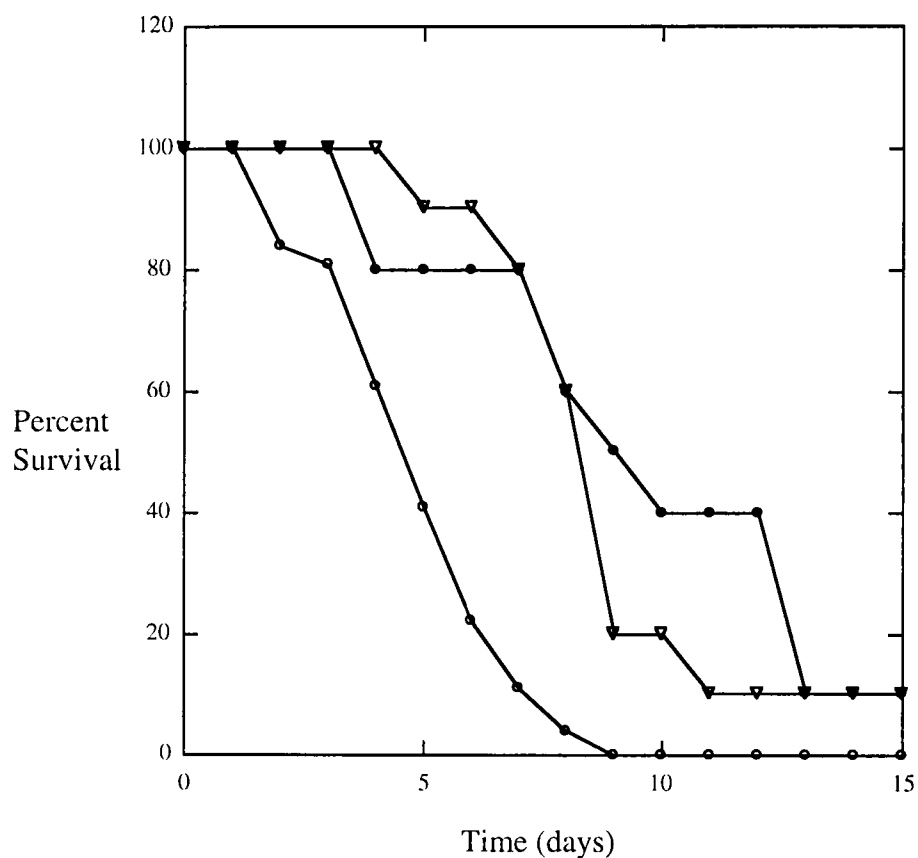


Figure 8. Survival curve of OPT1 disruptants in a murine model of systemic candidiasis. Five mice were injected with CAF2-1, HTA6, or HTC22 as described in Materials and Methods and percent survivability monitored over time. The experiment was repeated several times and the results presented are a summation of two such experiments. CAF2-1 (o), HTA6(●), and HTC22(▽).

of virulence while HTA6 exhibited levels comparable to the homozygous disruptants HTC22 and HTC49. HTC2 exhibited the most attenuation possibly due to the tandem integration of the Ura Blaster cassette (Table 3).

Kidneys and spleen were recovered from mice sacrificed on day 5 and day 10 and the number of CFU/g of tissue was calculated (Table 4). Large numbers of CFU/g of tissue were recovered from the kidney for all strains whereas very few cells were found in the spleen. Southern blots performed on CAF2-1, HTA6, and HTC22 isolates recovered from the kidney confirmed that the *OPT1* locus was not altered by passage through a murine host. Similarly, the same level of oligopeptide transport activity was maintained by each strain as determined by halo assays (data not shown).

Table 3: Day of 50% post-infection survival.

Strain	Day ^b	N ^a
CAF2-1	4.5	27
HTA6	8.0	24
HTA7	4.5	5
HTA14	5.5	5
HTA15	5.5	6
HTC22	7.0	24
HTC49	6.5	5
HTC2	11	5

^a Number of mice used in each experiment. For strains CAF2-1, HTA6, and HTC22 the values were obtained by combining the results of several independent experiments.

^b The differences observed between the various strains were found to have the following P-scores: CAF2-1 and HTA6 (P) .0001, CAF2-1 and HTC22 (P) .0001, and HTA6 and HTC22 (P) .5327.

Table 4: Recovery of *OPT1* disruptant strains from mice^a

Strain	Mouse	Day	CFU/g kidney	CFU/g spleen
CAF2-1	m1	5	2.1×10^5	0
CAF2-1	m2	5	4.1×10^4	3.3×10^2
CAF2-1	m3	5	3.1×10^5	4.4×10^4
HTA6	m1	5	9.4×10^4	0
HTA6	m2	5	7.3×10^3	0
HTA6	m3	5	2.2×10^5	8.9×10^2
HTA6	m4	5	2.3×10^5	0
HTC22	m1	5	7.2×10^4	281
HTC22	m2	5	1.6×10^5	367
HTC22	m3	5	2.1×10^5	3.8×10^2
HTC22	m4	5	6.9×10^4	0
HTA6	m5	10	1.5×10^6	0
HTA6	m6	10	1.9×10^6	0
HTA6	m7	10	8.8×10^4	0
HTC22	m5	10	4.4×10^5	0
HTC22	m6	10	1.2×10^6	0
HTC22	m7	10	1.3×10^4	94

^aMice were sacrificed on specified day and CFU/g of tissue calculated as described in Materials and Methods. The results were obtained from two independent experiments.

CHAPTER IV

DISCUSSION

We report here the construction and characterization of several *OPT1* disruptant strains. These strains are characterized by a decrease in sensitivity to toxic tri-, tetra-, and pentapeptides in a copy number dependent manner (Table 1, Fig. 3). That is, the homozygous disruptants are less sensitive than the heterozygous disruptants. Similarly, the accumulation of KLG-[³H]L over a four minute time course was dependent on the number of copies of *OPT1* present (Fig. 4). Furthermore, kinetic analyses of the homozygous disruptants HTC22 and HTC49 failed to detect any oligopeptide transport activity. Results of these experiments revealed that the activity observed in CAF2-1 had an apparent *K_m* of 11.5 and an apparent *V_{max}* of 0.45 nmoles/min/mg of dry wt (Fig. 5). Because no activity was observed in HTC22 and HTC49 under these experimental conditions, we believe that the activity observed in CAF2-1 is attributable to *OPT1*. Based upon these criteria, we propose that *OPT1* does indeed encode an oligopeptide transporter.

Furthermore, we report the detection of another oligopeptide transport system as indicated in the homozygous knockout disruptants by a decrease but not a complete loss of oligopeptide transport activity as measured by halo assays (Table 1, Fig. 3). The fact that this secondary system was not detected by kinetic analyses does not preclude the possibility that a peptide transporter with different kinetic properties, substrate preferences, or regulatory schemes exists. However, the finding that the apparent *K_i* for OLLG is identical by this experimental measurement to the apparent *K_m* for KLG-[³H]L suggests that the differences in apparent transport are not a function of substrate affinity. Uptake assays measure the influx of a substrate whereas halo assays measure transport as a function of sensitivity to a toxic compound. Thus, the accumulation of a radiolabeled

substrate over a short time course is not necessarily reflective of the total accumulation that occurs over a prolonged incubation period as measured by halo assays. Furthermore, neither a low affinity (K_m in mM range)-low capacity transporter nor a peptide inducible system would have been detected by the kinetic analyses performed but may have been detected by halo assay. The possible existence of a second transporter is further substantiated by Southern blot analyses. During the construction of the *OPT1* deletion strains, a 2.1 kb band was observed in over-exposed autoradiograms for all strains including CAF2-1 and CAI4. This band could be indicative of an *OPT1* homolog in *C. albicans*.

In Part 2 we demonstrated that *OPT1* expression in *S. cerevisiae* is dependent upon nitrogen source. To determine if this regulation was present in the native host, we measured the sensitivity of HTA6, HTA14, HTC22, and HTC49 to various toxic peptides on either MM or 0.1% proline media. Larger halos were observed for OLG, OLLG, and OLLLG when 0.1% proline was used as the nitrogen source for all strains tested. For example, CAF2-1 formed a small and faint halo to OLLLG and a 23-26 mm halo on proline medium. Similarly, CAF2-1 accumulated much higher levels of KLG- $[^3H]L$ over a four minute time course when grown in 0.1% proline than when grown in ammonium sulfate. The level of accumulation observed in CAF2-1 in proline medium decreased in HTA6 and HTC22 indicating that this nitrogen dependent oligopeptide transport activity is in part due to *OPT1*. Interestingly, the residual peptide transport activity detected in HTC22 and HTC49 by halo assays seems to be regulated at least in part by nitrogen source as indicated by the larger halos to OLLG and OLLLG when grown in proline medium.

It has been proposed that the multiple manipulations and selection of intrachromosomal recombinants can lead to additional undetected mutations in the genome (Bulawa, personal communication). Gow *et al.* (1994) and now our laboratory have

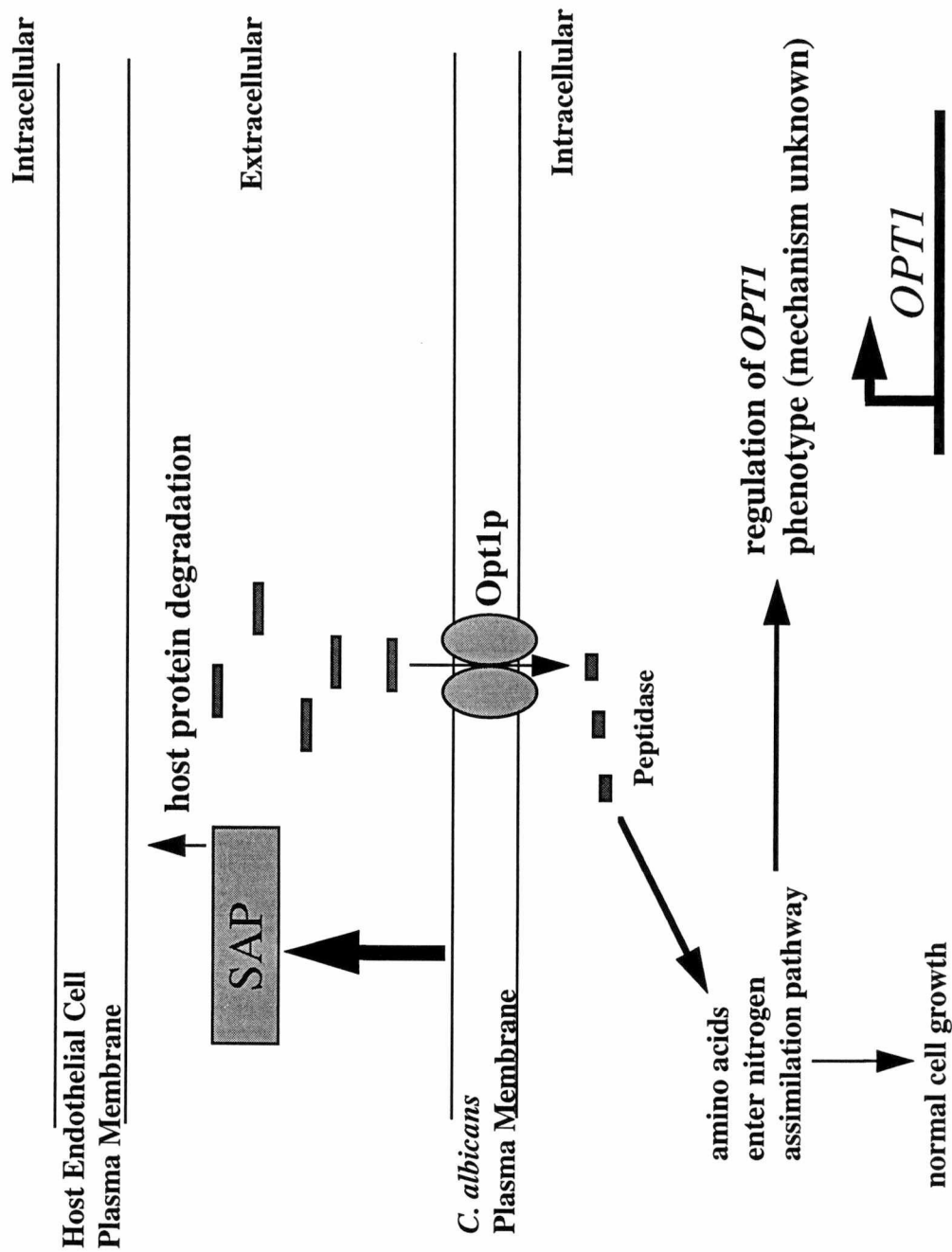
detected gene duplication events at the targeted locus that arose presumably during the construction of the disruptant. These observations should serve as a warning to investigators working in *Candida*. The induction of gene duplications may cause distal chromosomal effects and may potentially obfuscate the subsequent characterization of the disruptants. Therefore, *Candida* investigators would be wise to examine several independently generated lineages before drawing any conclusions from their experimental results. Furthermore, Bulawa *et al.* (1995) and our laboratory have observed multiple integrations of the Ura-Blaster cassette at the targeted locus. Once again the effect of such an event on chromatin structure or on distal regions is not known.

Both HTA6 and HTA14 as well as HTC22 and HTC49 had identical genotypes with respect to the *OPT1* locus. Similarly, HTC22 and HTC49 had identical oligopeptide transport phenotypes as measured by uptake and halo assays. On the other hand, HTA6 and HTA14 did not exhibit identical levels of oligopeptide transport as measured by halo assays. This discrepancy was further manifested in a murine model of systemic candidiasis. All homozygous knockouts exhibited very similar levels of virulence except HTC2 which appears to contain a tandem integration of the Ura-Blaster cassette. There are several possible explanations for these discrepancies. First a mutation, not present in the other heterozygotes, could have arisen in HTA6 during the transformation step or during subsequent passages such that this strain was now attenuated. Alternatively, it could be argued that HTA7, HTA14, and HTA15 acquired mutations that increased their virulence. However, we believe a gain of function mutation in three independent strains is much more unlikely than a loss of function mutation in one strain. The observation that the homozygous disruptants had levels of virulence similar to that of HTA6 raises a second possible explanation for the variability exhibited in the heterozygous disruptants. The difference in virulence levels in the HTA strains could be a function of allelic variation at the *OPT1* locus. If allelic variation does exist and if

virulence is affected by *OPT1*, then the virulence level would be a function of which allele was disrupted. In such a scenario, the homozygous disruptant would presumably exhibit the same level of virulence simply because both alleles were disrupted. Testing of such a hypothesis could be accomplished by a thorough kinetic analysis of the HTA strains.

Strain HTC22 and HTC49 did show slight levels of attenuation. Furthermore, both HTC22 and HTA6 were recovered in high numbers from the kidney indicating that the mouse is not able to clear the organism. Originally, we hypothesized that oligopeptide transport may directly or indirectly influence virulence by transporting a regulator of the SAP genes or an inducer of a virulence program. Since the completion of our study, Fallon *et al.* (1997) has proposed that the SAPs play a large role in early dissemination. They demonstrated that the SAPs were necessary for dissemination when a low inoculum was administered to neutropenic mice intranasally. However, the SAP requirement for virulence could be overcome by providing a larger inoculum intravenously thereby bypassing the rigors of initially establishing an infection. In their proposed model, the SAPs are produced early in dissemination primarily to provide nutrients for the organism. In such a model of infection, *C. albicans*, prior to reaching the vascular system of the host, is in a nitrogen limiting environment and the degradation products of the SAPs, *i.e.*, peptides, are internalized and used as a nitrogen source. Therefore, the possibility exists that oligopeptide transport may play a large role in virulence by providing the necessary nitrogen sources during the early stages of dissemination (Fig. 9). Such a role in infection would not have been detected by our murine model of systemic candidiasis simply because the large inoculum size injected into the vascular system circumvents the early stage of infection where *OPT1* would

Figure 9. Proposed model for the role of *OPT1* in virulence. Prior to dissemination or during the early stages, *C. albicans* is in a nitrogen limiting environment such as the lungs. SAPs are produced and hydrolysis of host proteins ensues. Simultaneously, the nitrogen limiting environment leads to the up-regulation of the OPT1 phenotype. The degradation products are transported by Opt1p, hydrolyzed, and used as a nitrogen source. The OPT1 phenotype is eventually down-regulated as the pathogen enters the vascular system where nitrogen is more readily available.



be required for nitrogen scavenging. In congruence with this hypothesis, a high affinity glutamine transporter from *S. typhimurium* is required for virulence because of its role in acquiring nitrogen (Klose and Mekalanos, 1997).

A search of the Expressed Sequence Tag (EST) database for sequences similar to *OPT1* identified three putative homologs in *Arabidopsis thaliana* and in rice. Although the EST databases are not complete, no mammalian ESTs were identified. If the OPT family is present only in fungi and plants then Opt1p could be exploited as a drug delivery mechanism. Such a unique transporter could be used as a vehicle for delivering a specific peptide-based antibiotic. By increasing specificity of the drug for its target, toxicity could be increased for fungal cells but decreased for mammalian cells. This mechanism could consist of "piggy-backing" a drug on a peptide that is specific for a fungal transporter. This concept has been termed "illicit transport" (Ames *et al.*, 1973; Fickel and Gilvarg, 1973) and several naturally occurring examples have been reported (Naider and Becker, 1987).

In summary, we report that *OPT1* encodes a high-affinity/low-capacity transporter that is regulated by nitrogen source. Furthermore, characterization of *OPT1* disruptants has revealed the presence of a second oligopeptide transport in *C. albicans* which is also regulated by nitrogen source. Finally, *OPT1* deletion strains exhibit slight attenuation of virulence, however, the possibility still exists that this gene plays a larger role in certain stages of virulence in specific animal models by assisting in nitrogen acquisition during early dissemination.

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PART 5

CONCLUSIONS AND PROSPECTUS

CHAPTER I

CONCLUSION

Through this dissertation I have attempted to tell the story of how our laboratory cloned and characterized an oligopeptide transporter from *C. albicans* and how the identification of this transporter led to the discovery of an oligopeptide transporter in *S. pombe* and the establishment of a novel gene family. Furthermore, we uncovered the presence of yet another oligopeptide transport system and explored the role of *OPT1* in virulence in one model of systemic candidiasis. In this final section I will pose some of the questions that might be addressed in future extensions of this work .

OPT Transporters

Through this work we were able to identify a new family of transport proteins based primarily upon sequence similarity, heterologous expression, and common function; however, definitive and rigorous proof that the OPT proteins are indeed transporters and not regulators of transport activity requires that one of the members of the family be reconstituted in a proteoliposome and assayed for peptide transport activity. Furthermore, several basic structural and functional questions were not addressed. In particular, 1) what is the nature of the energy source that is used to translocate the substrate? 2) what is the significance of the highly conserved SPY-EVR motif to the structure and/or function of Opt1p? 3) what is the topology of the transporters? and 4) what is the function of the two OPT proteins in *S. cerevisiae*? As stated earlier, searches of the EST and sequence databases have failed to identify any potential members of the OPT family in higher eukaryotes or prokaryotes, although it should be noted that these

databases certainly are not complete. This observation implies that these proteins may be unique to plants and fungi.

The observation that Isp4p could only utilize tetrapeptides as an auxotrophic supplement and only conferred sensitivity to toxic tetrapeptides, raises the possibility that this transporter has a very defined number of substrates. Is the small number of observed substrates indicative of specialized function, or were some of the substrates not hydrolyzed by the internal peptidases?

Multiplicity

Two different peptide transporters have now been cloned from *C. albicans*: the di-/tripeptide transporter *CaPTR2* and the oligopeptide transporter *OPT1*. Studies with *OPT1* disruptant strains revealed the presence of at least one more peptide transport system. These observations have led to the following questions: why is the phenomenon so redundant? Is the redundancy a function of the large number of possible substrates, is it indicative of specialized function, or does it simply underscore the importance of the phenomenon in scavenging? In an analogous manner, many prokaryotes have redundant peptide transport systems with either different regulatory schemes, specialized functions, or different substrate preferences, and, like *C. albicans*, many organisms have at least two evolutionary distinct families operating in peptide transport.

All three systems detected in *C. albicans* appear to be regulated in part by nitrogen source, implying that at least one function of these systems is in nitrogen acquisition. Furthermore, we have previously shown that *CaPTR2* is inducible by amino acids and in Part IV we suggested that the third system may be peptide inducible. These additional levels of regulation may be indicative of specialized function or, alternatively, may simply reflect the complicated nature of nitrogen catabolism. Nitrogen catabolism has not been extensively studied in *C. albicans*. However, the recent finding that genes involved in

nitrogen acquisition and metabolism are necessary for virulence in some bacterial pathogens (Klose and Mekalanos, 1997) and the recent suggestion that SAPs liberate nitrogen from the host in the form of peptides (Fallon *et al.*, 1997) will undoubtedly bring this phenomenon under closer scrutiny. The cloning of two nitrogen regulated genes, *CaPTR2* and *OPT1*, may provide the tools necessary for beginning to understand nitrogen catabolite repression (transcriptional regulation) and nitrogen catabolite inactivation (protein regulation) in *C. albicans*. Future experiments and promoter analyses of *CaPTR2* and *OPT1* may help to identify the regions and promoter sequences involved in nitrogen catabolite repression. Furthermore, as stated in Part I, regulation of peptide transport induction appears to also occur at the protein level. The identification of the two transporters CaPtr2p and Opt1p will allow us to identify the nature of this protein regulation and to determine the contribution of each of the levels of regulation to the inducible phenotype. These studies and/or findings could be further substantiated/facilitated by cloning the third peptide transport system. Creation of individual and combinatorial disruptants of all the peptide transport systems will allow the dissection of the regulatory and biochemical characteristics of each individual system.

Peptide transport and virulence

OPT1 disruptant strains exhibited only a slight decrease in virulence in our model of systemic candidiasis. However, apparent attenuation levels can be dramatically influenced by the nature of the model. Our model bypasses the stages of initial infection. If one views virulence as a developmental program, then our model circumnavigates all the early developmental requirements. In Part IV we suggested that the role of peptide transport may be to scavenge the SAP degradation products in order to acquire nitrogen. If such a model is correct, then one would predict that SAP production and peptide

transport activity are coordinately regulated. Are SAPs regulated by nitrogen source? Is SAP production and peptide transport activity inducible by a common peptide?

Whereas the ideas and questions put forward here certainly are not exhaustive, they will serve as a starting point for future endeavors into the basic regulatory, biochemical, and structural nature of peptide transport as well as into future explorations of peptide transport in different models or stages of infection.

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VITA

Mark was born on October 18, 1968 in Huntsville, Alabama to Axel and Maria Lubkowitz. At age 18 months the family moved to Signal Mountain, Tennessee where Mark spent the remainder of his youth trekking in the remote regions of the Cumberland Plateau. Mark graduated from the Baylor School for Boys in 1987 where as the captain he led the swim team to its sixth consecutive state title. His undergraduate studies were completed at Washington and Lee University in 1991 where he received a B.S. in biology and graduated *cum laude*. While attending W&L, Mark worked in the laboratory of Dr. Maryanne Simurda where he performed research on the parasite *Schistosoma mansoni*. Late in high school Mark developed a passion for kayaking that persisted through college. After graduating from college in 1991, Mark traveled throughout the world in search of bigger and better rapids before entering a Ph. D. program at the University of Tennessee. In January of 1993 Mark joined the laboratory of Dr. Jeffrey M. Becker where he has completed his graduate studies. In 1995 Mark married his long time companion Virginia Tobin Gay.