# GENETIC AND BIOCHEMICAL STUDIES ON THE UREASE ENZYME SYSTEM OF *SCHIZOSACCHAROMYCES POMBE*

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requirements for the degree of Doctor of Philosophy in
Genetics at Massey University

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12 residues of the N-terminal sequence, extending the N-terminal sequence to 18 residues. The 18 N-terminal amino acids had 55.6% identity and 83.3% similarity (exact plus conservative replacements) with the jack bean urease N-terminal sequence. The seven amino acids of T21 had 42.9% identity and 100% similarity with the urease from *Klebsiella aerogenes*. Peptide T40 (25 amino acids) had only very poor identity with other sequenced ureases.

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## ABSTRACT

Two indicator media were developed to detect urease activity in *Schizosaccharomyces pombe* colonies. These media were more sensitive than previously published media, permitted the rapid identification of urease mutants, were suitable for identifying urease positive transformants and were not affected by amino acid and nucleotide supplements.

Four genes, designated *ure1*, *ure2*, *ure3*, and *ure4*, are required for urease activity in *S. pombe*. Each of the genes was mapped to an approximate genetic location by induced haploidization and meiotic recombination: *ure1* on the left arm of chromosome III, 32 cM from *fur1* and 50 cM from *ade6*; *ure2* on the right arm of chromosome I, 69 cM from *ura2* and 100 cM from *ade4*; *ure3* on the right arm of chromosome I, 31 cM from *ade4* and 91 cM from *ura2*; *ure4* on the left arm of chromosome I, 100 cM from *lys1*.

The lithium chloride method for *S. pombe* transformation was modified to improve the transformation frequency up to 100-fold by using carrier DNA and resuspending the cells in 0.9% NaCl after transformation. Urease mutants for each of the four *ure* genes were transformed with a *S. pombe* gene bank. Three different plasmid clones, each of which specifically complemented one of the *ure1*, *ure3*, or *ure4* mutants, were isolated by complementation of the *ure*$^-$ phenotype. A gene bank clone complementing the *ure2* mutant was not found.

*S. pombe* urease was purified and characterized. The enzyme was intracellular and only one urease enzyme was detected by non-denaturing PAGE. The urease was purified 3,939-fold, with a 34% yield, by acetone precipitation, ammonium sulfate precipitation and DEAE-Sepharose ion exchange column chromatography. The native enzyme had $M_r$ = 212,000 (Sepharose CL6B-200 gel filtration). One subunit was detected, with $M_r$ =102,000 (SDS-PAGE), indicating the undissociated enzyme contains two identical subunits. The specific activity was 709 μmol urea per min/mg protein. The enzyme was stable between pH 5.0 and pH 9.0. The optimum pH range for enzyme activity was pH 7.5 - pH 8.5. The $K_m$ for urea was 1.03 mM. The sequences of the amino-terminus and three tryptic peptides of the enzyme were determined: N-terminus - Met Gln Pro Arg Glu Leu His Lys Leu Thr Leu His Gln Leu Gly Ser, peptide T21 - Phe Ile Glu Thr Asn Glu Lys, peptide T40 - Leu Tyr Ala Pro Glu Asn Ser Pro Gly Phe Val Glu Val Leu Glu Gly Glu Ile Glu Leu Leu Pro Asn Leu Pro, peptide T43 - Glu Leu His Lys Leu Thr Leu His Gln Leu Gly Ser Leu Ala. The sequence of T43 overlaps the last

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# INTRODUCTION

## 1 THE ETHYL CARBAMATE PROBLEM

### 1.1 Introduction

Ethyl carbamate (also known as urethane, ethylurethane, carbamic acid ethyl ester) is a known animal carcinogen (Nettleship *et al.*, 1943; Mirvish, 1968; IARC, 1974; McCann *et al.*, 1975; Schmähl *et al.*, 1977). In 1985 high levels of ethyl carbamate were discovered in alcoholic beverages by the Liquor Control Board of Ontario, Canada. Ethyl carbamate concentrations as high as 13,400 µg/l were found and as a result a number of sherries, ports, sakes, fruit brandies, liqueurs, bourbons and wines were removed from sale (Food Chemical News 15 September 1986). In 1986 excessive ethyl carbamate levels were also found in alcoholic beverages in the United States of America (Food Chemical News 8 September 1986).

The majority of ethyl carbamate in wine is probably formed when urea, present at the end of fermentation, reacts spontaneously with ethanol (Monteiro *et al.*, 1989; Ough *et al.*, 1988a; Ough *et al.*, 1988b). Urea is produced from the catabolism of arginine by the wine yeast and is lost to the medium during fermentation (Ough *et al.*, 1988b). Urease is an enzyme that catalyzes the hydrolysis of urea to ammonia and carbon dioxide (Schlegel and Kaltwasser, 1974). A wine yeast which had an active urease enzyme may be expected to rapidly break down urea and, therefore, produce wine with lower levels of ethyl carbamate. Wine yeasts do not have urease; however, a urease-producing wine yeast could be obtained by the transfer of urease genes from a suitable donor. The yeast *Schizosaccharomyces pombe* was considered a suitable urease gene donor so the genetics and biochemistry of *S. pombe* urease were investigated in this study.

### 1.2 Toxicology of Ethyl Carbamate

Ethyl carbamate was used for many years as a sedative and general anesthetic for animals and humans. It was even considered as an antineoplastic agent for cancer patients; however, studies revealed that it could also cause carcinogenesis when administered to healthy tissues (Mirvish, 1968).

The carcinogenic action of ethyl carbamate was first noted by Nettleship *et al.* (1943). A single dose of ethyl carbamate, administered as an intraperitoneal injection, was

sufficient to cause pulmonary tumors (adenomas) in mice. Since then, it has been shown that ethyl carbamate can induce malignant lymphomas of the thymus, hepatomas, mammary carcinomas, and hemangiomas in mice (Mirvish, 1968). Furthermore, it can also act as a carcinogen in isolated rat and hamster tissues, and in *Drosophila* (Mirvish, 1968). It can induce sister chromatid exchange in mice (Conner, 1986). Breakdown products of ethyl carbamate, in particular the epoxide of vinyl carbamate, rather than ethyl carbamate itself, are thought to be responsible for the carcinogenic action (Mirvish, 1968; Park *et al.*, 1990; Ashby, 1991; Wild, 1991). A component in wine appears to inhibit the tumorogenic action of ethyl carbamate in mice; however, it is unclear whether ethanol or some other compound is responsible for the observed inhibition (Stoewsand *et al.*, 1991; Kurata *et al.*, 1991; Altmann *et al.*, 1991).

### 1.3 Ethyl Carbamate in Wine

Ethyl carbamate has been a known trace component of wine, and other alcoholic beverages and fermented foods, for more than twenty years (Löfroth and Gejvall, 1971; Walker *et al.*, 1974, Ough, 1976a). The presence of ethyl carbamate in wine has become a matter of concern twice in recent history. Through the 1960's and early 1970's diethyl pyrocarbonate (DEPC) was widely used as an antimicrobial food additive for beverages such as fruit juices, wine and beer; however, DEPC can react with ammonia in aqueous solutions to form ethyl carbamate. In the early 1970's it was shown that DEPC addition to wines and other products was contributing to excessive levels of ethyl carbamate (Löfroth and Gejvall, 1971; Fischer, 1972), although the amount of ethyl carbamate formed was disputed (Ough, 1976b). The use of DEPC in beverages was banned (in the United States of America) in 1972. The natural occurrence of ethyl carbamate in a limited number of various foods and beverages was subsequently investigated. A number of fermented foods such as yogurt, soy sauce, olives and bread contained ethyl carbamate at concentrations of 1.2 to 3.9 µg/l or µg/kg (Ough, 1976a). The ethyl carbamate concentration in wines was 1.2 - 5.8 µg/l and in sake was 154 - 170 µg/l. No ethyl carbamate was detected in unfermented foods.

In 1985 the discovery of much higher levels of ethyl carbamate resulted in the withdrawal from sale of a number of sherries, ports, fruit brandies, liqueurs, and bourbons. The Canadian Department of Health and Welfare established the following limits for ethyl carbamate concentrations in alcoholic beverages: table wines - 30 ug/l, fortified wines (sherries and ports) - 100 ug/l, distilled spirits - 150 ug/l,

fruit brandies and liqueurs - 400 ug/l (Liquor Control Board of Ontario, 1986). An extensive study of 196 white and 57 red wines showed that 15% and 5.9%, respectively, contained ethyl carbamate at a concentration greater than the limits recommended by the Canadian government (Clegg *et al.*, 1988). Excessive ethyl carbamate in American alcoholic beverages was discovered in 1986 (Food Chemical News 8 September 1986). Guidelines were set in 1988 to limit the ethyl carbamate concentrations in imported and locally produced products (Food Chemical News 4 January 1988; Food Chemical News 1 February 1988; Food Chemical News 16 May 1988). In the guidelines it was agreed that, starting with grapes from the 1988/1989 harvests, table wines (14% alcohol or less), desert wines (over 14% alcohol) and spirits should contain less than 15 µg/l, 60 µg/l and 125 µg/l ethyl carbamate, respectively. Furthermore, commencing in 1995, no more than 1% of table or dessert wines should have ethyl carbamate at greater than 25 ug/l and 90 ug/l, respectively. The industries also promised to conduct annual surveys, continue intensive research programs and try to reduce ethyl carbamate to the lowest concentration possible (Food Chemical News 18 January 1988). The analysis of beer, white wines, red wines, fortified wines, distilled spirits, fruit brandies, and liqueurs showed that ethyl carbamate concentrations exceeding the limits were common for all categories of beverages except beer (Fünch and Lisbjerg, 1988; Food Chemical News 20 October 1986, Food Chemical News 8 September 1986).

## 1.4 Ethyl Carbamate Formation

The use of DEPC, known to cause high ethyl carbamate in wine, has been banned and need no longer be considered as a precursor of ethyl carbamate. The potential of a variety of commonly used yeast food types to form ethyl carbamate during wine fermentation has been investigated. The following yeast foods were tested: casamino acids, diammonium phosphate, glycine, urea, and yeast extract. Only urea resulted in high ethyl carbamate levels, and only when the wine was heated after fermentation. Before heat treatment no ethyl carbamate was detected (Ingledew, *et al.*, 1987). The use of urea as a yeast food in wine manufacture was banned in 1986.

The ethanolysis of various compounds naturally present in wine is another potential source of ethyl carbamate (Ough, 1976a; Baumann and Zimmerli, 1986; Ough *et al.*, 1988a). Carbamyl compounds are expected to react with ethanol to form ethyl carbamate (Ough *et al.*, 1988a; Ough, 1976a). Carbamyl compounds that are expected to be present in wine include carbamyl phosphate, urea, citrulline, allantoin, N-carbamyl α-amino acids, and N-carbamyl α-isobutyrate (Baumann and Zimmerli,

1986; Ough *et al.*, 1988a). Of these compounds, only urea and carbamyl phosphate react with ethanol to form significant amounts of ethyl carbamate (Ough *et al.*, 1988a; Baumann and Zimmerli, 1986). Citrulline can also form ethyl carbamate, but to a lesser extent. Carbamyl phosphate is more reactive than urea and rapidly forms ethyl carbamate at room temperature. Urea requires heating for maximum ethyl carbamate formation (Ough *et al.*, 1988a; Ingledew *et al.*, 1987; Baumann and Zimmerli, 1986). Although carbamyl phosphate is very reactive, only small amounts are present in yeast cells and this compound probably accounts only for the very low levels of ethyl carbamate found in freshly fermented foods and beverages (Ough *et al.*, 1988a). Citrulline can be present in wines at up to 50 mg/l (Ough, 1988); however, it reacts about eight times slower than urea (Ough *et al.*, 1988a; Ough, 1988) and so would give eight times less ethyl carbamate at any given time and temperature. Urea may be present at even higher concentrations than citrulline (Ough and Trioli, 1988). Monteiro *et al.*, (1989) observed that radioactively labeled urea added to wine became incorporated into ethyl carbamate. Ough *et al.*, (1988b) showed that urea can account for the majority of ethyl carbamate formed in wine; however, other unidentified precursors of ethyl carbamate are also present in unfermented grape juice (Tegmo-Larsson and Henick-Kling, 1990). Urea produced by the yeast during fermentation appears to be the main, if not the only, source of all detectable ethyl carbamate in sake (Kitamoto *et al.*, 1991).

The very high level of ethyl carbamate found in some stone fruit brandies appears to be formed by the reaction of hydrocyanic acid with dicarbonyl compounds and ethanol (Baumann and Zimmerli, 1986). Hydrocyanic acid is released from fruit-stones broken during the must preparation. Ethyl carbamate in grain-based spirits seems to be primarily formed from a reaction between cyanide compounds and ethanol (McGill and Morley, 1990). The cyanide compounds are formed when the cyanohydrin of isobutyraldehyde, a substance present in malted barley wort, is heated during distillation (Cook *et al.*, 1990).

## 1.5 Urea Formation in Wine

Urea can be formed in wine during fermentation (Ough, 1988; Ough *et al.*, 1988b). Urea may be produced by two catabolic processes in yeasts: purine degradation and arginine degradation (Cooper, 1982; Large, 1986). The concentration of purines in grape juice is too low to account for the observed accumulation of urea (Monteiro *et al.*, 1989); however, arginine is one of the main amino acids present in grape juice, with concentrations frequently higher than 500 mg/l (Monteiro *et al.*, 1989; Castor

1953a; Kliewer, 1970). Radioactive tracer studies have shown that arginine is degraded to urea which is released into the medium during fermentation (Monteiro *et al.*, 1989). Arginine additions to grape juice increase the final yield of ethyl carbamate (Ough *et al.*, 1988b).

Arginine transport into the cell and the arginase enzyme, which catalyzes the breakdown of arginine to ornithine and urea, are both controlled by nitrogen catabolite repression (Large, 1986; Cooper, 1982). Therefore, arginine degradation is repressed when a more readily utilized nitrogen source, such as ammonium or glutamate, is available. Ough *et al.* (1988b) observed that ethyl carbamate formation in arginine-supplemented grape juice was reduced by the addition of moderate amounts of ammonium or glutamate to the fermentation.

Urea can be lost from the cell by facilitated diffusion (Cooper, 1982). Additionally, urea can be lost from the cell due to permeabilization caused by the ethanol produced during fermentation or added during fortification (Salgueiro *et al.*, 1988; Monteiro *et al.*, 1989). Urea can also be metabolized to carbon dioxide and ammonium by the two-step urea amidolyase reaction (Whitney and Cooper, 1972). Urea breakdown in wine yeast is subject to nitrogen catabolite repression (Cooper, 1982).

The factors that determine the amount of urea at the end of fermentation are complex and poorly understood. The following account is a possible explanation for urea accumulation during wine fermentation. Grape juice contains inorganic (ammonia) and organic nitrogen sources (Amerine *et al.*, 1967; Ough, 1969). The inorganic nitrogen is the preferred nitrogen source and is rapidly used up within the first two degrees brix drop, at which time the organic nitrogen is taken up and used. The two main amino acids in wine are arginine and proline; however, only arginine is a very important nitrogen source used by yeast during vinification (Castor, 1953b). Arginine is taken into the cell when the ammonia is depleted. As the arginine concentration increases within the cell, arginase production is induced. This enzyme hydrolyzes arginine to ornithine and urea. Ornithine is converted to proline and used as a nitrogen source and for protein synthesis (Cooper, 1982). Ornithine is also used for putrescene and polyamine synthesis (Davis, 1986). Urea may be manufactured faster than it can be used, resulting in urea transport out of the cell. Eventually, urea may be taken back into the cell and converted to ammonia and carbon dioxide when other nitrogen sources have been depleted; however, fermentation may be stopped before this process can occur.

## 1.6 The Solution

The factors affecting the amount of ethyl carbamate that forms in wines are not well understood and many experiments have lacked reproducibility (Ough, 1988); however, some factors have been identified which may help reduce ethyl carbamate in wines. Ethyl carbamate appears to be gradually formed in wine and the reaction rate is greatly influenced by pH, time and temperature (Ough *et al.*, 1988a). Therefore, the storage conditions of wine may be important. Ough (1988) recommends fermenting, storing and shipping wine in cold conditions to reduce ethyl carbamate; however, Monteiro *et al.* (1989) found that incubating cells at low temperatures (4 °C) caused a greater leakage of urea from the yeast cells. Certain varieties of grapes tend to produce more ethyl carbamate (Ough, 1988). When these grapes are nitrogen starved ethyl carbamate is reduced (Ough, 1988). Some yeast strains tend to result in lower ethyl carbamate levels (Ough, 1988).

The use of grape and yeast varieties, and culture and storage conditions that promote lower ethyl carbamate levels may reduce the number of wines having high ethyl carbamate; however, these conditions are difficult to control, may affect the wine quality, and tend to produce widely varying results (Ough, 1988). Two alternative strategies for ensuring low ethyl carbamate have been suggested: (1) minimize urea production by using an arginase-deficient yeast strain, and (2) use urease to remove urea.

An arginase-deficient strain of *S. cerevisiae* has been used to make sake (Kitamoto *et al.*, 1991). This strain resulted in no detectable urea or ethyl carbamate in the final product, whereas sake prepared with the usual yeast contained urea and ethyl carbamate. Kitamoto *et al.* (1991) suggested that an arginase-deficient yeast may reduce ethyl carbamate in wine; however, arginine is one of the major sources of nitrogen for yeasts during vinification (Castor, 1953b) and may account for up to 50% of the total nitrogen in grape juice (Kliewer, 1969, 1970). Much of the arginine is used for growth, for example, in a must containing 1,500 mg/l arginine, and generally low amounts of other amino acids and ammonia, about 1,100 mg/l of arginine was metabolized (Ough, 1988). The use of an arginase-deficient yeast in wine manufacture may require the addition of an exogenous nitrogen source to support yeast growth. In sake, arginine is not the major amino acid and little arginine is metabolized (Kitamoto *et al.*, 1991). Many other amino acids are present at high levels and could act as alternative nitrogen sources.

Urea has been successfully removed from sake by the addition of an acid stable urease (Yoshizawa and Takahashi, 1988). Heat killed cells of *Lactobacillus fermentum*, which has a urease that is stable and active at low pH, were added to sake. The *L. fermentum* acid-stable urease was tested in a similar way on wine (Ough and Trioli, 1988). The sensory quality of the wine was not adversely affected; however, the urease was not very effective and large amounts were required (Ough and Trioli, 1988).

The acid stable urease is inhibited by fluoride ions and malic acid which are present in wine and the efficiency varies greatly from wine to wine (Ough and Trioli, 1988; Trioli and Ough, 1989; Famuyiwa and Ough, 1991). A more reliable way to reduce urea in wine, without adversely affecting the growth of the yeast, may be to use a wine yeast which produces intracellular urease. Urea may then be broken down rapidly, within the cell, as it was formed from the degradation of arginine. Such a wine yeast could be obtained by the transfer of the urease genes from a suitable donor.

A suitable donor must have several properties before it can be considered as an appropriate and convenient source for the urease genes. The organism should be non-bacterial because wine makers would be reluctant to use a yeast containing recombinant DNA of bacterial origin. Furthermore, most urease-positive bacteria studied are pathogens or opportunist pathogens of humans and it would be difficult to obtain approval to use a yeast containing DNA from these organisms. A yeast donor would be more acceptable. A genetically engineered bakers yeast, containing rearranged yeast DNA, has been approved for food use in Europe (Hodgson, 1990). The donor organism should be amenable to genetic and biochemical analysis and gene manipulation so that the urease genes could be readily identified, studied and isolated. The subsequent manipulation of the isolated urease genes required to obtain expression in *S. cerevisiae* may be simplified if the urease in the donor organism was produced intracellularly and was not controlled by nitrogen catabolite repression. An intracellular activity is essential because the urease is not expected to function efficiently at the low pH of wine. Also, grape must has high nitrogen levels and so genes controlled by nitrogen catabolite repression may not be very active (see above). It may also improve expression of the urease in *S. cerevisiae* if the donor genes shared codon biases with *S. cerevisiae*. The yeast *Schizosaccharomyces pombe* was selected as a potential urease donor. *S. pombe* (Snow and Gallander, 1979) and the closely related *Schizosaccharomyces malidevorans* 11 (Thornton and Rodriguez, 1992) have been added to wines to assist deacidification. Therefore, little resistance to using a wine yeast containing genes from *S. pombe* is expected. *S. pombe* has an active (Kocková-

Kratochvilová *et al.*, 1985; Booth and Vishniac, 1987) urease that is constitutively expressed (Fluri and Kinghorn, 1985a). Some aspects of *S. pombe* gene expression are conserved between *S. pombe* and *S. cerevisiae*, although there are also many differences, and several *S. pombe* genes have been shown to complement *S. cerevisiae* mutants (Russell and Hall, 1983; Russell, 1989). The two organisms also share many codon biases (Sharp *et al.*, 1988). The genetic manipulation of *S. pombe* is well established (Russell, 1989; Moreno *et al.*, 1991) and vectors suitable for the construction of recombinant industrial yeasts free of bacterial sequences have been described and successfully used on industrial strains of *Saccharomyces* (Casey *et al.*, 1988; Xiao and Rank, 1989). *S. pombe* seemed to be a suitable urease gene donor. Therefore, the genetics and biochemistry of *S. pombe* urease were further characterized in the present study.

## 2 *SCHIZOSACCHAROMYCES POMBE*

The genetics and physiology of *S. pombe* have been the subject of several recent reviews (Mitchison, 1970; Leupold, 1970; Gutz *et al.*, 1974; Egel *et al.*, 1980; Nasim *et al.*, 1989; Moreno *et al.*, 1991).

*Schizosaccharomyces pombe* is a unicellular eukaryote with a predominantly haploid life-cycle. *S. pombe* cells are cylinders with rounded ends. The diameter of haploid cells is about 3.5 µm and changes very little throughout the cell cycle. The length increases from about 7 µm, for the newly formed cell, to about 12 - 15 µm at the end of the cell cycle. Thus, growth is primarily by extension alone. Diploid cells are both longer and wider than haploid cells. Growth conditions can greatly influence cell size and shape. Cells divide by transverse binary fission. The length of the cell cycle varies greatly with culture conditions, but is about 2.5 hours in rich medium at 30 °C. Nuclear division begins at 0.75 of the cell cycle and the medial septum is formed at 0.85. The G1 phase of the cell cycle is very short. DNA replication occurs early and is completed in 10 - 15 minutes. The G2 phase occupies about 75% of the cell cycle. Cultures in stationary phase accumulate in G1 or G2, depending on whether nitrogen or carbon, respectively, is limiting. Cells conjugate in the G1 stage when approaching stationary phase, therefore, nitrogen limitation promotes conjugation. Glucose is limiting in the commonly used yeast extract (YE) and minimal (MM) media, and cells accumulate mostly in G2, although some conjugation can occur.

The sexual cycle is initiated through the conjugation of G1 cells of opposite mating types. When cells of opposite mating type are grown together, a strong sexual

agglutination occurs in response to nutrient limitation at the end of vegetative growth, followed by a pairwise copulation of cells to form the zygote. The zygote immediately proceeds through meiosis and sporulation to generate four haploid ascospores within the cell wall of the original zygote (zygotic ascus). Thus, the diploid phase is normally confined to the zygote. The spores germinate with high efficiency to form vegetative haploid cells. Under appropriate conditions, and with low frequency, a zygote may enter the mitotic cycle to produce vegetatively dividing diploid cells. The response of diploid cells to nutrient starvation depends on the mating type configuration. Diploids with heterozygous $h^+/h^-$ mating type (see below) undergo two meiotic divisions, without prior conjugation, to form four haploid spores in an ascus (azygotic ascus) which is morphologically distinct from the zygotic ascus. Stable diploid cells can arise due to mitotic recombination between the mating type locus and the centromere, to generate homozygous $h^+/h^+$ or $h^-/h^-$ cells. These cells cannot sporulate but may mate with diploid (or haploid) cells of opposite mating type. Nuclear fusion occurs and a tetraploid (or triploid) meiosis follows to generate four diploid spores in a zygotic ascus. Homothallic diploid strains with an $h^{90}/h^{90}$ configuration can sporulate (azygotic ascus with haploid spores) or mate with cells of all other mating types and generate asci, as above. Under conditions where conjugation is inefficient, nuclear fusion may not occur. Instead, both nuclei may undergo meiosis separately (twin meiosis) and generate a six or eight spored ascus. Diploid cells can also arise as mitotic segregants generated by endomitosis. These cells are homozygous for all genes, including the mating type, and cannot sporulate, but may mate with cells of opposite mating type as described above. All haploid cultures contain a few diploid cells generated by endomitosis. Both haploid and diploid strains are used for genetic, physiological and biochemical studies of *S. pombe*; however, diploid cells are generally less viable than haploid cells. Therefore, unless a diploid strain is specifically required, haploid strains are always used.

*S. pombe* has two mating types, $h^+$ and $h^-$. Strains used for most purposes are heterothallic $h^{+N}$ (N = normal) or $h^{-S}$ (S = stable) and the designation of these strains is usually shortened to $h^+$ and $h^-$. Heterothallic strains of opposite mating type can mate with each other, whereas the homothallic strain $h^{90}$ is self fertile, as well as able to mate with either heterothallic type. Homothallic strains are able to switch their mating type between $h^+$ and $h^-$ every few cell divisions. Any single cell is committed to one or the other mating type, but sister cells are nearly independent of each other with respect to mating type. Thus, any population of $h^{90}$ cells contains both $h^-$ and $h^+$ types which can mate and sporulate. At low frequency $h^{+N}$ strains switch to $h^{90}$. $h^{-S}$ strains are stable and do not switch to $h^+$ or $h^{90}$. Several other alleles of $h^+$

and $h^-$ have also been described: $h^{+R}$ (R = recombinant), $h^{-U}$ (U = unstable), and $h^{+S}$ (S = stable). These alleles, with the exception of $h^{+S}$, are generally not used unless specifically required. The $h^{+S}$ allele has recently been constructed by recombinant DNA techniques and is increasingly being used because it does not revert to $h^{90}$.

The progenitors of all strains of *S. pombe* currently used were isolated by Leupold (1950) from a culture of *S. pombe* Lindner str. *liquefaciens* Osterwalder which was obtained from the "Centraalbureau voor Schimmelcultures" in Delft, the Netherlands. The original wild type strains, isolated by Leupold, with $h^{90}$, $h^{+N}$ and $h^{-S}$ mating type have strain designations 968, 975, and 972, respectively. All other strains currently used were derived from these strains and, therefore, have a high degree of genetic homogeneity.

Cytological, genetic and electrophoretic data show that *S. pombe* has three chromosomes. The first extensive genetic map, showing the locations of 118 genes, was published by Kohli *et al.* (1977). This map is periodically revised and expanded. Currently 162 genes have been precisely located on the three chromosomes (Munz *et al.*, 1989). A list of 460 genes and the corresponding gene products or mutants phenotypes has been described Kohli (1987). The total length of the genetic map is 2,100 cM. This length is an underestimate, due to incomplete mapping of the chromosome. Interference between crossover events in *S. pombe* meiosis is largely absent. The DNA content per haploid spore is about $1.5 \times 10^{-14}$g, similar to the DNA content of *S. cerevisiae* and about three to four times the DNA content of *E. coli*. The length of the *S. pombe* genome is about 14,000 kilobases. *S. pombe* chromosomes have been separated by pulsed field electrophoresis and a *Not*I restriction map of the chromosomes has been published (Fan *et al.*, 1989).

Details of genetic, molecular biology and biochemistry techniques for *S. pombe* have been recently published (Moreno *et al.*, 1991).

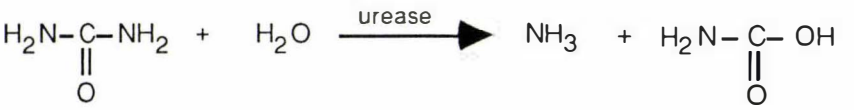
## 3 UREA AND UREASE

### 3.1 Introduction

Urea is formed from the degradation of purines and other nitrogenous compounds, such as amino acids (reviewed by Mobley and Hausinger, 1989). Enormous quantities of urea are released into the environment, for example, in the urine of mammals. Still more urea is formed from the degradation of uric acid which is excreted by birds,

reptiles and many insects; however, urea is rapidly broken down in the environment through the action of urease.

Urease (urea amidohydrolase; EC 3.5.1.5) catalyzes the hydrolysis of urea to yield ammonia and carbamate (Andrews *et al.*, 1984).




Carbamate spontaneously hydrolyzes to carbonic acid and a second molecule of ammonia.


In biological systems the ammonia becomes protonated, resulting in a pH increase, and the carbonic acid proton dissociates.



The first enzyme ever crystallized was the urease from jack bean (*Canavalia ensiformis*) (Sumner, 1926) and this enzyme remains the best characterized urease. Urease occurs in plants, algae, invertebrates, yeasts, filamentous fungi, and bacteria, including archaebacteria; however, only certain genera or species within each division have urease activity (Mobley and Hausinger, 1989). Urease activity is often a useful criterion for taxonomic assignment of bacteria and yeasts (Christensen, 1946; Seeliger, 1956; Sen and Komagata, 1979; Booth and Vishniac, 1987). Urease is important in the pathogenesis of animals and humans, in the removal of urea from the environment and in the ruminant metabolism. The urease activity of a number of pathogenic bacteria has been directly associated with the formation of infection stones and also contributes to a number of other pathogenic conditions of the urinary and gastro-intestinal systems (Mobley and Hausinger, 1989). Some of the more commonly involved organisms include *Proteus mirabilis*, *Providencia stuartii*, *Klebsiella* species, *Morganella* (*Proteus*) *morganii*, *Ureaplasma urealyticum*, and *Helicobacter* (*Campylobacter*) *pylori*. Microbial ureases are also important in the nitrogen metabolism of ruminants (Mobley and Hausinger, 1989). Ammonia, released from the hydrolysis of urea, is an important nitrogen source for ruminal bacteria. The ruminal bacteria are, in turn, an important nutrient for the ruminant. Urea is

widespread in the environment. Bacteria, yeast, filamentous fungi and algae are all important sources of environmental urease activity. The ammonia liberated by urease action can be assimilated by plants and soil microbes.

Very high concentrations of urease are found in some cells, for example, *Bacillus pasteurii*, *Aspergillus tamarii*, and the seeds of some *Leguminosae* (Mobley and Hausinger, 1989). It has been suggested that the prime function of urease in these organisms is as a storage protein (Zawada and Sutcliffe, 1981). The breakdown of urease would provide amino acids and ammonia necessary for growth.

Urease activity is subject to a variety of regulatory mechanisms. The synthesis of urease in many bacteria appears to be repressed in the presence of a preferred nitrogen source by the nitrogen regulatory system. In other bacteria urease synthesis may be directly induced by the presence of urea. Both nitrogen repression and urea induction control urease expression in some bacteria. A third class of ureases is expressed constitutively and is unaffected by the nitrogen source or the presence of urea. (for review see Mobley and Hausinger, 1989). Urease activity in *Aspergillus nidulans* is not inducible but is controlled by nitrogen repression (Mackay and Pateman, 1982), unlike the urease of *Neurospora crassa*, which appears to be neither induced nor repressed (Haysman and Howe, 1971). Urea transport systems in bacteria (Jahns *et al.*, 1988), filamentous fungi (Pateman *et al.*, 1982) and yeasts (Cooper and Sumrada, 1975) may also be controlled by nitrogen repression.

Urease is probably a cytoplasmic enzyme in yeasts, most bacteria and jack bean (Mobley and Hausinger, 1989). Urease from *Helicobacter pylori* is probably present on the cell surface (Bode *et al.*, 1989; Hawtin *et al.*, 1990; Dunn *et al.*, 1991).

Cell membranes are not generally freely permeable to urea (Mobley and Hausinger, 1989). Rosenstein *et al.*, (1981) observed similar levels of urease activity in whole-cell and membrane disrupted suspensions of *Proteus* species and concluded that the cell membrane did not present a permeability barrier to urea; however, urea transport systems have been observed in other bacteria, algae and fungi. For example, evidence for an energy-dependent urea permease was found for the bacteria *Alcaligenes eutrophus*, *Klebsiella pneumoniae*, *Peudomonas aeruginosa* and *Providencia rettgeri* (Jahns *et al.*, 1988). No urea transport system was observed in *Proteus vulgaris* and *Bacillus pasteurii* (Jahns *et al.*, 1988). The cyanobacteria *Anabaena doliolum* and *Anacystis nidulans* (Rai and Singh, 1987), the diatom *Phaeodactylum* (Rees and Syrett, 1979) and the algae *Chlorella fuesca* (Syrett and Bekheet, 1977) have energy

dependent urea transport systems. The *Aspergillus nidulans* urea active transport system, with a $K_m$ of 30 μM urea, is capable of concentrating urea 50-fold over the medium levels (Pateman *et al.*, 1982). In addition, *A. nidulans* may also have an energy-independent passive or facilitated diffusion system for urea transport (Pateman *et al.*, 1982). *Saccharomyces cerevisiae* also has two urea transport systems, an energy-dependent, inducible system with a $K_m$ of 14 μM urea and a constitutively produced facilitated diffusion system which is active at higher (0.5 mM) urea concentrations (Cooper and Sumrada, 1975). Similarly, *Chlorella* also has two urea transport systems (Syrett and Bekheet, 1977). The urea active transport systems for the bacteria, cyanobacteria, fungi, diatom and yeast described above are subject to nitrogen repression and, therefore, are expressed at higher levels under conditions of nitrogen limitation.

The urease enzymes from many bacteria (for review see Mobley and Hausinger, 1989; Kakimoto *et al.*, 1989; Kakimoto *et al.*, 1990; Hu *et al.*, 1990; Hu and Mobley, 1990; Dunn *et al.*, 1990; Evans *et al.*, 1991; Thirkell *et al.*, 1989) and one filamentous fungus, *Aspergillus nidulans* (Creaser and Porter, 1985) have been purified and characterized. Ureases from many other sources have been partially purified and/or characterized, including the urease from two other fungi, *Ustilago violacea* (Baird and Garber, 1981), and *Aspergillus tamarii* (Zawada and Sutcliffe, 1981). Early purification protocols involved a series of fractionation steps using precipitants such as ammonium sulfate, calcium phosphate, acetone, and chloroform (Sumner, 1926; Larson and Kallio, 1954; Magaña-Plaza *et al.*, 1971). More recent procedures have included various chromotographic techniques, often in addition to precipitations. Anion-exchange and hydrophobic chromatography have been very useful because ureases are negatively charged at neutral pH and more hydrophobic than many contaminant proteins. Therefore, they bind more tightly to anion-exchange and hydrophobic resins (Mobley and Hausinger, 1989).

Affinity chromatography has been used to purify urease from a number of sources, with various degrees of success. Urease binds specifically to long chain hydroxamic acids (Kobashi and Hase, 1966; Hase and Kobashi, 1967; Andrews *et al.*, 1984). Hydroxyurea resembles the functional ligand group of hydroxamic acids (Kobashi and Hase, 1966), as well as being a competitive inhibitor of urease itself (Andrews *et al.*, 1984), and for this reason it has been used as a ligand for affinity purification of urease (Wong and Shobe, 1974; Shobe and Brosseau, 1974). Hydroxyurea-substituted affinity resins have been very useful for the purification of urease from some organisms (*Morganella morganii* and jack bean, Wong and Shobe, 1974;

*Brevibacterium ammoniagenes*, Nakano *et al.*, 1984; *Lactobacillus reuteri*, Kakimoto *et al.*, 1989; *Lactobacillus fermentum*, Kakimoto *et al.*, 1990), of some use with other organisms (*Aspergillus nidulans*, Creaser and Porter, 1985; *Ureaplasma urealyticum*, Saada and Kahane, 1988) and of very little use for the purification of urease from bovine rumen (Mahadevan *et al.*, 1977) or *Bacillus pasteurii* (Christians and Kaltwasser, 1986). A urea-substituted affinity resin was successfully used to purify jack bean urease (Mendes *et al.*, 1988). An affinity resin substituted with anti-urease antibodies was very useful for the purification of urease from *Ureaplasma urealyticum* (Precious *et al.*, 1987). Although jack bean urease was the first enzyme ever crystallized (Sumner, 1926), only one other urease has since been crystallized, and then only low levels of activity were recovered (*Providencia rettgeri*, Magaña-Plaza *et al.*, 1971).

### 3.2 Urease Structural Properties

Bacterial ureases generally have two or three different subunits. The three-subunit urease enzymes have one large ($\alpha$) and two smaller ($\beta$, $\gamma$) subunits with sizes 61,000 to 73,000, 8,000 to 17,000 and 6,000 to 11,000, respectively (*Klebsiella aerogenes*, *Selenomonas ruminantium*, *Sporosarcina ureae*, Todd and Hausinger, 1987; *Ureaplasma urealyticum*, Thirkell *et al.*, 1989, Blanchard, 1990; *Lactobacillus reuteri*, Kakimoto *et al.*, 1989; *Lactobacillus fermentum*, Kakimoto *et al.*, 1990; *Proteus mirabilis*, Jones and Mobley, 1988; *Providencia stuartii*, Mulrooney *et al.*, 1988; *Morganella morganii*, Hu *et al.*, 1990). Two subunits of sizes about 62,000 to 66,000 and 26,500 to 30,000 have been identified for *Helicobacter pylori* (Labigne *et al.*, 1991; Hu and Mobley, 1990; Hawtin *et al.*, 1990; Dunn *et al.*, 1990) and *Helicobacter mustelae* (Costas *et al.*, 1991; Dunn *et al.*, 1991). *E. coli* (Collins and Falkow, 1988) and *Staphylococcus saprophyticus* (Gatermann and Marre, 1989; Gatermann *et al.*, 1989) ureases appear to have one large subunit, $M_r$ 67,000 - 70,000, and perhaps a smaller subunit $M_r$ 47,000 - 48,000. A single subunit has been reported for some bacteria, for example, *Bacillus pasteurii* (Christians and Kaltwasser, 1986) and *Brevibacterium ammoniagenes* (Nakano *et al.*, 1984); however, it is possible that additional small subunits were not identified because they would migrate with the dye front on SDS gels containing less than 10 % acrylamide. The enzymes from *Ureaplasma urealyticum* (Saada and Kahane, 1988) and *Selenomonas ruminantium* (Hausinger, 1986) were originally thought to be single-subunit ureases until they were examined on higher percent acrylamide gels (Thirkell *et al.*, 1989; Todd and Hausinger, 1987). Only single-subunit ureases have so far been observed for eukaryotes. The subunit size varies greatly between these ureases,

for example, $M_r$ = 90,770 for jack bean (by sequencing, Takishima *et al.*, 1988), $M_r$ = 40,000 for *Aspergillus nidulans* (SDS-PAGE gradient gels 2.5 - 27% acrylamide, Creaser and Porter, 1985), $M_r$ = 80,000 for *Ustilago violacea* (SDS-PAGE 5 % acrylamide gel, Baird and Garber, 1981). The data for *U. violacea* must be treated with caution because the polyacrylamide gels used to determine the molecular weight were low percent acrylamide and small subunits may have been missed.

The native molecular weights of ureases have been typically determined by gel filtration. Aggregation of enzymes and interaction with the filtration matrix have often given inaccurately high values for native molecular weight, especially for the earlier determinations. These effects can be reduced by including 0.1 M NaCl in the elution buffer (Jones and Mobley, 1988; Mobley and Hausinger, 1989). Most bacterial ureases have a native $M_r$ between 200,000 to 250,000 (*Brevibacterium ammoniagenes*, *Providencia stuartii*, *Proteus mirabilis*, *Lactobacillus reuteri*, *Lactobacillus fermentum*, *Ureaplasma urealyticum*, *Bacillus pasteurii*, *Klebsiella aerogenes*, (for details see above references), and *Arthrobacter oxydans* (Schneider and Kaltwasser, 1984). *Helicobacter* species have consistently been shown to have higher molecular weight urease. Recent estimates are 380,000 (Dunn *et al.*, 1990), 510,000 (Hawtin *et al.*, 1990), 535,000 (Dunn *et al.*, 1991), 550,000 (Hu and Mobley, 1990), 600,000 (Evans *et al*, 1991) and 625,000 (Mobley *et al.*, 1988). *H. pylori* urease subunits appear to aggregate to form disc shaped structures which may stack to form pairs or larger four-disc stacks (Austin *et al.*, 1991). These structures may explain the discrepancies observed in the molecular weight determinations. *Morganella morganii* (Hu *et al.*, 1990; $M_r$ = 590,000 ) and *Staphylococcus saprophyticus* (Gatermann *et al.*, 1989; $M_r$ = 420,000) urease are also large.

The subunit stoichiometry has generally been calculated from the relative intensities of the corresponding coomassie blue stained bands on polyacrylamide gels and the native molecular weight, therefore, some inaccuracies may result (Mobley and Hausinger, 1989). The subunit stoichiometry of urease from *Providencia stuartii* (Mulrooney *et al.*, 1988), *Klebsiella aerogenes* (Todd and Hausinger, 1987), and *Proteus mirabilis* (Jones and Mobley, 1988) is probably best represented by $(\alpha_1\beta_2\gamma_2)_2$. The subunits of *Lactobacillus* urease (Kakimoto *et al.*, 1989; Kakimoto *et al.*, 1990) probably have a $(\alpha_1\beta_2\gamma_1)_2$ stoichiometry. *Ureaplasma urealyticum* subunits appear to form a $\alpha_2\beta_2\gamma_2$ hexamer (Thirkell *et al.*, 1989) and the two subunits of *Helicobacter* urease form a $(\alpha_1\beta_1)_6$ enzyme (Hu and Mobley, 1990; Dunn *et al.*, 1991). Jack bean urease is a homopolymeric hexamer with native $M_r$ 590,000

(Andrews *et al.*, 1984). *Aspergillus nidulans* is a hexamer with native $M_r$ 240,000 (Creaser and Porter, 1985). The native molecular weight for *Ustilago violacea* urease is unknown.

Nickel is probably an essential component of urease. No active urease enzyme has been identified that does not contain nickel. A mechanism for urease activity that involves two nickel ions at the active site has been proposed and many urease inhibitors bind to nickel ions (Andrews *et al.*, 1984). In contrast, the adenosine triphosphate- and biotin-requiring urea amido-lyase system used by many yeasts and algae to degrade urea does not appear to require nickel (Roon and Levenberg, 1968; Whitney and Cooper, 1972; Hausinger, 1987).

Jack bean urease contains two nickel ions per $M_r$ 90,770 subunit. The nickel is tightly bound (Andrews *et al.*, 1984) and may be ligated by histidine residues (Hasnain and Piggott, 1983). A domain containing the urease active site has eight histidine and one cysteine residues that are conserved between jack bean and bacteria (Labigne *et al.*, 1991; Takishima *et al.*, 1988). At low pH, in the presence of EDTA, the nickel ion may be released and the resulting loss of activity is proportional to the amount of nickel removed (Andrews *et al.*, 1984). A similar loss of nickel has been observed for *Arthrobacter oxydans* (Schneider and Kaltwasser, 1984) and many other ureases are inhibited at low pH (Todd and Hausinger, 1989). Nickel has been quantified in a few bacterial ureases. The ureases from *Klebsiella aerogenes* (Todd and Hausinger, 1987) and *Providencia stuartii* (Mulrooney *et al.*, 1988) contain two nickel ions per $\alpha_1\beta_2\gamma_2$ structure (four nickel ions per native enzyme molecule) and it has been shown that one $\alpha_1\beta_2\gamma_2$ unit corresponds to a catalytic unit (Mobley and Hausinger, 1989). Therefore, two nickel ions may also be involved at the active site of bacterial ureases (Mobley and Hausinger, 1989).

Nickel has not been directly demonstrated in fungal ureases, although there is indirect evidence for its presence. Hydroxamic acids competitively inhibit urease, probably by binding to the nickel ions at the active site (Andrews *et al.*, 1984). Ureases from *Aspergillus* species (Zawada and Sutcliffe, 1981; Creaser and Porter, 1985) and *Rhodotorula pilimanae* (Akers, 1981) are inhibited by hydroxamic acids. Growth in the absence of nickel, or in the presence of nickel chelators, can inhibit urease activity. For example, the growth of *Arthrobacter oxydans* and other bacteria on urea, and the urease activity of cell-free extracts, were inhibited by including a strong chelator of nickel (EDTA) in the growth medium (Schneider and Kaltwasser, 1984). Urease activity and growth on urea were restored by the addition of nickel. Similar

observations have been noted for fungal ureases. Nickel is required for urea-supported growth of a *Penicillium* species (Hausinger, 1987). Growth in the presence of EDTA can inhibit active urease production by yeasts and activity can be restored by the addition of nickel (Booth and Vishniac, 1987). Histidine can chelate nickel and also inhibits urease activity in *Proteus mirabilis* (Rando *et al.*, 1990). Inhibition is reversed by the addition of nickel. Inhibition of urease activity by histidine has been observed for the filamentous fungi *Aspergillus tamarii* (Zawada and Sutcliffe, 1981) and *Aspergillus nidulans* (Mackay and Pateman, 1982). Mackay and Pateman offer no explanation for the apparent inhibition. Zawada and Sutcliffe suggest that histidine represses urease synthesis, although this was not specifically tested. An alternative explanation is that histidine chelated available nickel so that inactive urease was synthesized. The amount of nickel present in unsupplemented media can be limiting (Rando *et al.*, 1990). Histidine also inhibits the production of active nitrate reductase in *Neurospora crassa* (Premakumar *et al.*, 1979). Nitrate reductase is a flavoprotein containing both molybdenum and cytochrome b. It seems possible that histidine chelated essential metal ions so that inactive nitrate reductase apoenzyme was produced.

The mechanism of insertion of the nickel cofactor into urease is unknown; however, a nickel transport system and other cellular functions appear to be required for active urease production. Synthesis of inactive urease apoenzyme, lacking a nickel component, has been demonstrated for plants (Winkler *et al.*, 1983), algae (Rees and Bekheet, 1982), bacteria, (Mulrooney *et al.*, 1989; Lee *et al.*, 1990; Rando *et al.*, 1990), cyanobacteria (Mackerras and Smith, 1986) and purple sulfur bacteria (Bast, 1988) grown in nickel deficient media. Furthermore, the apourease could be activated by incubating whole cells in the presence of nickel, even in the absence of *de novo* protein synthesis (see above references). This process was shown to be energy dependent (Lee *et al.*, 1990). Therefore, a cellular factor appears to be necessary for nickel incorporation into urease (Mulrooney *et al.*, 1989; Rando *et al.*, 1990; Lee *et al.*, 1990). High affinity energy-dependent nickel-specific transport systems, have been described for bacteria, yeast and filamentous fungi (Hausinger, 1987; Jahns *et al.*, 1988). Mutation of the transport system can lead to a requirement for a higher nickel concentration for urease activity and may affect more than one nickel-requiring enzyme, for example, urease and hydrogenase (Eberz *et al.*, 1989). The gene for a nickel-specific transport system has been cloned and sequenced (Eitinger and Friedrich, 1991).

### 3.3 Urease Kinetic Characteristics

The kinetic characteristics for ureases from various sources have been determined under a variety of conditions of pH, temperature, and buffer systems, therefore, kinetic comparisons between different ureases must be made with caution. The $K_m$ for urea of bacterial ureases range from 0.1 mM to greater than 100 mM (reviewed by Mobley and Hausinger, 1989; Gatermann *et al.*, 1989; Hu and Mobley, 1990; Dunn *et al.*, 1990; Dunn *et al.*, 1991; Evans *et al.*, 1991; Hu *et al.*, 1990; Kakimoto *et al.*, 1989, 1990). The $K_m$ may reflect the environmental urea concentration for some organisms, for example, *Helicobacter* (*Campylobacter*) *pylori* ($K_m$ 0.17 - 0.8 mM) colonizes the gastric mucosa and must scavenge urea from serum. The concentration of urea in blood is 1.7 to 3.4 mM, therefore, a low $K_m$ is necessary for efficient utilization of urea by this organism (Mobley *et al.*, 1988; Hu and Mobley, 1990; Evans *et al.*, 1991). Urease from *Proteus mirabilis* or *Providencia stuartii*, two organisms which colonize the urinary tract, are 13 mM (Breitenbach and Hausinger, 1988) and 9.3 mM (Mulrooney *et al.*, 1988), respectively. These enzymes would be saturated at the high (400 mM) urea concentrations found in urine (Mobley *et al.*, 1988). The $K_m$ values for the eukaryote ureases: jack bean (2.9 mM, Andrews *et al.*, 1984), *Aspergillus nidulans* (1.33 mM, Creaser and Porter, 1985) and *Ustilago violacea* (2.8 mM, Baird and Garber, 1981) are all quite similar. The pH optimum for bacterial ureases is generally in the range pH 7.0 to 8.0 (Mobley and Hausinger, 1989), although there are some ureases with optimum activity at more extremes of pH, for example, pH 2 for urease from *Lactobacillus* species (Kakimoto *et al.*, 1989, 1990) and pH 8.7 for *Spirulina maxima* (Mobley and Hausinger, 1989). Jack bean urease has an optimum pH range of pH 7.0 to 7.5 (Andrews *et al.*, 1984). The pH optimum of *Aspergillus* urease is between pH 8.2 and 8.7 (Zawada and Sutcliffe, 1981; Creaser and Porter, 1985). The effect of pH on enzyme stability has been determined for some ureases. Irreversible inactivation below pH 4 to 5 was observed for urease from jack bean (Andrews *et al.*, 1984), *Klebsiella aerogenes* (Todd and Hausinger, 1987), *Bacillus pasteurii* (Larson and Kallio, 1954) and *Arthrobacter oxydans* (Schneider and Kaltwasser, 1984). A high pH inactivation, above pH 10, has been observed for *K. aerogenes* (Todd and Hausinger, 1987), *Proteus mirabilis* (Breitenbach and Hausinger, 1988) and *Brevibacterium ammoniagenes* (Nakano *et al.*, 1984). These last two ureases were inactivated below pH 7.0.

Other kinetic and structural aspects of microbial ureases have been reviewed by Mobley and Hausinger (1989).

3.4 Urease Genes

The urease genes from several bacteria have been cloned: *Ureaplasma urealyticum* (Blanchard and Barile, 1989; Blanchard, 1990), *Morganella morganii* (Hu *et al.*, 1990), *Providencia stuartii* (Mobley *et al.*, 1986; Mulrooney *et al.*, 1988), *Helicobacter pylori* (Labigne *et al.*, 1991), *Klebsiella aerogenes* (Lee *et al.*, 1990; Mulrooney and Hausinger, 1990), *Escherichia coli* (Collins and Falkow, 1988), *Proteus mirabilis* (Walz *et al.*, 1988; Jones and Mobley, 1988; Jones and Mobley, 1989), and *Staphylococcus saprophyticus* (Gatermann and Marre, 1989; Gatermann *et al.*, 1989). Comparison of the predicted amino acid sequence of the cloned genes and partial amino acid sequence of other purified ureases with the amino acid sequence of jack bean urease (Takishima *et al.*, 1988) reveals significant identity. For example, there are many conserved residues between a) the 11 kilodalton polypeptide of *Proteus mirabilis* urease and the N-terminal sequence of jack bean urease, b) the 12.2 kilodalton polypeptide of *Proteus mirabilis* urease and internal sequences of jack bean urease, and c) the 61 kilodalton polypeptide of *Proteus mirabilis* urease and the C-terminal residues of jack bean urease. Overall 58% exact matches and 73% exact plus conservative replacements were observed (Jones and Mobley, 1989). Alignments have been determined for *Ureaplasma urealyticum* (Blanchard, 1990), *Morganella morganii* (Hu *et al.*, 1990), *Klebsiella aerogenes* (Mulrooney and Hausinger, 1990) and *Helicobacter pylori* (Hu and Mobley, 1990; Evans *et al.*, 1991; Labigne *et al.*, 1991) ureases, with similar results. The *M. morganii* urease data used for sequence comparison with was based on 10 (6 kilodalton subunit) or 15 (63 kilodalton subunit) residues of N-terminal amino acid sequence. No homology could be inferred between the 15 kilodalton *M. morganii* urease subunit and jack bean or *P. mirabilis* urease, but was apparent with the two other subunits. *H. pylori* urease has two subunits. Overall these subunits have a high degree of sequence identity with the three *P. mirabilis* (56% exact matches) and the single jack bean (55.5% exact matches) urease subunits. Evolutionary relationships between these ureases have been suggested (Jones and Mobley, 1989; Labigne *et al.*, 1991; Hu *et al.*, 1990; Hu and Mobley, 1990).

Six genes have been identified which are associated with urease activity in *Staphylococcus saprophyticus* (Gatermann and Marre, 1989), *Providencia stuartii* (Mulrooney *et al.*, 1988), *Klebsiella aerogenes* (Lee *et al.*, 1990; Mulrooney and Hausinger, 1990) and *Proteus mirabilis* (Walz *et al.*, 1988); at least three more than are required to encode the urease enzyme subunits. *Helicobacter pylori* probably requires at least four genes for activity (Labigne *et al.*, 1991), two more than needed

to encode the urease subunits. Similarly, four genes are required for *Aspergillus nidulans* (Mackay and Pateman, 1982) and *Neurospora crassa* (Haysman and Howe, 1971; Benson and Howe, 1978) urease activity. The four *A. nidulans* genes code for the single subunit urease, a urease transport protein, a product involved in the synthesis or incorporation of the nickel cofactor and a product of unknown function (Mackay and Pateman, 1982). If *N. crassa* also has a single subunit urease, then it too has several genes contributing to other aspects of urease function. Furthermore, two urease genes of soybean are known to be involved in urease maturation (Meyer-Bothling *et al.*, 1987). The accessory genes required for urease activity in *K. aerogenes* are necessary for incorporation of the nickel cofacter and can act in trans when supplied on a plasmid separate from the urease subunit genes (Mulrooney and Hausinger, 1990). It appears that generally one or more genes may be required for urease maturation as well as for other urease-related functions. Urease maturation functions could include the synthesis and/or incorporation of the nickel cofactor and perhaps the assembly of the multiple subunit complex. Other functions may include regulation of urease synthesis and urea and nickel transport.

The expression of cloned urease genes has been investigated for *Proteus mirabilis* (Jones and Mobley, 1988), *Klebsiella aerogenes* (Mulrooney *et al.*, 1989) and *Providencia stuartii* (Mulrooney *et al.*, 1988). The genes for all bacteria tested were transcribed as a single polycistronic mRNA. *P. mirabilis* was regulated by urease induction. Regulatory sequences were identified upstream of the *P. mirabilis* subunit genes which conferred regulation in a manner consistent with the classic "*lac* operon" model for *Escherichia coli*. The *K. aerogenes* urease genes were regulated by nitrogen repression and the *P. stuartii* genes were regulated by both urea induction and nitrogen repression.

### 3.5 Urea breakdown in *Saccharomyces cerevisiae*

*Saccharomyces cerevisiae* does not have a urease enzyme. Urea is broken down by two separate enzymatic activities, collectively called urea amido-lyase (Whitney and Cooper, 1972). The first of these activities is urea carboxylase (urea: $CO_2$ ligase EC 6.3.4.6), which catalyzes the carboxylation of urea to allophanate. This reaction requires ATP and biotin:

$$\text{Urea} + \text{ATP} + HCO_3^- \xrightarrow[Mg^{2+}\quad K^+]{\text{urea carboxylase}} \text{allophanate} + \text{ADP} + \text{Pi}$$

The second reaction is the hydrolysis of allophanate and is catalyzed by allophanate hydrolase (allophanate amidohydrolase EC 3.5.1.13).

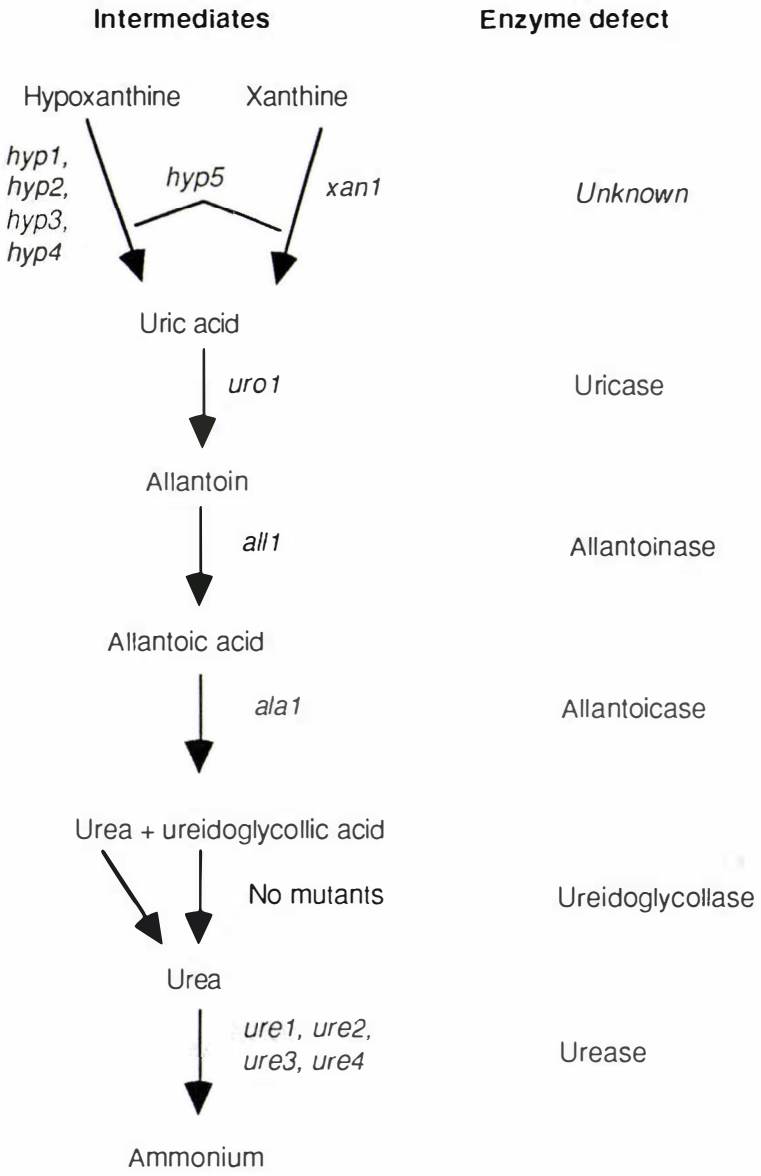
$$\text{Allophanate} + H_2O \xrightarrow[\text{hydrolase}]{\text{allophanate}} 2NH_3 + 2CO_2$$

Urea carboxylase has a high affinity for urea ($K_m$ = 0.4 mM) and $CO_2$ ($K_m$ = 1 mM) (Cooper, 1982). There is no net production or consumption of $CO_2$ in the overall reaction. The first reaction is the rate-limiting step (Whitney and Cooper, 1972). A single gene (*Dur1,2*) encodes urea carboxylase and allophanate hydrolase. Both enzyme activities are associated with a single monomeric 204 kilodalton polypeptide (Sumrada and Cooper, 1982).

Purine catabolism in *S. cerevisiae* occurs by the same series of reactions as in *S. pombe* (Figure 1), except that urea is degraded as described above. Urea can also be formed by arginine catabolism. Arginine hydrolysis, catalyzed by arginase, produces ornithine and urea. Ornithine is degraded via a proline degradation pathway. Arginine and purine catabolism are controlled by inducer exclusion, metabolite-specific induction, and nitrogen repression (reviewed by Cooper, 1982). Inducer exclusion regulates enzymes by blocking the entry of an inducer or metabolite into the cell. Metabolite induction is the specific induction of enzymes and transport systems by a metabolite. Nitrogen repression reduces the catabolic enzyme levels and/or transport activity for a poor nitrogen source when cells are provided with a higher quality nitrogen source, such as ammonium. Arginase is induced by arginine and ornithine and repressed by ammonia and most amino acids, except those which cannot be used as nitrogen sources (Large, 1986). Allophanate is an inducer of the active transport of urea, allantoinase, ureidoglycollase, urea carboxylase and allophanate hydrolase (Large, 1986). These enzymes and transport systems are also controlled by nitrogen repression and are, therefore, repressed by growth on quality nitrogen sources such as serine, asparagine and glutamine (Large, 1986). Allophanate is degraded five times faster (by allophanate hydrolase activity) than it is formed (by urea carboxylase activity). Therefore, the urea degradative enzymes will be induced only if the concentration of an appropriate precursor increases and remains above a threshold level. This situation would only exist under conditions of nitrogen starvation, when arginine would be released from vacuoles, or if arginine or allantoin is present (and metabolically efficient nitrogen sources absent), in the growth medium (Cooper, 1982).

Figure 1

Pathway of purine catabolism in *Schizosaccharomyces pombe*.




(Fluri and Kinghorn, 1985a).

### 3.6 *Schizosaccharomyces pombe* urease

Urease activity in *S. pombe* has been studied only for taxonomic purposes (Sen and Komagata, 1979; Booth and Vishniac, 1987; Kocková-Kratochvilová *et al.*, 1985) or as part of a study on purine catabolism (Kinghorn and Fluri, 1984; Fluri and Kinghorn, 1985b). The pathway of purine catabolism in *S. pombe* (Kinghorn and Fluri, 1984) and the filamentous fungi (Darlington *et al.*, 1965; Scazzocchio and Darlington, 1968) involves the sequential action of the following enzymes: uricase, allantoinase, allantoicase, ureidoglycollase, and urease (Figure 1; Fluri and Kinghorn, 1985a). These enzymes are controlled by nitrogen repression and metabolite induction. For *S. pombe*, uricase, allantoinase, allantoicase and ureidoglycollase are induced by their purine metabolic precursors. Specifically, uricase is induced by uric acid, allantoinase by allantoic acid (and perhaps allantoin), allantoicase by allantoic acid, and ureidoglycollase by ureidoglycollic acid. Thus, induction appears to occur in a sequential manner (Fluri and Kinghorn, 1985a). The *all2* gene is required for the induction of these enzymes (Fluri and Kinghorn, 1985b). Induction, at least for uricase, is by an increase in the level of transcription (Fluri and Kinghorn, 1985b). Urease activity is not induced, but remains at constant high levels irrespective of the nitrogen source. Urea does not induce any of the purine catabolic enzymes. Induction in the filamentous fungi differs from *S. pombe*. In filamentous fungi, allantoinase and allantoicase are both induced by uric acid and allantoin, rather than allantoic acid, and ureidoglycollase is constitutively active (Scazzocchio and Darlington, 1968; Reinert and Marzluf, 1975). Ureidoglycollase in *S. pombe* is highly regulated. Purine catabolism is also regulated by nitrogen repression. In *S. pombe*, uricase and ureidoglycollase activities are strongly repressed by ammonium, but urease activity is unaffected (Fluri and Kinghorn, 1985a). In *Aspergillus nidulans*, the activity of all of the purine degradation enzymes, including urease, is repressed by ammonium (Scazzocchio and Darlington, 1968). Urease and ureidoglycollase are not subject to nitrogen repression in *Neurospora crassa* (Reinert and Marzluf, 1975).

Kinghorn and Fluri (1984) isolated 55 mutants of *S. pombe*, after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, that were unable to use hypoxanthine as a sole nitrogen source. 27 of these mutants specifically lacked urease activity when permeabilized cell suspensions were assayed. The remainder of the mutants were deficient for uricase activity (*uro1* mutants), allantoinase activity (*all1* mutants), allantoicase activity (*ala1* mutants), or unidentified activities specifically affecting hypoxanthine (*hyp*) or xanthine (*xan*) degradation. The urease mutants were divided into four complementation groups (*ure1*, *ure2*, *ure3*, and *ure4*), based on the lack of

recombination between mutants. The *ure1* group had eight mutants designated *ure1-1* to *ure1-8*; *ure2* had fourteen mutants, *ure2-1* to *ure2-14*; *ure3* had three mutants, *ure3-1* to *ure3-3*; *ure4* had two mutants, *ure4-1* and *ure4-2*. Apart from the urease activity assays of permeabilized cells and the induction and nitrogen repression studies discussed above, no further work was reported on these mutants.

## 4 AIM OF THE PRESENT STUDY

*Schizosaccharomyces pombe* was considered to be a suitable organism from which to isolate the genes contributing to the urease function; however, the only data described for *S. pombe* urease was the identification of urease mutants and the lack of induction and nitrogen repression regulation observed for this urease. Furthermore, only one fungal urease, from *Aspergillus nidulans*, had previously been purified. The aim of this study was to characterize *S. pombe* urease, both biochemically and genetically, and isolate the urease genes.

# MATERIALS AND METHODS

## 1 MICROBIOLOGICAL METHODS

### 1.1 Microbial strains

The *Schizosaccharomyces pombe* and *Escherichia coli* strains used in this study are listed in Table 1.

### 1.2 Media

The media used for *S. pombe* and *E. coli* are described in Tables 2 and 3.

#### 1.2.1 Auxotroph supplements

Amino acid and nucleotide supplements were added as the solid form to media before autoclaving, or to the autoclaved medium using sterile stock solutions. Unless otherwise stated, stock solutions of the amino acid supplements (leucine, histidine, lysine, proline, arginine) were prepared as 1% solutions in water and autoclaved for 15 minutes at 121 $^{o}$C. The uracil stock solution was 0.5% in water, and was autoclaved as above. The adenine stock solution was 1% in 1 N NaOH and filter sterilized.

### 1.3 Strain maintenance

#### 1.3.1 *Schizosaccharomyces pombe* strain storage

*S. pombe* strains were frozen at -70 $^{o}$C in YES containing 15% glycerol, for long-term storage (Moreno *et al.*, 1991). For short-term storage (up to several months), strains were kept as patches on YES plates or slants at 4 $^{o}$C (Moreno *et al.*, 1991). Re-isolation of strains from frozen stocks or patches was performed as described by Moreno *et al.* (1991).

#### 1.3.2 *Escherichia coli* strain storage

*E. coli* strains were kept as frozen stocks at -70 $^{o}$C in L broth containing 15% glycerol. Short-term storage (1-2 weeks) was as patches on L plates at 18-25 $^{o}$C. Fresh *E. coli* cultures were revived from frozen stocks every 3 months.

### 1.4 Growing *S. pombe* cells

*S. pombe* cultures were incubated at 30 $^{o}$C. Liquid cultures were shaken on an orbital shaker at 180 rpm.

Table 1 Strains of *Schizosaccharomyces pombe* , *Escherichia coli* and bacteriophage M13 used in this study

| Strain | Genotype | Source |
|---|---|---|
| *Schizosaccharomyces pombe* 972 $h^-$ | Wild type | P. Thuriaux |
| 975 $h^+$ | Wild type | P. Thuriaux |
| *ura4-D18* $h^-$ and $h^+$ | 1.8 kb deletion of the whole *ura4* gene | J. Kohli |
| *lys1-131* $h^-$ and $h^+$ | *lys1-131* | P. Thuriaux |
| *ade6-704* $h^-$ | *ade6-704* | P. Thuriaux |
| 122 | *leu1-32 his2-245* $h^+$ | B. D. Hall |
| 131 | *leu1-32* $h^+$ | B. D. Hall |
| 2143 | *ade4-31 lys1-131 ura2-10* $h^+$ | NCYC |
| 2171 | *ade6-706 fur1-2* $h^+$ * | NCYC |
| 2173 | *ura1-171 his3-237 mat2-102 ade6-M210* | NCYC |
| *ure1-1* $h^-$ and $h^+$ | *ure1-1* | R. Fluri |
| *ure2-1* $h^-$ and $h^+$ | *ure2-1* | R. Fluri |
| *ure3-1* $h^-$ and $h^+$ | *ure3-1* | R. Fluri |
| *ure4-1* $h^-$ and $h^+$ | *ure4-1* | R. Fluri |
| LH162 | *ure2-1 leu1-32 his2-245* | This study, mutagenesis of 122 |
| XL1-1A to D (four strains) | *ure1-1 leu1-32* | This study, *ure1-1* $h^-$ x 131 |
| XL2-1A to D (four strains) | *ure2-1 leu1-32* | This study, *ure2-1* $h^-$ x 131 |
| XL3-1A to D (four strains) | *ure3-1 leu1-32* | This study, *ure3-1* $h^-$ x 131 |
| XL4-1A to D (four strains) | *ure4-1 leu1-32* | This study, *ure4-1* $h^-$ x 131 |

* *S. pombe* strain 2171 is described in the NCYC catalogue as also being *arg1-230*, however, the strain used in the present study did not require arginine for growth.

Table 1 continued.

| Strain | Genotype | Source |
|---|---|---|
| UL1-1A to D (four strains) | *ure1-1 leu1-32 ura4-D18* | This study, XL1-1A x *ura4-D18 h⁻* |
| XU1-1A to D (four strains) | *ure1-1 ura4-D18* | This study, XL1-1A x *ura4-D18 h⁻* |
| UL2-1A to D (four strains) | *ure2-1 leu1-32 ura4-D18* | This study, XL2-1A x *ura4-D18 h⁻* |
| XU2-1a to D (four strains) | *ure2-1 ura4-D18* | This study, XL2-1A x *ura4-D18 h⁻* |
| U1-1A to J (10 strains) | *ure1-1 ura4-D18* | This study, *ure1-1 h⁺* x *ura4-D18 h⁻* |
| U3-1A to J (10 strains) | *ure3-1 ura4-D18* | This study, *ure3-1 h⁺* x *ura4-D18 h⁻* |
| U4-1A to J (10 strains) | *ure4-1 ura4-D18* | This study, *ure4-1 h⁺* x *ura4-D18 h⁻* |
| UL1 | *ura4-D18 leu1-32* | This study, *ura4-D18 h⁻* x 131 |
| XK1-1X | *ure1-1 lys1-131* | This study, *ure1-1 h⁺* x *lys1-131 h⁻* |
| XK2-1A | *ure2-1 lys1-131* | This study, *ure2-1 h⁺* x *lys1-131 h⁻* |
| XK3-1A | *ure3-1 lys1-131* | This study, *ure3-1 h⁺* x *lys1-131 h⁻* |
| XK4-1A and B (two strains) | *ure4-1 lys1-131* | This study, *ure4-1 h⁺* x *lys1-131 h⁻* |

Table 1 continued

| Strain | Genotype | Source |
|---|---|---|
| *Escherichia coli* | | |
| DH1 | *supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*96 (*Nal*$^R$) *thi*-1 *relA*1 | MUCC |
| XL1-Blue | *supE*44 *hsdR*17 ($r_k$-$m_k$+) *recA*1 *endA*1 *gyrA*96 *thi* *relA*1 *lac*$^-$ F' [*proAB+ lacI*$^q$ *lacZ*ΔM15 Tn*10*(*tetr*)] | Promega |
| DB1318 | *recD*1014 *recA*938::Tn*9*-200 (*cam*$^R$) *hsdR*2 *zjj*-202::Tn*10* | EGSC |
| MC1022 | *lacZ*ΔM15 *str*$^R$ | MUCC |
| Bacteriophage M13 | | |
| M13KO7 | insertions in the intergenic region:: *kan*$^R$ and *ori*p15A, mutant gene II | Promega |

NCYC National Collection of Yeast Cultures, Institute of Food Research, Norwich, UK.

MUCC Massey University Culture Collection, Palmerston North, New Zealand.

EGSC *E. coli* Genetic Stock Center, Yale University, New Haven, Connecticut USA.

Promega Corp., Madison, Wisconsin, USA.

Table 2. *S. pombe* media

| Medium | Name | Use | Recipe/(Reference) | Notes |
|---|---|---|---|---|
| Yeast extract | YE | Vegetative growth, inhibits conjugation & sporulation | (Moreno *et al.*, 1991) | Difco yeast extract |
| Yeast extract + supplements | YES | Vegetative growth, includes supplements for auxotrophes | (Moreno *et al.*, 1991) | adenine lysine uracil leucine histidine included at 50-250 µg/ml |
| Yeast extract + phloxin B | YEP | Checking ploidy | (Moreno *et al.*, 1991) | YES + 20mg phloxin B (Gurr)per liter |
| Malt extract + supplements | MES | Conjugation & sporulation (genetic crosses) | (Moreno *et al.*, 1991) | Supplements as for YES |
| Minimal medium | MM | Vegetative growth | 0.67% Difco yeast nitrogen base without amino acids, 2% glucose | |
| Minimal Ammonium | MINA | Vegetative growth | 0.17% Difco yeast nitrogen base without amino acids or ammonium sulfate, 1% glucose, 2 mM ammonium (1 mM ammonium sulfate). (Kinghorn and Fluri, 1984) | |
| Minimal + phloxin B | MINAP | Selection of diploids for induced haploidization | MINA containing 20 mg phloxin B (Gurr) per liter | |
| Minimal hypoxanthine | MINH | Selective for *ure*$^+$ strains, *ure*$^-$ will not grow. | MINA with 2 mM hypoxanthine instead of ammonium sulfate. (Kinghorn and Fluri, 1984) | |

Table 2 Continued

| Medium | | Use | Reference/recipe | Notes |
|---|---|---|---|---|
| Indicator low glucose | ILG | Test for urease activity by replica plating or patching | 0.17% Difco yeast nitrogen base without amino acids or ammonium sulfate, 0.2% glucose, 1 mM ammonium sulfate, 3% agar, 0.0022% bromocresol green, pH adjusted to 4.5 with 1 M HCl, 0.2% urea added to autoclaved and cooled media from 10% stock solution, filter sterilized. (This study) | |
| Indicator high glucose | IHG | Test for urease activity by spread-plating cell suspensions | ILG with 1% glucose. (This study) | |
| Rapid urea hydrolysis broth | RUH | Miniaturized broth test for urease activity | (Booth and Vishniac, 1987) | |
| Fluorophenyl-alanine | FPA | Used for induced haploidization | MM containing 0.05% *m-fluorophenylalanine* | All required supplements included |

Solid media were made by adding 2% agar. Media were sterilized by autoclaving at 110 °C for 20 minutes. All % are w/v.

Table 3 *E. coli* media

| Medium | Name | Use | Reference |
|---|---|---|---|
| Luria | L | Vegetative growth | (Sambrook *et al.*, 1989) |
| SOC | SOC | Recovery medium after electro-transformation | (Sambrook *et al.*, 1989) |
| 2 x strength yeast extract tryptone | 2 x YT | Growth of cells for single-stranded DNA preparation from phagemids | (Sambrook *et al.*, 1989) |

Solid media were prepared by adding 1.5% agar to the medium.

Table 4 Antibiotics

| Antibiotic | Stock solution | Concentration in medium |
|---|---|---|
| Ampicillin | 5 mg/ml in water, filter sterilized | 100 µg/ml, unless otherwise stated |
| Tetracyclin | 10 mg/ml in methanol | 10 µg/ml |
| Streptomycin | 50 mg/ml | 30 µg/ml |
| Kanamycin | 50 mg/ml in water, filter sterilized | 50 µg/ml |

Antibiotics were added to autoclaved media cooled to below 55 °C.

1.5 Growing *E. coli* cells

*E. coli* cultures were incubated at 37 °C. Liquid cultures were incubated in conical flasks with a capacity five-times larger than the liquid volume and were shaken at 225 rpm.

1.6 Aseptic technique

Standard microbiological techniques were used: a wire inoculating loop was used to streak plates; cultures were patched onto plates using sterile wooden toothpicks; liquid cultures were spread onto solid media using alcohol-sterilized glass spreaders; and sterile velvets were used for replica plating. Dilution of *E. coli* and *S. pombe* cells was done in 0.9% NaCl. *S. pombe* cells were counted using a hemacytometer.

1.7 Filter sterilization

0.2 μm pore-size membrane filters were used for filter sterilization.

1.8 Testing the phenotype of *S. pombe* strains

Before any genetic, biochemical or molecular procedure was performed on a *S. pombe* strain, the phenotype was tested. Checked strains were stored as patches and used without further testing for up to two months.

1.8.1 Haploid/Diploid

*S. pombe* strains were streaked or replica plated onto YEP plates to check the ploidy, as described by Moreno *et al.* (1991). Diploid colonies stain darker pink than haploid colonies.

1.8.2 Mating Type

The presence of homothallic $h^{90}$ was tested by exposing single colonies on MES plates to iodine vapors (Moreno *et al.*, 1991). Sporulating $h^{90}$ colonies stain black, non-sporulating colonies stain light yellow. The mating type of non-sporulating heterothallic strains was determined by crossing to $h^{+}$ (975) or $h^{-}$ (972) tester strains. The crosses were examined for the presence of spores and asci by microscopic examination or by exposure to iodine vapors (Moreno *et al.*, 1991).

1.8.3 Auxotrophy and fluorouracil resistance

Strains were tested for auxotrophic markers by replica plating or patching colonies onto minimal medium (MM or MINA) with and without the appropriate supplement (Moreno *et al.*, 1991). Resistance to fluorouracil (*fur1-2* mutants) was tested by patching onto minimal medium (MM or MINA, with required supplements) containing

500 µg/ml 5-fluorouracil (Sigma). Fluorouracil was added to the autoclaved medium as the dry powder.

## 2 UREASE ACTIVITY TESTS FOR *S. POMBE* CULTURES

*S. pombe* cultures were tested for urease activity by the RUH test, color reaction on indicator plates or growth on MINH plates.

### 2.1 RUH test.

The RUH test was done as described Roberts *et al.* (1978): cell mass (eg. a large colony) was resuspended in 200 µl of RUH broth plate and incubated at 37 °C for four hours. A color change from straw to red or pink indicates a positive reaction. No color change indicates a negative reaction. Tests were done in 96-well microtiter plates.

### 2.2 Indicator plates.

Colonies were replica plated or patched onto ILG plates and incubated at 30 °C for up to three days. A positive reaction is indicated by a color change of the green medium to blue. No color change, or a change to yellow, indicates a negative reaction.

Cell suspensions were spread on IHG plates at a density of less than 200 cells per plate and incubated for 5 - 10 days. Color reactions around colonies were scored as for the ILG indicator medium.

### 2.3 MINH plates

MINH plates were streaked, patched or spread with cells, incubated at 30 °C and examined for growth. Only urease-positive *S. pombe* strains will grow on MINH plates.

## 3 *S. POMBE* GENETIC MAPPING

The general guidelines suggested by Gygax and Thuriaux (1984) were followed for the genetic mapping of the *ure* genes of *S. pombe*. Strains were first crossed to *lys1-131* $h^-$ and tetrads were examined for linkage to the mating type locus, *lys1* or a centromere, as discussed below.

### 3.1 Genetic crosses

*S. pombe* strains of checked phenotype were patched onto YES plates, incubated for two days at 30 °C, then mated on MES plates at 25 °C, as described by Moreno *et al.* (1991).

## 3.2 Tetrad Dissection

*S. pombe* asci from two days old crosses were dissected on YES agar slabs using a Prior micromanipulator, as described by Moreno *et al.* (1991).

## 3.3 Spore suspensions

Suspensions of spores from *S. pombe* crosses were prepared as described by Gutz *et al.* (1974).

## 3.4 Analysis of tetrad data

### 3.4.1 Genetic linkage

A zygote heterozygous at two loci, A/a B/b can produce three types of asci, parental ditype (PD) AB AB ab ab; non-parental ditype (NPD) Ab Ab aB aB; and tetratype (T) AB Ab aB ab. If PD are significantly greater than NPD, the two genes are linked and a map distance can be calculated. The Chi-square test was used to test if PD tetrads significantly exceeded NPD. Linkage was also confirmed by the assignment of the *ure* genes to their respective chromosomes by induced haploidization (see below). The relative frequencies of the three tetrad classes were used to calculate the genetic map distances according to the formula of Perkins (1949):

$$Xp = 50(T+6NPD)/(PD+NPD+T)$$

and were corrected for multiple crossovers over long genetic distances as described by Munz *et al.* (1989). Interference was assumed to be absent (Munz *et al.*, 1989).

### 3.4.2 Linkage to a centromere

Linkage to a centromere was determined by taking advantage of the close linkage of *lysI* to the centromere of chromosome I (Kohli *et al.*, 1977). If two unlinked genes are near their respective centromeres, tetratype asci will only occur if there has been a crossover between at least one of the genes and its centromere. Linkage to a centromere is indicated by a parental ditype:non-parental ditype:tetratype ratio of 1:1:<4. The frequency of tetratype (T) asci is related to the second division segregation frequencies, w and y, of the two genes by Perkins (1949) equation:

$$T = w + y - 3/2wy.$$

If one of the genes is very close to the centromere, which is true for *lysI*, the frequency of tetratypes can be taken to approximate the gene-centromere distance for the other gene (Mortimer and Hawthorne, 1969). The Chi-square test was used to test if PD = NPD and, if the gene of interest and *lys1* were not linked (PD ≈ NPD), the Chi-square test was used to determine if T = 4 x (average of PD and NPD).

3.4.3 Chromosome assignment by induced haploidization

The *ure* genes were assigned to their respective chromosomes by induced haploidization, as described by Kohli *et al.* (1977). The *mat2-102* (previously *mei1-102*) mutation was used to construct stable diploids. The *mat2-102* mutation abolishes the ability of cells to undergo meiosis and sporulation (Egel, 1973). Zygotes of the composition *mat2-102/h⁻* or *mat2-102/mat2-102* form stable vegetative diploids and do not undergo meiotic crossover. These diploids can be exposed to m-fluorophenylalanine to induce haploidization. This treatment induces nondisjunction of chromosomes during mitosis. Subsequently, chromosomes are spontaneously and successively lost, leading to the production of haploid segregants (Kohli *et al.*, 1977). The segregation of the gene of interest and marker genes on each chromosome can be used to assign the gene of interest to a chromosome. Stable diploids of the following composition were constructed by crossing *ure⁻ lys1 h⁻* strains with strain 2173 (*mat2-102 ade6 ura1 his3*):

$$\frac{lys1\ +}{+\ ura1} \qquad \frac{h^-\ +}{mat2\ \ his3} \qquad \frac{+}{ade6} \qquad \frac{ure}{+}\ \text{(chromosome unknown)}$$

I II III

Chromosome

Strains were crossed on MES for 16 - 30 hours at 25 °C and examined microscopically for zygotes. Zygotes were resuspended in 0.9% NaCl and plated onto MINAP. Plates were examined after 3 - 5 days incubation at 30 °C. All colonies were dark red, indicating they were diploid. A colony from each cross was spread across a FPA slope and incubated for five days at 30 °C. A suspension of cells from the FPA slope was plated onto YEP and incubated for 4 - 5 days at 30 °C. Plates contained a mixture of pink and red colonies, representing haploid and diploid colonies, respectively. For each diploid parent, 104 haploid segregants were patched onto plates to test for the *lys1, his3, ura1,* and *ure* markers. Kohli *et al.* (1977) suggest 100 segregants are sufficient for this analysis. For each *ure⁻* strain, a *lys1 ure⁻* derivative was crossed with 2173 and two resulting diploids were independently haploidized giving a total of at least 206 haploid segregants per analysis (for *ure4*, the two diploids were derived from two separate *lys1 ure4* parent strains). The *lys1 ure⁻* strains used for these experiments were XK1-1X, XK2-1A, XK3-1A, XK4-1A and XK4-1B. The segregation of each marker gene and the *ure* gene were examined pairwise. The data were interpreted, as described by Kohli *et al.* (1977), according to the following patterns: 1) If the four possible genotypes are present in roughly equal numbers, independent segregation is assumed and the markers are on different chromosomes.

2) If the two parental genotypes greatly exceed the recombinants, joint segregation, and thus linkage, is assumed.

3) Sometimes one parental and one recombinant class greatly exceed the other classes. This situation may arise if chromosomal nondisjunction occurred early in the outgrowth of the zygote or one allele was specifically selected against, therefore it is consistent with nonlinkage. Early mitotic crossover between two linked markers resulting in homozygousity of one of them would also lead to this situation but is less likely, especially because two zygotes were independently haploidized and the *lys1* and *ade6* markers are closely linked to their respective centromeres (Gygax and Thuriaux, 1984).

4). One parental class may greatly exceed all other classes. Selection against one allele and the concomitant loss of a linked marker gene is consistent with this pattern, therefore linkage is assumed.

5) In the present study one further pattern was observed: one recombinant class greatly exceeds all other classes. This situation can be explained by selection against alleles of two genes and probably indicates nonlinkage, although it is difficult to confidently interpret. Although the presence of recombinants could be taken to indicate nonlinkage, rare cases of mitotic recombination resulting in favorable recombinant genotypes, could conceivably outgrow the parental segregants due to a selective advantage. Fortunately data from other haploidization experiments enabled chromosome assignments without relying on this type of ambiguous data.

The 2 x 2 contingency test was used to confirm independent or joint segregation (Chatfield, 1983).

## 4 PURIFICATION AND CHARACTERIZATION OF *S. POMBE* UREASE

### 4.1 Buffers for urease purification

#### 4.1.1 PEB

PEB buffer was 0.02 M potassium phosphate buffer system ($K_2HPO_4/KH_2PO_4$, pH 7.0) containing 1 mM EDTA and 1 mM 2-mercaptoethanol, as described by Wong and Shobe (1974). Sodium azide (0.02%) was routinely added to this buffer as a preservative.

#### 4.1.2 PEBS

PEBS was PEB containing 0.1 M NaCl, prepared by mixing the two buffer components containing NaCl until the desired pH (7.0) was achieved..

4.1.3 0.2 M PEBS

0.2 M PEBS was PEB containing 0.2 M NaCl, prepared as above.

4.1.4 0.35 M PEBS

0.35 M PEBS was PEB containing 0.35 M NaCl, prepared as above.

4.2 Urease assay

Urease activity was measured by following the release of ammonia from urea. A modified Berthelot reaction was used, as described by Wong and Shobe (1974). All assays were done in duplicate or triplicate. The amount of ammonia released by urease activity was determined by subtraction of a reaction in which the substrate (urea) was ommitted. Reagent blanks were prepared as described by Wong and Shobe (1974). The standard curves were linear (usually regression coefficient = 1.00) over the range 0.00 to 0.50 µmol ammonia (absorbance at 625 nm is 2.0 for 0.5 µmoles of ammonium).

4.3 Protein assay

The concentration of protein in solution was determined using the coomassie blue dye binding method of Bradford (1976), for protein concentrations between 20 to 200 µg/ml. 5 ml of reagent was added to 0.5 ml of sample and the assay performed as described by Bradford (1976). For lower protein concentrations (10 to 50 µg/ml) the modified dye-binding assay described by Spector (1978) was used: 0.5 ml of reagent was added to 0.1 ml of protein solution. For all assays, bovine serum albumin (BSA fraction V, Sigma) was used to prepare the standard curve. The extinction coefficient of BSA ($E_{280}$ = 6.6) was used to determine the concentration of the BSA standard solution. The color reaction for all assays was stable after two minutes and for up to 30 minutes. All determinations were read within this time.

4.4 Preparation of *S. pombe* crude cell extracts for urease purification

1. Cells from a stationary-phase culture in YES were harvested by centrifugation (4000 g for 10 minutes at 4 °C), resuspended in about half the original volume of ice-cold water and recentrifuged. Cells were kept at 0 - 4 °C for all subsequent procedures.
2. The cell pellet was resuspended in twice the pellet volume of PEB and disrupted by two passages through a French press at a pressure of 9,000 pounds per square inch.
3. The disrupted cells were centrifuged at 110,000 g for one hour to remove all cell debris, including membranes, if the crude extract was to be used directly; or at 31,000 g for 40 minutes if further purification was to be carried out.

4.5 Preparation of jack bean urease crude extracts

One to three jack bean meal tablets (BDH) were crushed and resuspended in 5 - 10 ml PEB and incubated on ice for 15 minutes. The suspension was centrifuged (5000 g for ten minutes at 4 °C) and the supernatant was used as the crude urease extract.

4.6 Preparation of affinity adsorbents

4.6.1 Oxirane Hydroxyurea Agarose (OHA)

Hydroxyurea was coupled to agarose via a bisoxirane (Sundberg and Porath, 1974).

1. 29 g of suction-dried Sepharose 4B (Pharmacia) was activated with 1,4-butanediol diglycidyl ether as described by Sundberg and Porath (1974).
2. The degree of activation was determined: a sample of gel corresponding to 0.1 g dry weight (estimated from the suction-dried gel and confirmed after the analysis was completed) was removed, an equivalent weight each of sodium sulfite and sodium metabisulfite was added to the gel, along with about 2 ml of water and the reaction was incubated overnight with mixing by rotation. The sample was washed twice with water, twice with 1.0 N HCl, then five times with water. The washed sample was resuspended in about 2 ml water, about 0.5 g of NaCl was added and then the gel was titrated with 0.1 N NaOH. 0.0589 mmoles of NaOH neutralized the sample, therefore the degree of activation was 0.589 mmoles/g dry gel.
3. The remainder of the activated gel was coupled to hydroxyurea: 0.75 g of hydroxyurea was dissolved in 10 ml water, the pH was adjusted to pH 11 with 1 N NaOH. The suction-dried activated gel was resuspended in the hydroxyurea solution and incubated at 25 °C with mixing by rotation for 60 hours.
4. The coupled gel was washed extensively with water before use. The degree of substitution was determined by titration with 0.1 N NaOH after washing twice with 0.1 N HCl and then five times with water. The substitution was 0.57 mmoles hydroxyurea/g dry weight of gel.

4.6.2 Aminocaprylic acid hydroxyurea agarose (AHA)

Caprylic acid was coupled to 1,1'-carbonyldiimidazole activated (Bethell *et al.*, 1979) Sepharose CL6B (Pharmacia). Hydroxyurea was then coupled to the caprylic acid substituted agarose by a carbodiimide-mediated reaction (Cuatrecasas and Anfinsen, 1971).

1. 40 g of suction-dried Sepharose CL6B (Pharmacia) was washed on a glass filter-funnel several times with water and then solvent exchanged to 100% dioxane by washing twice each with 100 ml 25%, 50%, 75% dioxane, and finally five times with 100% dioxane.

2. The dry weight of the gel was estimated to be 2.4 g and an equal weight of 1,1'-carbonyldiimidazole (CDI, Sigma) was added to the gel, which was resuspended in about 10 ml of 100% dioxane. The reaction was incubated for two hours at room temperature with mixing by rotation.
3. The gel was solvent exchanged back to water by washing twice each with 100 ml 75%, 50%, 25% dioxane and finally five washes with water.
4. A greater than 4 M excess of caprylic acid (moles caprylic acid:moles CDI greater than 4:1, 1.68 g caprylic acid) was dissolved in 20 ml water, the pH was adjusted to pH 11 with 5 N NaOH and the solution was added to the suction-dried gel. The pH was maintained between pH 10.5 and 11 and the gel was incubated at room temperature, with mixing by rotation, for 16 hours.
5. The gel was washed five times with water. The degree of substitution was estimated by titration with 0.1 N NaOH exactly as described for measuring the bisoxirane activation of agarose (above). The degree of substitution was 0.57 mmoles/g dry weight.
6. A five molar excess of hydroxyurea (moles hydroxyurea:moles caprylic acid groups in the gel = 5:1, 0.53 g hydroxyurea) was dissolved in 10 ml water, added to the suction-dried gel and the pH was adjusted to pH 4.7 with 0.1 N HCl. A five molar excess of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC, 1.4 g) (moles EDC:moles caprylic acid groups in the gel= 5:1)was dissolved in water and the pH was adjusted to pH 5.0. The EDC solution was added dropwise to the stirred gel. The pH was maintained at pH 5.0 by the addition of 1 N HCl. After the rapid pH change had subsided the gel was incubated for a further 16 hours at room temperature with mixing by rotation.
7. The gel was washed twice with 1 N HCl and then five times with water and used.

4.6.3 Ethylenediamine-ethylenediamine hydroxyurea agarose (EHA)

This affinity resin is essentially the same as the "$I_{16}$-Hu" adsorbent described by Shobe and Brosseau (1974). Hydroxyurea is coupled to agarose via a 16 atom side chain consisting of two ethylenediamine residues linked together with a succinyl residue.

1. The matrix was prepared as described by Shobe and Brosseau except that the agarose was activated by CDI as described above for AHA, rather than activation by cyanogen bromide, and Sepharose CL6B, rather than Sepharose 4B, was used.
2. The degree of activation of the matrix, determined as described for AHA, was 1.08 mmoles/g resin (dry).
3. The degree of ethylenediamine-succinate-ethylenediamine-succinate substitution of the agarose, measured by titration with 0.1 N NaOH, was 0.23 mmoles/g and the

rapid pH change during the coupling of hydroxyurea to this spacer arm indicated good substitution of the matrix had occurred.

4.6.4 Substitution of the affinity resins with hydroxyurea.

The degree of substitution of the affinity resins with the oxirane, aminocaprylic acid, or ethylene diamine and succinic anhydride was determined by titration (above). The rapid pH change observed when hydroxyurea was reacted with the resins in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide indicated the coupling reaction had occurred. Each resin was further tested for substitution with amine derivatives using the 2,4,6-trinitrobenzenesulfonate-borate color test, as described by Cuatrecasas (1970). The AHA and EHA resins turned an intense orange color, implying very good substitution with hydroxyurea. The color reaction for the EHA resin could also have been caused by the presence of free amino groups of ethylene that escaped succinylation and further reaction. The color development for OHA was a less intense orange, indicating substitution may not have been as good as the other resins and may be below the value determined by titration (above).

4.7 Purification of *S. pombe* urease

Urease from *S. pombe* crude extracts was purified by acetone precipitation, followed by ammonium sulfate precipitation, DEAE ion exchange column chromatography, and sometimes Mono-Q ion exchange FPLC as a final step.

4.7.1 Acetone precipitation

Acetone precipitation was carried out on crude extracts according to the general guidelines suggested by Scopes (1987) for solvent precipitations. The pH of crude extracts was adjusted to pH 7 with a few drops of 3 N KOH, as required, and the following acetone cuts performed: 0-40%, 40-50%, 50-60% acetone. The temperature of all materials was maintained at 0 - 2 °C, acetone was added over a period of 15 - 30 minutes, and extracts were stirred a further 15 minutes after acetone addition was completed. Precipitated proteins were recovered by centrifugation at 20,000 g for ten minutes at 0 °C and dissolved in PEB (8 ml per liter starting culture to give a protein concentration of about 5 mg/ml). Each fraction was assayed for urease activity and protein content. Dialysis against PEB did not affect the specific activity of redissolved urease and, therefore, was not routinely done. The most active fraction (usually the 50-60% cut, sometimes the 40-50% cut) was used for ammonium sulfate precipitation.

4.7.2 Ammonium sulfate precipitation

Ammonium sulfate precipitation was carried out following the guidelines suggested by Scopes (1987). All materials and extracts were maintained at 0 - 2 °C, ammonium sulfate was added over a period of 15 - 30 minutes, and extracts were stirred a further 15 minutes after ammonium sulfate addition was completed. The ammonium sulfate cuts used were 0-35% and 35-45% saturation with ammonium sulfate. Precipitated proteins were recovered by centrifugation at 20,000 g for ten minutes at 0 °C. Enzymes are generally very stable when stored as ammonium sulfate pellets (Scopes, 1987). For some large scale purifications ammonium sulfate precipitated proteins were stored at 4 °C for up to one week until further purification on pooled samples could be done. No difference was observed in the urease activity of stored pellets compared to freshly prepared pellets. Ammonium sulfate pellets were resuspended in 0.66 ml PEB per liter starting culture and dialyzed with PEB until all detectable ammonium was removed. All samples were assayed for protein content and urease activity. Most urease was present in the 35-45% cut.

4.7.3 Diethylaminoethyl (DEAE) Sepharose ion exchange

All procedures were carried out following the manufacturers guidelines (Pharmacia handbook "Ion exchange chromatography : principles and methods"). The DEAE-Sepharose™ (Pharmacia) gel bed (8 cm x 1.6 cm, 16 ml total volume) was prepared in a glass column (Pharmacia). The resin was equilibrated in PEB. The urease sample was applied to the column and end pieces were fitted to minimize disturbance of the gel bed. The column was washed with several column volumes of PEB containing 0.2 M NaCl (0.2 M PEBS) and then the urease was eluted with a linear NaCl gradient of 0.2 M to 0.35 M (in PEB). The total volume of the gradient was at least six times the volume of the column. Fractions (2 ml) were collected and assayed for urease activity and protein content. Dialysis against PEB did not affect the specific activity of column fractions so was not routinely done. The conductivity of fractions was tested with a Radiometer conductivity meter to confirm that the NaCl gradient was linear. A flow rate of less than 10 ml per hour was maintained by gravity or a Masterflex™ (Coleman) peristaltic pump and all operations were performed at 4 °C. The most active fractions were pooled and then concentrated and desalted by ultrafiltration through a XM 50 membrane (Amicon). The filtrate was monitored for urease activity to confirm that the enzyme did not pass through the filter.

4.7.4 FPLC purification of urease.

FPLC was sometimes used as a final purification step when the standard methods (above) did not produce sufficiently pure urease. The concentrated and desalted

urease, in PEB, was applied to a Mono Q HR 5/5 ion-exchange column (Pharmacia) equilibrated with 0.2 M PEBS and eluted with a linear NaCl gradient of 0.2 M to 0.5 M (in PEB). The flow rate was 0.5 ml/minute and a total volume of 30 ml was used. The absorbance at 280 nm was monitored and fractions corresponding to absorbance peaks were collected and assayed for urease activity and protein content.

### 4.8 Ultrafiltration of protein solutions

Protein solutions were often concentrated and/or desalted by ultrafiltration. For very small volumes (less than 2 ml) a Centricon™ 30 apparatus (Amicon, 30,000 molecular weight cutoff) was used according to the manufacturers instructions. For larger volumes, solutions were ultrafiltered using Amicon ultrafiltration cells and XM50 membranes (molecular weight cutoff 50,000), according to the manufacturers instructions. Solutions were desalted by several cycles of dilution with buffer (usually PEB) and concentration by ultrafiltration.

### 4.9 Preparation of dialysis tubing

Dialysis tubing was prepared as described by Sambrook *et al.* (1989).

### 4.10 Polyacrylamide gel electrophoresis (PAGE)

For both native (non-denaturing) and SDS (denaturing and reducing) PAGE the buffer system of Laemmli (1970) was used, except that sodium dodecyl sulfate (SDS) was not included in the loading buffer, gel or running buffer for native-PAGE. Slab gels were used for all PAGE. All gels were discontinuous, containing a stacking gel as well as the separating gel. The gels were prepared from a stock solution of acrylamide containing 30% total monomer and 2.67% crosslinking monomer. Samples were loaded in 62.5 mM Tris-HCl pH6.8, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 0.05% bromophenol blue. Samples for SDS-PAGE also contained 2% SDS and were heated at 95$^{o}$C for 4 minutes immediately before loading. The manufacturers guidelines (Bio-Rad Protean™ II apparatus handbook) for preparing and running gels were followed. The Protean™ II (Bio-Rad) apparatus with 20 cm long plates was used for molecular weight determinations. Molecular weights of proteins were determined by comparison to a standard curve of log molecular weight vs relative mobility. The Bio-Rad high MW SDS-PAGE standards (sizes 200,000, 116,250, 97,400, 66,200, 45,000) and Bio-Rad low MW SDS-PAGE standards (sizes 97,400, 66,200, 31,000, 21,500, 14,400) were used to prepare standard curves.

4.11 Staining polyacrylamide gels for protein

Gels were stained for protein using either Coomassie Blue R-250 (for protein concentrations greater than 2 µg per band) or the silver stain method (for protein concentrations as low as 1 ng per band).

4.11.1 Coomassie Blue R-250 stain

Gels were stained for 1/2 hour with 0.1% Coomassie Blue R-250 in 40% (v/v) methanol, 10% (v/v) acetic acid. Gels were destained in 40% (v/v) methanol, 10% (v/v) acetic acid until the background was uncolored (usually 2 - 5 hours).

4.11.2 Silver stain

A modification of the method described by Morrissey (1981) was used. Gentle agitation and removal of solutions by aspiration were used:

1. Gels were prefixed in 10% (v/v) acetic acid, 10% trichloroacetic acid, 30% (v/v) methanol for 30 minutes.
2. Gels were fixed for 30 minutes in 10% (v/v) glutaraldehyde (Merck, electron microscopy grade 25% solution).
3. The gels were rinsed in water for at least four hours with at least five changes of water.
4. Gels were soaked for 30 minutes in 5 µg/ml dithiothreitol (DTT).
5. The DTT solution was removed and, without rinsing, replaced with 0.1% silver nitrate for 30 minutes.
6. Gels were rinsed for a total of five minutes with several changes of water.
7. Gels were briefly rinsed twice with 40 ml developer (3% sodium carbonate containing 100 µl 37% formaldehyde per 100 ml) and then soaked in developer until the desired level of staining was attained.
8. Development was stopped by adding a 1/10 volume of 2.3 M citric acid to the developer and agitating for 20 minutes.
9. Gels were washed with several changes of water for 30 minutes, soaked in 0.03% sodium carbonate for ten minutes and then rinsed with water.

Both silver stained and coomassie stained gels were photographed using Kodak Tecpan™ (high contrast) film.

4.12 Urease activity stain for native-PAGE gels

Urease activity stains were done as described by de Llano *et al.* (1989), with the following modifications: gels were soaked in 10 mM 2-mercaptoethanol for 45 minutes (15 minutes x three changes) rather than 30 minutes; incubation in 0.25 M urea was

for four minutes, rather than three minutes; Milli-Q™ purified water, rather than glass distilled water was used. This procedure allows the detection of 0.015 to 2.500 enzyme units per band (de Llano *et al.*, 1989) which corresponds to 5 to 850 ng protein for pure jack bean urease (de Llano *et al.*, 1989) or about 20 to 3,500 ng for *S. pombe* urease (based on specific activity of 709, Results section 3.4).

4.13 Determination of protein molecular weight by gel filtration

A Sepharose™ CL6B 200 (Sigma) gel bed was prepared in a glass column (1.6 cm x 100 cm, Pharmacia) fitted with end pieces. The resin was washed, resuspended, degassed, poured, equilibrated and run in PEBS buffer at 4 °C (PEB containing 0.1 M NaCl), using the procedures suggested in the Pharmacia handbook - "Gel filtration: theory and practice". The column was eluted in an upward flow configuration to reduce packing of the gel matrix. The packed gel bed dimensions were 1.6 cm x 92 cm and remained unchanged throughout the course of the experiments. A constant operating pressure of 100 cm of water was maintained by using a Mariotte flask. This pressure is well below the maximum recommended pressure (>200 cm ) for this matrix (Pharmacia handbook - "Gel filtration: theory and practice"). The flow rate was 12 ml/hr, which is less than the maximum rate recommended for determination of molecular weight using CL6B 200 (Sigma Technical Bulletin No. GF-3). The flow of the column was not stopped from the time the column was poured until the end of all molecular weight determinations. The column was calibrated using the protein standards in the MW-GF-1000 kit (Sigma) according to the manufacturers instructions. The protein standards supplied in this kit were Blue Dextran $M_r$ = 2,000,000, Thyroglobulin $M_r$ = 669,000, Apoferritin $M_r$ = 443,000, ß-Amylase $M_r$ = 200,000, Alcohol Dehydrogenase $M_r$ = 150,000, Albumin $M_r$ = 66,000 and Carbonic Anhydrase $M_r$ = 29,000. The elution volume ($V_e$) of blue dextran, determined by monitoring the absorbance of fractions at 620 nm, was taken as the void volume ($V_o$) of the column. Each standard was separately run through the column and the absorbance at 280 nm was used to follow the elution of the proteins. All samples were dissolved in PEBS, centrifuged at 13,000 g for five minutes at 4 °C, adjusted to 2 ml volume and loaded onto the column. Fractions (2.04 ml) were collected once the applied sample had reached the column surface. The amount of protein standard applied to the column was 10 - 16 mg for thyroglobulin, bovine serum albumin and apoferritin; 4 mg for carbonic anhydrase; 6 - 8 mg for alcohol dehydrogenase and ß-amylase; and 5 mg for blue dextran. The total protein content of *S. pombe* urease samples (crude extracts partially purified by acetone precipitation) applied to the column was about 8 mg, which is below the recommended maximum of 70 mg/ml (Pharmacia handbook - "Gel filtration: theory and practice"). *S. pombe* urease

samples were loaded and fractions collected, as for the standards. Fractions were assayed in triplicate for urease activity.

4.14 Purification of peptides by High Performance Liquid Chromatography (HPLC)

Peptides were purified by reversed-phase HPLC using a HY-TACH C18 column. This column has a micropellicular packing (2 μm diameter beads), with overall dimensions of 75 x 4.6 mm, therefore, it should irreversibly bind less material than the longer conventional columns usually used for peptide purification (D. J. Poll, personal communication). The following chromatography conditions were used:

Buffer A: 0.1% formic acid in 49:1 water:acetonitrile.

Buffer B: Same as buffer A except that the ratio of water:acetonitrile was 1:9.

Gradient: Linear, 0 - 100% Buffer B over 60 minutes at 1 ml/min, (100 - 0% Buffer A).

Detection: Absorbance at 214 nm (wavelength of strong absorbance for the peptide bond; Scopes, 1987), scale maximum = 0.4.

Temperature: 50 °C.

Chart speed: 200 mm/hr.

Peaks corresponding to eluted peptides were collected in tubes and frozen.

4.15 Protein sequencing

Purified proteins and peptides were sequenced by the sequential automated Edman degradation method with an Applied Biosystems™ model 470A apparatus.

5. POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION OF DNA

5.1 Oligonucleotide primer manufacture

Oligonucleotides were purchased from Oligos Etc. Inc. (Gullford, Connecticut). The oligonucleotides were redissolved in water and used without further purification.

5.2 PCR amplification of DNA

The DNA Thermal Cycler apparatus (Perkin Elmer Cetus) was used for PCR amplification of DNA. Temperature cycling was by the "Step Cycle" program and cycling parameters are given in Results 5.2 and 5.3. Taq polymerase (Promega) and the supplied buffer were used with 50 μM of dATP, dCTP, dTTP, and dGTP. The concentration of template DNA was 20 ng in a 50 μl reaction volume, which corresponds to the optimum (Innis and Gelfand, 1990) of about 1 x $10^6$ copies of the genome (15 fg DNA per *S. pombe* cell, Bostock, 1970). Reactions were overlaid with paraffin oil to reduce evaporation and the same set of wells in the temperature block

was used for each experiment to reduce variation due to uneven heating of the thermal cycler. 10% (5 µl) of each reaction was examined by agarose electrophoresis. Three controls were included in each PCR run: a) template DNA ommitted, b) one primer ommitted, c) the other primer ommitted.

### 5.3 Construction of T-vectors

PCR products often have a non-template directed single base 3' deoxadenosine overhang which can be efficiently ligated to a vector containing a single 3' thymidine base overhang (T-vector; Marchuk *et al.*, 1990). T-vectors were made as described by Marchuk *et al.* (1990). Briefly, the vector is cut with an enzyme that creates blunt ends and then incubated with Taq polymerase in the usual PCR buffer containing 2 mM dTTP (and no other nucleotides) for two hours at 70 °C. This results in the addition of a single base 3' T overhang. After phenol/chloroform extraction and ethanol precipitation the T-vector is used in ligations with gel-purified PCR products.

## 6 GENERAL DNA MANIPULATION TECHNIQUES

### 6.1 Preparation of plasmid DNA

#### 6.1.1 Small-scale plasmid isolation

Small-scale (5 - 50 µg DNA) plasmid isolations were performed using a modified version of the rapid boiling method originally described by Holmes and Quigley (1981). The procedure outlined by Sambrook *et al.* (1989) was used, with the following changes: the DNA was precipitated with 300 µl isopropanol, rather than 420 µl, sodium acetate was not used for DNA precipitation, and RNAase was not included in the TE used for redissolving the DNA.

#### 6.1.2 Large-scale plasmid isolation

Large-scale plasmid isolations were done using the alkali-lysis method described by Sambrook *et al.* (1989).

### 6.2 Purification of DNA

#### 6.2.1 Cesium chloride density gradient equilibrium centrifugation.

Equilibrium centrifugation in CsCl-ethidium bromide gradients was done as described by Sambrook *et al.* (1989). Ethidium bromide was removed by extraction with 1-butanol as described by Sambrook *et al.* (1989).

6.2.2 Phenol/chloroform extraction of DNA

Phenol/chloroform extraction of DNA was done as described by Sambrook *et al.* (1989).

6.3 Ethanol precipitation of DNA

DNA in aqueous solution was precipitated by adding 2 - 2.5 volumes of ethanol and 0.1 volume of 3 M sodium acetate, as described by Sambrook *et al.* (1989).

6.4 Agarose gel electrophoresis

TAE buffer was used for agarose gel electrophoresis. When accurate size determinations were not required mini-gels (6.5 cm long x 9.5 cm wide) were used and run at 5 V/cm for 1 - 1.5 hours. Large gels (20 cm long x 15 cm wide, Bio-Rad Sub Cell™) were used for blotting or accurate size determinations and were run at 1 V/cm for about 16 hours. DNA was visualized by immersing gels in 0.5 μg/ml ethidium bromide (in water) for 15 - 40 minutes and illuminating on an ultraviolet light transillumnator (Model TS-15; Ultra-Violet Products Inc.; 254 nm wavelength). Gels were photographed on Polaroid™ 665 or 667 film. DNA samples were mixed with an appropriate volume of load buffer or SDS dye buffer. BRL 1 Kb DNA Ladder (size range 298 bp - 12,216 bp) or λ DNA digested with *Hin*dIII (size range 600 bp - 23,130 bp) were used for molecular size markers. A mobility (distance moved) vs log molecular size standard curve, constructed from the mobility of the molecular size markers, was used to estimate the size of DNA fragments.

20x TAE stock solution: 96.8 g/l Tris, 14.8 g/l EDTA (disodium salt), 22.8 ml/l glacial acetic acid. The 1x working solution was prepared by appropriate dilution of the 20x stock solution.

11x agarose gel load buffer: 0.44% bromophenol blue, 0.44% xylene cyanol, 27.5% Ficoll (Type 400; Pharmacia). Stored at room temperature.

6.5 Restriction enzyme digestion of DNA

Restriction enzyme digests were done using approximately 10 U of enzyme per 2 μg of DNA. BRL brand enzymes and the recommended REact™ (BRL) buffer were used. Digests were incubated at the recommended temperature for 1 - 2 hours and reactions were stored on ice (for up to two hours) or frozen while an aliquot was examined by agarose electrophoresis. Alternatively, reactions were stopped by incubation at 65 °C for ten minutes (for heat labile enzymes), by the addition of 0.25 volumes of SDS dye buffer, or by phenol/chloroform extraction. Digests were treated with RNAase A, as

required, by adding 2 µl of DNAase free RNAase A and incubating for a further two minutes before the addition of SDS dye buffer.

SDS-dye buffer: 20% sucrose, 5 mM EDTA, 1% SDS, 0.2% bromophenol blue. Stored at 4 °C.

DNAase free RNAase was prepared as follows: a 2 mg/ml solution of RNAase A (Sigma) was prepared in sterile water, heated to 100 °C for ten minutes and frozen at -20 °C. The solution was periodically reheated to destroy DNAase.

### 6.6 Gel purification of DNA fragments

DNA fragments were recovered from agarose gels by excising the appropriate band from an ethidium bromide stained gel illuminated with low intensity long wavelength UV light (Model UVGL-58; Ultra-Violet Products Inc.; 360 nm wavelength) and spinning the liquid (containing DNA) out of the gel slice through silicanized glass wool, as described by Heery *et al.* (1990). The recovered DNA was concentrated by precipitation with ethanol and resuspended in TE ready for use directly in ligations.

### 6.7 DNA quantitation

The concentration of DNA in solution was determined by one of the following methods:

#### 6.7.1 Absorbance at 260 nm.

The $A_{260}$ was used to measure the concentration of DNA purified by CsCl gradient equilibrium centrifugation, as described by Sambrook *et al.* (1989). This method cannot be used if the samples contain RNA or protein. The presence of RNA was tested by agarose electrophoresis and the presence of protein and other contaminants was tested by the $A_{260}/A_{280}$ ratio (Sambrook *et al.*, 1989).

#### 6.7.2 Ethidium bromide dot quantitation

The concentration of DNA in dilute solutions was estimated by comparing the fluorescence of ethidium bromide stained samples to standards, as described by Selden and Chory (1991). Standards of 0, 1, 2.5, 5, 7.5, 10, and 20 µg/ml DNA and the test samples are each mixed with an equal volume of 1 µg/ml ethidium bromide and 8 µl aliquots are spotted onto plastic wrap, placed on a UV transilluminator and photographed. This method cannot be used if the samples contain RNA.

6.7.3 Fluorescence in agarose gels

DNA samples were run next to standards of known concentration in agarose gels. The gels were stained with ethidium bromide, illuminated on a UV transilluminator and the amount of DNA in the band of interest was estimated by comparison to the DNA standards. This method can be used if the samples contain RNA and other contaminants.

6.8 Ligation of DNA fragments

DNA fragments were ligated into vectors using T4 DNA ligase. The general guidelines outlined by Sambrook *et al.* (1989) for cohesive end ligations were followed. Ligase (1 U/µl) and 5x ligase buffer were from Promega. Ligations were carried out for approximately 18 hours at 14 °C and were examined by agarose gel electrophoresis before electro-transforming *E. coli* MC1022 with 1 µl of the ligation reaction.

6.9 Preparation of single-stranded DNA from phagemids (pUC118/119) for sequencing.

Single-stranded DNA templates for sequencing were prepared using the following method:

1. An overnight culture of *E. coli* XL1-Blue (Promega) transformed with the phagemid was prepared in 2 x YT containing 100 µg/ml ampicillin and 10 µg/ml tetracyclin.
2. The overnight culture was diluted 1/50 in the same medium and incubated for 30 minutes at 37 °C with shaking.
3. The helper phage M13KO7 (Promega, 1.35 x $10^{11}$ phage per ml stock solution) was added to give 1.35 x $10^{8}$ phage per ml (multiplicity of infection of about 10 phage per bacterium) and incubated for 30 minutes as above.
4. Kanamycin was added to give 50 µg/ml and the incubation was continued for a further 6 - 8 hours.
5. The culture was centrifuged for five minutes at 13,000 g, the supernatant was transferred to a new tube and recentrifuged as above.
6. A 0.25 volume of phage precipitation buffer (20% PEG 8000, 3.75 M ammonium acetate) was added to the supernatant (above) and incubated for 30 minutes on ice or overnight at 4 °C.
7. The precipitated phage were recovered by centrifugation at 30,000 g for 10 minutes.
8. Pelleted phage were resuspended in TE (100 µl per 5 ml culture), extracted twice with phenol/chloroform and once with chloroform, precipitated with ethanol and ammonium acetate and resuspended in 20 µl water.
9. The single-stranded template preparation was examined by agarose mini-gel electrophoresis and about 2 µg (2 - 5 µl) was used for sequencing.

6.10 DNA sequencing

Single-stranded DNA templates were sequenced by the dideoxynucleotides chain-termination reaction. The Sequenase II™ (United States Biochemical Corp.) sequencing kit was used according to the manufacturers instructions. The S2 (BRL) sequencing gel apparatus was used and 6% acrylamide sequencing gels were prepared and run as described for the 'Standard Protocol' by Slatko and Albright (1991). Gels were fixed in a 10% (v/v) acetic acid 10% (v/v) methanol solution for 45 minutes, dried using a Model 583 (Bio-Rad) apparatus at 80 °C and autoradiographed using Fuji Rx film.

6.11 Southern transfer of DNA

DNA was transfered to Hybond N™ nylon filters (Amersham) by vacuum transfer under alkaline conditions. The VacuGene XL™ (Pharmacia) apparatus was used according to the manufacturers 'Protocol No. 3' for alkaline transfer. DNA was depurinated with 0.2 N HCl for about 20 minutes (until the bromophenol blue marker dye had turned yellow) and denatured and transfered in 1 N NaOH for about 1.5 hours. A vacuum of 50 mbar was maintained throughout the procedure.

6.12 Hybridization of DNA probes to Southern blots

Nick-translation labeled DNA probes were hybridized to DNA immobilized on Hybond N nylon filters following the general guidelines described by Sambrook *et al.* (1989) but with the following modifications:

Prehybridization solution was 6x SSC, 10x Denhardt's reagent. A 50 ml volume was used.

Hybridization solution was 1 M NaCl, 0.05 M sodium phosphate buffer pH 6.5, 2 mM EDTA, 0.5% SDS, 10x Denhardt's reagent. A 5 ml volume was used.

Filters were prehybridized, hybridized and washed at 68 °C. A total of four post-hybridization washes were done, each for 15 - 30 minutes. The first two washes were with 200 ml of 2x SSC, 0.1% SDS and the last two with 200 ml of 1x SSC. Prehybridization, hybridization and washes were all carried out in glass hybridization tubes with screw caps and incubated in hybridization ovens (Bachofer Laboratoriumgeräte). Autoradiography using Fuji Rx film was done as described by Sambrook *et al.* (1989). Denhardt's reagent and SSC were prepared as described by Sambrook *et al.* (1989).

6.13 Hybridization of oligonucleotide probes to Southern blots

End-labeled oligonucleotide probes were hybridized to DNA immobilized on Hybond N nylon filters using the same general procedure as for nick-translation labeled probes but with the following differences:

Prehybridization solution was 6x SSC, 0.5% SDS, 0.05% Sodium pyrophosphate, 10x Denhardt's reagent.

Hybridization solution was 6x SSC, 0.05% Sodium pyrophosphate, 20x Denhardt's reagent.

Wash solution was 6x SSC, 0.05% Sodium pyrophosphate.
Prehybridization and hybridization were done at 41.5 °C. Three washes, each for ten minutes, were done at room temperature, followed by a final wash at 41.5 °C for two minutes.

6.14 Nick-translation labeling of DNA probes

DNA probes were radioactively labeled with [$\alpha$-$^{32}$P]dCTP using the following protocol:
1. The following were combined (on ice): 0.2 µg DNA, 2.5 µl 10x nick-translation buffer, 2.5 µl each of 0.1 mM dATP, dTTP, and dGTP, 5 µl [$\alpha$-$^{32}$P]dCTP (10 µCi/µl), 1 U DNA polymerase I, DNAasel (optimum amount determined empirically as described by Sambrook *et al.*, 1989) in a final volume made to 25 µl with water.
2. The reaction mixture was incubated at 15 °C for 15 minutes and the amount of incorporation of radiolabeled nucleotide in 1 µl of the reaction was estimated by thin layer chromatography using a polyethyleneimine (PEI) cellulose (Schleicher and Schüll GmbH, F1440/PEI/LS254) adsorbent developed with 2 N HCl. The unincorporated nucleotides migrate to the top of the PEI strip and the DNA remains at the origin. The radioactivity in the top half was compared to the bottom half, in a scintillation counter, to give an approximate estimation of the proportion of incorporation of radiolabel. Usually about 68% incorporation was achieved.
3. Unincorporated nucleotides were removed from the DNA by spin-column chromatography through Sephadex G-50, as described by Sambrook *et al.* (1989).
4. Salmon testes DNA (100 µl) and TNES (100 µl) were added to the probe which was then boiled for five minutes and immediately used for hybridization.

Salmon testes DNA (Sigma D1626) was prepared as described by Sambrook *et al.* (1989).
TNES was 10 mM NaCl, 10 mM Tris-HCl pH 8.0, 2 mM EDTA, 0.1% SDS.

6.15 End-labeling of oligonucleotide probes

Oligonucleotide probes were radioactively end-labeled with [$\gamma$-$^{32}$P]dATP using the following protocol:

1. The following components were combined (on ice): about 15 pmol oligonucleotide, 2 µl 10x kinase buffer, 10 U T4 polynucleotide kinase, 4 µl [$\gamma$-$^{32}$P]ATP, final volume to 20 µl with water.
2. The reaction was incubated at 37 °C for one hour and the degree of incorporation in a 1 µl sample was estimated by chromatography on PEI strips as for nick-translation except that the solvent was 1.2 M $K_2HPO_4$. The unincorporated nucleotides migrate to the top of the strip and the oligonucleotides stay at the origin. The counts in the top third and the bottom two thirds of the strip were compared. Incorporation was usually above 60%.
3. Salmon sperm DNA (100 µl) and TNES (100 µl) were added to the probe (as above) which was then used for hybridization without further processing.

6.16 Preparation of *S. pombe* chromosomal DNA

Chromosomal DNA from *S. pombe* was prepared as described by Moreno *et al.* (1991) with the following modifications: Zymolyase 60,000 U/mg, rather than 20,000 U/mg, was used (because of availability). The absorbance at 260 nm was found not to be an accurate way of determining the DNA concentration, due to the presence of a contaminant that also absorbed strongly at this wavelength. The DNA concentration was determined by comparing the staining intensity of DNA samples, run on agarose gels, with standards.

6.17 Plasmid vectors

The plasmid vectors used in this study are listed in Table 5

7 TRANSFORMATION WITH PLASMID DNA

7.1 *Schizosaccharomyces pombe* transformation

*S. pombe* strains were transformed using a modification of the lithium chloride procedure described by Bröker (1987).

1. An overnight culture in YES was diluted 1/10 in fresh YES and incubated for three hours.
2. The culture was harvested by centifugation (3000 g for five minutes at room temperature), resuspended in sterile water and recentrifuged, as above. The cells were resuspended in 0.6 ml of Buffer I and incubated at 30 °C for one hour, with gentle shaking.

Table 5

*E. coli* and *S. pombe* plasmid vectors used in this study

| Vector | Type | Selection markers *E. coli/S. pombe* | Reference |
|---|---|---|---|
| pDB262 | *E. coli*/yeast shuttle vector | $tet^r$/LEU2 | Russell (1989) |
| pDB248 | *E. coli*/yeast shuttle vector | $tet^r$ $amp^r$/LEU2 | Russell (1989) |
| pFL20 | *E. coli*/yeast shuttle vector | $tet^r$ $amp^r$/URA3 | Russell (1989) |
| pUC118/119 | *E. coli* phagemid vector | $amp^r$ *lacZ'* | Sambrook *et al.* (1989) |

$tet^r$ tetracycline resistance.

$amp^r$ ampicillin resistance.

LEU2 *Saccharomyces cerevisiae* LEU2 gene, complements *S. pombe leu1* mutants.

URA3 *Saccharomyces cerevisiae* URA3 gene, complements *S. pombe ura4* mutants.

*lacZ'* β-galactosidase gene for differentiation on plates containing X-gal.

3. The cells were dispensed as 200 µl aliquots in 1.5 ml polypropylene microfuge tubes, mixed with 10 µl transforming DNA (0.05 - 2 µg DNA) and 8 µl carrier DNA and incubated at 30 °C for 30 minutes, without shaking.
3. 700 µl of Buffer II was added to the cell suspension and incubated at 30 °C for 30 minutes, without shaking.
4. The tubes were placed in a water bath at 46 °C for 25 minutes.
5. Tubes were gently microfuged, without allowing the microfuge to attain full speed, for one minute. The supernatant was discarded and the cells were resuspended in a convenient volume of 0.9% NaCl (0.5 to 10 ml).
6. 50 - 100 µl aliquots of the transformed cells were spread onto pre-dried selective agar plates (usually MM without the appropriate supplement).

A negative control and a positive control were done for all transformations. Transforming DNA was ommitted in the negative controls, but carrier DNA and all other components were still included. DNA of a previously determined transforming ability was used for positive controls.

Carrier DNA for *S. pombe* transformations was prepared as follows: DNA from herring testes (Sigma D-6898) was dissolved in TE (pH 8.0) to a concentration of 5 mg/ml and sonicated for three minutes using a Soniprep 150 apparatus (MSE) with the 3 mm probe at amplitude 10 and then vortexed vigorously. Fragment sizes, measured by agarose electrophoresis, were about 2 - 15 kb. The DNA was placed in a boiling water bath for ten minutes, cooled in an ice bath and frozen at -20 °C. The DNA was periodically reheated to ensure it was single-stranded.

Buffer I: 20 mM Tris-HCl pH 7.5, 2 mM EDTA, 0.2 M LiCl, freshly prepared using Tris and EDTA stock solutions and filter sterilized.

Buffer II: 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1 M LiCl, 40% polyethylene glycol $M_r$ = 4000, freshly prepared and filter sterilized.

### 7.2 Stability test for *S. pombe* transformants

The plasmid stability test was used to distinguish between transformants containing an autonomously replicating plasmid, transformants containing an integrated plasmid, and revertants of the gene of interest. An autonomously replicating plasmid will be rapidly lost in the absence of selection for the vector marker gene, resulting in some colonies which have lost both the vector marker gene and the cloned gene of interest and other colonies which retain both phenotypes. An integrated plasmid will result in all

colonies retaining both phenotypes. A revertant for the gene of interest will result in colonies which lose the vector gene but retain the phenotype of the gene of interest. A modification of the method described by Moreno *et al.* (1991) was used:

1. An overnight YES culture (10 ml) of a transformant colony was appropriately diluted and plated for isolated colonies on YES plates.
2. The resultant colonies were replica plated onto media to test for the selective vector marker gene and the cloned gene of interest.

### 7.3 Recovering plasmids from *Schizosaccharomyces pombe*

Plasmids were recovered from *S. pombe* using the method described by Moreno *et al.*, (1991) with the following modifications: the density of cultures was determined by counting cells in a hemacytometer, rather than by using the optical density ($OD_{595}$ ); Zymolyase 60,000 U/mg, rather than 20,000 U/mg, was used (because of availability); 100 µl, rather than 50 µl, of cell extract was purified by the Geneclean procedure. The DNA was further concentrated two-fold by precipitation and *E. coli* strain DB1318 was electro-transformed with 2 µl DNA.

### 7.4 Electro-transformation of *E. coli*

#### 7.4.1 Preparation of competent *E. coli* cells for electro-transformation.

*E. coli* cells were prepared, frozen and used for electro-transformation using the protocol described in the manufacturers instruction manual for the Gene Pulser™ apparatus (Bio-Rad):

1. One liter of Luria broth was inoculated with 10 ml of a fresh overnight culture of *E. coli*.
2. The cultures was incubated at 37 °C with shaking at 250 rpm to an optical density of 0.5 at 600 nm.
3. The culture was chilled on ice for 25 minutes and centrifuged at 4000 g for 15 minutes at 4 °C.
4. The cell pellet was resuspended in 1 l of ice-cold sterile water and centrifuged as above.
5. The cell pellet was resuspended in 500 ml of ice-cold sterile water and centrifuged as above.
6. The cell pellet was resuspended in 20 ml of ice-cold sterile 10% (v/v) glycerol and centrifuged as above.
7. The cell pellet was resuspended to a final volume of 2 ml in ice-cold sterile 10% (v/v) glycerol. This resulted in a cell concentration of about 2 x $10^{10}$ cells/ml.
8. The cells were dispensed as 40 µl aliquots into chilled microfuge tubes, snap-frozen in liquid nitrogen and stored at -70 °C.

7.4.2 Electro-transformation procedure

Electro-transformation of *E. coli* was done using the Gene Pulser™ apparatus (Bio-Rad) according to the manufacturers suggested protocol:

1. 40 µl aliquots of electro-competent cells were gently thawed on ice, mixed with 1 to 2 µl of DNA and incubated on ice for about one minute. DNA was in TE buffer or water.

3. The DNA and cells were transfered to a 0.2 cm electro-transformation cuvette (on ice) and then pulsed once at the following settings:
Gene Pulser - 25 µF and 2.5 kV; Pulse Controller - 200 Ω. This produced a pulse with a time constant of 4 - 5 msec.

4. Immediately after pulsing, the cells were resuspended in 1 ml of SOC medium and incubated for one hour at 37 °C with shaking at 225 rpm.

5. Transformed cells were selected by plating on L plates containing the appropriate antibiotic (100 µg/ml ampicillin).

Positive and negative controls were included for all electro-transformations. A known quantity of pUC118 was used for the positive controls and TE was substituted for DNA in the negative controls. The transformation frequencies for *E. coli* strains MC1022 and DB1318 were $6 \times 10^{10}$ and $1 \times 10^{8}$ transformant colonies per µg pUC118, respectively.

## 8 ADDITIONAL BUFFERS, STOCK SOLUTIONS AND NOTES

All solutions were prepared in Milli-Q™ (Millipore Corporation) purified water unless otherwise stated and all % concentrations are w/v unless otherwise stated.

Buffers, reagents and stock solutions not described in other sections are listed below:

### 8.1 Tris stock solutions

1 M Tris stock solutions were prepared and sterilized as described by Sambrook *et al.* (1989). These stock solutions were used to prepare other buffers by appropriate dilution.

### 8.2 Ethylenediaminetetraacetic acid (EDTA, pH 8.0)

EDTA stock solutions were prepared as described by Sambrook *et al.* (1989), except a 0.2 M, rather than 0.5 M, stock was used. These stock solutions were used to prepare other buffers.

8.3 TE (Tris EDTA)

TE was 10 mM Tris-HCl, 1 mM EDTA; prepared by dilution of Tris and EDTA stocks and sterilized by autoclaving for 15 minutes at 121 °C.

# RESULTS

## 1 UREASE ACTIVITY SELECTION METHODS FOR *S. POMBE* STRAINS

Reliable and sensitive methods were required to detect urease activity in *S. pombe* cultures. A screening method able to differentiate between *ure*$^+$ and *ure*$^-$ colonies was also required. Several previously published methods were tested (Seeliger, 1956; Barnett *et al.*, 1990; Booth and Vishniac, 1987; Kinghorn and Fluri, 1984). Indicator plates which differentiated between urease$^+$ and urease$^-$ strains of *S. pombe* were developed.

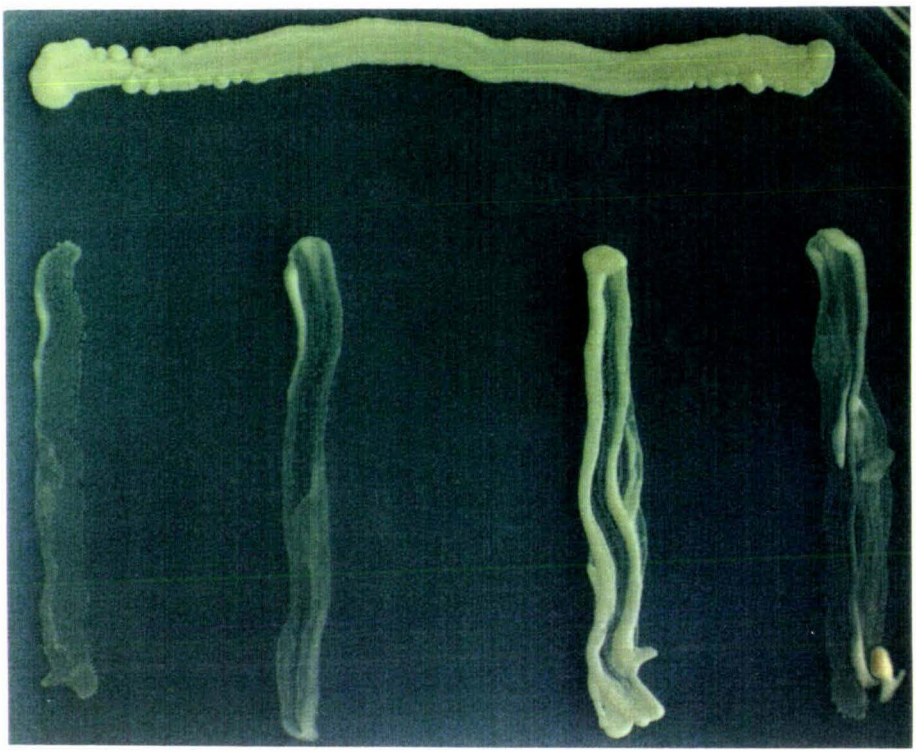
### 1.1 Nitrogen Source Plates

Urease mutants of *S. pombe* are unable to use hypoxanthine or urea as a sole nitrogen source (Kinghorn and Fluri, 1984). The growth of *ure*$^+$ and *ure*$^-$ strains was compared on solid media with ammonium sulfate, hypoxanthine, or urea as the sole nitrogen source. The effect of nutritional supplements, the purity of the media ingredients, the concentration of the nitrogen and carbon sources, and the method of media preparation were also examined.

The growth of the *S. pombe* urease mutants *ure1-1* $h^-$, *ure2-1* $h^-$, *ure2-1* $h^+$, *ure3-1* $h^-$, *ure3-1* $h^+$, *ure4-1* $h^-$ and the *ure*$^+$ strains 122 (*leu1 his2* ), 972 $h^-$, 975 $h^+$ were tested on MINH (2 mM hypoxanthine), MINA (1 mM ammonium sulfate), MINU (2 mM urea), MIN (no nitrogen source). Test media were streaked for single colonies and also were patched. Plates were periodically examined for growth over five days incubation at 30 °C. All strains produced isolated colonies of up to 0.3 mm diameter on MIN and very light growth where patched. No strains grew significantly more on MINU than on MIN. The *ure*$^+$ strains grew well on MINH (colonies up to 2 mm in diameter, heavy growth of patches). All strains grew well on MINA (colonies 2 - 3 mm diameter, heavy growth of patches). The *ure*$^-$ mutants grew poorly on MINH (colonies up to 0.5 mm diameter, very light growth of patches) with the exception of *ure3-1* (Figure 2). Mutant *ure3-1* frequently produced colonies up to 0.75 mm diameter and patches grew almost twice as much as those of the other *ure*$^-$ strains.

The background growth of all strains on MIN and the growth of *ure3-1* on MINH was not reduced by using Noble agar (Difco).

A



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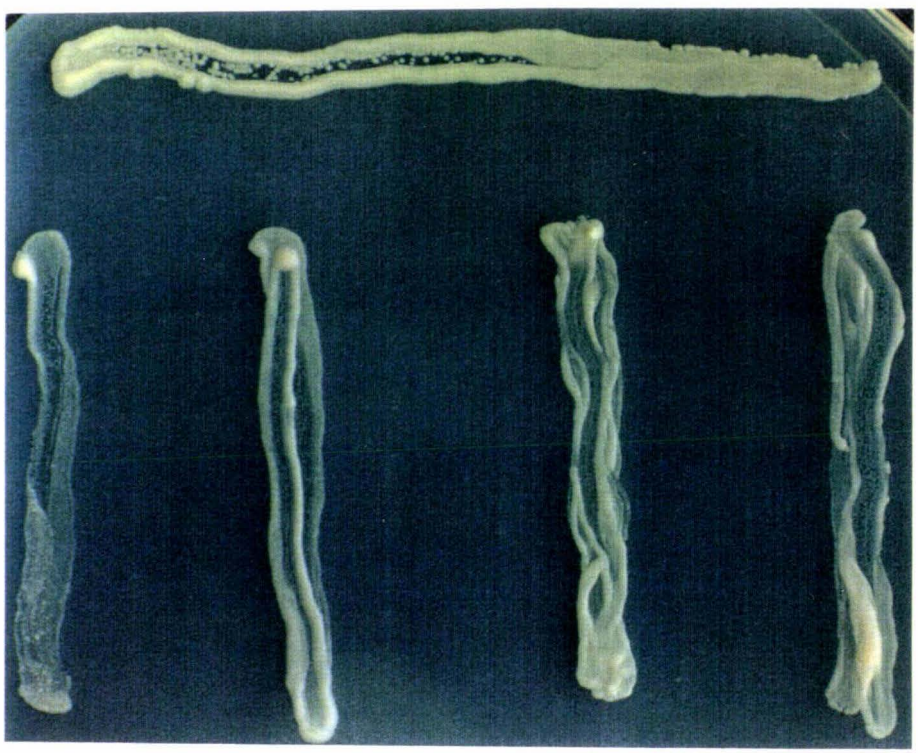


Background growth on MINH was not reduced by adding the hypoxanthine (40 mM stock solution in 0.1 N NaOH, filter sterilized) to the autoclaved and cooled medium, rather than before sterilization.

The minimal medium described by Gutz *et al.* (1974 , MMA) was prepared without any nitrogen source, with 1 mM ammonium sulfate, or with 2 mM hypoxanthine. No decrease of background growth or improvement of strain differentiation was obtained.

The glucose and nitrogen levels of MINA and MINH were increased to 3% and 4 mM, respectively. The differentiation of *ure*$^+$ and *ure*$^-$ strains was not improved.

The effect of amino acid and nucleotide supplements was examined. MIN, MINA, and MINH were prepared with or without 50 µg/ml each of leucine, histidine, and uracil. These supplements were added either before the media were autoclaved, after they were autoclaved and cooled to 50 $^{o}$C, or spread directly onto the surface of the agar plates from stock solutions. The 1% amino acid stock solutions were filter sterilized. The 0.5% uracil stock solution was prepared in 0.1 N NaOH and filter sterilized. In all cases the growth of *ure*$^-$ strains on MINH was slightly increased (Figure 2).

Summary

MINU did not support the growth of wild-type or *ure*$^-$ strains. MINH was useful in distinguishing between wild-type and *ure*$^-$ strains; however, the differentiation was not as good when the nutritional supplements leucine, histidine, and uracil were included in the medium and was not very good for *ure3-1*. Better selection was not obtained by using Noble agar, a different defined medium, adding filter sterilized hypoxanthine to the autoclaved medium, or increasing the glucose and nitrogen source concentrations.

## 1.2 Indicator Media

### 1.2.1 Standard methods

Christensen's urea agar (CUA; 0.1% peptone, 0.1% glucose, 0.5% sodium chloride, 0.2% potassium dihydrogen phosphate, 0.0012% phenol red, 2% urea, 2% agar; Kreger-Van Rij, 1984) or urea R broth (URB; 0.01% yeast extract, 0.0091% monopotassium phosphate, 0.0095% disodium phosphate, 2% urea, 0.001% phenol

red; Barnett *et al.*, 1990) are the media most commonly used to detect urease activity in yeasts. Roberts *et al.* (1978) miniaturized the URB test so that it could be carried out in a 96-well microtiter plate. Booth and Vishniac (1987) used RUH (Table 2), a modified URB, to avoid false positives with the miniaturized test.

*S. pombe ure*$^+$ and *ure*$^-$ strains were tested on CUA slopes, URB (3 ml), and RUH (200 µl in microtiter plate wells). The media were heavily inoculated with cells from fresh cultures on YES plates and incubated as described in the above references.

CUA supplemented with leucine and histidine was inoculated with 122 and LH162 (*ure2-1 leu1 his2*). No color change or growth occurred within 12 days incubation at 30 °C.

Strains 122, *lys1-131*, 972, 975, *ure1-1*, *ure2-1*, *ure3-1*, and *ure4-1*, were tested in RUH. The *ure*$^+$ strains were all strongly positive within two hours incubation. The *ure*$^-$ strains were all still negative after four hours. The medium used for the inoculum growth can affect the RUH test response (Booth and Vishniac, 1987). The strains 972 and *ure3-1* were tested after growth on MM; the RUH response was not affected.

1.2.2 Indicator plates

A medium was required that could be used to test for *ure*$^+$ transformants of *ure*$^-$ strains. The medium must not support the growth of *leu1* or *ura4* strains because the *S. pombe* vectors used for gene bank construction use *leu1* or *ura4* complementation for the selection of transformants (Moreno *et al.*,1991).

An agar medium based on URB with the following modifications was made: 0.67% yeast nitrogen base without amino acids (Difco) was used instead of yeast extract, 2% agar and 0.003% phenol red were used, urea (2%) was added to the autoclaved medium from a 20% stock solution (filter-sterilized). Nutritional supplements were included as required. The strains *ura4-D18*, 122, *lys1-131*, *ade6-704* were streaked onto the medium in plates. No strain grew sufficiently well for this medium to be used for differentiation of strains.

The inhibitory affects of phenol red and urea were examined. URB agar plates were prepared as above (0.003% phenol red, 2% urea), with various concentrations of urea (between 0 - 1.5%), or without phenol red and urea. The plates were streaked as

above for single colonies, incubated for four days at 30 °C and examined for growth (Figure 3). Phenol red did not significantly inhibit growth. Urea inhibited growth in a strain-dependent manner at levels as low as 0.1%.

Benson and Howe (1978) flooded agar plates with a phenol red + urea solution to detect urease activity in *Neurospora crassa* colonies. This technique was applied to *S. pombe* 122 colonies on YES and MM plates. Within 20 minutes at 30 °C or 37 °C colonies on YES had turned red; however, no color change occurred with colonies on MM plates, even after several hours incubation.

Colonies of 122, LH162, and mixtures of the two strains, were grown on YES plates and overlaid with a molten RUH + agar solution. The RUH overlay was prepared using single strength or double strength RUH with 0.5% or 2% agar, and single strength RUH containing 6% urea and 0.5% agar. Overlaid plates were incubated at 20 °C, 30 °C, or 37 °C. A color difference between the two strains was noted; however, it was too diffuse to allow differentiation of colonies that were within 1 cm of each other.

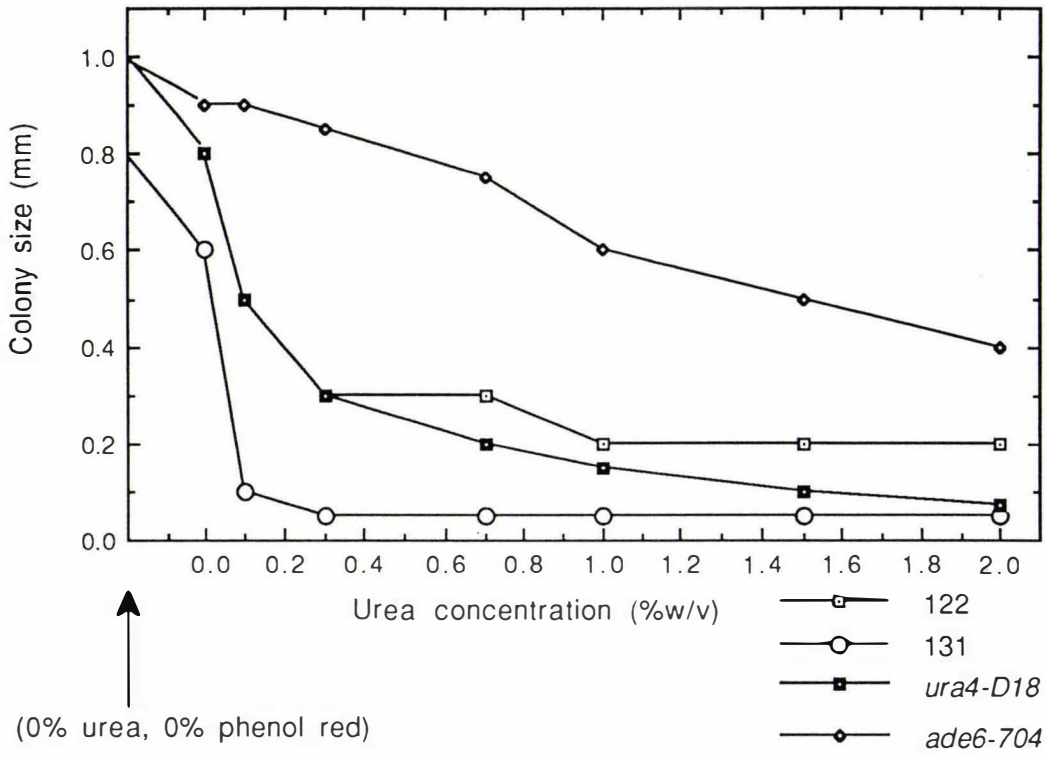
*S. pombe* 122 colonies on YES were overlaid with a molten 2% urea + 0.5% agar solution, incubated at 18 - 22 °C for five minutes, spread with the PN and AH reagents used for ammonia determination (Creno *et. al*, 1970) and incubated at 37 °C for several hours. No color change occurred around any colonies.

Indicator media with different indicator dyes and various concentrations of glucose and urea were tested for growth and color differentiation (Table 6). Cellulose was included in medium A to limit the diffusion of acidic and basic products (Cavin *et al.*, 1989). The medium was streaked for single colonies and patched with *ure2-1*, 972, and a mixture of the two strains. The growth of both strains and the color differentiation were poor.

Media B, C, D, IHG and ILG were each inoculated as above and also spread with a mixture of *ure2-1* and 972 cells at about 100:10 and 200:10 colonies per plate (*ure2-1*:972). Similar mixtures of cells were also spread onto YES and the resultant colonies replica plated onto ILG, IHG, MINH and MINA. All plates were incubated at 30 °C and examined periodically over ten days. IHG gave the best growth and color differentiation for spread plates. The *ure*$^+$ colonies could be easily identified after five days (Figure 4 A); however, differentiation of replica plated colonies was poor. Growth on ILG was poor but the color differentiation of patches and replica plated colonies was very good (Figure 4B). Provided that the ratio of *ure*$^-$ colonies:*ure*$^+$

Figure 3

Effect of urea and phenol red on *S. pombe* growth




Indicator media with various concentrations of urea were streaked with *S. pombe* strains 122, 131, *ura4-D18*, and *ade6-704*. The colony size was examined after four days incubation at 30 °C. Data for the medium without urea or phenol red are marked with an arrow.

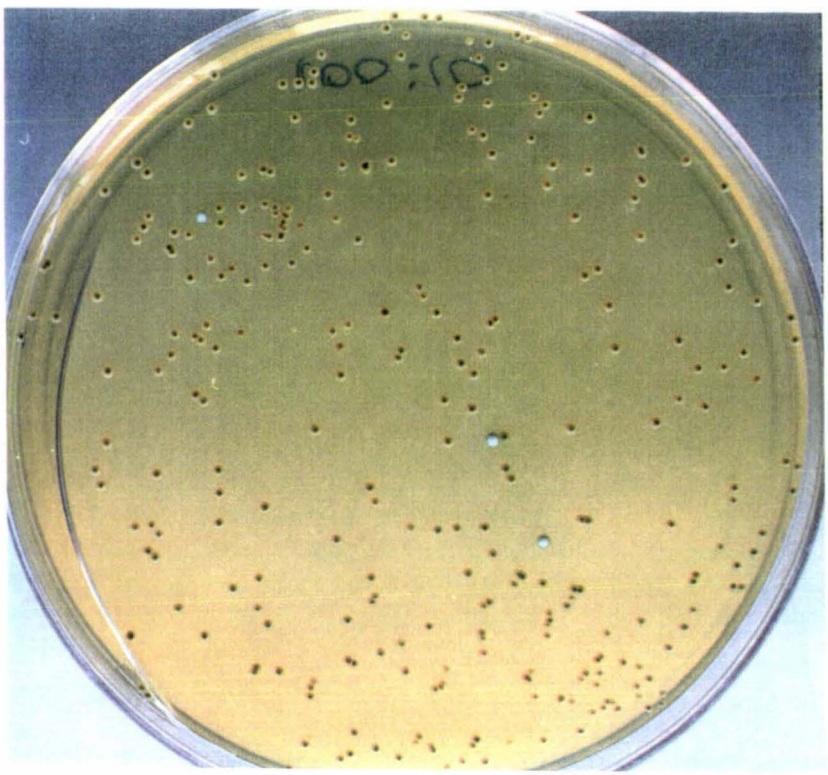
Table 6

Urease Indicator Plates.

| Medium | Glucose | Urea | Dye |
|---|---|---|---|
| A | 1.0% | 0.1% | BCG 0.01% |
| B | 1.0% | 0.1% | BCG 0.0022% |
| C | 0.1% | 0.1% | BCG 0.0022% |
| D | 1.0% | 0.2% | PR 0.003% |
| E | 0.2% | 0.2% | PR 0.003% |
| IHG | 1.0% | 0.2% | BCG 0.0022% |
| ILG | 0.2% | 0.2% | BCG 0.0022% |

All percentages are w/v. Urea was added to autoclaved media from 10% filter sterilized stock solution. Medium A contained 10% cellulose powder and 2% agar. All other media contained 3% agar. All media contained 1 mM ammonium sulfate, 0.17% yeast nitrogen base without amino acids or ammonium sulfate (Difco), and the pH was adjusted to pH 4.5 with 0.1 N HCl. BCG = bromocresol green, PR = phenol red.

A



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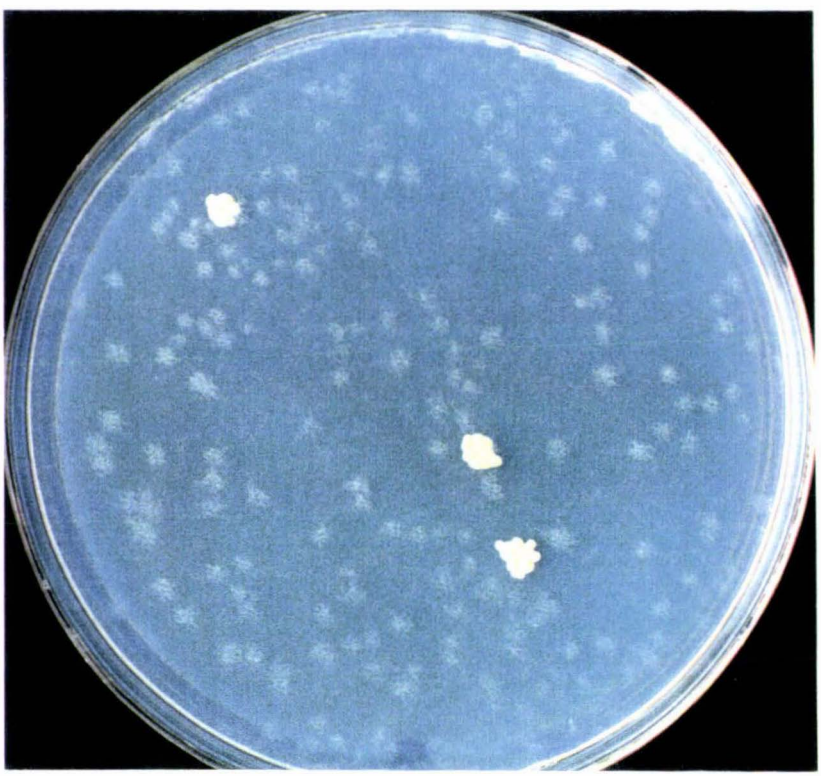

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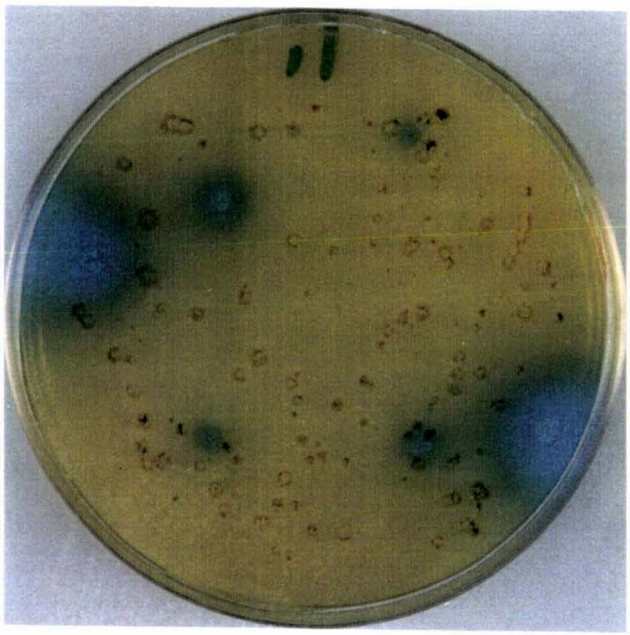

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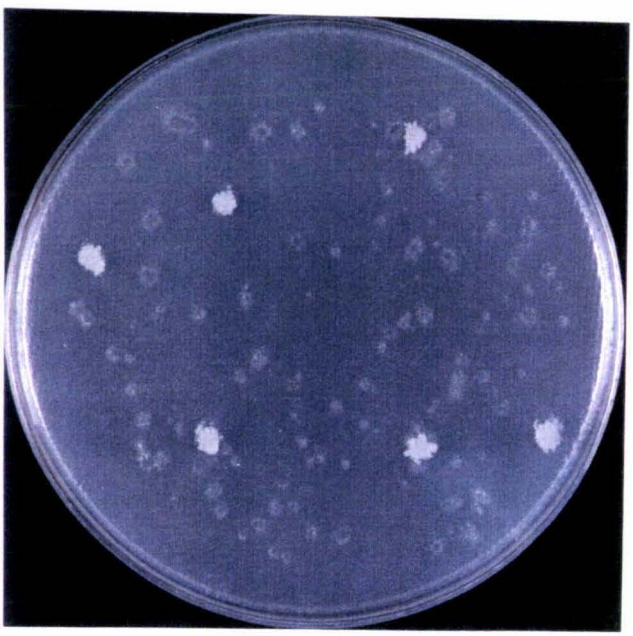

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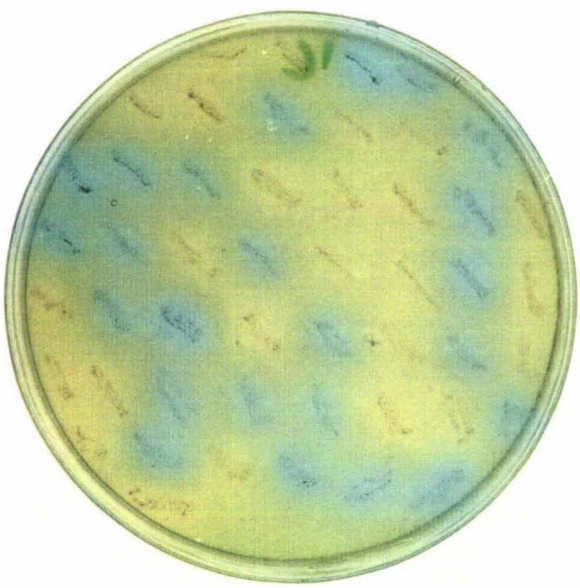


colonies was > 3:1, urease activity could be correctly scored on ILG and IHG for up to 200 colonies per plate. Differentiation of urease activity was not possible on any medium when the different strains were streaked as a mixture. When urea was omitted from the media no color differentiation occurred.

## Summary

Urease$^+$ colonies could easily be identified among a background of urease$^-$ colonies by replica plating onto ILG or by spreading IHG plates. MINH could also be used to identify urease$^+$ colonies by replica plating or streaking; however, nutritional supplements and the background growth of strain *ure3-1* decreased the clarity of differentiation. MINH could be used with higher colony densities and higher ratios of *ure*$^+$ to *ure*$^-$ colonies than the indicator plates.

## 2 CHARACTERIZATION OF THE UREASE GENES

The urease mutants used in the present study were characterized and the *ure* genes were genetically mapped.

### 2.1 Characterization of *ure* mutants

The *ure* mutant strains used in this study were tested for their complementation behavior, urease activity of cell-free extracts, and response to nickel and manganese.

#### 2.1.1 Urease mutant complementation groups

The ability of the different *ure*$^-$ strains to form prototrophic progeny when crossed to each other was tested to confirm the strains belonged to the four complementation groups described by Kinghorn and Fluri (1984). Reciprocal crosses were carried out between *ure1-1*, *ure2-1*, *ure3-1* and *ure4-1* strains in all 16 possible combinations. Each cross was examined microscopically for the presence of asci, streaked onto MINH, and examined for growth after incubation. Growth on MINH was assumed to mean that the mutations were non-allelic and, therefore, belonged to different complementation groups. The growth patterns showed that each *ure* genotype represented a separate complementation group (Table 7).

#### 2.1.2 The urease activity of cell-free extracts

Permeabilized-cell suspensions of *ure* mutant strains have no urease activity (Kinghorn and Fluri, 1984). The urease activity of cell-free extracts of *ure*$^-$ strains was tested to confirm the absence of an active urease. Crude extracts of *S. pombe* 972, *ure1-1* $h^-$, *ure2-1* $h^-$, *ure3-1* $h^-$ and *ure4-1* $h^-$ were assayed for urease activity. No activity was detected in any of the *ure*$^-$ extracts and high activity was detected in the 972 extract.

#### 2.1.3 Effect of nickel and manganese on urease activity

The addition of nickel, or nickel and manganese to the growth medium can restore urease activity in a *ure*$^-$ mutant of *Aspergillus nidulans* (Mackay and Pateman, 1980) and increase urease activity in wild-type *Lactobacillus fermentum* (Kakimoto *et al.*, 1990). MINH plates with 0%, 0.0005%, 0.005% or 0.05% each of nickel

Table 7

Complementation of *ure*$^-$ strains

| Crossed strains | | |
|---|---|---|
| $h^-$ strain | $h^+$ strain | Growth on MINH |
| *ure1-1* | *ure1-1* | - |
| *ure1-1* | *ure2-1* | + |
| *ure1-1* | *ure3-1* | + |
| *ure1-1* | *ure4-1* | + |
| *ure2-1* | *ure1-1* | + |
| *ure2-1* | *ure2-1* | - |
| *ure2-1* | *ure3-1* | + |
| *ure2-1* | *ure4-1* | + |
| *ure3-1* | *ure1-1* | + |
| *ure3-1* | *ure2-1* | + |
| *ure3-1* | *ure3-1* | - |
| *ure3-1* | *ure4-1* | + |
| *ure4-1* | *ure1-1* | + |
| *ure4-1* | *ure2-1* | + |
| *ure4-1* | *ure3-1* | + |
| *ure4-1* | *ure4-1* | - |

*ure*$^-$ strains were reciprocally crossed on MEA plates for two days at 25 $^{o}$C. Each cross was then streaked onto MINH and examined for growth after five days incubation at 30 $^{o}$C.

\+ Growth

\- No growth

sulfate tetrahydrate and manganese sulfate were patched with 972, *ure1-1 h⁻*, *ure2-1 h⁻*, *ure3-1 h⁻* and *ure4-1 h⁻*. No improvement of growth was noted with any of the *ure⁻* strains and the growth of 972 was inhibited at the highest nickel and manganese concentration (Figure 5).

## 2.2 Chromosome assignment and linkage relationships of *ure1*, *ure2*, *ure3*, and *ure4*

The *ure1*, *ure2*, *ure3*, and *ure4* genes were genetically mapped by induced haploidization and meiotic recombination.

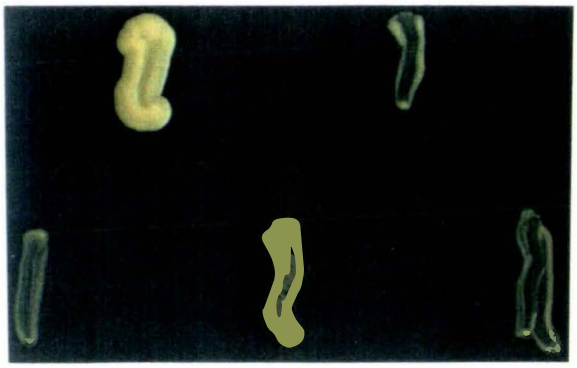
### 2.2.1 Linkage to *lys1*, the *mat* locus, or a centromere.

The strains *ure1-1 h⁺*, *ure2-1 h⁺*, *ure3-1 h⁺*, and *ure4-1 h⁺* were each crossed with *lysI-131 h⁻* to obtain the *ure⁻ lys1⁻ h⁻* mutant required for the induced haploidization step. At least 25 tetrads from each cross were also analyzed for potential linkage of the *ure* gene to the *mat* locus, *lysI*, or a centromere. Linkage to *lys1* or *mat* was assumed if parental ditype (PD) tetrads significantly exceeded non-parental ditype (NPD) tetrads. Linkage to a centromere was determined by examining the ascus type ratios of each *ure* gene relative to *lys1*. *lys1* is closely linked to the centromere of chromosome I. If *lys1* and the *ure* gene were not linked, and the ratio of tetratype (T) asci to PD and NPD asci was significantly lower than 4:1:1 (T:PD:NPD), then linkage of the *ure* gene to a centromere was assumed and the approximate gene to centromere distance was taken to equal the tetratype frequency. The following potential linkages were revealed by the above analyses: *ure1* - a centromere, and *ure4* - *lys1*. Very weak potential linkages, not significant at the 10% level, were found for *ure3* - *lys1* and *ure2* - centromere (Table 8).

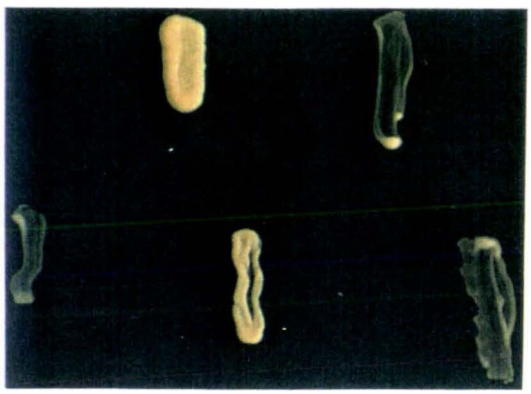
### 2.2.2 Assignment to linkage groups by induced haploidization

Each of the four *ure* genes was assigned to a chromosome by induced haploidization (Kohli *et al.*, 1977). Stable, diploid *S. pombe* strains can be induced to haploidize, without undergoing meiotic divisions, by growth in the presence of m-fluorophenylalanine. The haploid segregants are identified by their color (light pink) on YEP plates. The segregation patterns of the gene of interest and marker genes on each chromosome can be used to assign the gene of interest to a specific chromosome. Stable diploid strains were obtained by crossing *lys1 ure⁻ h⁻* strains with 2173 (*mat2-102 ade6 ura1 his3*). Diploid colonies (red on MINAP) were haploidized on FPA. The haploid segregants were tested for the *lys1* (chromosome I), *ura1*

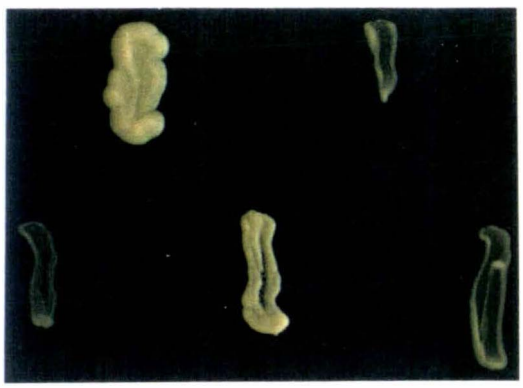
A



B



C



D

Table 8

Analysis of linkage of *ure* genes to *lys1*, the *mat* locus, or a centromere

| Gene pair | Number of tetrads[a] PD | NPD | T | Linkage between genes[b] | Linkage to centromere[b] |
|---|---|---|---|---|---|
| *ure1* *lys1* | 7 | 7 | 12 | PD = NPD, no linkage | $\chi^2$ = 4.9<br>0.05 < *P* < 0.1<br>potential linkage 46 cM |
| *ure1* *mat* | 4 | 3 | 19 | $\chi^2$ = 0.14<br>*P* > 0.2, no linkage | |
| *ure2* *lys1* | 5 | 8 | 17 | PD < NPD, no linkage | $\chi^2$ = 2.25<br>0.1< *P* < 0.2<br>potential weak linkage |
| *ure2* *mat* | 4 | 8 | 16 | PD < NPD, no linkage | |
| *ure3* *lys1* | 18 | 10 | 29 | $\chi^2$=2.29<br>0.1 <*P* < 0.2<br>potential weak linkage | (potential linkage to *lys1* established) |
| *ure3* *mat* | 13 | 8 | 35 | $\chi^2$ = 1.19, *P* > 0.2,<br>no linkage | |
| *ure4* *lys1* | 9 | 3 | 24 | $\chi$2 = 3.<br>0.05 < *P* < 0.10<br>potential linkage | (potential linkage to *lys1* established) |
| *ure4* *mat* | 8 | 8 | 20 | PD = NPD, no linkage | |

[a] Data are from crosses between the *ure* strain and *lys1-131* $h^-$

[b] The principles of the analyses are outlined in the text and in Materials and Methods 3.4.1 and 3.4.2.

(chromosome I), *his3* (chromosome II), *ade6* (chromosome III), and *ure* phenotypes. The data were analyzed as described in Materials and Methods 3.5. The 2 x 2 contingency test (Chatfield, 1983) was used to confirm independent or joint segregation of two markers. *ure1* segregated with *ade6* and, therefore, was assigned to chromosome III; *ure2, ure3,* and *ure4* all segregated with *lys1* and *ura1* and, therefore, were assigned to chromosome I (Tables 9 and 10).

### 2.2.3 Mapping by meiotic recombination

Once the *ure* genes were assigned to their respective chromosomes, each *ure*$^-$ strain was crossed with a mapping strain having marker genes on the appropriate chromosome. *ure1* was crossed with 2171 (chromosome III; centromere linked markers *ade6* and *fur1* ) and with *ade6-704*; *ure2, ure3,* and *ure4* were each crossed with 2143 (chromosome I; *ade4 lys1 ura2*). Asci were dissected and tetrads producing three or four viable colonies were tested and designated T, PD or NPD. Tetrads with less than three viable spores cannot be scored. The viability of spores for each cross is presented in Table 11. A significant excess of PD over NPD tetrads indicated linkage of two markers and confirmed the chromosome assignments established by induced haploidization. Map distances were calculated using Perkins' (1949) formula:

$$Xp = 50(T+6NPD)/(PD+NPD+T)$$

These map distances were corrected for multiple crossovers by using a conversion table (Munz *et al.*, 1989). The data from the *ure*$^-$ x *lys1-131* crosses (above) were included in the analysis, as appropriate. Interference was assumed to be absent. *ure1* was mapped 32 cM from *fur1* and 50 cM from *ade6*; *ure2* was mapped 69 cM from *ura2* and 100 cM from *ade4*; *ure3* was mapped 31 cM from *ade4* and 91 cM from *ura2*; and *ure4* was mapped 100 cM from *lys1* and an even greater distance from *ura2* (Table 12).

Table 9

Observed numbers of the genotypes among the haploid segregants from induced haploidization.

| | | [a]I | | [a]II | [a]III | | [b]Observed number | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Genotype | | *lys1* | *ura1* | *his3* | *ade6* | *ure* | *ure1* | *ure2* | *ure3* | *ure4* |
| 1 | | + | - | + | + | + | 1 | 79 | 53 | 103 |
| 2 | | + | - | + | + | - | 78 | 0 | 0 | 0 |
| 3 | | + | - | + | - | + | 114 | 47 | 67 | 98 |
| 4 | | + | - | + | - | - | 0 | 0 | 0 | 0 |
| 5 | | + | - | - | + | + | 0 | 15 | 22 | 0 |
| 6 | | + | - | - | + | - | 0 | 0 | 0 | 0 |
| 7 | | + | - | - | - | + | 0 | 16 | 29 | 1 |
| 8 | | + | - | - | - | - | 0 | 0 | 0 | 0 |
| 9 | | - | + | + | + | + | 0 | 1 | 0 | 0 |
| 10 | | - | + | + | + | - | 6 | 11 | 1 | 2 |
| 11 | | - | + | + | - | + | 3 | 0 | 0 | 0 |
| 12 | | - | + | + | - | - | 0 | 9 | 1 | 4 |
| 13 | | - | + | - | + | + | 0 | 0 | 0 | 0 |
| 14 | | - | + | - | + | - | 0 | 18 | 25 | 0 |
| 15 | | - | + | - | - | + | 0 | 0 | 0 | 0 |
| 16 | | - | + | - | - | - | 0 | 10 | 9 | 0 |
| [c]Frequency | *ure1* | 93.2 | 6.8 | 100 | 41.1 | 59.4 | 207 | 206 | 206 | 208 |
| of wild- | *ure2* | 76.2 | 23.8 | 71.4 | 60.2 | 76.7 | (Total number of | | | |
| type | *ure3* | 83.0 | 17.0 | 59.2 | 49.0 | 83.0 | haploid segregants | | | |
| alleles (%) | *ure4* | 97.1 | 2.9 | 99.5 | 50.5 | 97.1 | analyzed) | | | |

[a] I, II, and III refer to the chromosome the listed markers are located on.

[b] The observed number of each genotype (1 - 16) among the haploid segregants is listed for each of the four *ure* genes analyzed by induced haploidization.

[c] The frequency of haploid segregants for the indicated *ure* gene data that are wild type for the marker gene (*lys1*, *ura1*, *his3*, or *ade6*) or the *ure* gene, as indicated.

Table 10

Evaluation of haploidization data[a]

Assignment of *ure1* to one of the defined linkage groups

* = Parental genotypes

| | | *lys1/ura1* | | | |
|---|---|---|---|---|---|
| | | + / - | - / + | Total | |
| *ure1* | + | 115* | 8 | 123 | $\chi^2$ = 0.031 |
| | - | 78 | 6* | 84 | $P > 0.10$ |
| | total | 193 | 14 | 207 | No linkage |
| | | *his3* | | | |
| | | - | + | Total | |
| *ure1* | + | 0* | 123 | 123 | Parental and |
| | - | 0 | 84* | 84 | recombinant |
| | total | 0 | 207 | 207 | class absent. |
| | | | | | No linkage |
| | | *ade6* | | | |
| | | - | + | Total | |
| *ure1* | + | 122* | 1 | 123 | $\chi^2$ = 203 |
| | - | 0 | 84* | 84 | $P < 0.005$ |
| | total | 122 | 85 | 207 | Linkage |

[a] Data are the number of haploid segregants in each of the four possible genotype classes

Table 10 cont.

Assignment of *ure2* to one of the defined linkage groups

* = Parental genotypes

| | | *lys1/ura1* | | | |
|---|---|---|---|---|---|
| | | + / - | - / + | Total | |
| *ure2* | + | 157* | 1 | 158 | $\chi^2$ = 205 |
| | - | 0 | 48* | 48 | $P < 0.005$ |
| | total | 157 | 49 | 206 | Linkage |
| | | *his3* | | | |
| | | - | + | Total | |
| *ure2* | + | 31* | 127 | 158 | Excess of one |
| | - | 28 | 20* | 48 | recombinant |
| | total | 59 | 147 | 206 | class, similar |
| | | | | | numbers of other |
| | | | | | classes. |
| | | | | | No linkage |
| | | *ade6* | | | |
| | | - | + | Total | |
| *ure2* | + | 63* | 95 | 158 | $\chi^2$ = 0.001 |
| | - | 19 | 29* | 48 | $P > 0.10$ |
| | total | 82 | 124 | 206 | No linkage |

Table 10 cont.

Assignment of *ure3* to one of the defined linkage groups

* = Parental genotypes

| | | *lys1/ura1* | | | |
|---|---|---|---|---|---|
| | | + / - | - / + | Total | |
| *ure3* | + | 171* | 0 | 171 | $\chi^2$ = 207 |
| | - | 0 | 36* | 36 | $P < 0.005$ |
| | total | 171 | 36 | 207 | Linkage |
| | | *his3* | | | |
| | | - | + | Total | |
| *ure3* | + | 51* | 120 | 171 | Excess of one recombinant class, low number of one parental class. No linkage |
| | - | 34 | 2* | 36 | |
| | total | 85 | 122 | 207 | |
| | | *ade6* | | | |
| | | - | + | Total | |
| *ure3* | + | 96* | 75 | 171 | Excess of one parental and one recombinant class. No linkage |
| | - | 10 | 26* | 36 | |
| | total | 106 | 101 | 207 | |

Table 10 cont.

Assignment of *ure4* to one of the defined linkage groups

* = Parental genotypes

| | | *lys1/ura1* | | | |
|---|---|---|---|---|---|
| | | + / - | - / + | Total | |
| *ure4* | + | 202* | 0 | 202 | $\chi^2$ = 208 |
| | - | 0 | 6* | 6 | $P < 0.005$ |
| | total | 202 | 6 | 208 | Linkage |
| | | *his3* | | | |
| | | - | + | Total | |
| *ure4* | + | 1* | 201 | 202 | Excess of one |
| | - | 0 | 6* | 6 | class, which is |
| | total | 1 | 207 | 208 | recombinant. |
| | | | | | Probably no linkage |
| | | *ade6* | | | |
| | | - | + | Total | |
| *ure4* | + | 99* | 103 | 202 | $\chi^2$ = 0.95 |
| | - | 4 | 2* | 6 | $P > 0.10$ |
| | total | 103 | 105 | 208 | No linkage |

Table 11

Fractional viability of spore tetrads used for the linkage studies

| Cross | Fractional viability[a] 4/4 | 3/4 | 2/4 | 1/4 | 0/4 | Unscoreable tetrads[b] (%) |
|---|---|---|---|---|---|---|
| *ure1-1* $h^-$ x *ade6 704* $h^+$ | 40 | 9 | 4 | 0 | 3 | 13 |
| XK1-1X $h^-$ x 2171 | 25 | 17 | 7 | 1 | 0 | 16 |
| *ure2-1* $h^+$ x *lys1-131* $h^-$ | 26 | 6 | 3 | 0 | 0 | 9 |
| *ure2-1* $h^-$ x 2143 | 30 | 13 | 6 | 2 | 2 | 19 |
| *ure3-1* $h^-$ x 2143 | 22 | 19 | 18 | 5 | 1 | 37 |
| *ure3-1* $h^+$ x *lys1-131* $h^-$ | 43 | 15 | 7 | 2 | 0 | 13 |
| *ure4-1* $h^+$ x *lys1-131* $h^-$ | 30 | 6 | 2 | 0 | 0 | 5 |
| *ure4-1* $h^-$ x 2143 | 47 | 16 | 7 | 1 | 3 | 15 |

[a] Fractional viability is the proportion of spores in each successfully dissected ascus that germinated to form a colony.

[b] Unscoreable tetrads is the proportion of tetrads with less than three or four viable spores.

Table 12

Linkage Relationships

| Gene Pair | Cross | Number of tetrads PD | NPD | T | Map distance (cM) $^{a}x_p$ | $^{b}x$ |
|---|---|---|---|---|---|---|
| *ure1 - fur1* | XK1-1X $h^-$ x 2171 | 27 | 2 | 13 | 29.8 | 32 |
| *ure1 - ade6* | *ure1-1* $h^+$ x *ade6-704* $h^-$ | 26 | 3 | 20 | | |
| | XK1-1X $h^-$ x 2171 | 16 | 3 | 23 | | |
| | Total = | 42 | 6 | 43 | 43.4 | 50 |
| *ure2 - ura2* | *ure2-1* $h^-$ x 2143 | 16 | 4 | 23 | 54.7 | 69 |
| *ure2 - ade4* | *ure2-1* $h^-$ x 2143 | 11 | 5 | 25 | *67 | *100 |
| *ure2 - lys1* | *ure2-1* $h^+$ x *lys1-131* $h^-$ | 5 | 8 | 17 | | |
| | *ure2-1* $h^-$ x 2143 | 11 | 7 | 25 | | |
| | Total= | 16 | 15 | 42 | *90 | *>200 |
| *ure3 - ade4* | *ure3-1* $h^-$ x 2143 | 17 | 0 | 24 | 29.3 | 31 |
| *ure3 - ura2* | *ure3-1* $h^-$ x 2143 | 9 | 4 | 28 | *63.4 | *91 |
| *ure3 - lys1* | *ure3-1* $h^+$ x *lys1-131* $h^-$ | 18 | 10 | 29 | | |
| | *ure3-1* $h^-$ x 2143 | 7 | 8 | 27 | | |
| | Total= | 25 | 18 | 56 | *82.8 | *>200 |
| *ure4 - lys1* | *ure4-1* $h^+$ x *lysI-131* $h^-$ | 9 | 3 | 24 | | |
| | *ure4-1* $h^-$ x 2143 | 13 | 8 | 40 | | |
| | Total= | 22 | 11 | 64 | †67.0 | †100 |
| *ure4 - ura2* | *ure4-1* $h^-$ x 2143 | 10 | 7 | 43 | *70.8 | *115 |
| *ure4 - ade4* | *ure4-1* $h^-$ x 2143 | 9 | 8 | 44 | *75.4 | *135 |

$^{a}x_p$ = map distance (cM) calculated using Perkins (1949) formula. $^{b}x$ = map distance (cM) corrected for multiple crossovers (Munz *et al.*, 1989). PD tetrads significantly exceed NPD tetrads at the 1% level, unless marked. If marked (*), $P > 0.1$ and if marked (†), $0.1 > P > 0.05$. See text for details.

# 3 *S. POMBE* UREASE PURIFICATION AND CHARACTERIZATION

Factors which could affect urease assays and the yield of urease in crude extracts were investigated. *S. pombe* urease was purified to homogeneity, characterized, and partially sequenced. Kinetic, structural, and sequence data were obtained for comparison with ureases from other sources.

## 3.1 Factors affecting the urease activity assay

The urease assay used was the method described by Wong and Shobe (1974). This method was originally used for measuring the activity of jack bean and *Morganella* (*Proteus*) *morganii* urease . The appropriate urea concentration and incubation time for measuring *S. pombe* urease activity were determined in the present study. Potential urease inhibitors were also investigated.

### 3.1.1 Urea concentration of the substrate buffer

The urea saturation level of *S. pombe* urease was determined to ensure assays were not carried out at urea concentrations that would limit the reaction rate.

Substrate buffers (0.02 M phosphate, pH 7.0) containing various concentrations of urea were used to assay the urease activity of a *S. pombe* 972 crude extract. The substrate buffers contained no EDTA, 2-mercaptoethanol or sodium azide. All other conditions of the standard assay remained the same. Saturation of the urease was achieved at 25 mM urea (Figure 6). A urea concentration of 50 mM was used for subsequent substrate buffers.

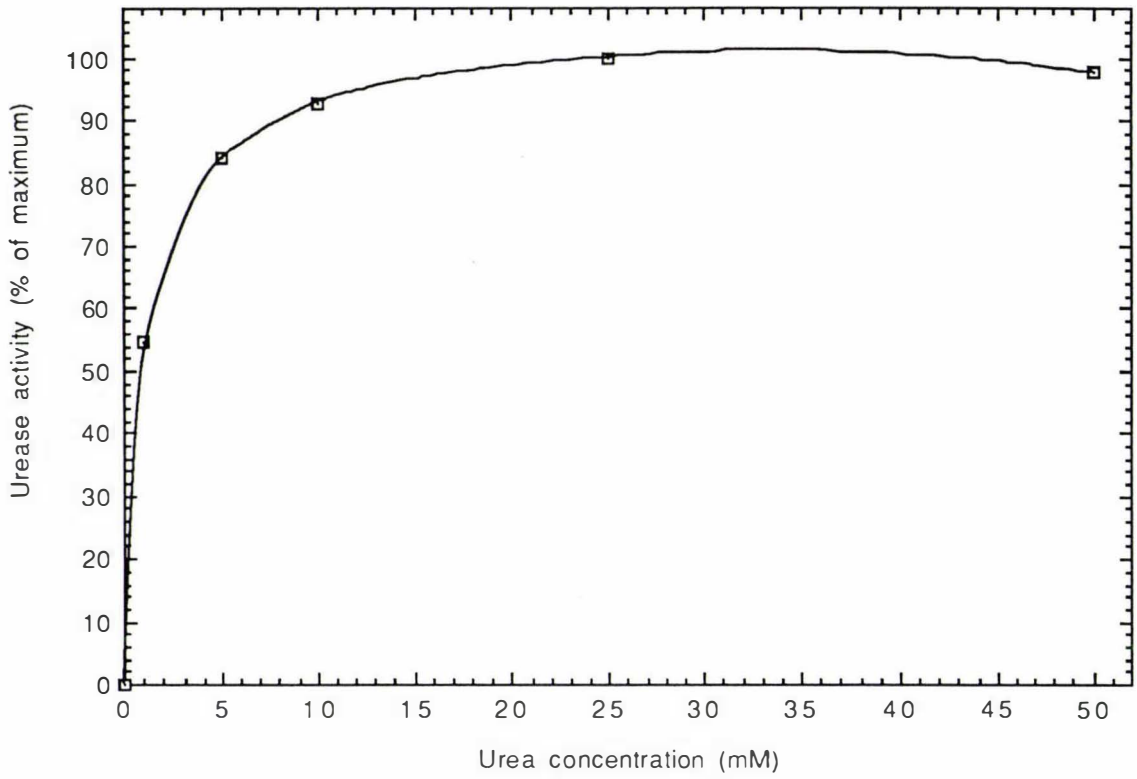
### 3.1.2 Urease assay incubation time

The incubation time used for the urease assay was examined. Two minutes was the standard length of incubation used by Wong and Shobe (1974). Various incubation times were tested to determine if *S. pombe* urease activity was constant over two minutes.

Aliquots of a *S. pombe* 972 crude extract were incubated in substrate buffer and the reaction stopped by the addition of PN reagent after 0, 30, 60, 90, or 120 seconds incubation. The amount of ammonia released by urea hydrolysis was determined.

Figure 6

Urea saturation curve of *S. pombe* urease.




A crude extract of *S. pombe* urease was assayed at urea concentrations of 0, 1, 5, 10, 25, and 50 mM urea in 0.02 M potassium phosphate buffer, pH 7.0.

The reaction rate was constant over two minutes (Figure 7). Two minutes incubation was used for subsequent urease assays.

### 3.1.3 Effect of 2-mercaptoethanol, EDTA, and sodium azide on urease activity

EDTA and 2-mercaptoethanol increase the stability of ureases but may also be inhibitors of urease activity (Mobley and Hausinger, 1989). Sodium azide (0.02%) is often added to buffers as a preservative (Scopes, 1987). The effect of including 1 mM EDTA, 1 mM 2-mercaptoethanol, and 0.02% sodium azide in the substrate buffer was tested. The urease activity of a 972 crude extract was assayed in the usual substrate buffer with the following modifications: a) no EDTA, 2-mercaptoethanol and sodium azide; b) no sodium azide; or c) all components included. Similar levels of activity were observed in all buffers. Sodium azide (0.02%), EDTA (1 mM) and 2-mercaptoethanol (1 mM) were added to extraction and substrate buffers in subsequent experiments.

## 3.2 Preparation of the crude extract

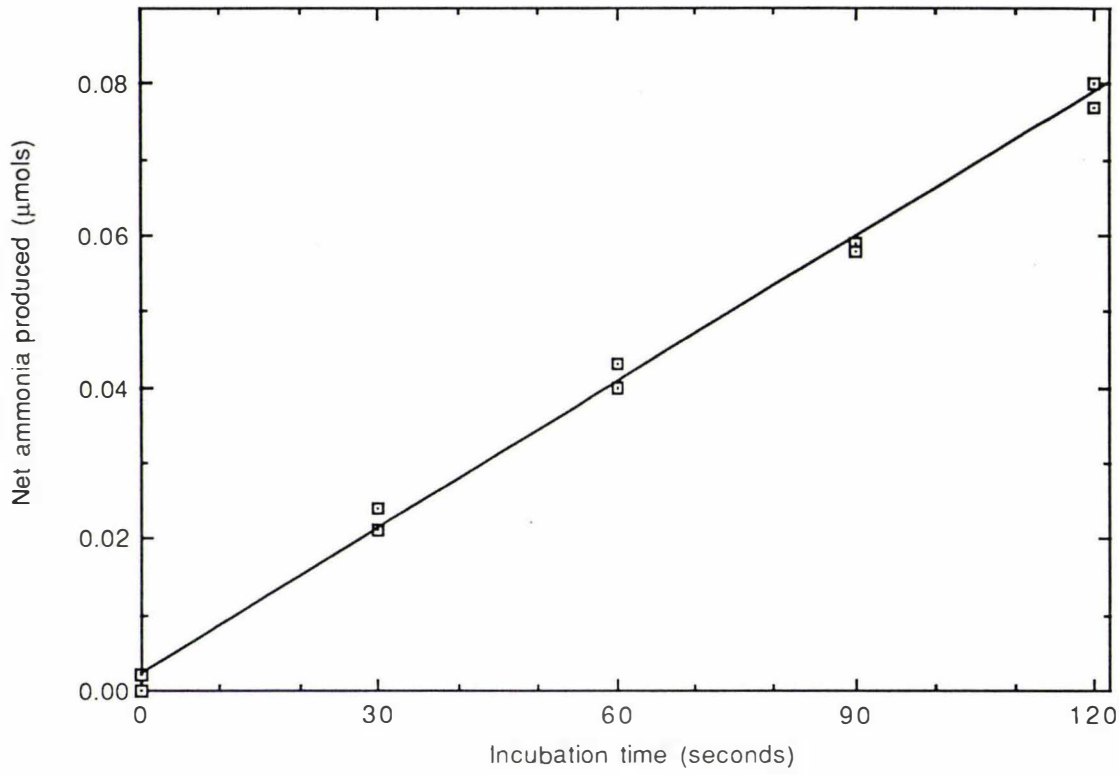
The following factors which could affect the yield of urease recovered in crude extracts were investigated: the cellular location of urease activity; the inclusion of urea, nickel, and manganese in the growth medium; urease isozymes; the growth-phase of the culture; the method of cell disruption; and the inclusion of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) in the extraction buffer.

### 3.2.1 Location of Urease Activity

The cellular location of *S. pombe* urease was determined by fractionating a culture of 972 by filtration and centrifugation. An aliquot of an early stationary-phase 972 culture was filtered through a 0.45 µm pore-size membrane filter. The filtrate was kept for analysis (sample 1) and the rest of the culture was centrifuged (6,000 g, five minutes, 4 $^{o}$C). The cell pellet was resuspended in PEB and washed by shaking vigorously for five minutes. An aliquot of the cell suspension was filtered as above and the filtrate was kept for analysis (sample 2). The rest of the cell suspension was centrifuged as above. The cell pellet was resuspended in PEB and an aliquot was kept for analysis (sample 3). The rest of the cells were disrupted by two passes through a French pressure cell and centrifuged as above. An aliquot of the supernatant, which would have contained organelles, membranes and cellular debris, was kept for analysis (sample 4). The remainder of the supernatant was ultracentrifuged

Figure 7.

The activity of *S. pombe* urease over a two minute time course.




A crude extract of *S. pombe* urease was assayed using the standard method with the following incubation times: 0, 30, 60, 90, and 120 seconds.

(110,000 g, one hour, 4 °C) to remove all particulate matter, including membranes. A clear supernatant, a gelatinous pellet, and a very thin pellicle were obtained. The pellet was resuspended in PEB (sample 5) and the supernatant was kept for analysis (sample 6). Aliquots of each sample were assayed for urease activity. The urease activity was associated with the soluble, non-particulate fraction of the cytoplasm (Table 13).

### 3.2.2 *S. pombe* urease isozymes

The presence of multiple forms of *S. pombe* urease was investigated. A crude extract of *S. pombe* 972 was examined by native-PAGE. The gel was stained for urease activity. One band of urease activity was observed (Figure 8). This indicates that probably a single urease was present, although the possibility of multiple forms with the same electrophoretic mobility cannot be discounted.

### 3.2.3 Effect of urea, nickel sulfate, and manganese sulfate on the yield of urease activity

Urea, nickel sulfate, and manganese sulfate, when included in the culture medium, can increase the yield of urease activity in crude extracts of some microorganisms (eg. Bast, 1988; Kakimoto *et al.*, 1990; Rando *et al.*, 1990). The effect of these substances on the yield of *S. pombe* urease was tested. Crude extracts were prepared from cultures of *S. pombe* 972 grown in YE with the following additives: a) no urea (the usual YE); b) 0.1% urea; or c) 0.1% urea, 0.005% $MnSO_4.4H_2O$ and 0.005% $NiSO_4$. No significant difference in the total urease activity of the extracts was observed. The specific activity was not directly determined; however, the same number of cells and the same degree of cell disruption was observed for each treatment.

### 3.2.4 Growth phase of the culture

The effect of the growth-phase of a culture on the yield of urease activity was investigated. Crude extracts of *S. pombe* 972 were prepared from a stationary-phase culture (40 hours incubation, $10^8$ cells/ml) and from a log-phase culture (15 hours incubation, $10^7$ cells/ml). The total urease activity per volume of culture was about five-fold greater in the stationary-phase culture extract.

Table 13

Urease activity in a fractionated *S. pombe* cell extract.

| Sample | Fraction | Total Activity(U) |
|---|---|---|
| 1 | Culture medium | NA |
| 2 | Wash buffer | NA |
| 3 | Washed cell pellet (whole cells) | 55 |
| 4 | Crude lysate | 934 |
| 5 | Cell debris, particulate fraction | 7 |
| 6 | Soluble non-particulate fraction | 943 |

Details are in the text. Activity is expressed in units of enzyme activity, one unit is the amount of enzyme which hydrolyzes 1 μmol of urea/min. Total activity calculations are for the entire amount of material that would have been present in each fraction and were adjusted for the amount of material removed for samples. NA = no activity detected.

Figure 8

Native-PAGE of *S. pombe* crude urease extract stained for urease activity.

The *S. pombe* crude extract sample contained 200 µg protein. The non-denaturing (native) separating gel was 6% acrylamide. Urease activity was visualized by using a specific urease stain (Materials and Methods 4.12).

### 3.2.5 Method of cell disruption

Ultrasound, glass-bead, and French press methods of disrupting *S. pombe* cells were compared. *S. pombe* 972 cultures were washed with water, resuspended in PEB and disrupted by one of the methods below. Cell disruption with ultrasound was done with a Soniprep 150 (MSE) apparatus using the 9.5 mm probe. The following series of energy pulses was used: five minutes at amplitude 22, followed by five minutes, 15 minutes, and ten minutes at amplitude 26, with a one minute pause between pulses. The extract was kept on ice throughout the procedure to ensure it did not heat. Glass bead cell disruption was carried out by adding glass beads (BDH, 80 mesh) to a cell suspension in a bottle so that almost all of the suspension was full of beads. The bottle was shaken by hand for four minutes at 4 °C. The glass beads were removed by vacuum filtration through Whatman number 54 filter paper and washed twice with a small volume of ice-cold PEB. French press cell disruption was carried out as described in Materials and Methods 4.4. The sonication and French press methods produced extracts with similar amounts of disrupted cells and urease activity. Disruption by glass beads produced greater cell breakage but less than one fifth of the total activity and the total protein obtained by the other two methods. The French press was used for all subsequent urease preparations.

### 3.2.6 Phenylmethylsulfonyl fluoride (PMSF) protease inhibitor.

PMSF is often included in extraction buffers to reduce the digestion of proteins by proteases released from disrupted cells. The effect of PMSF on the yield of urease activity was tested. Crude extracts were prepared in extraction buffers (PEB) containing no PMSF, or containing 0.02 mM PMSF. A 0.1 M PMSF stock solution in 95% ethanol was used. PMSF did not affect the yield of urease activity or the enzyme stability. Without PMSF, the enzyme retained over 90% of original activity after storage for seven days at 4 °C or overnight at 18 - 22 °C. PMSF was not included in subsequent crude extracts.

## 3.3 Affinity column purification of *S. pombe* urease

Affinity chromatography by using hydroxyurea-derivatized resins has been successful in purifying ureases from a variety of sources (Wong and Shobe, 1974; Shobe and Brosseau, 1974; Mendes *et al.*, 1988; Creaser and Porter, 1985). Three affinity resins were prepared and used in trial purifications of jack bean (*Canavalia ensiformis*) and *S. pombe* urease. Jack bean urease was used as a model enzyme to test

the affinity resins because it was readily available in crude form (BDH) and had previously been purified by affinity chromatography (Wong and Shobe, 1974; Shobe and Brosseau, 1974; Mendes *et al.*, 1988).

### 3.3.1 Oxirane Hydroxyurea Agarose (OHA)

An affinity adsorbent was prepared by introducing a reactive oxirane group into a beaded agarose matrix (Sepharose 4B, Pharmacia). The $NH_2$ group of hydroxyurea was then coupled to the oxirane-agarose (Figure 9; Materials and Methods 4.6.1). The degree of activation of the gel matrix was 0.59 mmol oxirane per gram (dry weight) of gel, although the substitution with hydroxyurea may have been considerably less than this (Materials and Methods 4.6.4).

A crude jack bean extract was applied to an OHA column (8.5 cm x 1.6 cm) and eluted with 40 ml of 0.02 M or 0.002 M phosphate buffer (pH 7.0) containing 1 mM 2-mercaptoethanol (0.02 M PB or 0.002 M PB, respectively). The column was equilibrated in the elution buffer before use and the jack bean extract was prepared in buffer with the same phosphate concentration and pH as the equilibration buffer. The urease activity was eluted at an effluent volume of 14 mls, which is less than the total volume (17 ml) of the column. No further activity was eluted when the elution buffer strength and acidity were increased to 0.2 M PB pH 4.6, or 0.4 M PB pH 4.6.

Urease may be inactivated by phosphate at low pH (Mobley and Hausinger, 1989). Therefore, eluted urease may have been inactivated by 0.2 M PB pH 4.6 and 0.4 M PB pH 4.6. To test for inactivation, jack bean urease was diluted in the elution buffers and assayed for activity. A jack bean extract in 0.002 M PB was diluted ten fold with 0.02 M PB, 0.2 M PB pH 4.6 or 0.4 M PB pH 4.6. The activity of the diluted enzyme was assayed in the usual assay buffer. The activity of urease diluted in 0.2 M PB pH 4.6 and in 0.4 M PB pH 4.6 was 84% and 71%, respectively, of the activity obtained in 0.02 M PB pH 7.0. The reduction in activity was not sufficient to prevent the detection of urease activity in the column effluents.

Inhibition of urease by hydroxamic acids is progressive with time (Kobashi *et al.*, 1962). The inhibitory group of hydroxamic acids is the same as that of hydroxyurea (-CONHOH) so binding to the affinity resin may occur slowly. The binding of jack bean urease to the OHA column was not improved by delaying elution until one and a half hours after the enzyme was loaded into the column.

Figure 9.

Structure of adsorbents made for affinity chromatography purification of urease

Oxirane Hydroxyurea Agarose (OHA)

agarose-O-$CH_2$-CH(OH)-$CH_2$-O-$(CH_2)_4$-O-$CH_2$-CH(OH)-$CH_2$-NH-CO-NH-OH

Aminocaprylic acid Hydroxyurea Agarose (AHA)

agarose-NH-$(CH_2)_7$-CO-NH-CO-NH-OH

Ethylenediamine Ethylenediamine Hydroxyurea Agarose (EHA)

agarose-NH-$(CH_2)_2$-NH-CO-$(CH_2)_2$-CO-NH-$(CH_2)_2$-NH-CO-$(CH_2)_2$-CO-NH-CO-NH-OH

The ligand portion of the adsorbents, derived from hydroxyurea, are underlined.

3.3.2 Aminocaprylic acid hydroxyurea agarose (AHA).

Urease specifically binds to caprylohydroxamic acid, one of the most potent specific inhibitors of urease (Kobashi *et al.*, 1962; Hase and Kobashi, 1967). An affinity adsorbent with a structure similar, but not identical, to caprylohydroxamic acid was prepared. Aminocaprylic acid was introduced into Sepharose CL6B (Pharmacia). Hydroxyurea was coupled to the aminocaprylic acid-agarose (Figure 9; Materials and Methods 4.6.2). The degree of derivation of the agarose matrix with caprylic acid was 0.57 milliequivalents per gram dry weight and good substitution with hydroxyurea was indicated by the 2,4,6-trinitrobenzenesulfonate-borate color test (Materials and Methods 4.6.4).

Crude extracts of jack bean and of *S. pombe* 972 were passed through a column of AHA using the elution conditions described for the OHA matrix. No binding of urease activity occurred.

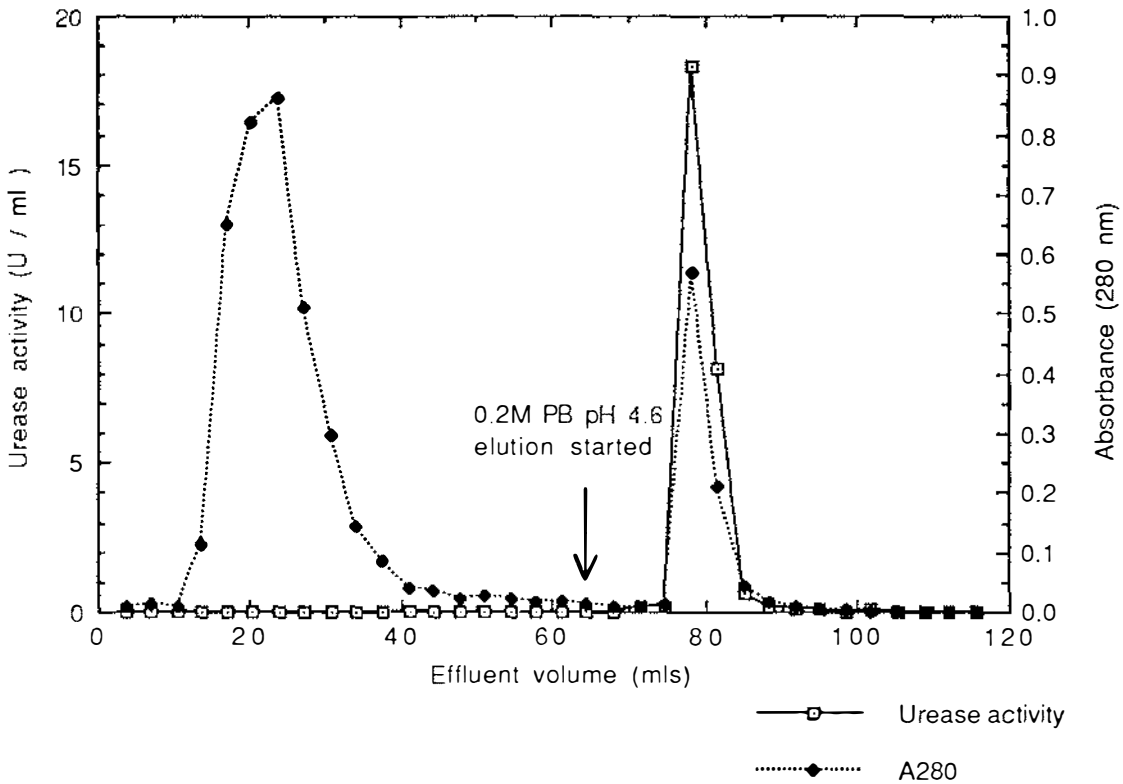
3.3.3 Ethylenediamine-ethylenediamine Hydroxyurea Agarose (EHA).

A third affinity column was made by introducing ethylenediamine into Sepharose CL6B and succinylating the resultant aminoethyl-agarose. A second reaction with ethylenediamine and subsequent succinylation were done to produce a matrix with a 16-unit side chain. The resultant matrix was reacted with hydroxyurea to form the EHA affinity resin (Figure 9; Materials and Methods 4.6.3). The degree of substitution of the agarose by the 16-unit side chain was 0.23 mmol per gram dry weight and good substitution with amine derivatives (hydroxyurea or unsuccinylated ethylene diamine) was indicated by the 2,4,6-trinitrobenzenesulfonate-borate color test (Materials and Methods 4.6.4).

A crude jack bean extract was applied to a column of EHA (7.5 cm x 1.6 cm). The column was then extensively washed with 0.02 M PB and fractions were monitored for urease activity. No urease activity was eluted. When the elution buffer was changed to 0.2 M PB pH4.6 urease activity was eluted as a single peak (Figure 10). No further activity was eluted when the elution buffer was changed to 0.4 M PB pH 4.6. The most active fractions, containing about 80% of the total eluted activity, were pooled. The specific activity of the pooled fractions was 21.6 U/mg. A 3.2-fold increase in purity was achieved. A second pass through the column increased the specific activity to about 170 U/mg protein. This specific activity is well below the value expected for a

Figure 10

Purification of jack bean urease by EHA affinity chromatography with 0.2 M PB elution




A crude jack bean extract was applied to an EHA column and washed with 0.02 M PB (pH 7.0). The urease activity was eluted with 0.2 M PB pH 4.6.

homogeneous preparation of urease purified by affinity chromatography using this adsorbent (3,000 U/mg; Shobe and Brosseau, 1974). Jack bean urease purified by two passes through the column was examined by SDS-PAGE. The urease subunit was a small fraction of the total protein.

A linear gradient of 0 to 50 mM urea (200 ml total volume) was used to elute a crude jack bean extract from the EHA column (Figure 11). The urease activity was eluted as a single peak at about 0.2 mM urea. The pH of the eluted fractions rose as high as pH 9.3, due to the action of urease on the elution buffer. The most active fractions were examined by SDS-PAGE. The urease was still a minor component of the total protein in the preparation (Figure 12).

Jack bean urease has previously been purified to homogeneity using an EHA adsorbent (Shobe and Brosseau, 1974). The jack bean urease applied to the column was more pure (specific activity 120 U/mg) than the crude extract used in this study (specific activity about 5 to 7 U/mg). To test whether the EHA column would be useful in purifying an extract containing less contaminant protein, a crude jack bean extract was first partially purified by DEAE-Sepharose ion-exchange chromatography, and then passed through the EHA column.

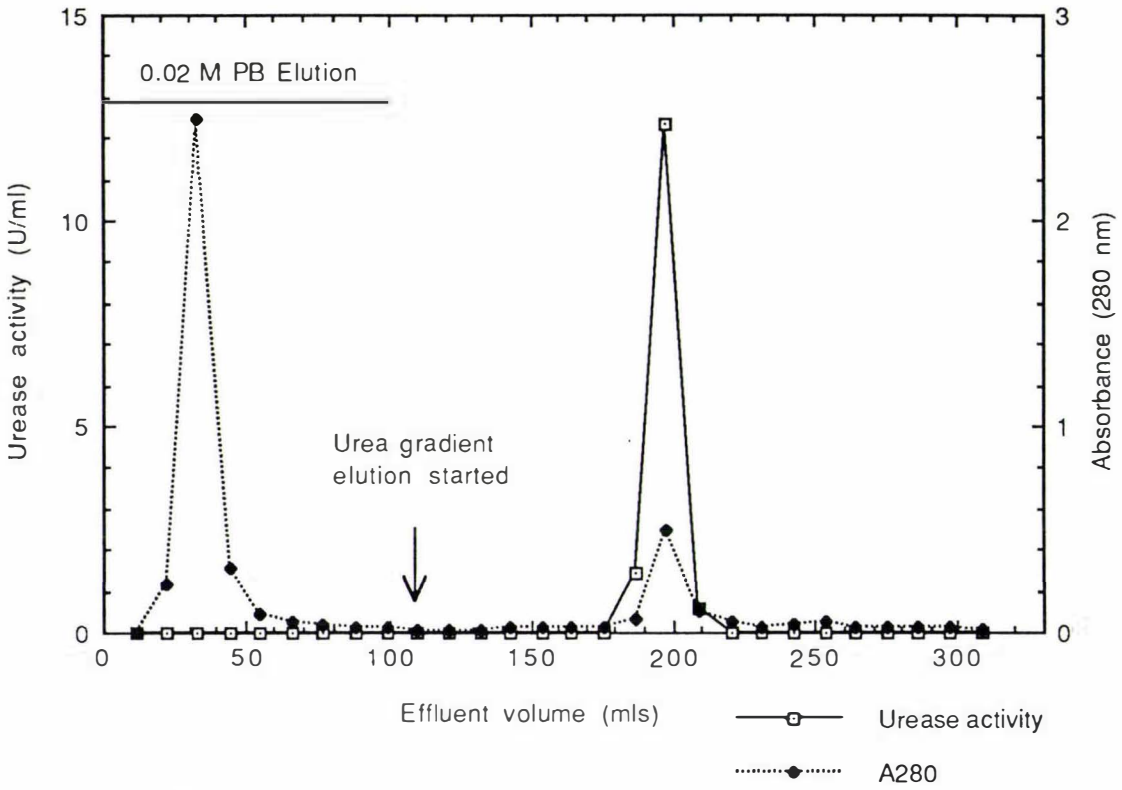
A crude jack bean extract was applied to a DEAE-Sepharose column (30 cm x 1.5 cm, equilibrated in 0.02 M PB) and eluted with a 0 to 30 mM NaCl linear gradient (800 ml total volume in PEB buffer). Effluent fractions were monitored for urease activity (Figure 13). The most active six fractions together comprised 64% of the total activity and had an average specific activity of 68 U/mg, representing an increase in purity of about 15 fold over the crude extract (4.6 U/mg). These fractions were pooled, then concentrated and desalted by ultrafiltration (XM 50 membrane, Amicon). An aliquot of the concentrated fractions was applied to the EHA column and eluted with 0.2 M PB pH 4.6, as before. The absorbance profile indicates that a small amount of non-urease material was separated from the enzyme (Figure 14), although the specific activity of the most active fraction was only 26.4 U/mg. Probably loss of activity had occurred. The EHS column did not significantly improve the purity of the partially purified jack bean urease.

## Summary

Oxirane-hydroxyurea-agarose (OHA) and aminocaprylic acid-hydroxyurea-agarose (AHA) did not bind urease. Ethylenediamine-ethylenediamine-hydroxyurea-agarose

Figure 11

Purification of jack bean urease by EHA affinity chromatography using urea gradient elution.




A crude jack bean extract was applied to a EHA column and washed with 0.02 M PB (pH 7.0). The urease activity was eluted with a linear urea gradient (0 - 50 mM).

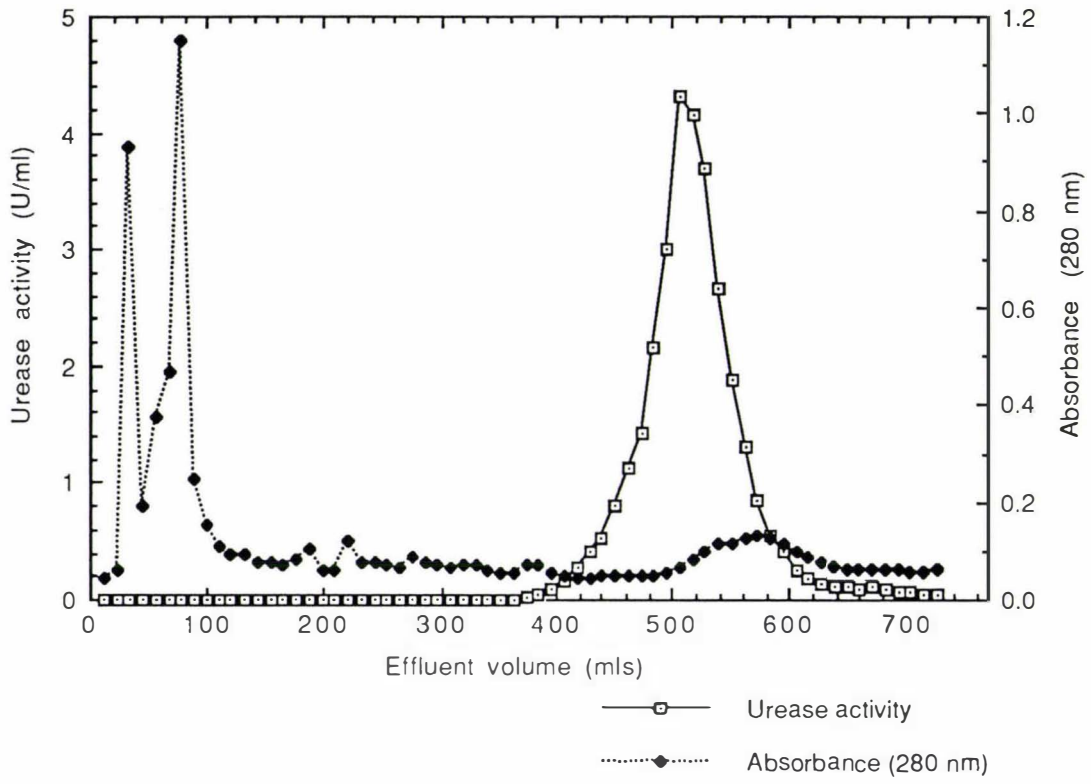
Figure 12

SDS-PAGE of jack bean urease purified by EHA affinity chromatography using urea gradient elution.


A 7.5% acrylamide separating gel (SDS-PAGE) was used and proteins were visualized by silver staining. (A) crude jack bean urease extract; (B) most active fraction (comprising about 85% of the total activity recovered) eluted from EHA by a urea gradient. The band corresponding to the size expected for jack bean urease (96,600 by SDS-PAGE, Andrews *et al.*, 1984) is marked by an arrow.

Figure 13

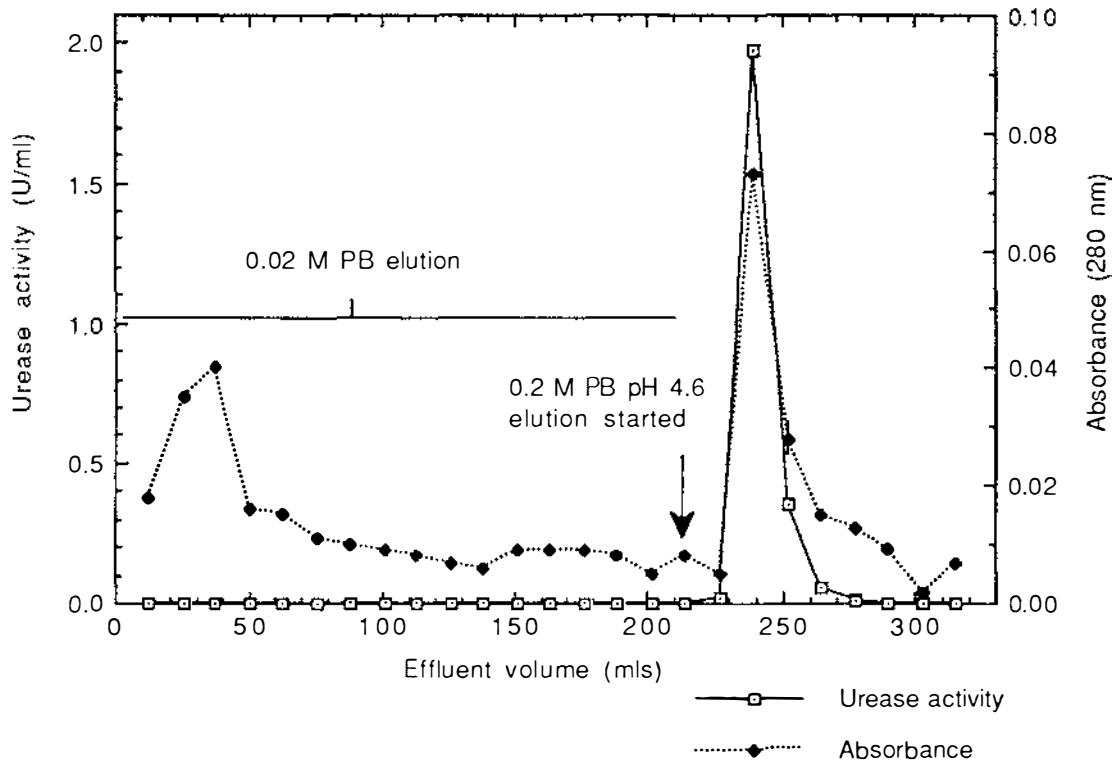
Purification of jack bean urease by DEAE-Sepharose ion-exchange chromatography.




Crude jack bean extract was applied to a DEAE-Sepharose column and urease activity was eluted with a linear 0 - 30 mM NaCl gradient in PEB.

Figure 14

Purification of partially purified jack bean urease by EHA chromatography.




Jack bean urease was partially purified by DEAE-Sepharose ion-exchange and then applied to an EHA column. The column was first washed with 0.02 M PB (pH 7.0) and then the urease activity was eluted with 0.2 M PB pH 4.6.

(EHA) did bind urease but with poor specificity. Many other proteins also co-eluted with urease and the degree of purification was less than that obtained by conventional ion-exchange chromatography. The majority of contaminants which co-eluted with urease from DEAE-Sepharose also co-eluted with urease from EHA.

## 3.4 Precipitation and ion-exchange purification of *S. pombe* urease

Acetone and ammonium sulfate precipitation, and DEAE-Sepharose (column chromatography) and Mono-Q (Fast Protein Liquid Chromatography) ion-exchange were optimized and used for the purification of *S. pombe* urease.

### 3.4.1 Acetone Precipitation

Crude extracts of *S. pombe* 972 were partially purified by precipitation with acetone (Materials and Methods 4.7.1). The crude extracts had protein concentrations within the optimum range for precipitation by organic solvents (5 - 30 mg/ml; Scopes, 1987). Most of the urease activity was precipitated in the 50-60% acetone fraction. Typical data are presented in Table 14. Occasionally high levels of activity were precipitated in the 40-50% acetone fraction (Preparation 3 in Table 14). The pH of the crude extracts was sometimes as low as pH 6.2. When the pH was increased to pH 7.0, with a few drops of potassium hydroxide, little urease activity precipitated in the 40-50% fraction.

### 3.4.2 Ammonium Sulfate Precipitation

Trial ammonium sulfate precipitations were carried out on *S. pombe* crude extracts (Materials and Methods 4.7.2). The protein concentration of crude extracts was above the minimum concentration recommended for ammonium sulfate precipitations (1 mg/ml; Scopes, 1987). The following two series of precipitations were done: 0-30%, 30-50%, 50-70%; and 0-35%, 35-45% (% = % saturation with ammonium sulfate at 0 $^{o}$C). The best specific activity was obtained with the 35-45% saturation fraction (Table 15).

### 3.4.3 Acetone precipitation followed by ammonium sulfate precipitation

*S. pombe* urease preparations partially purified by acetone precipitation (50-60% acetone fraction) were diluted to a protein concentration of about 5 mg/ml and precipitated with ammonium sulfate. The following series of precipitations was used:

| Fraction | Total Activity (U) | | | Total Protein (mg) | | | Specific Activity (U/mg) | | | Purification (-fold) | | | Yield (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| Crude Lysate | 85.5 | 465 | 1,000 | 636 | 2,632 | 6,032 | 0.13 | 0.18 | 0.17 | 1 | 1 | 1 | 100 | 100 | 100 |
| 0-40 % Acetone | 2.4 | ND | ND | 257 | ND | ND | 0.01 | ND | ND | 0.08 | ND | ND | 2.8 | ND | ND |
| 40-50 % Acetone | 3.3 | ND | 753 | 75 | ND | 795 | 0.04 | ND | 0.95 | 0.31 | ND | 5.59 | 3.9 | ND | 75.3 |
| 50-60 % Acetone | 65.6 | 281 | ND | 53 | 210 | ND | 1.24 | 1.34 | ND | 9.54 | 7.4 | ND | 76.7 | 60.4 | ND |
| 60-70 % Acetone | 1.6 | ND | ND | 9 | ND | ND | 0.17 | ND | ND | 1.31 | ND | ND | 1.9 | ND | ND |

A

| Fraction | Total Activity (U/mg) | Total Protein (mg) | Sp Act (U/mg) | Purification | Yield(%) |
|---|---|---|---|---|---|
| Crude | 34 | 112 | 0.30 | 1.0 | 100 |
| 0-40% saturation | 0.22 | 0.9 | 0.24 | 0.8 | 0.6 |
| 30-50% saturation | 26.1 | 37 | 0.71 | 2.4 | 76.8 |
| 50-70% saturation | 0.17 | 19 | 0.01 | 0.03 | 0.5 |

B

| Fraction | Total activity (U) | | Total protein (mg) | | Sp act (U/mg) | | Purification (-fold) | | Yield (%) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 |
| Crude | 43.1 | 59.5 | 154 | 260 | 0.28 | 0.23 | 1.0 | 1.0 | 100 | 100 |
| 0-35% saturation | 2.2 | 12.6 | 12.7 | 54.5 | 0.18 | 0.23 | 0.6 | 1.0 | 5.1 | 21.2 |
| 35-45% saturation | 20.8 | 39.5 | 12.3 | 33.1 | 1.69 | 1.19 | 6.0 | 5.2 | 48.3 | 66.4 |
| Supernatant of 45% saturation | 9.6 | 4.74 | 80.6 | 127.1 | 0.12 | 0.04 | 0.4 | 0.2 | 22.3 | 8.0 |

0-35%, 35-45%, 45-55%, 55-65%, 65-80% saturation with ammonium sulfate. The 35-45% saturation fraction gave good purification of urease, although the yield varied between preparations. Typical data are presented in Table 16.

### 3.4.4 Ion-exchange purification of *S. pombe* urease

Ion-exchange chromatography using DEAE-Sepharose was used to purify *S. pombe* urease to near homogeneity. Fast Protein Liquid Chromatography (FPLC) ion-exchange was used as a final purification step with urease preparations that contained a significant amount of contaminant protein after the DEAE-Sepharose purification step.

#### 3.4.4.1 DEAE-Sepharose purification

Partially purified *S. pombe* urease (Results 3.4.3) was applied to a DEAE-Sepharose column (8 cm x 1.6 cm). The column was washed with 0.2 M NaCl in PEB (0.2 M PEBS) and then the urease activity was eluted with a linear gradient of 0.2 - 0.35 M NaCl in PEB (Materials and Methods 4.7.3). The most active effluent fractions, containing about 80% of the eluted activity, were pooled. The purified urease had a specific activity of 709 U/mg and the purification factor was 3,939-fold. Typical data are presented in Figure 15 and Table 17.

The combined fractions were concentrated by ultrafiltration (Amicon XM50 membrane) and analyzed by native-PAGE using protein and urease activity stains (Materials and Methods 4.12 ). The urease preparation contained one major active band and a few barely detectable contaminants (Figure 16).

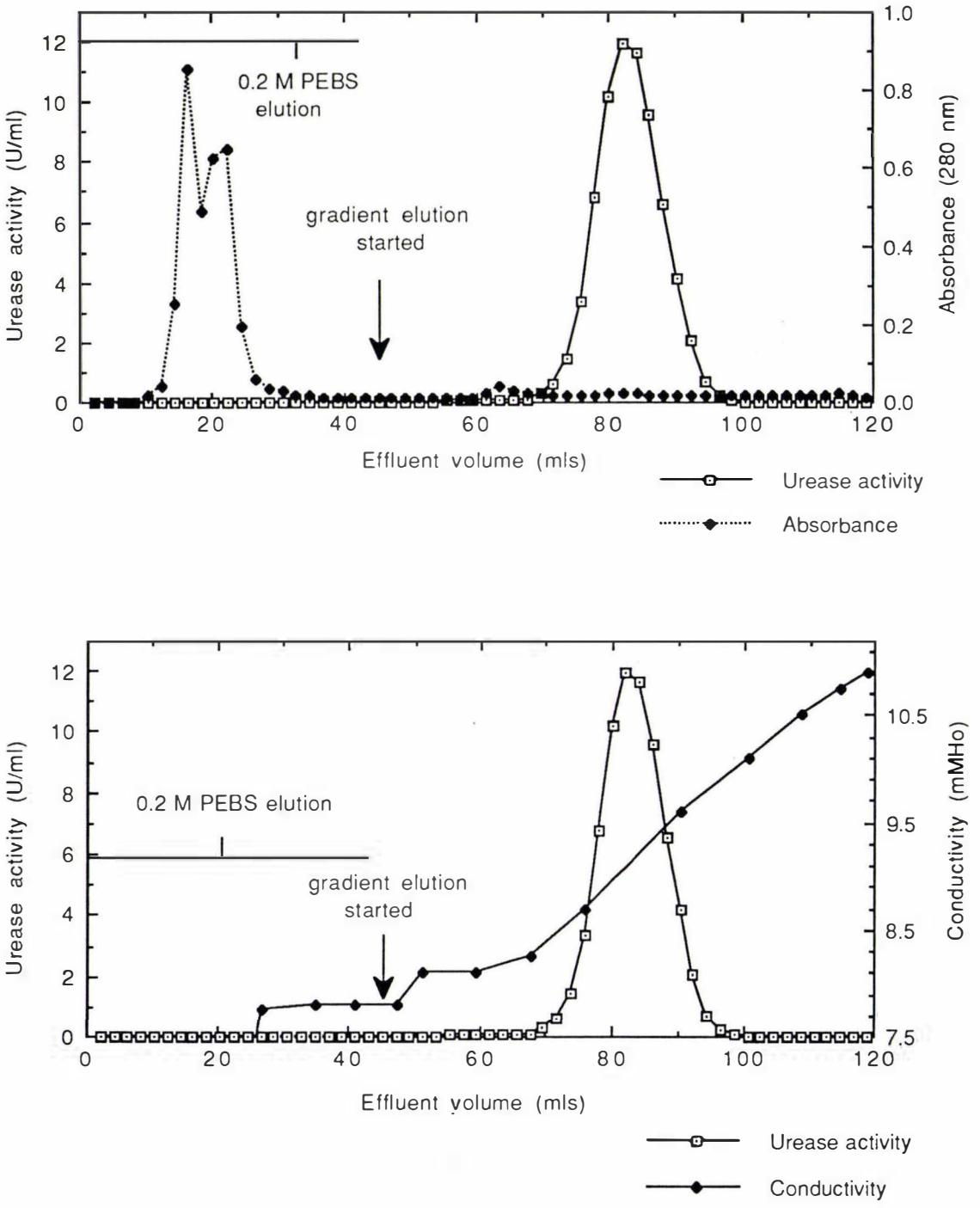
#### 3.4.4.2 FPLC-Mono-Q purification

Some urease preparations purified by the above method contained a significant amount of contaminant protein. A second, identical, DEAE-Sepharose ion exchange step improved the purity of these preparations; however, it was necessary to use another purification step to remove all contaminant protein. The likely cause of poor purification was the deterioration of the DEAE-Sepharose. Subsequent to the observed poor purification, the urease binding capacity of the DEAE-Sepharose column dramatically decreased, despite efforts to regenerate the resin according to the manufacturer's instructions.

| Fraction | Total activity (U) | | | Total protein (mg) | | | Sp act (U/mg) | | | Purification (-fold) | | | Yield (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| 50-60% acetone | 42.13 | 219 | 653 | 34 | 163 | 690 | 1.24 | 1.34 | 0.95 | 1.0 | 1.0 | 1.0 | 100 | 100 | 100 |
| 0-35% $(NH_4)_2SO_4$ | 3.38 | ND | 55 | 1.4 | ND | 40.7 | 2.41 | ND | 1.36 | 1.9 | ND | 1.4 | 8.1 | ND | 8.4 |
| 35-45% $(NH_4)_2SO_4$ | 28.43 | 205 | 270 | 1.6 | 7.7 | 17.6 | 17.76 | 26.40 | 15.34 | 14.3 | 19.7 | 16.1 | 67.5 | 93.6 | 41.3 |
| 45-55% $(NH_4)_2SO_4$ | 0.38 | ND | ND | 3.5 | ND | ND | 0.11 | ND | ND | 0.09 | ND | ND | 0.9 | ND | ND |
| 55-65% $(NH_4)_2SO_4$ | 0.15 | ND | ND | 7.5 | ND | ND | 0.02 | ND | ND | 0.02 | ND | ND | 0.4 | ND | ND |
| 65-80% $(NH_4)_2SO_4$ | 0.03 | ND | ND | 4.2 | ND | ND | 0.007 | ND | ND | 0.006 | ND | ND | 0.1 | ND | ND |

Figure 15

Purification of *S. pombe* urease by DEAE-Sepharose ion-exchange chromatography




*S. pombe* urease, partially purified by acetone and ammonium sulfate precipitations, was applied to a DEAE-Sepharose column, washed with 0.2 M PEBS and then the urease activity was eluted with a 0.2 M - 0.35 M NaCl linear gradient.

Table 17

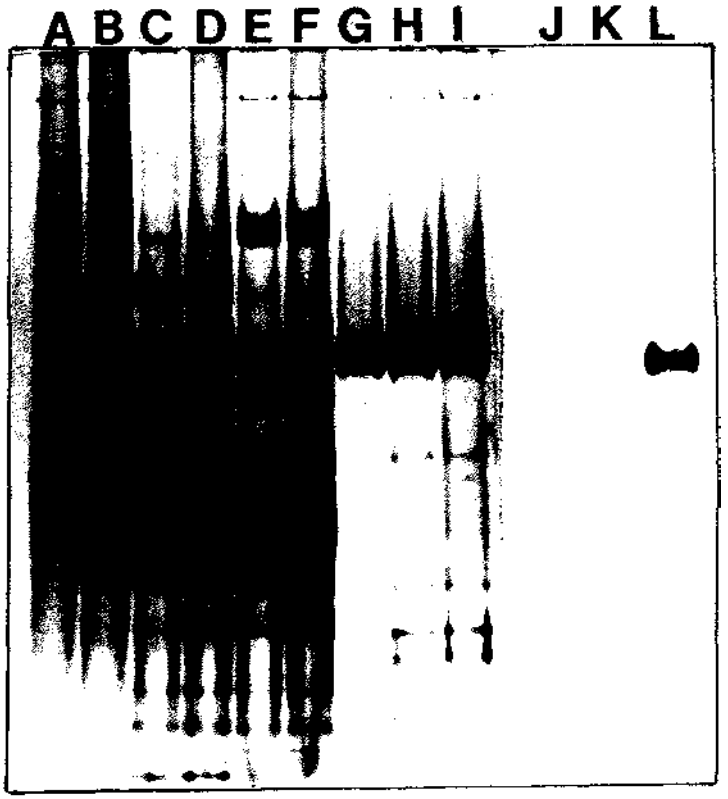
DEAE-Sepharose ion-exchange purification of *S. pombe* urease

| Fraction | Total activity (U) | Total Protein (mg) | Sp Act (U/mg) | Purification (-fold) | Yield (%) |
|---|---|---|---|---|---|
| Crude extract | 252 | 1428 | 0.18 | 1 | 100 |
| Acetone precip. | 152 | 113.8 | 1.34 | 7.40 | 60.3 |
| $(NH_4)_2SO_4$ precip. | 143 | 5.40 | 26.40 | 146.7 | 56.7 |
| DEAE - Sepharose | 85.50 | 0.12 | 709 | 3938.9 | 33.9 |

A crude extract of *S. pombe* was purified to near homogeneity by acetone precipitation, ammonium sulfate precipitation and DEAE-Sepharose ion-exchange. Abbreviations and definition of units are the same as for Tables 14 and 15.

Figure 16.

Native-PAGE of *S. pombe* urease purified by acetone precipitation, ammonium sulfate precipitation and DEAE-Sepharose ion-exchange chromatography




The separating gel was 6% polyacrylamide without SDS (native-PAGE). *S. pombe* urease was purified by acetone precipitation, ammonium sulfate precipitation and DEAE-Sepharose ion-exchange column chromatography. The amount of protein loaded per lane was as follows: (A) 1.5 μg crude extract; (B) 3.0 μg crude extract; (C) 1.5 μg acetone precipitate; (D) 3.0 μg acetone precipitate; (E) 1.8 μg ammonium sulfate precipitate; (F) 3.6 μg ammonium sulfate precipitate; (G) 0.6 μg pooled DEAE-Sepharose chromatography fractions; (H) 1.2 μg pooled DEAE-Sepharose chromatography fractions; (I) 1.8 μg pooled DEAE-Sepharose chromatography fractions; (J) 3.0 μg crude extract; (K) 3.6 μg ammonium sulfate precipitate; (L) 0.6 μg pooled DEAE-Sepharose chromatography fractions. Proteins in lanes A to I were visualized by silver staining. Urease activity in lanes J, K, and L was visualized by the specific urease activity staining method.

FPLC was used to further purify urease preparations which still contained contaminant protein after the DEAE-Sepharose step (Materials and Methods 4.7.4). Partially purified urease (acetone precipitation, ammonium sulfate precipitation then two DEAE-Sepharose ion-exchange steps) was concentrated and desalted by ultrafiltration (Amicon XM50 membrane) and then applied to a Mono-Q column. The urease activity was eluted with a 0.2 - 0.5 M NaCl gradient in PEB. Urease activity was eluted as a single peak (Figure 17 and Table 18). A single major band and one barely detectable minor contaminant band were observed when the purified urease was analyzed by native-PAGE (Figure 18). No further activity was eluted when the column was washed with 1 M NaCl in PEB.

## 3.5 Urease Enzyme Characterization

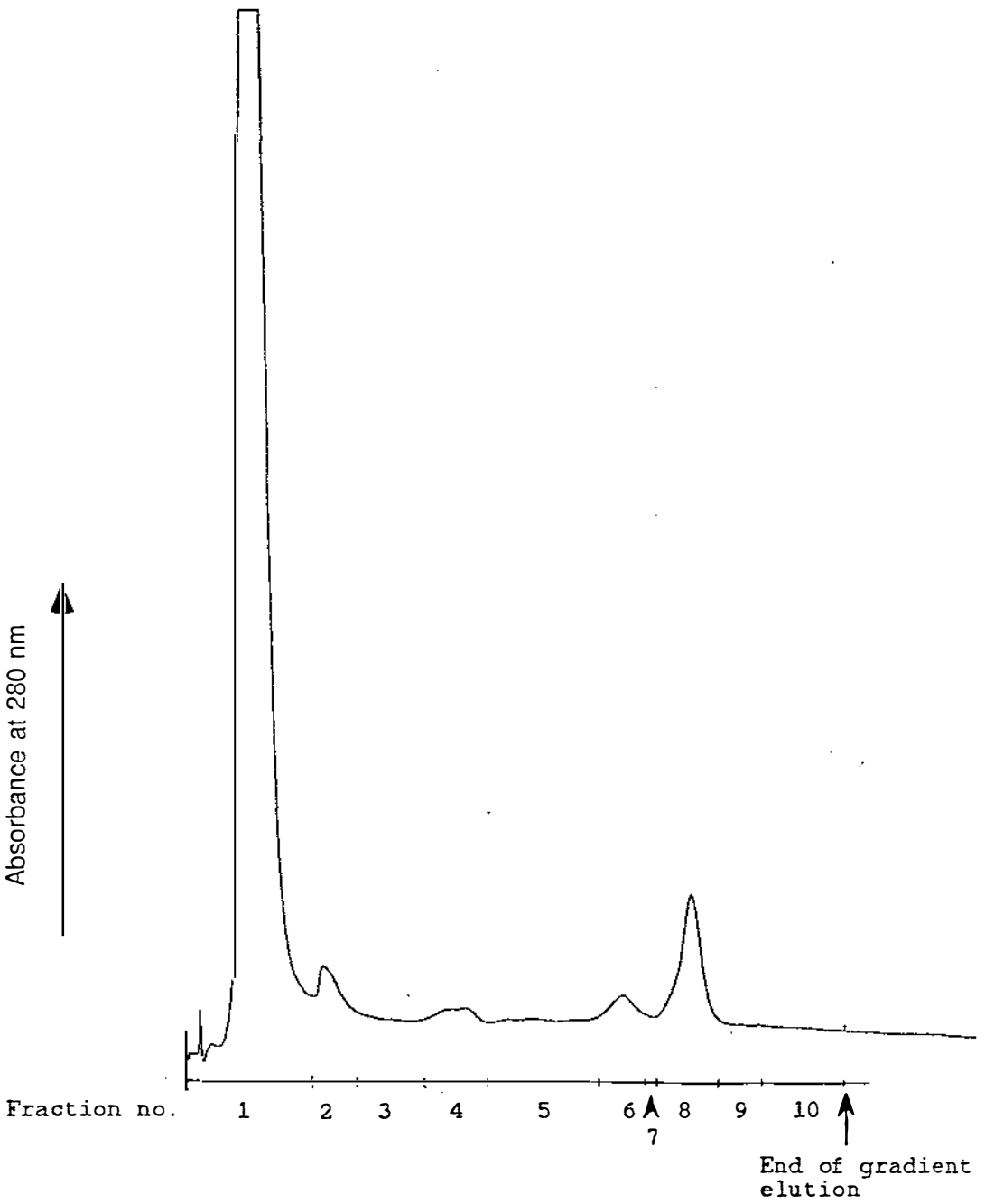
Physical and kinetic properties of *S. pombe* urease were characterized; the native and subunit molecular weights, pH stability, pH optimum, and $K_m$ for urea were determined.

### 3.5.1 Native Molecular Weight

The native molecular weight was determined by gel filtration using a calibrated Sepharose CL6B 200 column. The column (92 x 1.6 cm) was calibrated using the protein standards in the Sigma MW-GF-1000 molecular weight standards kit according to the manufacturers instructions (Materials and Methods 4.13). The void volume ($V_o$, 73.0 ml) of the column and the elution volume ($V_e$, 124.0 ml) of β-amylase were periodically checked and remained unchanged throughout the course of the experiment. Partially purified *S. pombe* urease (acetone precipitated, specific activity about 1 U/mg) was passed through the calibrated column and effluent fractions were monitored for urease activity. Three independently prepared urease extracts were used for a total of four determinations. Urease assays were done in triplicate and the average urease activity for each fraction was graphed. The elution volume, $V_e$ (122.8 ml) was estimated from the point of maximum activity (Figure 19). The elution parameter, $V_e/V_o$, was 1.682 for all four determinations. The native molecular weight, estimated from the standard curve (Figure 20) was about 212,000 daltons.

Figure 17

Purification of *S. pombe* urease by using FPLC Mono-Q ion-exchange as the final purification step




*S. pombe* urease purified by acetone and ammonium sulfate precipitation and DEAE-Sepharose ion-exchange, but still containing some contaminant protein, was further purified by Mono-Q ion-exchange (FPLC). A linear gradient of 0.2 M - 0.5 M NaCl in PEB was used to elute the urease from the Mono-Q column. The numbered fractions were collected.

Table 18

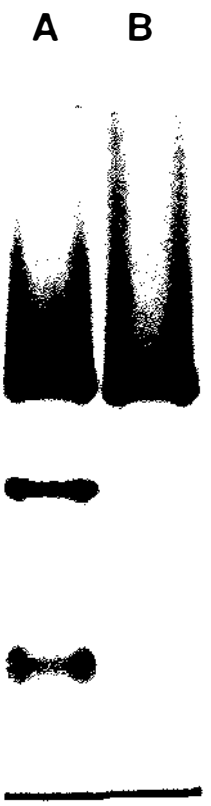
The activity of *S. pombe* urease fractions obtained from the FPLC-Mono-Q purification step

| [a]Fraction number | Urease Activity U/ml |
|---|---|
| 1 | 0.00 |
| 2 | 0.00 |
| 3 | 0.00 |
| 4 | 0.00 |
| 5 | 0.00 |
| 6 | 0.17 |
| 7 | 0.77 |
| 8 | 8.24 |
| 9 | 0.22 |
| 10 | 0.00 |

*S. pombe* urease fractions collected from the Mono-Q ion-exchange purification step (FPLC) were assayed for urease activity. [a] Fraction number corresponds to the fractions marked in Figure 17. Activity units (U) are as for Table 14.

Figure 18.

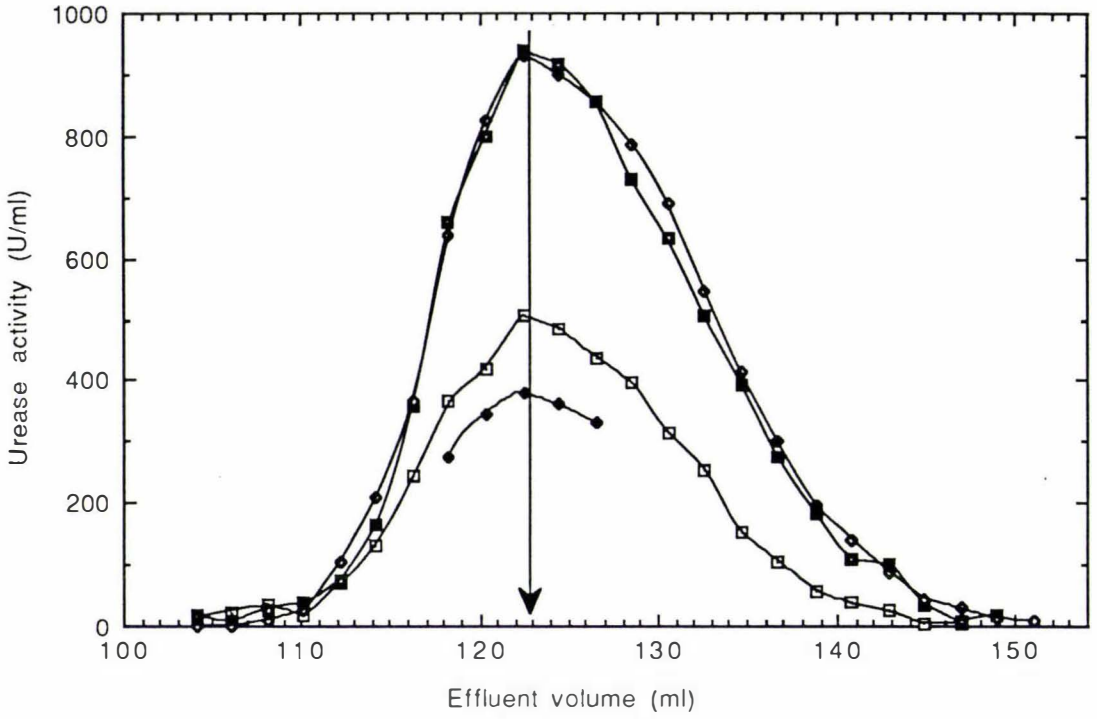
Native-PAGE of *S. pombe* urease purified by using Mono-Q FPLC as the last purification step




The separating gel was 6% polyacrylamide without SDS (native-PAGE). Proteins were visualized by silver staining. *S. pombe* urease was purified by acetone precipitation, ammonium sulfate precipitation, DEAE-Sepharose ion-exchange column chromatography and FPLC using a Mono-Q column. Details are in the text. (A) urease sample before purification by FPLC; (B) urease after FPLC purification - the most active fraction, containing about 90% of the total recovered activity.

Figure 19

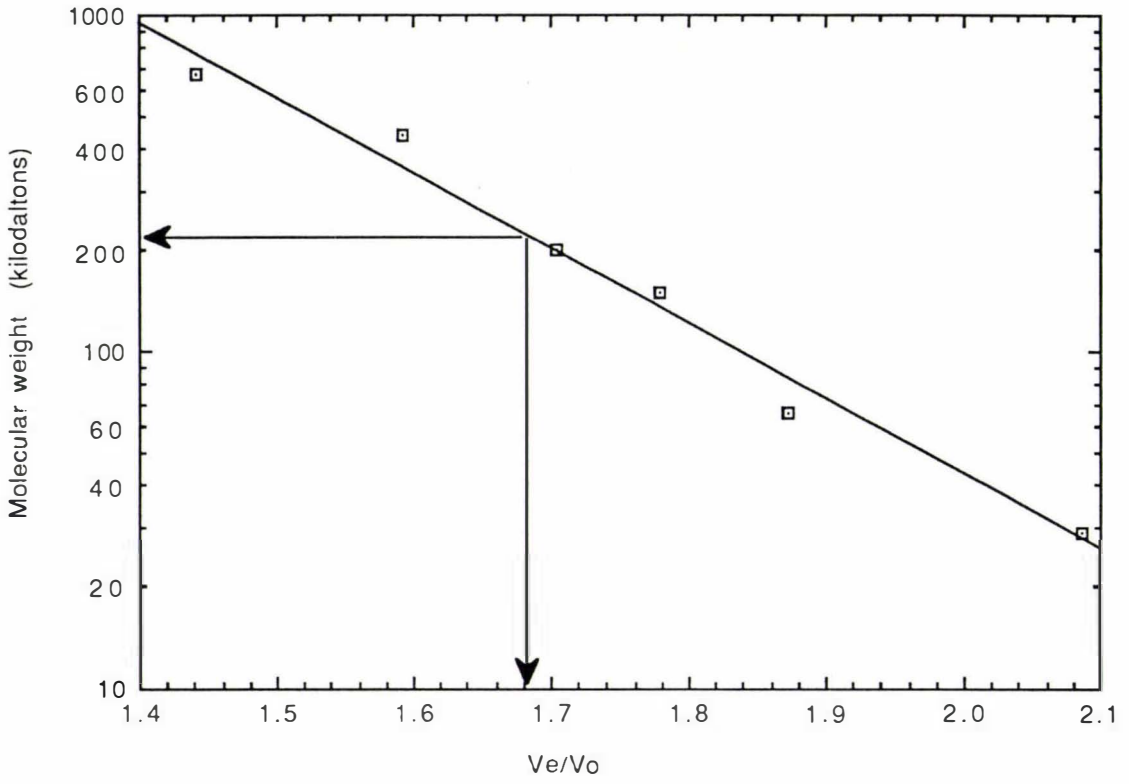
Elution volume ($V_e$) of *S. pombe* urease determined by gel filtration through a calibrated Sepharose CL6B 200 column




Each curve represents an independent passage of *S. pombe* urease through the calibrated Sepharose CL6B 200 column. Urease activity assays for each determination were done in triplicate. The interpolated peak of the curves represents the $V_e$ of *S. pombe* urease and is marked by the arrow.

Figure 20

Calibration curve of Sepharose CL6B 200 column.




Calibration of the Sepharose CL6B 200 column is described in Materials and Methods 4.13. The $V_e/V_o$ and corresponding molecular weight for *S. pombe* urease are marked with arrows.

### 3.5.2 Subunit Size

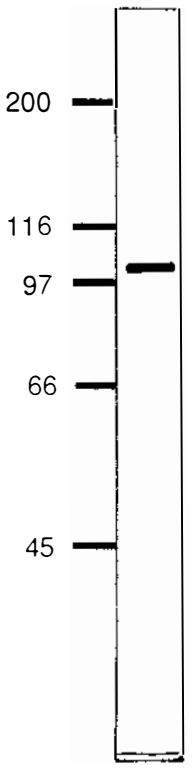
The subunit size of purified *S. pombe* urease was examined by using SDS-PAGE. A single major band with $M_r$ = 102,000, and a few minor contaminant proteins, were identified by using a 7.5% acrylamide separating gel (Figure 21). Many bacterial ureases have one large and two small subunits. The small subunits are poorly resolved on gels containing less than 10% acrylamide and they have been inadvertently overlooked (Mobley and Hausinger, 1989). No small subunits were seen when a *S. pombe* urease preparation, free of contaminants, was run on a 15% acrylamide gel (Figure 22). *S. pombe* urease appears to have a single subunit that is present as a dimer, to give a native $M_r$ of about 212,000.

### 3.5.3 pH Stability

The enzyme stability was tested between pH 4 and 11. The set of buffers used by Todd and Hausinger (1987) for pH studies of *Klebsiella aerogenes* was used in the present study for testing *S. pombe* urease. Partially purified *S. pombe* urease (purified by acetone precipitation, specific activity about 1 U/mg) was diluted ten-fold in buffers at various pH values and incubated for 30 minutes on ice. Aliquots were then assayed for urease activity in a 20-fold volume of 25 mM HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid), 5 mM EDTA, 50 mM urea. The test buffers were: acetate pH 4.0, 5.0, 6.0; MES (2-morpholino)ethane sulfonic acid) pH 6.0, 7.0; HEPES pH 7.0, 8.0; CHES (2-(N-cyclohexylamino)ethane sulfonic acid) pH 8.0, 9.0, 10.0; CAPS (cyclohexylaminopropanesulfonic acid) pH 10.0, 11.0, 12.0 at a concentration of 50 mM. In addition all test buffers contained 10 mM EDTA. Changes in pH caused by the addition of enzyme to the test buffers or to the assay buffer were measured. *S. pombe* urease appeared to show irreversible loss of activity below about pH 4.5 and above about pH 9.5 (Figure 23 A); however, the addition of urease diluted in test buffer, to the assay buffer, altered the pH (Figure 23 B). This may have affected the urease activity. Several of the assays were done at similar pH, eg. test buffers CHES pH 9 and CAPS pH 10 were both assayed at pH 7.07. Direct activity comparisons were made between such data. This analysis confirms the urease was inactivated above about pH 9.5 but lack of data prohibits confirmation of a low pH inactivation. The color development of the ammonium assay is reduced by CHES and CAPS buffers (data not shown). This accounts for the lower activity measured in the CHES buffer with the same pH as a HEPES buffer.

Figure 21

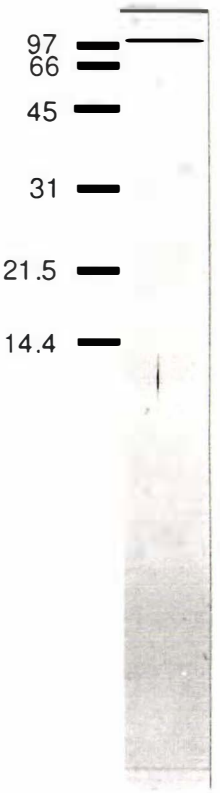
SDS-PAGE of *S. pombe* urease (7.5% acrylamide)




A 7.5% acrylamide separating gel (SDS-PAGE) was used. Proteins were visualized by silver staining. Molecular size standards (Bio-Rad high and low molecular weight SDS-PAGE standards) are marked in kilodaltons. *S. pombe* urease was purified by acetone and ammonium sulfate precipitations and DEAE-Sepharose ion-exchange.

Figure 22

SDS-PAGE of *S. pombe* urease (15% acrylamide)

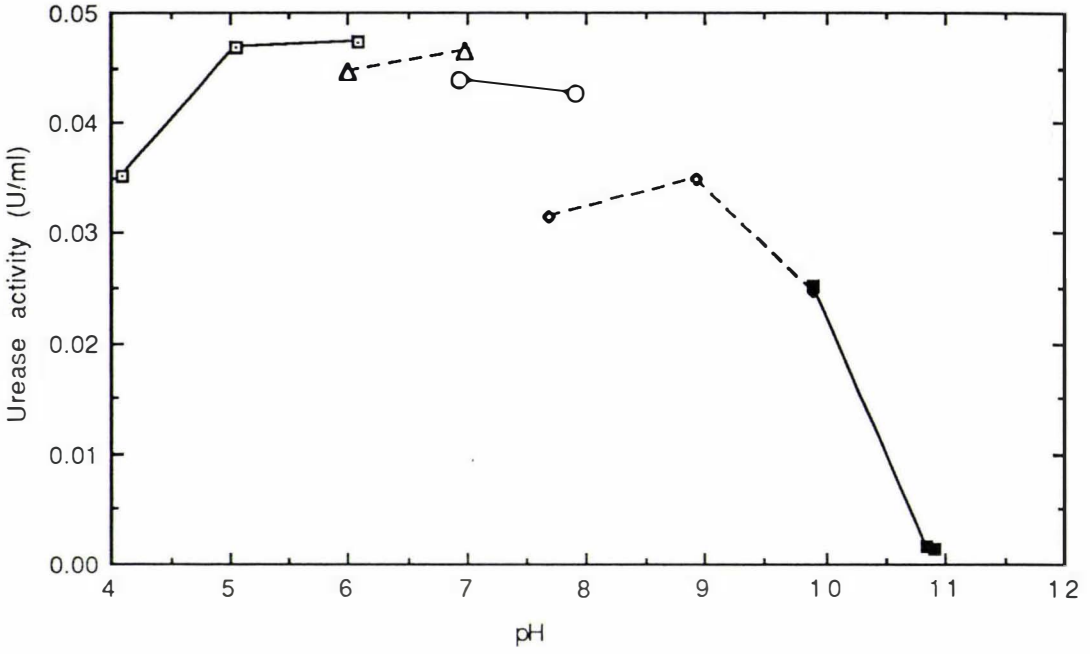



A 15% acrylamide separating gel (SDS-PAGE) was used. Proteins were visualized by silver staining. Molecular size standards (Bio-Rad low molecular weight SDS-PAGE standards) are marked in kilodaltons. *S. pombe* urease was purified by acetone and ammonium sulfate precipitations, DEAE-Sepharose ion-exchange and Mono-Q ion-exchange (FPLC).

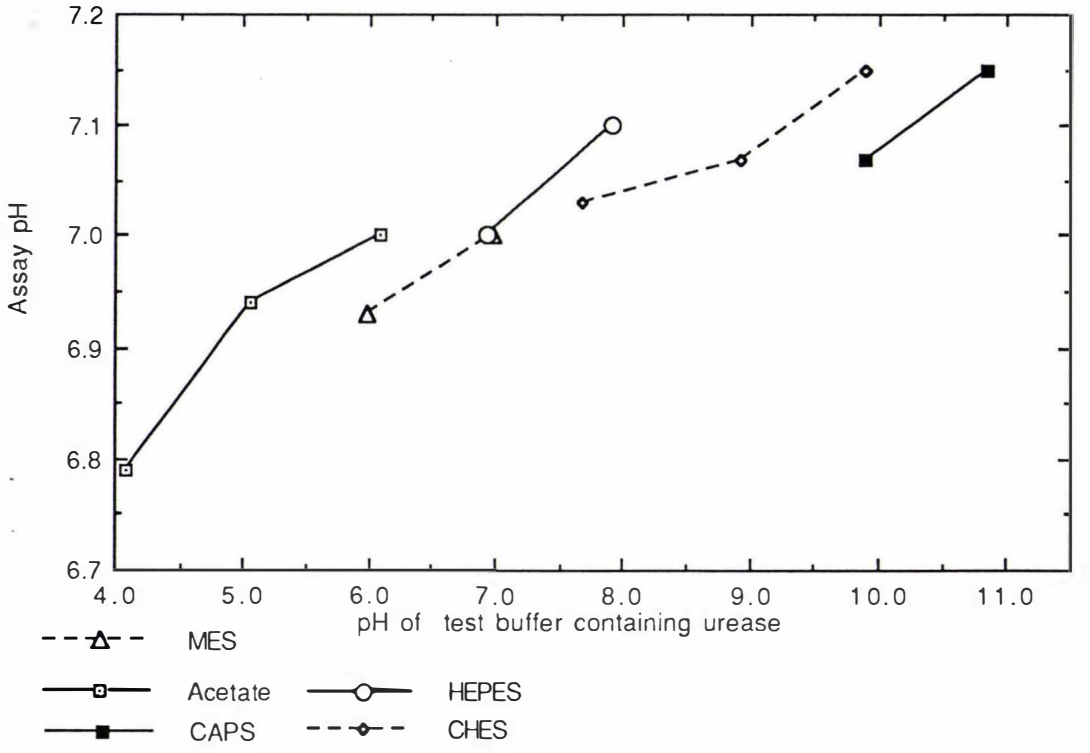
Figure 23

pH stability of *S. pombe* urease.

**A.** Activity after incubation in buffers with various pH.




B. Effect of the pH of the test buffer on the assay pH.




Details are in the text.

### 3.5.4 pH optimum

The activity of urease was determined between pH 4 and 10. A *S. pombe* crude extract was assayed for activity in the following substrate buffers: acetate pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5; phosphate pH 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5. All buffers were 0.02 M and contained 1 mM EDTA and 50 mM urea. Some of the pH values were outside the effective buffering range of these buffers; however, alternative buffers , eg Tris-HCl, CHES, inhibited the color development of the ammonia assay. Any change in pH caused by the addition of enzyme was estimated by diluting PEB 20-fold in each substrate buffer. A single pH optimum, occurring between pH 7.5 - 8.5, was observed (Figure 24).

### 3.5.5 $K_m$ for urea

A crude extract was assayed in substrate buffers (0.02 M phosphate, pH 7.5) containing 0.1, 0.15, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 5.0, 10.0, or 25.0 mM urea. All buffers contained 1 mM EDTA. Activity was determined for 15, 30, 45, 90, and 120 seconds incubation at 25 °C. Reactions were done in duplicate. 2-mercaptoethanol was not included because it may competitively inhibit urease, although it did not affect activity at a high (50 mM) urea concentration. A pH of 7.5 was used because it is within the effective buffering range of phosphate and is close to the optimum pH for activity. The $K_m$, estimated from an Eadie-Hofstee plot, was 1.03 mM urea (Figure 25). An Eadie-Hofstee plot, rather than a Lineweaver-Burk plot, was used to estimate the $K_m$, because it magnifies departures from linearity and the $K_m$ can be determined more accurately (Lehninger, 1975).

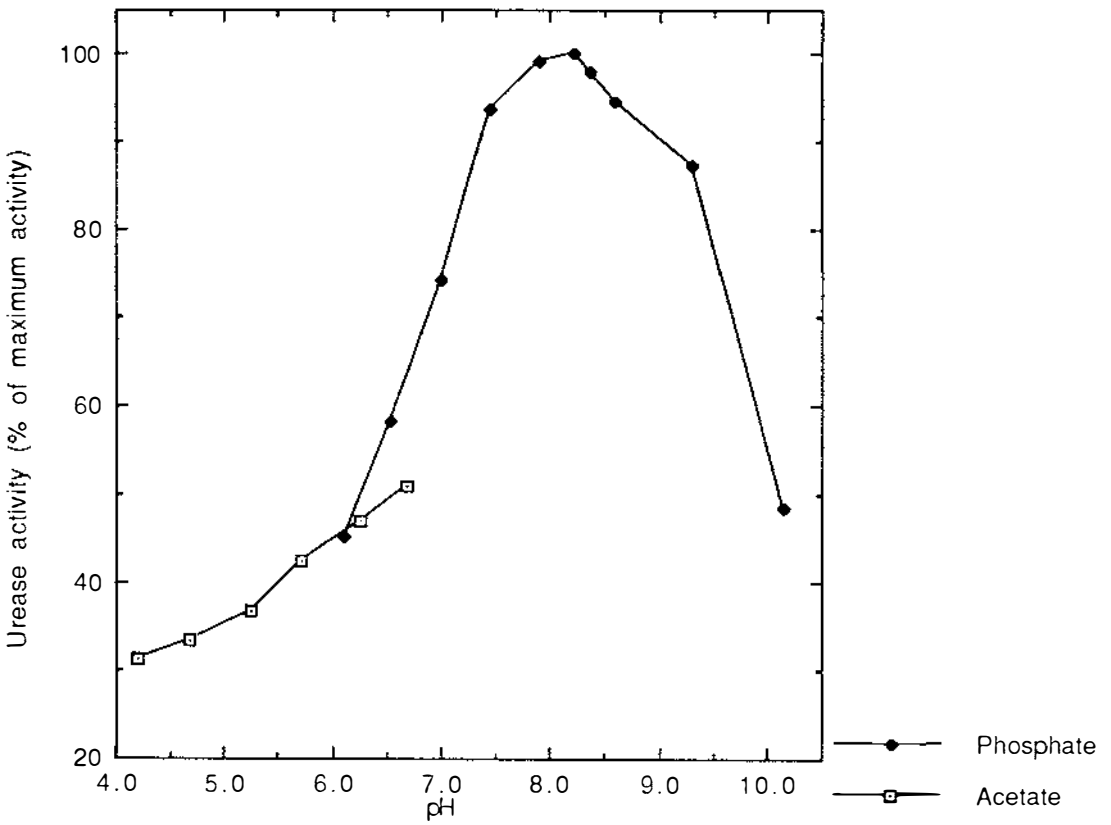
### 3.5.6 Partial sequence of *S. pombe* urease

#### N-terminal sequence

*S. pombe* urease was purified. Two samples (47 µg and 300 µg) were sequenced by the sequential automated Edman degradation method with an Applied Biosystems model 470A apparatus. The sequence of the first 16 N-terminal amino acids was obtained (Figure 27).

Figure 24

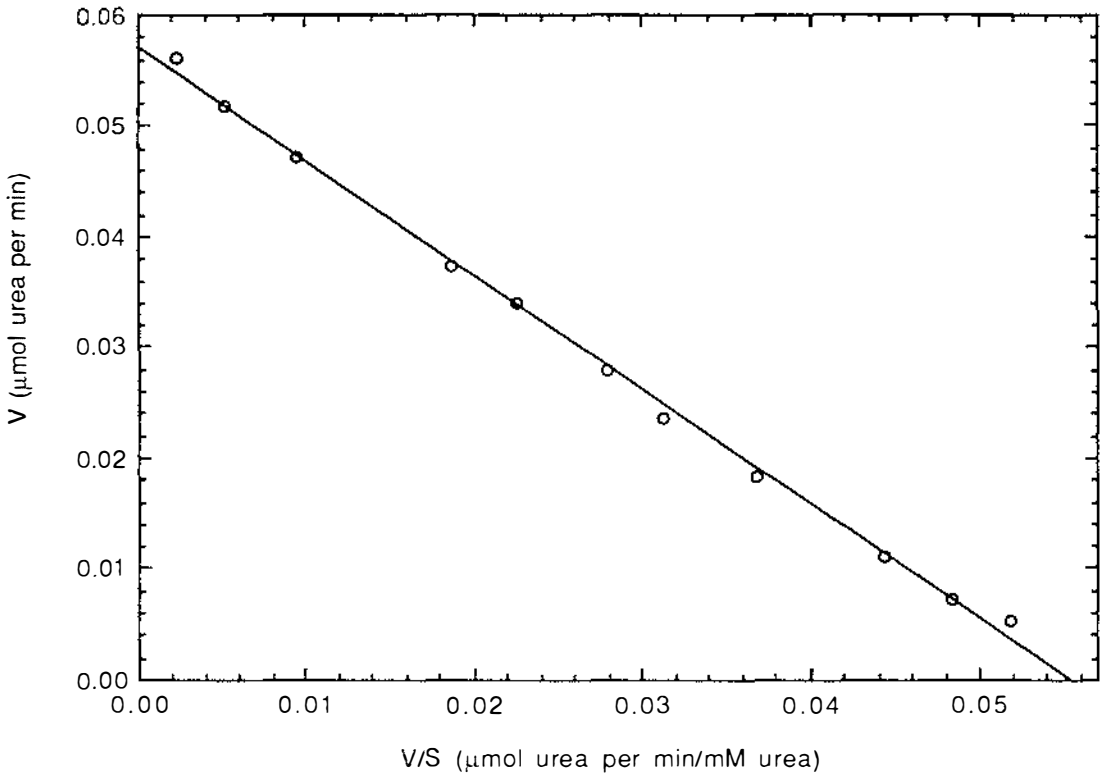
Activity of *S. pombe* urease at various pH




Crude *S. pombe* urease was assayed at various pH in 0.02 M potassium phosphate and acetate buffers containing 1 mM EDTA and 50 mM urea. Details are in the text.

Figure 25

Eadie-Hofstee plot of *S. pombe* urease activity.



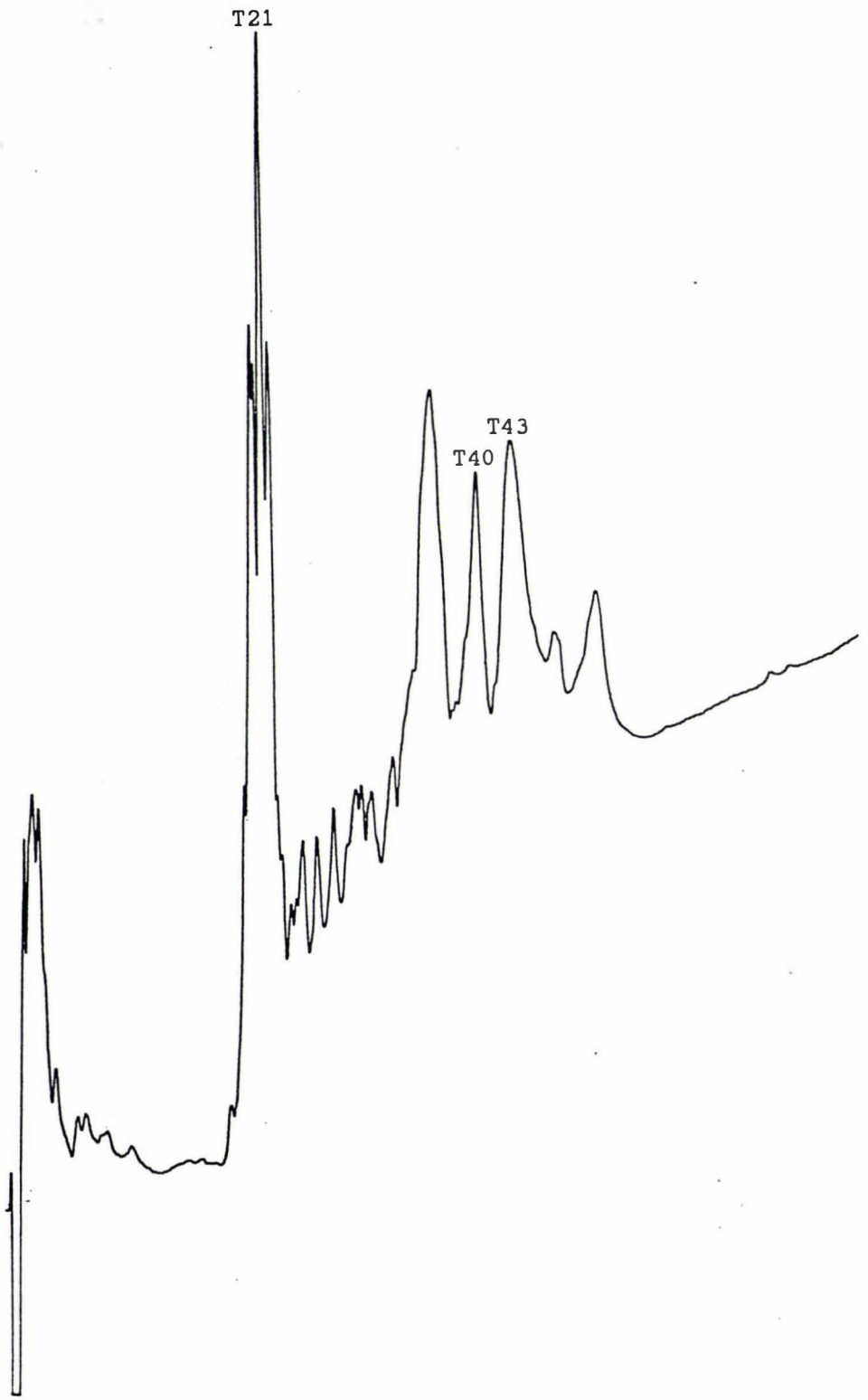

Crude *S. pombe* urease was assayed at various urea concentrations in 0.02 M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA. The $K_m$ (given by the positive slope of the plot) is 1.033 mM urea.

Internal sequences.

Short segments of the amino acid sequence of internal parts of the enzyme were obtained by sequencing peptides produced by proteolytic cleavage of the enzyme. About 300 µg of purified *S. pombe* urease in 150 µl of 0.1 M ammonium bicarbonate was heated to 80 °C for five minutes to denature the enzyme. The denatured urease was incubated with 3 µg trypsin (PFCK treated, Sigma) for 4.5 hours at 37 °C. The resulting peptides were separated and recovered by High Performance Liquid Chromatography (HPLC). A HY-TACH C18 column was used for HPLC and peptides were eluted with a 2 - 90% acetonitrile gradient (Materials and Methods 4.14). The HY-TACH column was used because it is small (4.6 x 75 mm) and has a micropellicular adsorbent (2 µm diameter beads). It irreversibly adsorbs less material than the longer columns with porous packing material that are more commonly used. Effluent peaks were collected and fractions T21, T40, and T43 (Figure 26), were sequenced as above (Figure 27). The sequence of T43 overlapped the N-terminal sequence. All sequences were compared to bovine trypsin, to ensure a fragment of trypsin had not been sequenced. No sequence similarity was found.

Figure 26

Purification of *S. pombe* urease tryptic peptides by High Performance Liquid Chromatography (HPLC)




HPLC was done using a HY-TACH C18 column and an acetonitrile gradient. The elution of peptides was monitored by the absorbance at 214 nm.

Figure 27

Amino acid sequences of *S. pombe* urease N-terminus and tryptic peptides

| | Amino acid sequence |
|---|---|
| N-terminus | Met Gln Pro Arg *Glu Leu His Lys Leu Thr Leu His Gln Leu Gly Ser |
| Peptide T21 | Phe Ile Glu Thr Asn Glu Lys |
| Peptide T40 | Leu Tyr Ala Pro Glu Asn Ser Pro Gly Phe Val Glu Val Leu Glu Gly Glu Ile Glu Leu Leu Pro Asn Leu Pro |
| Peptide T43 | *Glu Leu His Lys Leu Thr Leu His Gln Leu Gly Ser Leu Ala |

*Sequences T43 and N-terminus overlap at this point.

## 4 PCR AMPLIFICATION USING PRIMERS TO THE UREASE PROTEIN SEQUENCE

A segment of the urease subunit gene could be used as a DNA probe to identify corresponding clones in a *S. pombe* gene bank. The polymerase chain reaction (PCR) could be used to isolate a section of the urease gene. PCR is used to amplify the DNA sequence between two primers. These primers hybridize to opposite strands of the target sequence and are oriented so that the DNA polymerase extends the primers across the intervening region. Repeated cycles of heat denaturation of the template DNA and extension products, annealing of the primers to their complementary sequences, and extension of the annealed primers results in an exponential accumulation of target sequence.

The N-terminal and peptide amino acid sequences of *S. pombe* were used to make PCR primers. The primers were used to amplify a product, which was then cloned and sequenced.

### 4.1 PCR primer design

PCR primers to the N-terminal and T40 amino acid sequences were made. PCR primers should not have too many degenerate nucleotide positions (Compton, 1990). Therefore, amino acid sequences were used that contained the smallest number of degenerate codons. Degeneracy on the 3' end of the primer is also undesirable (Compton, 1990) and was avoided. To further reduce degeneracy, the codon bias tables described by Sharp *et al.* (1988) and Russell (1989) were used to exclude the less frequently used codons. *S. pombe* preferentially uses different subsets of codons, depending on whether the gene is expressed at high or low levels; however, it is unknown at what level *S. pombe* urease is expressed. The codon bias tables were combined to give a single table showing the overall codon usage frequencies, regardless of whether the genes have high or low levels of expression. This table was used to avoid including less frequently used codons in the primer sequences. Also, primers were chosen so that some amino acid sequence at both ends of the region between the primers was known. This would allow positive identification of the correct PCR product, by comparing the PCR product nucleotide sequence to the known amino acid sequence. Primers were purchased from Oligos Etc. Inc. (Guilford, Connecticut). Primer sequences and the codon frequencies are described in Figure 28.

Figure 28

PCR primer sequences and codon usage frequency tables.

Primer ML1

N-terminal amino acid sequence and codon frequencies.

| Met | Gln | Pro | Arg | Glu | Leu | His |
|---|---|---|---|---|---|---|
| ATG 1.00 | CAA 0.86 | CCT 0.43 | CGT 0.59 | GAG 0.54 | CTT 0.33 | CAC 0.51 |
| | CAG 0.14 | CCC 0.35 | AGA 0.13 | GAA 0.46 | TTG 0.25 | CAT 0.49 |
| | | CCA 0.17 | CGC 0.09 | | TTA 0.17 | |
| | | CCG 0.05 | CGA 0.08 | | CTC 0.12 | |
| | | | AGG 0.07 | | CTA 0.07 | |
| | | | CGG 0.04 | | CTG 0.05 | |

| Lys | Leu | Thr | Leu | His | Gln | Leu |
|---|---|---|---|---|---|---|
| AAG 0.66 | CTT 0.33 | ACT 0.43 | CTT 0.33 | CAC 0.51 | CAA 0.86 | CTT 0.33 |
| AAA 0.34 | TTG 0.25 | ACC 0.40 | TTG 0.25 | CAT 0.49 | CAG 0.14 | TTG 0.25 |
| | TTA 0.17 | ACA 0.12 | TTA 0.17 | | | TTA 0.17 |
| | CTC 0.12 | ACG 0.05 | CTC 0.12 | | | CTC 0.12 |
| | CTA 0.07 | | CTA 0.07 | | | CTA 0.07 |
| | CTG 0.05 | | CTG 0.05 | | | CTG 0.05 |

| Gly | Ser |
|---|---|
| GGT 0.65 | TCT 0.38 |
| GGA 0.19 | TCC 0.27 |
| GGC 0.12 | AGT 0.13 |
| GGG 0.04 | TCA 0.12 |
| | AGC 0.05 |
| | TCG 0.05 |

ML1 primer nucleotide sequence

5' ATG CAA CC(ATC) CGT GA(GA) TT(GA) CA 3'

The codon frequency data was calculated by combining the high and low expression codon frequency tables as described in the text. Codons within the boxed sections were included in the primer sequence. For the primer sequences, parentheses () indicate redundant positions. Nucleotides grouped in three constitute a codon.

Figure 28 continued.

Primer ML2

T40 amino acid sequence and codon frequencies

| Leu | Tyr | Ala | Pro | Glu | Asn | Ser |
|---|---|---|---|---|---|---|
| CTT 0.33 | TAC 0.58 | GCC 0.50 | CCT 0.43 | GAG 0.54 | AAC 0.59 | TCT 0.38 |
| TTG 0.25 | TAT 0.42 | GCT 0.35 | CCC 0.35 | GAA 0.46 | AAT 0.41 | TCC 0.27 |
| TTA 0.17 | | GCA 0.11 | CCA 0.17 | | | AGT 0.13 |
| CTC 0.12 | | GCG 0.04 | CCG 0.05 | | | TCA 0.12 |
| CTA 0.07 | | | | | | AGC 0.05 |
| CTG 0.05 | | | | | | TCG 0.05 |

| Pro | Gly | Phe | Val | Glu | Val | Leu |
|---|---|---|---|---|---|---|
| CCT 0.43 | GGT 0.65 | TTC 0.55 | GTT 0.46 | GAG 0.54 | GTT 0.46 | CTT 0.33 |
| CCC 0.35 | GGA 0.19 | TTT 0.45 | GTC 0.37 | GAA 0.46 | GTC 0.37 | TTG 0.25 |
| CCA 0.17 | GGC 0.12 | | GTA 0.13 | | GTA 0.13 | TTA 0.17 |
| CCG 0.05 | GGG 0.04 | | GTG 0.04 | | GTG 0.04 | CTC 0.12 |
| | | | | | | CTA 0.07 |
| | | | | | | CTG 0.05 |

| Glu | Gly | Glu | Ile | Glu | Leu | Leu |
|---|---|---|---|---|---|---|
| GAG 0.54 | GGT 0.65 | GAG 0.54 | ATT 0.56 | GAG 0.54 | CTT 0.33 | CTT 0.33 |
| GAA 0.46 | GGA 0.19 | GAA 0.46 | ATC 0.34 | GAA 0.46 | TTG 0.25 | TTG 0.25 |
| | GGC 0.12 | | ATA 0.10 | | TTA 0.17 | TTA 0.17 |
| | GGG 0.04 | | | | CTC 0.12 | CTC 0.12 |
| | | | | | CTA 0.07 | CTA 0.07 |
| | | | | | CTG 0.05 | CTG 0.05 |

| Pro | Asn | Leu | Pro |
|---|---|---|---|
| CCT 0.43 | AAC 0.59 | CTT 0.33 | CCT 0.43 |
| CCC 0.35 | AAT 0.41 | TTG 0.25 | CCC 0.35 |
| CCA 0.17 | | TTA 0.17 | CCA 0.17 |
| CCG 0.05 | | CTC 0.12 | CCG 0.05 |
| | | CTA 0.07 | |
| | | CTG 0.05 | |

Primer ML2 nucleotide sequence

5' - AA ACC (GA)GG (GA)GA (GA)TT (TC)TC (GA)GG - 3'

The reverse complement sequence was used for primer ML2.

4.2 Optimization of PCR

PCR was carried out as described in Materials and Methods 5.2 using primers ML1 and ML2. A variety of denaturation, annealing and extension conditions were tested (Table 19). Three negative controls were included in each set of PCR reactions: (a) template DNA (*S. pombe* genomic DNA) not included; (b) ML1 primer not included; and (c) ML2 primer not included. In all cases no products were obtained with controls (a) and (c). Primer ML2 without ML1, (control b), was able to produce a very weak product under some conditions.

A single 1.1 kb major PCR product and two smaller minor products were obtained when annealing temperatures between 50 and 54 $^{o}$C were used (Figure 29, conditions 1 - 4). At 55 or 56 $^{o}$C no products were obtained (Figure 29, conditions 5). When the primer concentrations were increased four-fold, to 80 pmol per reaction, the yield and specificity were slightly reduced (Figure 29, conditions 2 ). Using a short (30 seconds) annealing time decreased the product yield (Figure 29, conditions 6 and 7). One minute annealing was not as good as two minutes (Figure 29, conditions 9). Decreasing the extension time to one minute (Figure 29, conditions 8) or even 30 seconds (not shown) did not affect the product yield or the specificity. Shortening the denaturation time to 30 seconds improved the yield (conditions 10). A 45 seconds annealing and extension time with 41 cycles amplification gave a good yield but increased the priming of ML2 on its own (Figure 29, conditions 11). For all conditions, the same PCR products were obtained; the two small minor products never disappeared. The ratio of the major band to the minor bands was similar for all conditions, except for annealing at 50 $^{o}$C. When annealing at 50 $^{o}$C was used, slightly more of the smallest minor product was produced. Tetramethylammonium chloride (TMAC) or formamide can improve the specificity of PCR, especially when degenerate primers are used (Mody and Paul, 1990; Sarkar *et al.*, 1990). TMAC ($10^{-6}$M) or formamide (1.25%) were included in a PCR reaction. The thermal cycling conditions were as follows: denaturation - first cycle 94 $^{o}$C for 2 minutes, then 92 $^{o}$C for 30 seconds; annealing - 45 $^{o}$C for two minutes; extension - 72 $^{o}$C for one minute. A low annealing temperature was used because TMAC and formamide lower the maximum temperature at which annealing will occur. Poor yield and specificity were obtained with these conditions.

Table 19

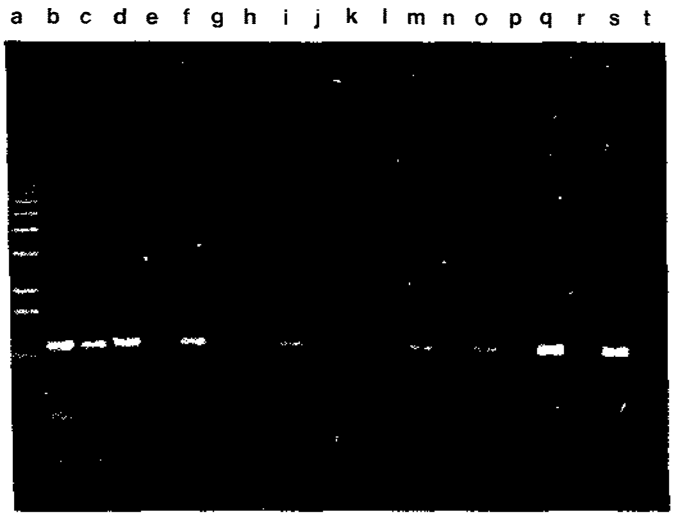
PCR thermal-cycling conditions

| Conditions | Denaturation | Annealing | Extension |
|---|---|---|---|
| 1 | *94 °C x 2.5 min first cycle, then 94 °C x 1 min | 50 °C x 2 min | 72 °C x 1.5 min |
| 2 | *94 °C x 2.5 min first cycle, then 94 °C x 1 min | 52 °C x 2 min | 72 °C x 1.5 min |
| 3 | 94 °C x 2.5 min first cycle, then 94 °C x 1 min | 52 °C x 2 min | 72 °C x 1.5 min |
| 4 | 94 °C x 2.5 min first cycle, then 94 °C x 1 min | 54 °C x 2 min | 72 °C x 1.5 min |
| 5 | 94 °C x 2.5 min first cycle, then 94 °C x 1 min | 56 °C x 2 min | 72 °C x 1.5 min |
| 6 | 94 °C x 3 min first cycle, then 94 °C x 30 secs | 50 °C x 30 sec | 72 °C x 1.5 min |
| 7 | 94 °C x 3 min first cycle, then 94 °C x 30 secs | 52 °C x 30 sec | 72 °C x 1.5 min |
| 8 | 94 °C x 3 min first cycle, then 94 °C x 1 min | 52 °C x 1 min | 72 °C x 1 min |
| 9 | 94 °C x 3 min first cycle, then 94 °C x 1 min | 52 °C x 1 min | 72 °C x 1.5 min |
| 10 | 94 °C x 2.5 min first cycle, then 94 °C x 30 secs | 52 °C x 2 min | 72 °C x 1 min |
| 11 | 93 °C x 2.5 min first cycle, then 92 °C x 30 secs | 52 °C x 45 sec | 72 °C x 45 sec |

Primers ML1 and ML2 were used at 20 pmol per reaction unless marked *. If marked * 80 pmol per reaction was used. 31 cycles of amplification were used for conditions 1 - 10, 41 cycles were used for conditions 11.

Figure 29

Agarose gel electrophoresis of PCR amplified *S. pombe* DNA using primers ML1 and ML2




5 µl aliquots of PCR reactions were loaded onto the 1% agarose mini-gel. DNA was visualized by staining with ethidium bromide. Lane a, BRL 1 Kb Ladder (fragment sizes in kb: 12.2, 11.2, 10.2, 9.2, 8.1, 7.1, 6.1, 5.1, 4.1, 3.1, 2.0, 1.6, 1.0); lanes b - d, conditions 1 - 3; lane e, conditions 3 control b; lane f conditions 4; lane g, conditions 4 control b; lane h, conditions 5; lane i, conditions 6; lane j, conditions 6 control b; lane k, conditions 7; lane l, conditions 7 ML2 control b; lane m, conditions 8; lane n, conditions 8 control b; lane o conditions 9; lane p, conditions 9 control b; lane q, conditions 10; lane r, conditions 10 control b; lane s, conditions 11; lane t conditions 11 control b. Control b reactions contained primer ML2 but not primer ML1. Refer to Table 19 for a description of the cycling conditions.

4.3 Cloning of the PCR product.

A simple procedure for cloning blunt-ended PCR products has recently been described (Marchuk *et al.*, 1991). This procedure takes advantage of the single non template-directed deoxyadenosine (A) residue that is often added to the 3' end of duplex PCR products during the amplification reaction. The PCR products can be efficiently ligated into a T-vector, a blunt-ended vector which has had a single deoxythymidine (T) residue added to the 3' ends of the duplex DNA. A pUC118 T-vector was constructed and used to clone the 1.1 kb PCR product.

PCR was carried out using primers ML1 and ML2 and the following thermal cycling conditions: initial denaturation was 92 °C x 2 minutes, then 35 cycles of 92 °C for 30 seconds, 53 °C x 2 minutes, 72 °C x 30 seconds; followed by a final incubation for five minutes at 72 °C. Six identical reactions were pooled, precipitated with ethanol, and resuspended in 50 µl TE. A 20 µl aliquot of the PCR products was subjected to electrophoresis through a 1% Seaplaque agarose gel. The 1.1 kb PCR product, designated ML12, was recovered from the gel, precipitated with ethanol, and resuspended in TE (see Materials and Methods for details of DNA manipulation techniques). pUC118 was cut with *Sma*I to generate blunt ends, purified by phenol-chloroform extraction, precipitated with ethanol and resuspended in TE. A single thymidine was added to the 3' ends of the cut pUC118 (Materials and Methods 5.3). ML12 and the T-tailed pUC118 were ligated. Analysis by agarose gel electrophoresis showed ligation was successful. *E. coli* strain MC1022 was electrotransformed with the ligation and transformant colonies were grown on Luria plates containing ampicillin, streptomycin and X-gal (5-Bromo-4-chloro-3-indolyl-ß-D-galactopyranoside). Twenty four white colonies were picked and plasmid DNA from each was prepared by the small-scale procedure. The plasmid DNA was digested with *Hin*dIII and analyzed by agarose electrophoresis. Two of the plasmid clones, designated pML12.4 and pML12.7, containing the ML12 fragment were selected for sequencing. *E.coli* strain XL1-Blue was transformed with pML12.4 and pML12.7. Transformant colonies were grown on Luria plates containing ampicillin, tetracycline, and X-gal. For each transformation, plasmid DNA was prepared from six white colonies and analyzed as above. All transformants contained the expected pML12.4 or pML12.7 plasmid. Single-stranded DNA templates were prepared from a pML12.4 and a pML12.7 transformant. The ML12 fragments in pML12.4 and pML12.7 were sequenced for about 370 nucleotides in one direction using the universal -40 primer (Sequenase II, United States Biochemical).

The ML12 sequences from pML12.4 and pML12.7 were identical except for three changes in the ML1 primer region, one at the ninth nucleotide position (T in ML12.4, C in ML12.7) one at the fifteenth nucleotide (G in ML12.4, A in ML12.7) and one at the 18th nucleotide position (A in ML12.4, G in ML12.7). These variations are consistent with different primer species, from the ML1 primer pool (Figure 28), having been used in the PCR amplification. The sequence was translated in the three forward frames. Multiple stop codons were present in each reading frame and no potential coding sequence was identified. The translated sequences were examined for identity with the amino acid sequences of the N-terminus, T21, T40, and T43 using the TFASTA computer program (Deveraux *et al.*, 1984). Apart from the first 6 N-terminal amino acids, which correspond to the ML1 primer, no significant identity was found.

The ML12 fragments from pML12.4 and pML12.7 were cloned into pUC119 to enable the other end of ML12 to be sequenced. pUC119 contains the same multiple cloning site as pUC118 but in the opposite orientation. A fragment cut out of pUC118 with two different restriction enzymes and ligated into the same two restriction enzyme sites in the pUC119 multiple cloning site will be in the opposite orientation, relative to the universal primer site. This allows the two ends of the fragment to be sequenced using the universal -40 primer. pML12.4 and pML12.7 were digested with several restriction enzymes to identify suitable enzymes for cloning into pUC119. The following enzymes were used: *Hin*dIII, *Pst*I, *Eco*RI, *Bam*HI, and *Kpn*I. The products of each restriction enzyme digest were analyzed by agarose gel electrophoresis. *Hin*dIII and *Bam*HI each cut ML12 once, and *Pst*I, *Eco*RI, and *Kpn*I did not cut ML12. pML12.4 and pML12.7 were each digested with *Eco*RI and *Pst*I to release ML12 from the pUC118 vector. The products were separated by agarose gel electrophoresis using a 1% Seaplaque agarose gel and the ML12 fragments were recovered. pUC119 was digested with the same two enzymes as above. After digestion, the enzymes were inactivated by heating to 65 ºC for 20 minutes. The DNA was then precipitated with ethanol and resuspended in TE. The purified ML12 fragments, one from pML12.4 and one from pML12.7, were each ligated to the *Eco*RI/*Pst*I cut pUC119. *E.coli* strain strain XL1-Blue was transformed with each of the ligations. Transformant colonies were grown on Luria plates containing ampicillin, tetracycline and X-gal. Six white colonies from each transformation were selected. Plasmid DNA was prepared from each clone and digested with *Eco*RI. Analysis by electrophoresis showed all plasmid clones contained ML12 ligated to pUC119. One clone from each transformation was selected for sequencing. The clones were designated pML12.4R (R for reverse orientation) and pML12.7R. Single stranded DNA of pML12.4R and pML12.7R was prepared from the

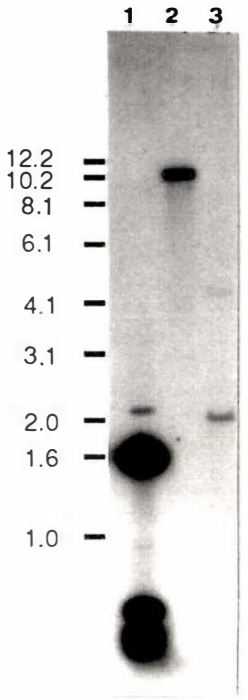
corresponding XL1-Blue transformants and sequenced (about 380 nucleotides), as above. The sequences of pML12.4R and pML12.7R were identical except for one difference at the position corresponding to the 18th nucleotide of primer ML2, (A in ML12.4R, G in ML12.7R). The sequence difference is consistent with different primer species from the ML2 primer pool (Figure 28) having been used in the PCR amplification. The sequence was translated in the three reverse-direction frames. One of the frames contained no stop codons and the sequence corresponding to ML2 was in-frame; however, the sequence after the end of ML2 did not correspond to the expected amino acid sequence. The other two frames contained nine or more stop codons each. Therefore, they are not potential coding sequences. The putative amino acid sequence in all three frames and the nucleotide sequence were examined for identity with other sequences, as was done for ML12.4. No significant identity was found with any of the *S. pombe* urease sequences, or any sequence in the GenBank and EMBL databases.

The PCR product, ML12, does not appear to be part of the urease-subunit gene, nor has any significant identity with any other sequenced gene been found. ML12 was hybridized to *S. pombe* genomic DNA to test if ML12 is a sequence that was amplified from the *S. pombe* genome, or from some other contaminant DNA. *S. pombe* genomic DNA was isolated and 5 μg was digested with *Eco*RI or with *Hin*dIII. The *S. pombe* DNA was a different preparation from that used for the PCR amplifications. The digested DNA was precipitated with ethanol, resuspended in TE, and subjected to electrophoresis through a 1% agarose gel. The gel was blotted onto Hybond-N nylon membrane (Amersham) using the Vacugene XL apparatus (Pharmacia) and the alkaline transfer method. The gel blot was probed with pML12.4, labeled with $^{32}P$ by nick-translation. A single 10.5 kb *Eco*RI fragment and to two *Hin*dIII fragments of 4.4 and 2.1 kb hybridized to pML12.4 (Figure 30). The blot was stripped and probed with pUC118. No *S. pombe* sequences hybridized and BRL 1Kb ladder fragments, which have some homology to pUC118, hybridized strongly (not shown). The hybridization pattern is consistent with the expected number of fragments from ML12; *Eco*R1 does not cut ML12, and *Hin*dIII cuts ML12 into two fragments. These data confirm that ML12 is a *S. pombe* sequence.

*S. pombe* genomic DNA was probed with primers ML1 and ML2 to test how specifically they hybridize. Two identical blots were prepared, as above. For each blot, 30 μg of *Eco*RI and *Hin*dIII digested genomic DNA, 10 ng of ML12 (positive control) and 5 ng of pUC119 (negative control) were used. ML1 and ML2 were end-labeled with $^{32}P$ and used to probe the duplicate blots. Hybridization and the final two minute wash were carried out at 41.5 $^{o}C$ (Materials and Methods 6.13). ML2 hybridized strongly to a

Figure 30

Autoradiograph of *S. pombe* genomic DNA probed with pML12.4




pML12.4 was radiolabeled with $^{32}P$ by nicktranslation and used to probe *S. pombe* genomic DNA. Lane 1, BRL 1 Kb Ladder molecular weight weight markers, the 1.6, 0.5, 0.4, 0.3 kb fragments contain vector sequences which hybridize to the vector sequences of pML12.4 . Lane 2, *S. pombe* genomic DNA (5 µg) digested with *Eco*RI; lane 3, *S. pombe* genomic DNA (5 µg) digested with *Hin*dIII. Marker sizes in kilobases are indicated.

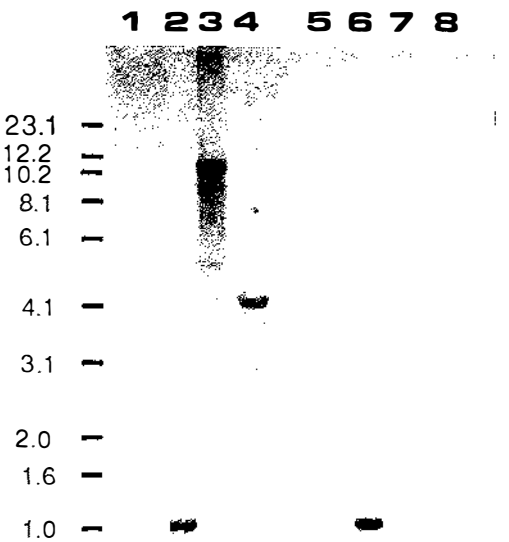
single 11.2 kb *Eco*R1 fragment and a single 4.2 kb *Hin*dIII fragment. ML1 did not hybridize strongly to any *S. pombe* DNA but did hybridize very weakly to a number of bands in both digests (Figure 31). ML1 and ML2 both hybridized strongly to the ML12 fragment. The two *S. pombe* DNA fragments which hybridize to ML2 are significantly different in size from any of the fragments that hybridize to pML12.4. It is possible that the rate of *S. pombe* DNA migration, relative to the BRL 1kb ladder fragments, was different between the two gels used for the blots. This could affect the estimated size of the hybridizing fragments. Several distinct, more intensely staining bands were visible in the *Hin*dIII and *Eco*RI cut DNA (Figure 32). A band from each digest was selected and the sizes were estimated by comparison to the molecular weight standards. The estimated sizes of the selected *Eco*RI and *Hin*dIII bands in the gel used for pML12.4 hybridization were 11 kb and 3.5 kb, respectively, and 11 kb and 3.4 kb in the gel used for ML2 hybridization. This variation is insufficient to account for the different sizes estimated for the fragments hybridizing to the two different probes. pML12.4 and ML2 appear to hybridize to different *S. pombe* genomic sequences.

## Summary

PCR using primers ML1 and ML2 consistently produced one major and two minor products. The major product (ML12) was cloned and sequenced. It did not correspond to the urease-subunit gene, or any other sequence in the GenBank or EMBL sequence databases; however, the PCR product sequence was present in the *S. pombe* genome. ML2 hybridized to a *S. pombe* genomic sequence; however, the sequence was not the same as the PCR product. ML1 did not strongly hybridize to any *S. pombe* sequence, although it did hybridize very weakly to a number of sequences.

Figure 31

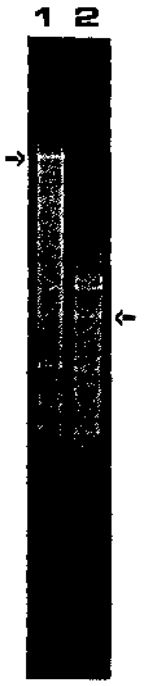
Autoradiograph of *S. pombe* genomic DNA probed with PCR primers ML1 and ML2




PCR primers were radiolabeled with $^{32}P$ using polynucleotide kinase and used to probe *S. pombe* genomic DNA. Lanes 1 to 4 were probed with ML2, lanes 5 to 8 were probed with ML1. Lanes 1 and 5, pUC119 vector (5 ng); lanes 2 and 6, pML12.4 (10 ng); lanes 3 and 7, *S. pombe* genomic DNA (30 µg) digested with *Eco*RI; lanes 4 and 8, *S. pombe* genomic DNA (30 µg) digested with *Hin*dIII. Marker sizes in kilobases are indicated.

Figure 32

Ethidium bromide stained agarose gel Southern blotted for pML12.4 hybridization




A 1% agarose gel was used and DNA was visualized by ethidium bromide staining. BRL 1 Kb Ladder molecular weight standards (sizes 12.2, 11.2, 10.2, 9.2, 8.1, 7.1, 6.1, 5.1, 4.1, 3.1, 2.0, 1.6, 1.0 kb) were used for size determination but are not included in the figure; lane 1 , *S. pombe* genomic DNA (30 µg) digested with *Eco*RI; lane 2, *S. pombe* genomic DNA ( 5 µg) digested with *Hin*dIII. The 11 kb *Eco*RI fragment and the 3.5 kb *Hin*dIII fragment used for gel mobility comparisons between the pML12.4 and ML2 hybridization experiments are marked with arrows.

## 5 ISOLATION OF *URE* GENES BY COMPLEMENTATION

*S. pombe ure*$^-$ strains marked with the *leu1-32* and/or *ura4-D18* alleles were obtained for transformation with *S. pombe* gene banks. The lithium chloride transformation procedure described by Bröker (1987) was improved and used to transform these *ure*$^-$ strains with a gene bank. Gene bank plasmids which complemented the *ure*$^-$ mutations were recovered from the *S. pombe* transformants and tested for homology to the PCR primers ML1 and ML2.

### 5.1 Construction of *ure*$^-$ strains for transformation

*S. pombe ure*$^-$ strains marked with the *leu1*$^-$ and *ura4*$^-$ mutations were required for transformation with *S. pombe* gene banks. *S. pombe* gene banks in the plasmid vectors pDB262, pDB248 and pFL20 were available. The first two vectors contain the *S. cerevisiae LEU2* gene and complement *S. pombe leu1* mutants. pFL20 contains the *S. cerevisiae URA3* gene and complements *S. pombe ura4* mutants.

*S. pombe* strains *ure1-1* $h^-$, *ure2-1* $h^-$, *ure3-1* $h^-$, and *ure4-1* $h^-$ were each crossed with *S. pombe* 131 $h^+$ (*leu1-32*). Spore suspensions from each cross were plated onto YES. The resultant colonies were replica plated onto MM, MINH containing leucine (MINH + leu), and MM containing leucine (MM + leu). Four *leu1 ure*$^-$ colonies from each cross were selected and designated as follows: *leu1 ure1-1* strains were named XL1-1A to D, *leu1 ure2-1* strains were named XL2-1A to D, *leu1 ure3-1* strains were named XL3-1A to D, and *leu1 ure4-1* strains were named XL4-1A to D. The mating type of all *leu1 ure*$^-$ strains was determined by crossing to *S. pombe* 972 $h^-$ and 975 $h^+$. All strains mated with 972 and not with 975 indicating they were $h^+$. XL1-1A and XL2-1A were each crossed with *S. pombe ura4-D18* $h^-$. Spore suspensions from each cross were plated onto YES. The spore-clone colonies were replica plated onto MINH + leu + ura (uracil), MM + leu, MM + ura, and MM + leu + ura. Four colonies of each of four genotypes were selected and named as follows: *leu1 ura4 ure1-1* designated UL1-1A to D; *ura4 ure1-1* designated XU1-1A to D; *leu1 ura4 ure2-1* designated UL2-1A to D; *ura4 ure2-1* designated XU2-1A to D. The strains *ure1-1* $h^+$, *ure3-1* $h^+$, *ure4-1* $h^+$ were each crossed with *ura4-D18* $h^-$ and *ure*$^-$ *ura4* progeny were identified by replica plating, as above. Ten strains of each genotype were selected and named as follows: *ure1-1 ura4* designated U1-1A to J, *ure3-1 ura4* designated U3-1A to J, *ure4-1 ura4* designated U4-1A to J. U1-1 and XU1-1 strains have the same genotype. The U1-1 strains were isolated because the XU1-1 strains were found to be non-transformable. A *ura4 leu1 ure*$^+$ strain,

named UL1, was also obtained from a cross between *ura4-D18 h*$^-$ and 131 (*leu1-32*) as above.

## 5.2 Improvement of the lithium chloride transformation method

The lithium chloride method of transformation (Bröker, 1987) did not work efficiently with the strains of *S. pombe* used in this study. Several modifications were made to the method to increase the frequency of transformation. A negative control reaction, in which no transforming DNA was included, was done for all transformations.

*S. pombe* strain 122 (*leu1-32 his2-245 h*$^+$) was transformed with 0.8 µg of CsCl-gradient purified pDB262, using the procedure described by Bröker (1987). No transformants were obtained. A low transformation frequency (≤7.9 x $10^1$ transformants per µg DNA) was obtained when YE medium, rather than YEPD, was used. The transformation frequency was not improved by gently centrifuging the transformed cell suspension for a few seconds and then resuspending the cells in a smaller volume of TE or Li-PEG buffer (Buffer II, Materials and Methods 7.1) before plating; however, it was possible to spread more transformant cells per plate if they were resuspended to a small volume. Transformation was not improved by increasing the lithium chloride treatment to three hours, instead of one hour, or using 0.1 M LiCl in 1 x TE, instead of 0.2 M LiCl in 2 x TE. Ito *et al.* (1983) resuspended *S. cerevisiae* cells to a concentration of 5 x $10^8$ cells/ml for LiCl transformation. A culture grown to 5 x $10^6$ cells/ml, rather than 5 x $10^7$ cells/ml, and resuspended to 5 x $10^8$ cells/ml, rather than 5 x $10^9$ cells/ml, did not improve transformation. Slightly better transformation was obtained with a fresh overnight culture (about $10^7$ cells/ml), subsequently diluted five-fold as usual, rather than a 24 - 48 hour culture. Ito *et al.* (1983) used a five minute heat shock time. Transformation was not improved by decreasing the length of the heat shock from 25 minutes to five minutes. A different pDB262 preparation, CsCl-gradient purified as before, gave an even lower transformation frequency. Transformation frequencies were dependent on the amount of pDB262 DNA and the strain used. *S. pombe* strains 122 and XL2-1A were each transformed with 1.7 µg or 3.4 µg pDB262. The transformed cell suspensions were gently centrifuged, resuspended in 100 µl TE and plated onto MM + his (histidine). Transformation frequencies of 4.5 x $10^0$ and 1.5 x $10^1$ transformants/µg pDB262 were obtained when 122 was transformed with 1.7 µg and 3.4 µg pDB262, respectively. XL2-1A gave higher transformation frequencies, 1.8 x $10^1$ and 3.1 x $10^1$ transformants/µg DNA for 1.7 µg and 3.4 µg pDB262, respectively.

Carrier DNA increased the transformation frequency at least 100-fold. A 5 mg/ml solution of high molecular weight DNA from herring testes (Sigma D-6898) was prepared in sterile TE, sonicated to break the DNA to fragments of about 2 - 15 kilobases, and denatured by heating at 100 $^{o}$C for ten minutes (Materials and Methods 7.1). A solution of crude DNA oligonucleotides from herring sperm (Sigma D3159) was also prepared as above. *S. pombe* strains XL2-1A and 122 were transformed with 1.7 µg pDB262. Carrier DNA (8 µl) was added to each transformation, including the negative controls (Materials and Methods 7.1). The transformed-cell suspension was gently centrifuged, resuspended in 100 µl TE and plated onto MM + his. The oligonucleotides did not affect the transformation frequency; however, the higher molecular weight DNA increased the frequency by up to 100-fold. About 1,000-2,000 transformants were obtained per plate, for both strains, which corresponds to a frequency of up to 1.2 x $10^{3}$ transformants/µg pDB262. The higher molecular weight carrier DNA was included in all subsequent transformations.

Often transformant colonies did not grow where the transformed cells were thickly spread-plated. The strain UL1 was transformed with 1.7 µg pDB262 or 0.075 µg pFL20. Transformed cells were resuspended in 100 µl TE to give a final volume of 200 µl, and then plated onto MM + ura (for pDB262) or MM + leu (for pFL20). The transformation frequency for pDB262 appeared to be similar to that for 122 and XL2-1A; however, growth was inhibited where the inoculum was more thickly spread. Inhibition was even more pronounced with the pFL20 transformed cells, colonies only grew near the edge of the plate where the inoculum was thinly spread. This type of inhibition was less pronounced, although still evident, in previous transformations using different strains. More uniform growth was obtained when a smaller volume of cells was plated, 50 µl or 100 µl instead of 200 µl, but inhibition was not entirely eliminated. Inhibition was absent when the cells were diluted ten-fold in TE before plating, but the transformation frequency was also slightly decreased. Incubation in PEG and alkali cations, eg. lithium, will eventually kill *S. cerevisiae* cells (Ito *et al.*, 1983). Also, lithium acetate and PEG cause visible alterations to the cell surface (Hong, 1987). It seemed possible that newly-transformed *S. pombe* cells may be temporarily rendered osmotically fragile and also killed or inhibited by continued exposure to LiCl and/or PEG. Perhaps cell viability was decreased by plating the cells without first diluting or removing the Li-PEG buffer (Buffer II, Materials and Methods 7.1) or by diluting the transformed cells in hypotonic buffer (TE). Sorbitol (1.2 M) is used to stabilize osmotically fragile *S. pombe* protoplasts (Beach and Nurse, 1981). Sorbitol and several other isotonic or hypotonic solutions were used to

resuspend transformed *S. pombe* cells before they were plated. XL4-1A and UL1 were transformed with 1.7 µg pDB262. Several identical transformations were done for each strain. The transformed-cell suspensions were gently centrifuged and most of the supernatant was removed, leaving 100 µl of Li-PEG buffer and cells per transformation. Each transformation was resuspended in 900 µl of one of the following solutions: TE, TE containing 1.2 M sorbitol, $H_20$, or 0.9 % saline. The saline solution gave the best transformation frequency, for both strains (Table 20). Only slight improvement of transformation was observed when cells were resuspended in TE containing sorbitol rather than TE alone. UL1 was transformed as above and resuspended in saline or saline containing 1.2 M sorbitol. A slightly lower transformation frequency was obtained when sorbitol was included in the saline. Subsequently all transformed cells were resuspended in 0.9% saline.

The transformation frequency could be increased further by using a culture in earlier log-phase of growth. When the strain UL2-1A was transformed with 0.075 µg of pFL20 a transformation frequency of $7.3 \times 10^4$ transformants/µg DNA was obtained. A transformation frequency of $1.5 \times 10^5$ transformants/µg pFL20 was obtained when the overnight culture (about $10^7$ cells/ml) was diluted ten-fold, instead of five-fold, and incubated for a further three hours, instead of four to five hours, before harvesting the cells for transformation. This transformation frequency is about 17 to 40-fold higher than the frequency obtained by Bröker ($4 - 9 \times 10^3$, 1987) with the same plasmid and is better than the frequency obtained with the protoplast method of transformation ($1 - 5 \times 10^4$, Moreno *et al.*, 1991).

Transformation frequencies were not always reproducible. Variations of up to five-fold were observed between identical transformations carried out at different times, despite great care being taken to ensure conditions were identical. Duplicate transformations done at the same time always gave similar transformation frequencies.

Not all strains could be transformed. Strains U4-1A, U4-1B, U4-1C, U4-1D and U4-1G were all *ure4-1 ura4* spore-clones isolated from the same cross. Strains XU1-1C and UL1-1B are *ure1-1 ura4* spore-clones from another cross (Results 5.1). The U4-1 strains were each transformed with 0.050 µg of pFL20. XU1-1C and UL1-1B were transformed with 0.075 µg pFL20. Transformations were done as described in Materials and Methods 7.1 and plated in duplicate. U4-1B, U4-1C, U4-1C and U4-1G transformed well, with frequencies ranging between $1.3 \times 10^4$ and $1.7 \times 10^5$ transformants per µg pFL20 (Table 21); however, U4-1A, 4-1D, XU1-1C and UL1-1B did not transform. The non-transformable strains also appeared to

Table 20

The effect on the transformation frequency of resuspending transformed cells in various isotonic and hypotonic solutions

| | [a]Transformation frequency | |
|---|---|---|
| Resuspension solution | UL1 | XL4-1 |
| TE | $5.5 \times 10^2$ | $4.7 \times 10^1$ |
| TE + 1.2 M sorbitol | $8.7 \times 10^2$ | $2.5 \times 10^2$ |
| $H_2O$ | $6.2 \times 10^2$ | $1.1 \times 10^2$ |
| 0.9% NaCl | $2.3 \times 10^3$ | $1.1 \times 10^3$ |

*S. pombe* strains UL1 and XL4-1 were transformed with 1.7 µg pDB262 by the lithium chloride method. The transformation buffer, Buffer II, was replaced with 900 µl of various resuspension solutions before cells were plated, as described in the text. [a] Transformation frequency is the number of transformants obtained per µg pDB262. TE and $H_2O$ are hypotonic solutions. TE + 1.2 M sorbitol and 0.9% NaCl (saline) are isotonic solutions.

Table 21

Transformation frequency of *S. pombe* strains

| Strain | *No. of colonies per plate | | [a]Transformation |
|---|---|---|---|
| | negative control | transformed cells | Frequency |
| [b]U4-1A | 2 | 8 | NT |
| [b]U4-1B | 0 | 63 | $1.3 \times 10^4$ |
| [b]U4-1C | 0 | 255 | $5.1 \times 10^4$ |
| [b]U4-1D | 10 | 9 | NT |
| [b]U4-1G | 0 | 847 | $1.7 \times 10^5$ |
| [c]XU1-1C | 44 | 46 | NT |
| [c]UL1-1B | 18 | 29 | NT |

*S. pombe* strains were transformed as described in Materials and Methods 7.1.

* Data are the average number of colonies on two plates.

[a] Transformation frequency is the number of transformants obtained per µg vector DNA.

[b] 0.05 µg pFL20 used for transformation.

[c] 0.075 µg pFL20 used for transformation.

NT = no transformants.

'revert' to *ura4*$^+$; similar numbers of colonies were obtained in the negative controls and the pFL20 transformations. Aliquots of the XU1-1C and UL1-1B cultures were plated onto selective media before the cultures were centrifuged and washed for transformation. Colonies were obtained, indicating that the *ure*$^+$ 'revertants' were present in the cultures before transformation. The other strains were not tested. Non-transformation was also observed with the pDB262 vector. XL1-1A and XL1-1B (Results 5.1) could not be transformed with pDB262, although XL1-1D, which is a spore-clone isolated from the same cross, could be transformed with good efficiency. Furthermore, XL1-1A is a parent of two strains that could not be transformed with pFL20 (XU1-1C and UL1-1B). No 'reversion' of *leu*$^-$ to *leu*$^+$ was observed with any strains.

## Summary

Carrier DNA was essential for high efficiency transformation of the strains used in this study. The frequency of transformation by the 2 μm-based plasmid pDB262 was increased from about $10^1$ to about $10^3$ transformants/μg DNA by including 40 μg (8 μl of a 5 mg/ml solution) of denatured, fragmented (size 2 - 15 kb) Herring DNA in transformations. The effect of carrier DNA on transformation by the *ars1*$^+$-*stb*$^+$-based vector pFL20 was not determined; however, the transformation frequencies obtained were higher (1.5 x $10^5$ transformants/μg DNA) than those previously reported for this vector (4 - 9 x $10^3$ transformants/μg DNA, Bröker, 1987). The frequency of transformation was increased a further 4 to 23-fold, depending on the strain, by resuspending transformed cells in 0.9% NaCl before plating onto selective media. This effect did not appear to be due to osmotic fragility of the cells. Younger cultures gave better transformation. Transformation was strain-dependent and some strains did not transform at all.

## 5.3 Transformation of *ure*$^-$ strains with a *S. pombe* gene bank

A *S. pombe* gene bank was amplified in *E. coli*. The frequency and size of inserts in the gene bank were determined. Gene bank DNA was prepared and used to transform *S. pombe ure*$^-$ mutants. Gene bank plasmid clones that complemented three of the four *ure*$^-$ mutants were recovered. The clones were hybridized to the PCR primers ML1 and ML2.

### 5.3.1 *S. pombe* gene bank amplification, insert size and frequency, and DNA preparation

A *S. pombe* gene bank was obtained, as a gift, from Dr Takashi Toda, Kyoto University, Japan. The gene bank was constructed in the *Bam*HI site of pDB248 by ligating with *Sau*3A partially digested *S. pombe* genomic DNA. The gene bank was received as purified DNA and it was necessary to amplify the DNA in *E. coli*.

*E. coli* strain DH1 was electrotransformed with the gene bank DNA and transformant colonies were grown on Luria plates containing 50 μg/ml ampicillin. About $3.7 \times 10^5$ DH1 transformant colonies were resuspended from agar plates. The resuspended gene bank clones were stored in glycerol at -70 °C.

The size and frequency of inserts in the gene bank were examined. Gene bank clones stored at -70 °C were gently thawed and plated onto Luria containing 50 μg/ml ampicillin. Plasmid DNA was prepared from 48 gene bank clones by the rapid boiling (small-scale) method and digested with one of the following restriction enzymes: *Eco*RI, *Sph*I, or *Hin*dIII. The digested and undigested plasmids were analyzed by agarose electrophoresis. Out of 48 clones tested, 29 had inserts. The insert sizes were 5 kb or larger. Small inserts (less than 2 kb) that were not cut with the restriction enzyme would not have been detected. Two other gene banks were tested in the same way. One gene bank was in the vector pDB262 and the other gene bank was in the vector pFL20. Both of these gene banks contained less than 10% inserts so were considered not useful.

Gene bank DNA suitable for transforming *S. pombe* strains was prepared from colonies resuspended from plates. Gene bank colonies were plated as above and about $2 - 3 \times 10^5$ colonies were resuspended in ice-cold STE buffer (1.5 ml per plate). Cells were pelleted by centrifugation and resuspended in 100 ml ice-cold STE. The cells were centrifuged again and resuspended in 18 ml ice-cold STE. The DNA was prepared by the alkali-lysis method (Materials and Methods 6.1.2). The DNA pellet was dissolved in 3 ml TE and purified by phenol, phenol/chloroform, and chloroform extractions. The DNA was precipitated with ethanol and redissolved in 1 ml TE. Examination using agarose electrophoresis showed plasmid DNA of about the expected size for pDB248 containing inserts had been purified.

5.3.2 Transformation of *ure*$^-$ strains

The *ure*$^-$ strains XL1-1D (*ure1 leu1*), XL2-1A (*ure2 leu1*), XL3-1A(*ure3 leu1*), and XL4-1A (*ure4 leu1*) were transformed with the gene bank. For XL1-1D, XL2-1A, and XL4-1A, transformed cells were plated onto MM. Colonies appeared after five to seven days and were replica plated onto MINH to select for *ure*$^+$ transformants. Colony densities of up to 1,000 colonies per plate were used. Colonies which grew on MINH were selected for further analysis. XL3-1A transformants grew enough on MINH to affect the differentiation of *ure*$^+$ and *ure*$^-$ colonies. The IHG indicator medium was used to identify *ure*$^+$ XL3-1A transformants. Transformed XL3-1A cells were plated directly onto IHG. A density of less than 50 colonies per plate was used so that the identification of colonies expressing only weak urease activity would not be masked by nearby *ure*$^-$ colonies. Blue colonies were selected for further analysis. The transformation frequency was reduced about nine-fold by plating onto IHG, rather than MM.

Potential *ure*$^+$ transformants were streaked onto MINH for single colonies. Large isolated colonies from MINH were patched onto ILG to confirm the *ure*$^+$ phenotype. pDB248 is rapidly lost from cells during growth under non-selective conditions (Beach and Nurse, 1981). The stability test (Materials and Methods 7.2) was used to show that complementation of *ure*$^-$ was due to a gene function present on the plasmid. YES was inoculated with a single colony of a *ure*$^+$ transformant and incubated overnight. The cells were then plated onto YES and the resultant colonies were replica-plated onto two plates. One replica plate screened for the *LEU2* gene and the other plate screened for the *ure*$^+$ phenotype (Materials and Methods 2.2, 2.3).

Out of $2 \times 10^4$ XL1-1D colonies transformed with the gene bank, seven *ure*$^+$ transformants were isolated: they were designated T1-1A, T1-1B, T1-1D, T1-1E, T1-1F, T1-1G, T1-1N. One *ure*$^+$ transformant of XL3-1A, designated T3-1A, was obtained out of $3.5 \times 10^3$ colonies screened. One *ure*$^+$ transformant of XL4-1A, designated T4-1A, was obtained out of $5 \times 10^3$ colonies screened. No *ure*$^+$ transformants of XL2-1A were obtained out of $7 \times 10^4$ colonies screened. All *ure*$^+$ transformants were tested for segregation of the *ure*$^+$ phenotype with the plasmid *LEU2* gene, as described above. Loss of the *ure*$^+$ phenotype was always associated with loss of the *leu*$^+$ phenotype. Therefore, the *ure*$^+$ phenotype was due to a plasmid-borne gene for all of the *ure*$^+$ transformants.

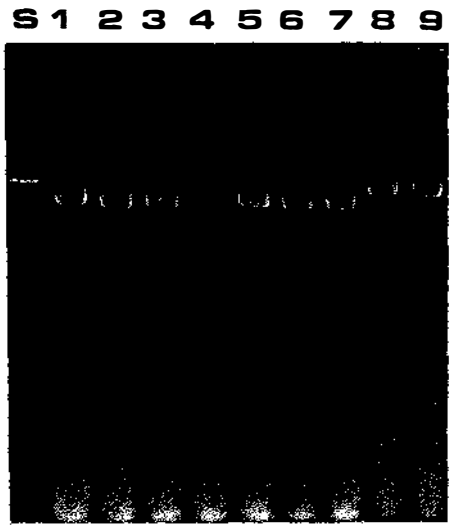
Plasmids containing the the *ure*$^-$-complementing genes were recovered by plasmid rescue in *E. coli*. *E. coli* DB1318 was electrotransformed with DNA isolated from the following yeast transformants: T1-1A, T1-1B, T1-1E, T1-1F, T1-1G, T1-1N, T3-1A, and T4-1A. T1-1D was not used due to poor growth. Transformant colonies were grown on Luria plates containing 100 µg/ml ampicillin. *E. coli* transformants were obtained for all DNA preparations, except T1-1E, and were given the same names as the corresponding *S. pombe* transformants. For each transformation, plasmid DNA was prepared from several colonies and examined by agarose electrophoresis. Two basic plasmid forms were observed: Form One, a good yield of DNA was obtained and the size was consistent with supercoiled pDB248 containing an insert (Figure 33, all lanes except 4); Form Two, a low yield of a much larger product was obtained (Figure 33, lane 4). The Form Two pattern was initially observed for all *E. coli* transformants of T1-1A, T1-1B, T1-1G, three out of four transformants of T1-1N, and two out of eight transformants of T1-1F; however, subsequent rapid boil plasmid preparations often gave the Form One pattern. Plasmids prepared from *E. coli* transformants of T3-1A and T4-1A always gave the Form One pattern. Multimeric forms of pDB248 are commonly formed in *S. pombe* (Heyer *et al.*, 1986). To test if the Form Two plasmids were multimeric, pT1-1F #1 (Form Two), pT1-1F #2 (Form One) and other Form One plasmids were digested with *Hin*dIII (Figure 34), and with *Hin*dIII and *Sal*I together (not shown) and examined by agarose electrophoresis (the prefix 'p' denotes plasmid DNA from the corresponding *E. coli* transformant). All pT1-1 plasmids had a similar band pattern in both digests, indicating Form Two plasmids may be multimers of Form One plasmids.

More importantly, the *Hin*dIII digest and the *Hin*dIII - *Sal*I double digest patterns show that the pT1-1 plasmids, originally from five different *ure*$^+$ transformants of XL1-1D, were all the same gene bank clone and that pT3-1A and pT4-1A were unique clones.

*S. pombe* strains XL1-1D, XL2-1A, XL3-1A and XL4-1A were each transformed with pT1-1G, pT3-1A and pT4-1A. XL1-1D was also transformed with plasmid DNA from all of the other T1-1 *E. coli* transformants. *S. pombe* transformants were grown on MM plates and ten colonies from each transformation were patched onto MINH and ILG to test for the *ure*$^+$ phenotype (Table 22). All XL1-1D colonies that were transformed with the T1-1 clones were *ure*$^+$, and those transformed with pT3-1A and pT4-1A were *ure*$^-$. All XL2-1A transformants were *ure*$^-$. All XL3-1A colonies that were transformed with pT3-1A were *ure*$^+$, and those transformed with pT1-1G and pT4-1A were *ure*$^-$. All XL4-1A colonies that were transformed with pT4-1A were *ure*$^+$, and

Figure 33

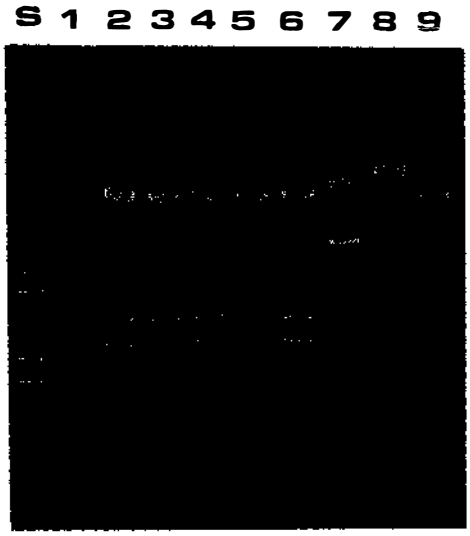
Agarose gel electrophoresis of *S. pombe* gene bank plasmids which complement *ure*$^-$ mutants.




A 1% agarose gel was used and DNA was visualized by staining with ethidium bromide. Plasmid DNA was prepared from *E. coli* cells transformed with DNA from *S. pombe* *ure*$^+$ transformants. Lane S, molecular weight standards, lambda DNA digested with *Hin*dIII (fragment sizes in kb: 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.6); lane 1, pT1-1A; lane 2, pT1-1B; lane 3, pT1-1F #1; lane 4, pT1-1F #2; lane 5, pT1-1G; lanes 6, pT1-1N #1; lane 7, pT1-1N #2; lane 8, pT3-1A; lane 9, pT4-1A. Where data for more than one *E. coli* transformant corresponding to a single original *S. pombe* transformant are shown, the different plasmid isolates are denoted by an identifying number, eg. #1.

Figure 34

Agarose gel electrophoresis of *ure*$^-$-complementing gene bank clones digested with *Hin*dIII.




A 1% agarose gel was used and DNA was visualized by staining with ethidium bromide. Plasmids were digested with *Hin*dIII. Lane S, BRL 1 Kb molecular weight standards (sizes in kb: 12.2, 11.2, 10.2, 9.2, 8.1, 7.1, 6.1, 5.1, 4.1, 3.1, 2.0, 1.6, 1.0); lane 1, pT1-1F #2; lane 2, pT1-1B; lane 3, pT1-1A; lane 4, pT1-1F #1; lane 5, pT1-1G; lane 6, pT1-1N #1; lane 7, pT3-1A; lane 8, pT4-1A; lane 9, pDB248. Plasmids correspond to those shown in Figure 33.

Table 22

Complementation of *ure*⁻ mutants by transformation with gene bank plasmid clones.

| | Gene bank clones | | |
|---|---|---|---|
| Strain | pT1-1G | pT3-1A | pT4-1A |
| XL1-1D (*ure1*) | + | - | - |
| XL2-1A (*ure2*) | - | - | - |
| XL3-1A (*ure3*) | - | + | - |
| XL4-1A (*ure4*) | - | - | + |

*S. pombe ure*⁻ strains were transformed with gene bank plasmid clones and tested for complementation of the urease deficient phenotype by growth on MINH and the color reaction on ILG.

'+' transformants were urease positive, complementation.

'-' transformants were urease deficient, no complementation.

those transformed with pT1-1G and pT3-1A were *ure*$^-$. One *ure*$^+$ *S. pombe* colony from each transformation was tested to show that *ure* complementation was due to a plasmid borne gene (stability test, Materials and Methods 7.2). This analysis confirmed that pT1-1G specifically complemented the *ure1-1* mutation, pT3-1A specifically complemented the *ure3-1* mutation, and pT4-1A specifically complemented the *ure4-1* mutation.

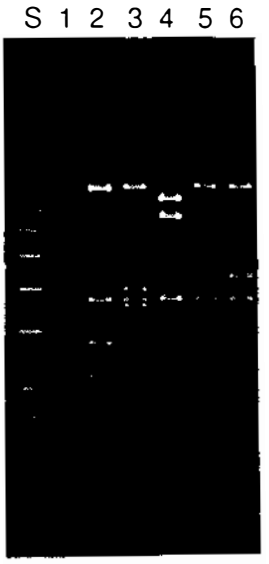
The gene bank clones were tested for homology to the PCR primers ML1 and ML2. pT1-1F, pT3-1A, pT4-1A, and the vector pDB248 were each digested with both *Hin*dIII and *Sal*I (Figure 35). A clone randomly picked from the gene bank, designated #23, was also digested as above. The digested plasmids and the PCR product ML12 were subjected to electrophoresis and vacuum blotted, in duplicate, onto Hybond N (Amersham) nylon filters. About 2 µg of each digest and 100 ng of ML12 were used for each blot. ML1 and ML2 were used to probe separate blots (Materials and Methods 6.13). The ML12 fragment hybridized strongly to both probes (Figure 36). No other bands hybridized significantly to either probe. Very weak hybridization to many bands, including the lambda DNA molecular weight markers, was obtained with both ML1 and ML2.

## Summary

Seven *ure*$^+$ transformants of XL1-1D, one *ure*$^+$ transformant of XL3-1A and one *ure*$^+$ transformant of XL4-1A were isolated by complementation with a gene bank. The *ure*$^+$ transformants were obtained at a frequency of about one per 3,000 transformants for *ure1* and *ure3*, and one per 5,000 transformants for *ure4*. No *ure*$^+$ transformants were obtained for *ure2* , out of about 70,000 transformants screened. Five identical *S. pombe* gene bank plasmid clones that complemented the *ure1-1* mutation, one clone that complemented the *ure3-1* mutation, and one clone that complemented the *ure4-1* mutation were isolated from the transformants by plasmid rescue in *E. coli*. The *ure1*, *ure3*, and *ure4* clones did not hybridize to the PCR primers ML1 and ML2.

Figure 35.

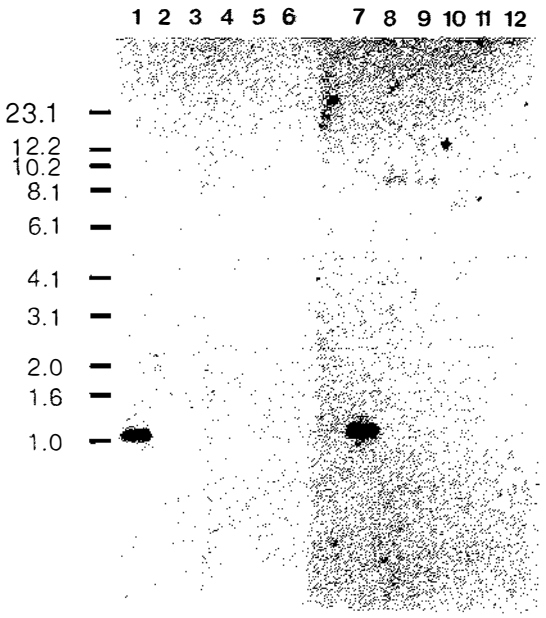
Agarose electrophoresis of *ure*$^-$-complementing gene bank clones, digested with *Hin*dIII and *Sal*I.




A 1% agarose gel was used and DNA was visualized by staining with ethidium bromide. Plasmid DNA (2 μg) was digested with *Hin*dIII and *Sal*I. Lane S, BRL 1 Kb molecular weight standards (sizes in kb: 12.2, 11.2, 10.2, 9.2, 8.1, 7.1, 6.1, 5.1, 4.1, 3.1, 2.0, 1.6, 1.0); lane 1, ML12; lane 2, pT1-1F #1; lane 3, pT3-1A; lane 4, pT4-1A; lane 5, pDB248; lane 6, #23 (random gene bank clone).

Figure 36

Autoradiograph of *ure*$^-$-complementing gene bank plasmid clones hybridized with PCR primers ML1 and ML2.




PCR primers were radiolabeled with $^{32}P$ using polynucleotide kinase and used to probe the *Hin*dIII and *Sal*I digested gene bank clones, as shown in Figure 35. Lanes 1 to 6 were probed with ML1, lanes 7 to 12 were probed with ML2. Lanes 1 and 7, ML12; lanes 2 and 8, pT1-1F #1; lanes 3 and 9, pT3-1A; lanes 4 and 10, pT4-1A; lanes 5 and 11, pDB248; lanes 6 and 12, #23 (random gene bank clone). Marker sizes in kilobases are indicated.

# DISCUSSION

## 1 GROWTH AND DIFFERENTIATION OF *S. POMBE URE+* AND *URE-* STRAINS ON HYPOXANTHINE, UREA, AND INDICATOR MEDIA

*Schizosaccharomyces pombe* can use the purine hypoxanthine as a sole nitrogen source by degrading it to ammonium (LaRue and Spencer, 1968). Urease and at least four other enzymes are required for this breakdown (Kinghorn and Fluri, 1984). All of the *ure-* mutants used in this study were previously isolated from wild type *S. pombe,* following *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) mutagenesis and nystatin enrichment. Mutants deficient in hypoxanthine breakdown were identified by replica plating onto MINH, a medium with hypoxanthine as the only nitrogen source. Urease mutants were identified among the colonies defective in hypoxanthine utilization by their inability to use urea as a sole nitrogen source in liquid media (Kinghorn and Fluri, 1984) or by a negative RUH reaction (mutant LH162, this study, data not shown).

Transformation and genetic mapping of the *ure-* strains required strains containing one or more auxotrophic markers. Therefore, selective media were required that could differentiate between *ure+* and *ure-* colonies when amino acid and nucleotide supplements were included in the test medium. Solid media containing urea (MINU) or hypoxanthine (MINH) as the sole nitrogen source were initially considered good candidates. MINH gave excellent differentiation of *ure+* and *ure-* strains when no supplements were included, with the exception of *ure3-1* strains. *ure3-1* strains grew significantly on MINH. Transformant colonies of XL3-1A (*leu1 ure3-1*) grew so well on MINH that the medium could not be used to detect urease activity. When the supplements leucine, histidine and uracil were included in the medium the clarity of differentiation of *ure+* and *ure-* strains was reduced, but still acceptable, for all strains except XL3-1A. It is likely that the background growth of *ure-* strains on MINH was caused by a small amount of nutrient impurities in the media, despite analytical-grade reagents, Noble agar and Milli-Q-purified water being used. All *S. pombe* strains could grow slightly when no nitrogen source was included in the medium. A similar effect has been noted with a medium used for *Saccharomyces* strain differentiation. A defined medium with lysine as the sole nitrogen source contained enough impurities to support limited growth of *Saccharomyces* species that cannot use lysine (Morris and Eddy, 1957). Pyrimidines, eg. uracil, and certain amino acids, including leucine but not histidine, can be used as a sole nitrogen source by *S. pombe* (LaRue and Spencer, 1967; LaRue and Spencer, 1968). Uracil is used as efficiently

as hypoxanthine and leucine is used poorly. In the present study, the supplements were used at the standard concentration of 50 µg/ml (Gutz *et al.*, 1974), which corresponds to about 0.3 - 0.4 mM for each of the amino acids and about 0.5 mM for uracil. The total nitrogen contribution for the uracil and leucine supplements was about 1.2 mM, 0.3 mM nitrogen from leucine and 0.9 mM nitrogen from uracil (two nitrogens per molecule). This should be sufficient nitrogen to support some growth of *S. pombe*, since good growth was obtained with 2 mM nitrogen.

The background growth of *ure3-1* and XL3-1A on MINH may have been due to a very low level of urease activity. *Neurospora crassa* mutants with partial urease activity can still use urea as a sole nitrogen source (Haysman and Howe, 1971). *Aspergillus nidulans* mutants with 5 - 8% of the wild-type urease activity can grow on plates with urea as the sole nitrogen source, producing colonies with half the diameter of the wild-type. In the present study, the lowest urease activity that could have been detected in *S. pombe* cell-free extracts was about 2% of the wild-type activity. Although no urease activity was detected in *ure3-1* cell-free extracts, if a urease activity lower than 2% of the wild-type was present it would not have been detected and may still have been sufficient to allow some growth on MINH.

Wild-type *S. pombe* can grow well in MINU liquid media (Kinghorn and Fluri, 1984); however, poor growth was observed when solid MINU medium in petri plates was tested (Results 1.1). It is possible that a localized pH change around *ure*$^+$ colonies on MINU, due to the action of urease on urea, inhibited growth. Such an effect would not be observed in liquid media because any pH change would not be localized, but would be rapidly dissipated in the medium. Jones and Mobley (1988) found it necessary to reduce the urea concentration in a urease indicator medium to avoid inhibiting urease-positive bacteria that were sensitive to pH rises (Mobley and Hausinger, 1989). The urea concentration in MINU (2 mM = 0.012%) is well below the urea concentration in the IHG indicator plate (0.2%). Growth on IHG was not as good as on MINA or MINH but was much better than the growth on MINU. The basal medium of IHG was similar to that of MINU, MINH, and MINA, apart from the different nitrogen sources. However, IHG did have several differences from these other media which may have affected the growth: the agar concentration was 3% rather than 2%, the acidity was higher, pH 4.5 rather than pH 5.5, and bromocresol green indicator was included. The higher agar concentration, lower pH, and the pH indicator in IHG may have exerted a buffering effect which inhibited a local pH increase enough to allow growth. It took several days for the bromocresol green in IHG to turn blue around growing colonies, indicating a significant pH change occurred only after colonies had reached a reasonable size.

Another possibility is that the ammonium sulfate (2 mM) in IHG repressed the urease activity so that less ammonium was produced and, therefore, a smaller pH increase occurred. This is unlikely because *S. pombe* urease is not controlled by nitrogen catabolite repression (Discussion 2.2). The possibility of a pH rise causing inhibition of growth on MINU could be easily tested by increasing the phosphate buffer concentration in the medium and including a pH indicator to visualize a pH rise.

The standard solid medium for identifying urease activity in yeasts is Christensen's urea agar (Kreger-Van Rij, 1984). This medium was not sensitive enough to detect urease activity of *S. pombe* in the present study, requires five days incubation before a negative test can be scored, and is used in slants rather than plates. Indicator media ILG and IHG, which enabled differentiation between *ure*$^+$ and *ure*$^-$ *S. pombe* colonies on plates, were developed in the present study. A pH rise around *ure*$^+$ colonies on these media, due to the hydrolysis of urea, changed a pH indicator (bromocresol green) from green to blue. The medium around *ure*$^-$ colonies changed from green to yellow, presumably due to the production of acid end-products from glucose metabolism. Higher levels of glucose produced more yellowing of the medium by *ure*$^-$ strains and less blue color change by *ure*$^+$ strains. The strains *ure3-1* and XL3-1A grew significantly on MINH and also produced less yellowing of the indicator medium than other *ure*$^-$ strains. This may indicate a low level of urease activity in these strains. Both of these strains grew as quickly as the other *ure*$^-$ strains so there is no reason to suspect that a lower rate of glucose breakdown caused less yellowing of the medium. A blue color change was dependent on both urea being present in the medium and the test strain having urease activity. Therefore, these media appear to specifically identify urease activity. These media were not affected by nutritional supplements and colonies patched or replica plated onto ILG could be confidently scored within two days incubation at 30 °C.

## 2 CHARACTERIZATION OF *URE* MUTANTS

### Introduction

Four unlinked urease (*ure*) loci have been identified in *S. pombe* (Kinghorn and Fluri, 1984; and Results 2). Four loci are also required for *Aspergillus nidulans* (Mackay and Pateman, 1982), and *Neurospora crassa* urease activity (Haysman and Howe, 1971; Benson and Howe, 1978). Another fungal urease similarly studied is that of *Ustilago violacea*, which requires two loci for urease activity (Baird and Garber, 1981). The functions of some of these genes are known. For *A. nidulans*, *ureA* appears

to be a structural gene coding for the urea active transport protein (Pateman *et al.*, 1982), *ureB* codes for the single urease enzyme subunit (Mackay and Pateman, 1982), the function of *ureC* is unknown and *ureD* is probably involved in the synthesis or incorporation of the nickel cofactor required for urease activity (Mackay and Pateman, 1980). The functions of the four *ure* genes of *N. crassa* are unknown; however, they all appear to be structural genes which code for proteins essential for urease activity (Haysman and Howe, 1971). The *U. violacea ure1* gene codes for the single urease subunit and the *ure2* gene probably has a urea transport function.

## 2.1 Urea transport

A urea transport role was considered for one or more of the *S. pombe ure* genes. Urea transport systems are found in algae, bacteria and fungi (Mobley and Hausinger, 1989). Fungal mutants which have an inactive urea transport system still appear to have urease activity. *U. violacea ure2* mutants have a non-functional urea transport system and do not accumulate urea intracellularly (Baird and Garber, 1981). Undisrupted cells of these mutants show no urease activity; however, cell-free extracts have wild-type urease activity. *U. violacea ure1* mutants have a non-functional urease enzyme and accumulate urea. Both whole cells and cell-free extracts of *U. violacea ure1* mutants have no urease activity. *A. nidulans* mutants which have a non-functional urea active transport system (*ureA* mutation) are able to grow on urea if the extracellular urea concentration is sufficiently high (>3 mM) to allow urea to enter the cell (Pateman *et al.*, 1982). Therefore, they must still have an active urease and cell-free extracts would have urease activity.

In the present study, no urease activity was detected in any of the cell-free extracts of *S. pombe ure* mutants. Similarly, none of the *N. crassa ure* mutants has wild-type urease activity in cell-free extract (Haysman and Howe, 1971). Therefore, none of the four *ure* genes identified for these organisms appears to have a urea transport function. The apparent absence of urea transport mutants of *S. pombe* is expected because the *ure* mutants were initially identified by their inability to use hypoxanthine as a sole nitrogen source, not by their inability to use urea. It is possible that one or more as yet unidentified genes in *S. pombe*, and perhaps in *N. crassa* as well, encodes a urea transport system.

The measurement of whole-cell urease activity involves four processes: entry of urea into the cell, hydrolysis, exit of ammonia from the cell, and quantitation of the released ammonia. Utilization of the ammonia released by urea hydrolysis may also affect the

measurement of urease activity, although for the *S. pombe* urease assays in the present study the effect would be negligible because assays were carried out for only two minutes. The whole-cell urease activity of wild-type *S. pombe* was less than 6% of the cell-free extract activity (Table 13). The rate-limiting step may be the entry of urea into the cell. It is less likely that ammonia diffusion is the rate limiting step. For bacteria without a urea transport system, the permeability of the cell membrane to urea is $10^4$ to $10^5$ times lower than the permeability to ammonia (Jahns *et al.*, 1988). A urea concentration of at least 100 mM would be required for diffusion-supported growth of bacteria on urea (Jahns *et al.*, 1988). In contrast, bacteria (Jahns *et al.*, 1988) and *A. nidulans* (Pateman *et al.*, 1982), which have an energy-dependent urea transport system can grow well on urea at concentrations below 5 mM. *A. nidulans ureA* mutants lack active transport of urea and grow poorly on urea below a concentration of 3 mM, but they grow well on urea above 3 mM. Urea transport of these mutants at the higher urea concentrations is likely to be facilitated by a second, energy-independent, passive or facilitated diffusion transport system (Pateman *et al.*, 1982). Wild-type *S. pombe* can grow well on low concentrations of urea (2 mM; Kinghorn and Fluri, 1984) so a urea transport system probably exists for this yeast.

## 2.2 Urease Inducibility

The possibility of a urease induction or repression function for the *S. pombe ure* genes was considered. The activity of urease in *S. pombe*, unlike the activity of the other purine catabolic enzymes, is not induced by the purine inducers hypoxanthine, uric acid, allantoin, or allantoic acid (Fluri and Kinghorn, 1985a). Constitutive levels of activity are obtained with all of the purine inducers. It was known that urease activity was present when urea was not included in the media (for example see Fluri and Kinghorn, 1985a); however, it was unknown if urea could increase the level of urease activity. The urease activity of *S. pombe* cell-free extracts was unaffected by including 0.1% urea in the growth medium (YE) (Results 3.2.3). *S. pombe* urease activity is also unaffected by growth in the presence of nitrogen sources known to cause nitrogen repression. Similar levels of activity were obtained for cells grown on glutamate or glutamate and ammonium (Fluri and Kinghorn, 1985a), or ammonium (Kinghorn and Fluri, 1984). Glutamate is a neutral nitrogen source and does not cause nitrogen repression in *S. pombe* (Fluri and Kinghorn, 1985a), whereas ammonium is known to cause nitrogen repression (Davis, 1986). *S. pombe* urease does not appear to be induced by urea or controlled by nitrogen repression. For comparison, *A. nidulans* urease is repressed by growth on ammonium or glutamine, but not by growth on glutamate or urea (Mackay and Pateman, 1982). The regulatory gene

involved in *A. nidulans* nitrogen repression has been cloned, sequenced and studied (Kudla *et al.*, 1990). Also, urea does not induce *A. nidulans* urease. There does not appear to be an obvious regulatory role for any of the *S. pombe ure* genes; however, it is still possible one of the genes encodes a positive, essential activator of urease transcription.

## 2.3 Nickel transport/cofactor

A role in the synthesis, incorporation or transport of a nickel cofactor was considered for the *S. pombe ure* genes. Although no direct involvement of nickel in *S. pombe* urease was tested, to date, all ureases tested have contained nickel. 2-mercaptoethanol competitively inhibits urease by a charge-transfer to a nickel ion (Andrews *et al.*, 1984; Todd and Hausinger, 1989). The absence of any effect on *S. pombe* urease activity by including nickel in the growth medium or 1 mM 2-mercaptoethanol in the assay buffer is insufficient to suggest nickel is absent from *S. pombe* urease. The low concentration of 2-mercaptoethanol (1 mM) may not significantly inhibit *S. pombe* urease at high (50 mM) urea concentrations (Discussion 4.1) and supplementation of the medium with nickel may not be required (Discussion 4.4).

Nickel has been directly demonstrated in plant and bacterial ureases, and implicated in fungal ureases (for review, see Hausinger, 1987; and Mobley and Hausinger, 1989). A nickel transport system and a cellular component for nickel incorporation into protein are required for urease activity. Synthesis of inactive urease apoenzyme, lacking a nickel component, has been demonstrated for many organisms grown in nickel deficient media or in the presence of a nickel chelator. Furthermore, the apourease could be activated by incubating whole cells in the presence of nickel, even in the absence of protein synthesis (see above reviews and references therein; Rando *et al.*, 1990; Lee *et al.*, 1990). Apoenzyme reconstitution appeared to be energy dependent. Nickel added to cell-free extracts could not reconstitute apourease, therefore, a cellular factor (as well as the presence of nickel) appears to be necessary for nickel incorporation into protein (Mulrooney *et al.*, 1989; Rando *et al.*, 1990; Lee *et al.*, 1990).

The ability of *S. pombe ure* mutants to grow on hypoxanthine was not restored by including 0.0005% to 0.05% (about 0.02 to 2 mM) nickel sulfate and manganese sulfate in the medium (Results 2.1.3). Both manganese and nickel were used because these metals increased the urease activity of *Lactobacillus fermentum* in a synergistic manner (Kakimoto *et al.*, 1990). The lack of response of *S. pombe ure* mutants to

nickel is in contrast to results obtained in similar tests with *Aspergillus nidulans ureD* mutants (Mackay and Pateman, 1980). The urease activity and urea utilization of *A. nidulans ureD* mutants is partially restored by including 0.1 mM $NiSO_4$ in the growth medium. The *ureD* gene may be responsible for the production or incorporation of a nickel cofactor essential for urease activity (Mackay and Pateman, 1980). Mackay and Pateman (1980) have discussed similarities between *cnx* mutants and the nickel mutants of *A. nidulans*. The *cnx* mutants are all involved in the synthesis or incorporation of a molybdenum cofactor, yet only one out of the seven classes of *cnx* mutants responds to the addition of molybdenum. Therefore, for *S. pombe ure* mutants, the lack of response to nickel does not necessarily rule out a nickel-related function for the *ure* genes. It also seems possible that the *A. nidulans ureD* mutant that exhibited partial restoration of urease activity was a leaky mutant. Perhaps a defective gene product responsible for incorporation of the nickel cofactor had an altered $K_m$ for nickel, or some other defect, that could be overcome with increased nickel concentrations. Alternatively, *ureD* may be a nickel transport mutant.

Nickel transport systems in several bacteria, *N. crassa*, and *S. cerevisiae* have been characterized (Hausinger, 1987; Jahns *et al.*, 1988). Generally they appear to be energy-dependent systems and some have a very high affinity for nickel. In addition to a nickel-specific transport system, nickel often appears to be transported by a lower affinity magnesium-specific transport system (Hausinger, 1987). Mutations in a nickel-specific transport system lead to a higher requirement for nickel in *Alcaligenes eutrophus*. Nickel was probably transported by the second lower affinity transport system (Eberz *et al.*, 1989). If *S. pombe* can transport nickel via two separate systems, as above, then it is unlikely that any of the *ure* genes has a transport function. If one transport system is non-functional, nickel transport should still occur via the second system, although perhaps only at higher nickel concentrations. The defect should be overcome by increasing the extracellular nickel concentration. Each of the *ure* genes maps to a single locus, as shown by the meiotic recombination data (Results 2.2); therefore, no *ure* class would represent mutations in two transport systems unless the systems shared a common component or the *ure* mutant had mutations in two adjacent transport genes.

## 2.4 Urease subunits

The subunit composition of *S. pombe* urease was investigated to determine how many *ure* genes would be required to code for the structural parts of the enzyme. One subunit, probably present as a dimer, was present in active *S. pombe* urease

(Discussion 4.8). A single *ure* gene would be sufficient to encode the urease enzyme. A single urease subunit and corresponding gene have been identified for two other fungi, *Ustilago violacea* (Baird and Garber, 1981) and *Aspergillus nidulans* (Mackay and Pateman, 1982; Creaser and Porter, 1985). In contrast, bacterial ureases usually have two or three different subunits which are encoded on separate genes and transcribed, with other genes involved in urease activity, as a single polycistronic messenger RNA (Mobley and Hausinger, 1989; Jones and Mobley, 1989; Labigne *et al.*, 1991).

## 3 GENETIC MAP OF THE *S. POMBE URE* GENES

The frequency of intragenic recombination is extremely low when compared to recombination over much larger intergenic distances. The high frequency of prototrophic progeny observed when different *ure*$^-$ strains of *S. pombe* were crossed (Kinghorn and Fluri, 1984; this study, Results 2.1.1) indicates *ure1, ure2, ure3,* and *ure4* represent different complementation groups and are not allelic. Each of the *ure* genes was assigned to a chromosome by induced haploidization and then mapped by meiotic recombination. Analysis of haploidization data has been discussed by Kohli *et al.*, (1977). For each *ure* gene mapped, two diploid heterozygotes were independently haploidized and equal numbers of haploid segregants from each were analyzed. Linkage of the *ure* genes to the respective chromosome markers was indicated by a near or total absence of the two recombinant configurations. The *ure1* gene was allocated to chromosome III, *ure2, ure3*, and *ure4* were allocated to chromosome I. For all haploidized diploids, there was an excess of *lys1*$^+$ *ura1*$^-$ genotypes among the haploid segregants (Table 9). *lys1* and *ura1* are on chromosome I. The most likely explanation for the bias towards *lys1*$^+$ *ura1*$^-$ is a selection against *lys1*$^-$ genotypes. It does not seem likely that the wild-type *ura1* allele would be selected against. Selection against *lys1* would account for the observed under-representation of *ure*$^-$ genotypes for *ure2, ure3,* and *ure4* because these genes are all on chromosome I. There is also a bias in favor of *his3*$^+$ for all haploid segregants, except those from *ure3*. For *ure3*, the frequency of *his3*$^+$ haploid segregants from the two diploids was 92% and 26%, giving an overall frequency of 59% (Table 9). Selection against *his3*$^-$ (or a *his3*-linked gene) may have occurred for all haploid segregants except those from one of the *ure3* diploids. Selection against *lys1*$^-$ and *his3*$^-$ (or *his3* - *lys1* - linked genes) can account for the excess of one recombinant class (*his3*$^+$ *ure*$^+$) amongst the *ure2, ure3,* and *ure4* haploidized segregants (Table 10). Selection against *his3*$^-$ can also account for the absence of the parental *ure1*$^+$ *his3*$^-$ and the recombinant *ure1*$^-$ *his3*$^-$ genotypes. No bias was observed with the *ade6* genotype.

Another explanation for the observed biases towards particular genotypes is that chromosomal nondisjunction occurred during the out growth of the zygote; however it does not seem likely that this would occur for both of the diploids that were haploidized for each of the *ure* genes. Chromosomal non-disjunction could account for the difference in bias noted for segregants from the two *ure3* diploids.

Tetrad analysis was used to determine the linkage relationships between *ure1* and the chromosome III marker genes *fur1* and *ade6*; and between *ure2, ure3,* and *ure4* and the chromosome I marker genes *lys1, ura2* and *ade4*. All genetic distances, including those previously published, were corrected for multiple crossovers, as described by Munz *et al.* (1989).

The distance between *ure1* and *fur1* or *ade6* was 32 cM and 50 cM, respectively. These distances correspond well to the known distance between *fur1* and *ade6* (21.6 cM, Kohli *et al.*, 1977; Munz *et al.*, 1989) and the centromere linkage observed for *ure1* (Table 8). *ure1* can be placed on the left arm of chromosome I, distal to *fur1* (Figure 37).

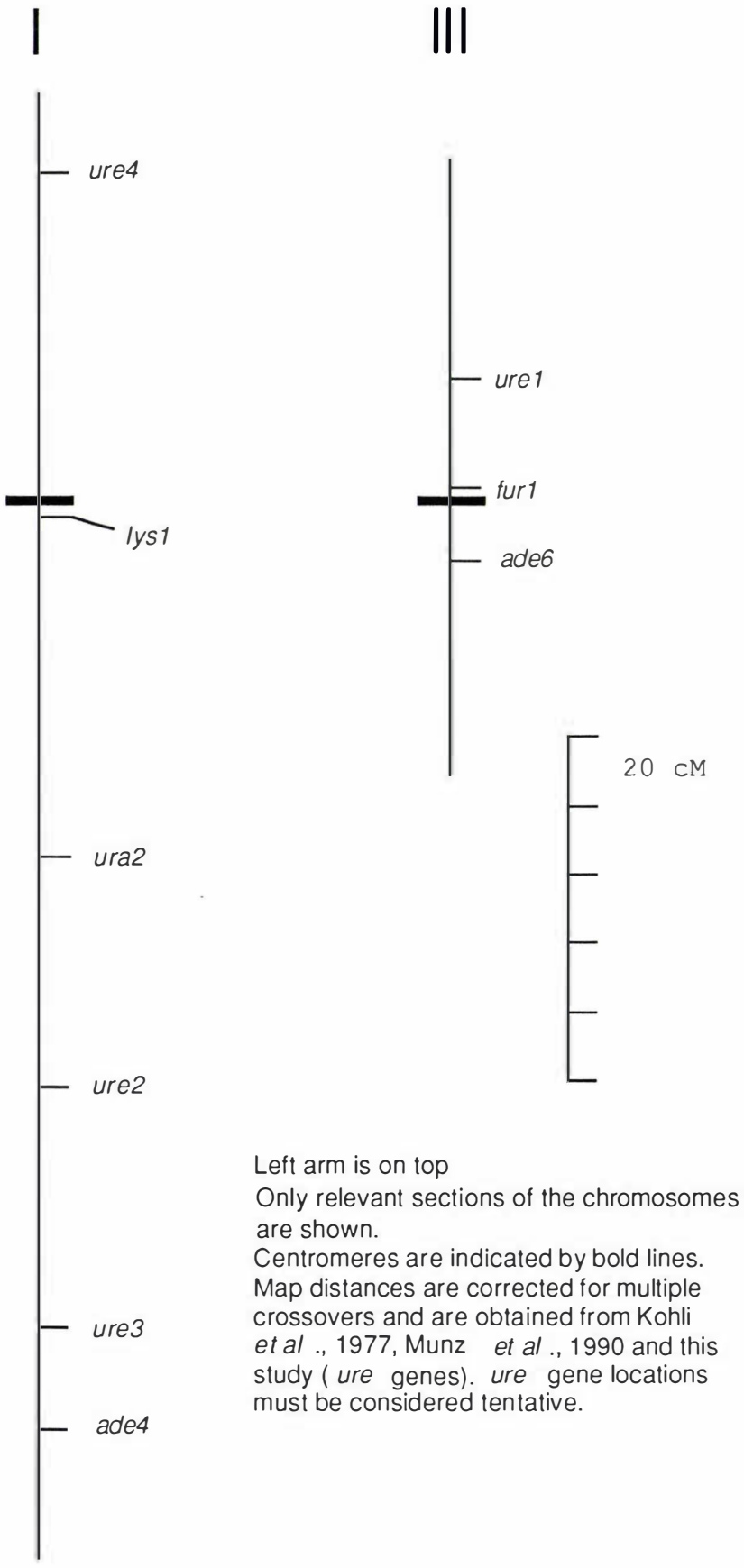
The map distance between *ure2* and *lys1, ura2,* or *ade4* was >200 cM, 69 cM, and 100 cM, respectively. The previously published map distance between *lys1* and *ura2* is 100 cM (Munz *et al.*, 1989), and between *ura2* and *ade4* is 168 cM (Kohli *et al.*, 1977; Munz *et al.*, 1989). Therefore, *ure2* can be placed between *ura2* and *ade4* (Figure 37).

The map distance between *ure3* and *lys1, ura2,* or *ade4* was >200 cM, 91 cM, and 31 cM, respectively. Therefore, *ure3* can be placed between *ura2* and *ade4* (Figure 37).

The map distance between *ure4* and *lys1, ura2,* or *ade4* was 100 cM, 115 cM, and 135 cM, respectively. Therefore, *ure4* can be tentatively assigned to the left arm of chromosome I (Figure 37). The closest linkage, between *ure4* and *lys1,* was only significant at the 10% level, although it is assumed greater significance would be obtained with larger numbers of tetrads analyzed. The calculated map distances are very large and, therefore, may be subject to considerable error (Munz *et al.*, 1989). For comparison, the four *ure* genes of *Neurospora crassa* are present as two sets of closely linked genes (Kolmark, 1969; Haysman and Howe, 1971). The four *ure* genes of *Aspergillus nidulans* are present as one set of two closely linked genes and two other unlinked genes (Mackay and Pateman, 1982). The two *ure* genes of *Ustilago violacea* are unlinked (Baird and Garber, 1981).

Figure 37

Genetic map of *S. pombe* chromosome I and II, showing the positions of the *ure* genes.




Left arm is on top
Only relevant sections of the chromosomes are shown.
Centromeres are indicated by bold lines.
Map distances are corrected for multiple crossovers and are obtained from Kohli *et al* ., 1977, Munz *et al* ., 1990 and this study ( *ure* genes). *ure* gene locations must be considered tentative.

The genetic distances estimated in the present study are subject to several sources of error. Firstly, only a small number of tetrads was analyzed, between 41 and 99 (Table 12). Therefore, small changes in the number of tetrads in a particular class can dramatically affect the map distance, eg. one more NPD tetrad for the *ure3-1* - *ade4* data would increase the calculated map distance from 31 cM to 40 cM. Secondly, the map distances for the larger intervals will only be a rough estimate of the true genetic distance (Munz *et al.*, 1989). Thirdly, interference was assumed to be absent because, overall, the interference constant ($k$) in *S. pombe* is close to one; however, interference may vary between particular segments of the chromosomes, with extremes of $k = 0.2$ to $k > 2.0$ (Munz *et al.*, 1989). Interference can dramatically affect the map distance, eg. if interference is taken into account, the *ade4* - *ura2* distance is 207.8 cM instead of 168 cM (Munz *et al.*, 1989). A further source of error could come from the selective viability of spores. Asci containing two or more non-viable spores cannot be included in the tetrad analysis. If certain classes of spores, eg. particular recombinants, had poor viability, then the map distances could be affected. For example, if the asci not included in the analysis because of poor spore viability were more likely to contain a recombinant spore class the calculated map distance would be underestimated. The fractional spore viabilities for each cross are presented in Table 11. The proportion of asci containing two or more non-viable spores was between 5% and 19% for all crosses except *ure3-1* $h^-$ x 2143. For this cross, 37% of the asci contained two or more non-viable spores. The accuracy of the recombination data was examined by comparing gene-pair genetic distances calculated from the data of the present study with the previously published distance for the same gene pair. The *fur1* - *ade6* distance was 35 cM (this study), compared to the previously published value of 21.6 cM. The *lys1* - *ura2* and *ura2* - *ade4* intervals estimated from the data of this study were as follows: *ure1* data - 86 cM and 128 cM, *ure2* data - 106 cM and ≥ 200 cM, and *ure3* data - 70 cM and 73 cM, respectively. The previously published values are *lys1* - *ura2*, 100 cM and *ura2* - *ade4*, 168 cM. Obviously the map distances calculated in this study may be inaccurate and should be treated as a general guide only. Interestingly, the data from the crosses with the lowest spore viability (*ure3* data) gave better estimates of the map distances than the crosses with higher viability. Spore inviability does not appear to have been associated with the *lys1, ura2,* or *ade4* genotypes, although the effect on the *ure* genotypes cannot be determined.

# .4 PURIFICATION AND CHARACTERIZATION OF *S. POMBE* UREASE

## 4.1 Urease assay method and inhibitors

The urease assay method described by Wong and Shobe (1974) was used to quantify urease activity in the present study. This method is based on the very sensitive and technically simple indophenol assay. The urea concentration (50 mM) of the substrate buffer was above the saturation concentration for *S. pombe* urease and the reaction rate was constant over the standard two-minute incubation period (Results 3.1). Ureases are susceptible to inactivation by oxidation and heavy metals (Mobley and Hausinger, 1989); therefore, 2-mercaptoethanol and EDTA were included in extraction and substrate buffers. Inhibition of urease by 2-mercaptoethanol has been demonstrated for the enzymes from jack bean (Andrews *et al.*, 1984) and *Klebsiella aerogenes* (Mobley and Hausinger, 1989; Todd and Hausinger, 1989). In both cases spectral evidence showed that 2-mercaptoethanol bound to a nickel ion at the active site. *S. pombe* urease might also be expected to be competitively inhibited by 2-mercaptoethanol because all ureases probably contain nickel (Hausinger, 1987); however, it was shown that *S. pombe* urease was not inhibited by 1 mM 2-mercaptoethanol when the urea substrate concentration was 50 mM (Results 3.1.3). The effect of a competitive inhibitor on the activity of an enzyme is represented by the following equation:

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \left( 1 + \frac{[I]}{K_i} \right) \left( \frac{1}{[S]} \right)$$

(Stryer, 1981)

The $K_m$ for urea of *S. pombe* urease (in the absence of 2-mercaptoethanol) was 1.03 mM. Therefore, at 50 mM urea and a 1 mM 2-mercaptoethanol, the equation can be rewritten:

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{1.03}{V_{max}} \left( 1 + \frac{1}{K_i} \right) \left( \frac{1}{50} \right)$$

If the term $(1+1/K_i)(1/50)$ is about 3/50 or less, $1/V$ will be within 6% of $V_{max}$ and, therefore, within the range of experimental error. This is true if $K_i \geq 0.5$ mM 2-mercaptoethanol. The $K_i$ for 2-mercaptoethanol of jack bean and *K. aerogenes* urease is 0.72 mM and 0.55 mM, respectively. The absence of inhibition of *S. pombe* urease under the conditions tested implies that if 2-mercaptoethanol is a competitive inhibitor of this urease, as expected, then the $K_i$ is no less than that of jack bean or *K. aerogenes* urease.

## 4.2 Cellular localization of urease

*S. pombe* urease was associated with the non-particulate soluble fraction of the cytosol (Results 3.2.1). This is in agreement with the intracellular location determined for ureases from bacteria and jack bean (Mobley and Hausinger, 1989) and *Aspergillus* species (Creaser and Porter, 1985; Zawada and Sutcliffe, 1981). It is possible that *S. pombe* urease is located in organelles, such as peroxisomes, that were disrupted during the isolation procedure. An extracellular location of urease has been demonstrated for *Helicobacter pylori* (Bode *et al.*, 1989; Dunn *et al.*, 1990).

## 4.3 Urease isozymes

Only one active form of *S. pombe* urease was detected by native polyacrylamide gel electrophoresis (native PAGE) (Results 3.2.2). Similar results have been observed for *A. nidulans* (Creaser and Porter, 1985). The single urease species observed for *S. pombe* is consistent with the genetic data. A mutation in any of the four *ure* genes abolishes urease activity (Kinghorn and Fluri, 1984). This would not be expected if two or more enzymes, encoded by different genes, needed to be inactivated before all urease activity was destroyed. Interestingly, bacteria with one active urease often exhibit two or more bands on native PAGE gels.(Senior *et al.*, 1980; Jones and Mobley, 1987). Usually one major band of higher mobility and one or two minor, more slowly migrating, bands were present. The multiple bands did not represent multiple urease isozymes and were not the result of self-aggregation (Mobley and Hausinger, 1989). Various treatments which would affect the charge on the enzyme, the self-aggregation or aggregation with other cellular components, the glycosylation or phosphorylation, the enzyme conformation or the nickel cofactor did not affect the banding pattern (Mobley and Hausinger, 1989). The reason for the multiple banding pattern of these bacterial ureases is unknown. In contrast, the multiple bands observed with jack bean urease are aggregate forms (Blattler, 1967).

## 4.4 Effect of nickel and manganese on urease activity

Complex media may contain insufficient available nickel for the synthesis of fully active urease. Booth and Vishniac (1987) observed that urease activity of yeasts could be increased by including nickel in the growth medium. A similar effect has been noted for various bacteria (Schneider and Kaltwasser, 1984; Mackerras and Smith, 1986; Bast, 1988; Rando *et al.*, 1990) and algae (Rees and Bekheet, 1982). Kakimoto *et al.*

(1990) demonstrated that nickel and manganese can act synergistically to increase the urease activity of *Lactobacillus fermentum*. Rando *et al.* (1990) propose that nickel chelating compounds may be present in complex media. Data from the present study show that the urease activity from *S. pombe* cultures grown in YE was not affected by including nickel and manganese in the medium, suggesting that the medium contained sufficient available nickel for urease synthesis. Although it is possible that *S. pombe* urease does not contain nickel, this is considered unlikely, given the ubiquity of nickel in urease (Discussion 2.3).

### 4.5 Urease stability

Crude *S. pombe* urease, like many bacterial ureases (Mobley and Hausinger, 1989), was very stable in buffer containing EDTA and 2-mercaptoethanol. The protease inhibitor phenylmethylsulfonyl fluoride (PMSF) did not improve the yield or stability of urease. PMSF inhibits serine proteases and some thiol proteases and carboxypeptidases (Scopes, 1987). The stability of *S. pombe* urease without PMSF indicates these proteases may not be active in *S. pombe* or are not active against urease.

### 4.6 Affinity chromatography

Three hydroxyurea derivatized resins were prepared and used for trial affinity chromatography purifications of urease from jack bean and *S. pombe* (Results 3.3). The adsorbents tested were (a) an oxirane-hydroxyurea derivatized resin (OHA), (b) an aminocaprylic acid-hydroxyurea derivatized resin (AHA) and (c) an adsorbent prepared by two rounds of reaction with ethylene diamine and succinic anhydride, followed by reaction with hydroxyurea (EHA adsorbent). The degree of substitution of AHA (with hydroxyurea) and EHA (with hydroxyurea or unsuccinylated ethylene diamine) was good. The substitution of OHA with hydroxyurea was lower than AHA and EHA (Materials and Methods 4.6.4).

The EHA adsorbent has been previously described (Shobe and Brosseau, 1974). EHA was tested for ability to purify jack bean urease (Results 3.3.3). The urease was eluted by increasing the ionic strength and acidity of the buffer, as described by Shobe and Brosseau, or with a linear urea gradient. The purification obtained with both eluants was poor. Better purification was obtained by conventional ion exchange chromatography using the anion-exchanger DEAE-Sepharose. Most of the contaminant proteins that co-eluted with urease from DEAE-Sepharose also co-eluted with urease from EHA. The EHA resin may have been acting as a anion exchanger with properties

similar to DEAE. This would occur if positively charged residues were present on the resin. Ethylene diamine residues that escaped succinylation may carry a positive charge at neutral pH ($pK_a$ of $-CH_2-CH_2-NH_3^+$ is about 10.5) and may be responsible for the apparent ion exchange effect. Shobe and Brosseau (1974) did not observe non-specific binding of proteins to their EHA adsorbent and cell-free extracts of *Morganella* (*Proteus*) *morganii* and partially purified jack bean urease were purified to homogeneity. The urease binding activity was shown to be specific for a hydroxyurea ligand; a hydroxylamine ligand bound very little urease (Shobe and Brosseau, 1974). Another explanation for the low degree of purification achieved using the EHA adsorbent prepared in the present study could be the large amount of contaminant proteins in the crude jack bean extract. For the purifications done by Shobe and Brosseau, purification factors of only 22 - 30 fold for jack bean and 51 - 99 fold for *M. morganii* were required to give pure urease. A purification factor of 400 - 500 fold would be required to purify the crude jack bean urease (specific activity 6 - 7 U/mg) used in the present study to the specific activity of pure urease (3000 U/mg, Shobe and Brosseau, 1974). Perhaps the contaminant proteins that co-elute with urease from DEAE-Sepharose and from EHA were not present in the partially purified jack bean urease used by Shobe and Brosseau.

The oxirane-hydroxyurea derivatized resin (OHA) did not bind any urease activity (Results 3.3.1). The substitution of this matrix may have been low but binding of some urease would be expected. A similar adsorbent with a urea ligand, rather than hydroxyurea has been successfully used to purify jack bean urease (Mendes *et al.*, 1988).

The aminocaprylic acid-hydroxyurea derivatized resin (AHA) did not bind urease activity. Shobe and Brosseau (1974) have suggested that a minimum of 8 - 13 atoms in the side chain between the hydroxyurea ligand and the agarose backbone may be required for binding of jack bean urease. The number of atoms in the side chain of AHA was nine. AHA may not have bound urease due to steric hindrance caused by insufficient distance between the agarose backbone and the hydroxyurea ligand. However, *S. pombe* urease is much smaller than jack bean urease, about 212 kilodaltons (Results 3.5.1) versus 590 kilodaltons (Andrews *et al.*, 1984) and, therefore, may be subject to less steric hindrance than jack bean urease. Interestingly, a hydroxyurea substituted resin with an 8-atom side chain bound urease from *Aspergillus nidulans* (240 kilodaltons; Creaser and Porter, 1985) and *Ureaplasma urealyticum* (150 kilodaltons; Saada and Kahane, 1988).

Affinity chromatography has been used to purify ureases from a number of sources, with various degrees of success. A hydroxyurea-substituted Affinose AF 202 adsorbent (13 atom side chain) was successfully used to purify to homogeneity urease from cell-free extracts of *M. morganii* and partially purified jack bean urease (Wong and Shobe, 1974). The same affinity resin was also useful when used as one of several steps to purify urease from *Brevibacterium ammoniagenes* (Nakano *et al.*, 1984), *Lactobacillus reuteri* (Kakimoto *et al.*, (1989) and *Lactobacillus fermentum* (Kakimoto *et al.*, 1990), but was not useful in purifying urease from bovine rumen (Mahadevan *et al.*, 1977) or from *Bacillus pasteurii* (Christians and Kaltwasser, 1986). A hydroxyurea substituted CH-Sepharose adsorbent was successfully used as one of several steps to purify urease from *A. nidulans* (Creaser and Porter, 1985) and *U. urealyticum* (Saada and Kahane, 1988; Mobley and Hausinger, 1989). In all of the above examples, including the unsuccessful purifications, the degree of purification required to give homogeneous urease was much less (between 25 and 1,000-fold) than that required to give homogeneous *S. pombe* urease from the cell-free extract (nearly 4,000-fold).

## 4.7 Purification of urease by precipitation and ion exchange chromatography

Acetone and ammonium sulfate precipitation are methods commonly used to partially purify proteins (Scopes, 1987) and have been used to purify ureases from a number of sources, eg. *Bacillus pasteurii* (Larson and Kallio, 1954). Acetone and ammonium sulfate precipitation of *S. pombe* urease increased the purity of the enzyme nearly 150-fold. Ureases are anionic (negatively charged) at neutral pH (Mobley and Hausinger, 1989). An anion exchange chromatography step is included in the purification of many ureases. The very good purification of *S. pombe* urease observed with the DEAE-Sepharose ion exchange procedure (Results 3.4.4.1) has also been observed for the purification of ureases from other sources, eg. recombinant urease from *Klebsiella aerogenes* (Lee *et al.*, 1990). The degree of purification required to give pure *S. pombe* urease in the present study was very high (3,939 fold); therefore, urease appears to be a poorly represented enzyme in *S. pombe*. Other organisms appear to have much higher concentrations of urease, although this may reflect the tendency to study organisms with high urease activity.

## 4.8 Urease structural properties

The native molecular weight of *S. pombe* urease was determined by gel filtration of partially purified urease extracts (Results 3.5.1). Aggregation of urease with

contaminants in impure preparations can cause inaccurately high estimations of native $M_r$; however, the inclusion of 0.1 M NaCl in the gel filtration buffer should have minimized aggregation (Jones and Mobley, 1988) in the estimations performed in the present study. The estimated native $M_r$ of *S. pombe* urease (212,000) was similar to that of most bacterial ureases (200,000 to 250,000; Mobley and Hausinger, 1989) and *Aspergillus nidulans* (240,000; Creaser and Porter, 1985). A single subunit of $M_r$ 102,000 was identified for *S. pombe* (Results 3.5.2). The subunit size is similar to that of jack bean urease (90,770 by sequencing, Takishima *et al.*, 1988); however, the subunit composition is different. Jack bean urease is a hexamer with $M_r$ 590,000 (Andrews *et al.*, 1984), whereas, the native molecular weight of *S. pombe* urease indicates it is a dimer of the $M_r$ 102,000 subunit. *A. nidulans* urease is a hexamer of a single subunit of $M_r$ 40,000 (Creaser and Porter, 1985). *Ustilago violacea* urease has a single subunit with $M_r$ 80,000, but the native molecular weight is unknown (Baird and Garber, 1981). Most bacterial ureases examined have three subunits: an $\alpha$ subunit of $M_r$ 67,000 to 73,000, a $\beta$ subunit of $M_r$ 8,000 to 17,000, and a $\gamma$ subunit of $M_r$ 6,000 to 11,000 (Mobley and Hausinger, 1989; Jones and Mobley, 1989; Kakimoto *et al.*, 1989; Kakimoto *et al.*, 1990; Hu *et al.*, 1990). The subunit stoichiometry is probably best represented by $(\alpha_1\beta_2\gamma_2)_2$ (Mobley and Hausinger, 1989). *Helicobacter pylori* (Dunn *et al.*, 1990; Hawtin *et al.*, 1990; Labigne *et al.*, 1991) and *Helicobacter mustelae* (Costas *et al.*, 1991) require only two subunits, of $M_r$ 61,000 to 65,000 and $M_r$ 26,000 to 29,000 with a 1:1 stoichiometry, for active urease. There have been reports of bacterial ureases with a single subunit; however, this observation may be the result of small subunits having been overlooked on SDS PAGE gels (Mobley and Hausinger, 1989). An example of this is the urease from *Ureaplasma urealyticum*, which has recently been shown to have not one but three subunits (Thirkell *et al.*, 1989). *S. pombe* urease was carefully examined by SDS PAGE using high percent acrylamide gels. No small subunits were observed.

The single subunit of *A. nidulans* is less than half the size of other eukaryotic urease subunits and the combined size of the multiple bacterial subunits. Therefore, it probably has less sequence complexity than other ureases. However, the active enzyme has $M_r$ 240,000, which is similar in size to ureases from *S. pombe*, most bacteria, and the active, trimeric form of jack bean urease (Andrews *et al.*, 1984). Perhaps, to a degree, an overall conformation and size, rather than a complex sequence, is required for urease activity. This requirement may be fulfilled by a smaller number of large subunits or a larger number of small subunits.

## 4.9 pH stability and pH optimum

pH stability studies of *S. pombe* urease (Results 3.5.3, 3.5.4) were done using a range of buffers at various pH. The CAPS and CHES buffers reduced the color development of the ammonia assay. The same buffers and ammonia assay have been used by others (eg. Todd and Hausinger, 1987, Breitenbach and Hausinger, 1988) for pH studies of urease. No comment on the inhibition of the ammonia assay is made by these authors. In the present study, the use of sets of buffers with overlapping pH ranges enabled the data to be meaningfully interpreted. *S. pombe* urease was irreversibly inactivated above about pH 9.5 and may have been inactivated below about pH 4.5 (Results 3.5.3). Inactivation below about pH 4 - 5 has been observed for urease from *Arthrobacter oxydans* (Schneider and Kaltwasser, 1984), *Klebsiella aerogenes* (Todd and Hausinger, 1987), jack bean (Andrews *et al.*, 1984) and *Bacillus pasteurii* (Larson and Kallio, 1954). For jack bean and *A. oxydans* loss of activity was correlated with loss of nickel at low pH. Inactivation above about pH 10 has been observed for *K. aerogenes* (Todd and Hausinger, 1987), *Brevibacterium ammoniagenes* (Nakano *et al.*, 1984) and *Proteus mirabilis* (Breitenbach and Hausinger, 1988). The optimum pH for *S. pombe* urease activity was between pH 7.5 and pH 8.5 (Results 3.5.4). Bacterial neutral ureases have pH optima between about pH 7.0 and pH 8.0 (Mobley and Hausinger, 1989). The pH optimum for *Aspergillus tamarii* (Zawada and Sutcliffe, 1981), *Ustilago violacea* (Baird and Garber, 1981) and jack bean (Andrews *et al.*, 1984) urease, in phosphate buffer, is pH 8.2 - 8.65, pH 7.0, and pH 7.0 - 7.5, respectively. The pH optimum for *Aspergillus nidulans* is given as pH 8.5 in the abstract of Creaser and Porter (1985); however, no reference is made to the pH optimum in the text, tables or figures of the article. Ureases have been shown to have different pH optima, depending on the buffer system in which the activity is measured (Reithel, 1971; Nakano *et al.*, 1984). Fully protonated phosphoric acid is a competitive inhibitor of urease, therefore, phosphate buffers at low pH can cause urease inhibition (Mobley and Hausinger, 1989). To avoid inhibition, acetate, rather than phosphate, buffer was used for the low pH range in the present study.

The $K_m$ (for urea) values of purified urease from microbial sources are similar to the values observed for crude cell extracts (Mobley and Hausinger, 1989). The $K_m$ of crude *S. pombe* urease was 1.03 mM urea (Results 3.5.5). This is close to the value observed for *A. nidulans* urease (1.33 mM; Creaser and Porter, 1985). Jack bean (Andrews *et al.*, 1984) and *Ustilago violacea* (Baird and Garber, 1981) urease have a $K_m$ for urea of 2.9 mM and 2.8 mM, respectively. The $K_m$ of bacterial ureases range from 0.1 mM to >100 mM (Mobley and Hausinger, 1989). The effect of pH on the $K_m$

has been examined for a number of bacterial ureases (Mobley and Hausinger, 1989). The $K_m$ varies with the buffer and pH conditions for some bacteria only, others are not affected. The effect of pH on the $K_m$ of *S. pombe* urease was not tested.

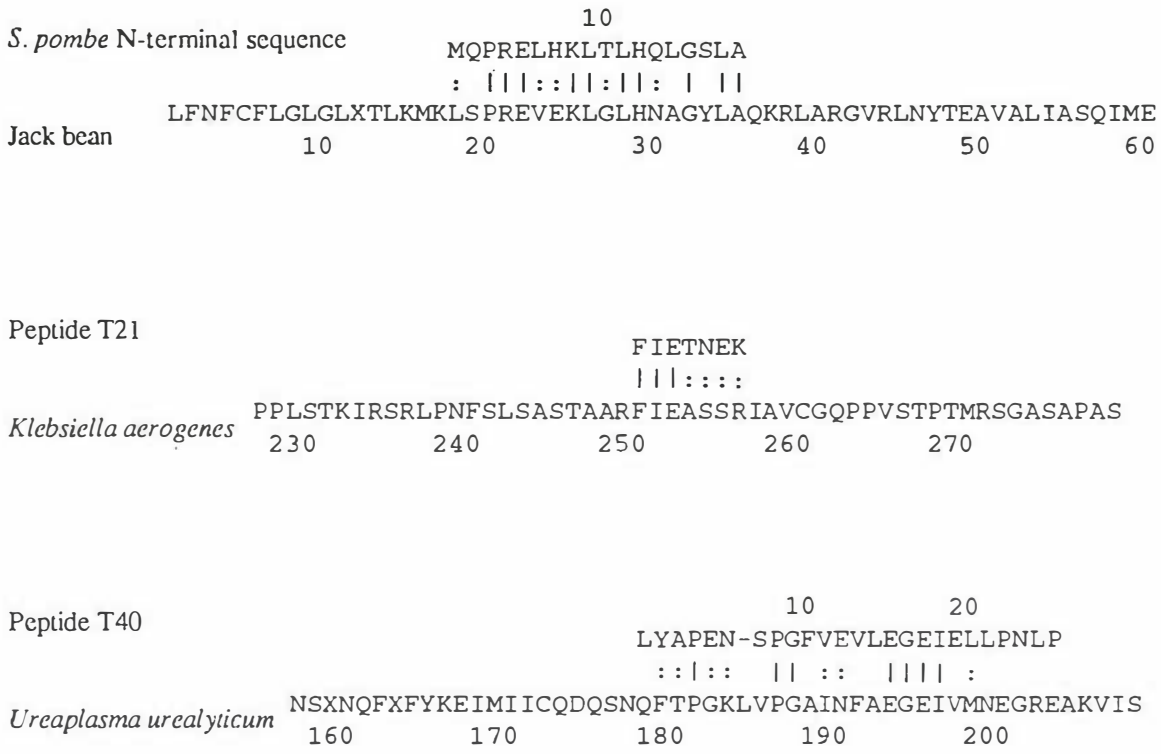
Urease is probably only present in small amounts in *A. nidulans* (Creaser and Porter, 1985) and in *S. pombe* (Discussion 4.7). The most important function of urease in both of these organisms may be in purine degradation (Kinghorn and Fluri, 1984; Scazzochio and Darlington, 1968). A urea rich environment has not been reported for these organisms. *S. pombe* growth was inhibited by urea concentrations much above 17 mM (0.1%; Results 1.3.2). Arginine degradation is another potential source of urea. *S. pombe* can grow on arginine (LaRue and Spencer, 1967). The majority of urea degraded by *Neurospora crassa* comes from purine, rather than arginine, breakdown (Davis, 1970). The relatively high affinity for urea and low concentration of *S. pombe* urease may reflect the low intracellular urea concentrations expected from purine degradation.

## 4.10 Urease sequence analysis

Extensive similarity exists between jack bean urease and many bacterial ureases eg. all three urease polypeptides from *Proteus mirabilis* (Jones and Mobley, 1989), two of the three polypeptides from *Morganella (Proteus) morganii* (Hu *et al.*, 1990), and the two subunits of *Helicobacter pylori* (Labigne *et al.*, 1991). For *P. mirabilis*, the similarity was distributed along the entire length of the jack bean amino acid sequence, with 58% exact matches and 73% exact plus conservative replacements. The degree of similarity appears to be similar for the other bacterial ureases. The 18 amino acid N-terminal sequence determined for *S. pombe* urease (Results 3.5.6) had 55.6% identity and 83.3% similarity (exact plus conservative replacements as determined by the TFASTA program, Deveraux *et al.*, 1984) with the jack bean N-terminal sequence (Figure 38). The seven amino acids of *S. pombe* urease peptide T21 had 42.9% identity and 100% similarity with the urease from *Klebsiella aerogenes* (Figure 38). *S. pombe* urease peptide T40 (25 amino acids) had only very poor identity with other sequenced ureases (Figure 38). While, overall, extensive homology between ureases exists (above), part of the *H. pylori* urease was shown to contain little similarity to other ureases and the 15 kilodalton subunit of the *M. morganii* urease has no similarity to any other urease (Hu *et al.*, 1990). The N-terminal sequence has been shown to be conserved in bacteria (as above), plants (jack bean) and yeasts (this study). The T40 peptide may represent urease sequence unique to *S. pombe*, as has been shown for some bacteria.

Figure 38

Comparison of *S. pombe* urease amino acid sequence with other ureases.




*S. pombe* urease sequences (Results 3.5.6) were compared to all urease sequences in the GenBank and EMBL nucleotide databases and the SwissProt peptide database. The TFASTA program was used (Deveraux *et al.*, 1984) and the best alignment for each of the *S. pombe* sequences is shown.

'|' (vertical line) denotes identities and ':' (colon) denotes conservative replacements.

For the bacteria with three subunits, the largest subunit (α) is similar to the C-terminal region of jack bean urease, the smallest subunit (γ) is similar to the N-terminal region, and the β subunit of *P. mirabilis*, but not *M. morganii*, is similar to internal sections of jack bean urease. The bacterial α, β and γ polypeptides are transcribed as a single polycistronic mRNA (Mulrooney *et al.*, 1988; Jones and Mobley, 1988; Mobley and Hausinger, 1989). The order of the subunit transcription corresponds to the order of the corresponding sequences in jack bean urease and may indicate an evolutionary relationship exists between bacterial and jack bean urease genes. It has been proposed that a two subunit urease was the evolutionary precursor of the three subunit urease. Jones and Mobley (1989) identified sequences at the junction of the *P. mirabilis ureA* (γ subunit) and *ureB* (β) genes which were very similar to a eukaryotic intron splice acceptor consensus sequence. This may represent the remnants of an ancestral organization that allowed these two domains to be spliced, resulting in a fused UreA-UreB subunit (Jones and Mobley, 1989). For the bacteria with two subunits (*Helicobacter*) the smallest subunit corresponds to both the β and γ subunits of *P. mirabilis*. The *Helicobacter* type urease may be the precursor of three-subunit bacterial ureases (Dunn *et al.*, 1990; Hu and Mobley, 1990). Alternatively, Labigne *et al.* (1991) suggest that *Helicobacter* urease is more similar to jack bean urease than are the three-subunit bacterial ureases and, therefore, the ancestral urease probably had a three-subunit configuration. A single nucleotide mutation in the stop codon between the two *Helicobacter* urease genes could produce a single-subunit urease (Labigne *et al.*, 1991). The single subunit demonstrated for *S. pombe* urease (the present study) supports previous findings that eukaryotic ureases have a single subunit.

## Summary

The pH optimum, $K_m$ , native molecular weight, and number of subunit types is similar between *S. pombe* and other eukaryotic ureases (fungi and jack bean). Bacterial ureases vary widely in their kinetic properties and have more than one subunit type. The N-terminal sequence of *S. pombe* urease is similar to other ureases.

## 5 AMPLIFICATION OF *S. POMBE* DNA USING PRIMERS TO THE UREASE AMINO ACID SEQUENCE

A single major product was obtained by PCR amplification of *S. pombe* genomic DNA using primers to the urease amino acid sequence. The product (ML12) was cloned and sequenced. The nucleotide sequence did not correspond in any way to the amino acid

sequence determined for the urease enzyme (Results 5.3). The sequence did show that ML12 was the result of the extension of the two primers.

Amplification artifacts have been described in which PCR products have contained extensive arrays of the primer sequences in various arrangements (Cooper and Baptist, 1991). The only primer sequences seen in ML12 were at either end of the fragment, as expected. Therefore, primer artifacts were not responsible for the observed product.

Hybridization to *S. pombe* genomic DNA showed that the ML12 sequence was present in the *S. pombe* genome. The lack of the expected N-terminal coding sequence could be explained by the presence of an intron splice site within each of the primer target sequences. The sequence after the primers would correspond to an intron and would not code for the expected amino acids. The sequence of one end of ML12 contains many stop codons in all three reading frames, therefore, it is a non-coding region and could be intron sequence. The other end of ML12 contained an open reading frame (orf1) for the entire sequenced region (386 nucleotides), therefore, it may be a coding sequence and not part of an intron. None of the consensus sequences for *S. pombe* introns (Russell, 1989) was identified near the ends of ML12. A more likely explanation is that the primers annealed to an unknown sequence in the *S. pombe* genome that is not part of a urease gene. No significant similarity was found between the predicted amino acid sequence of orf1 or any of the nucleotide sequence of ML12 and any other sequence in the GenBank and EMBL databases.

It is possible that the primer target sequences on the urease gene were too far apart to allow efficient amplification of the intervening sequence. The observed product (ML12) may have been amplified only because the annealing temperature was low enough to allow the primers to anneal to an unrelated sequence of low homology. The molecular weight of the urease subunit was $M_r$ 102,000 (Results 3.5.2). The average molecular weight of the more common amino acids in a polypeptide chain is about 108.7 daltons (Sambrook *et al.*, 1989). Therefore, the urease subunit probably contains about 940 amino acids. A DNA sequence of 2.8 kb is required to encode 940 amino acids and a longer sequence would be required if introns were present; however, introns in *S. pombe* are generally only 36 to 129 nucleotides long and usually only 0 to 5 introns are present (Russell, 1989). A large product of over 3 kb was often weakly amplified in PCR reactions when primer ML2 was used alone (negative control b, Results 4.2). Therefore, 3 kb was not outside the limit of amplification under the

conditions tested. An excessive distance between the primer target sequences probably does not account for the lack of amplification of the urease gene sequence.

PCR amplification can still occur when there is a very limited homology to the target sequence. Sommer and Tautz (1989) have observed amplification when one of the 17 nucleotide primers had as many as eight mismatched positions with the target sequence. Wilks *et al.* (1989) have shown efficient priming can occur at temperatures well above the calculated melting temperature ($T_m$) of the primer. Efficient amplification can be obtained with primers which are up to 262,144-fold degenerate and that incorporate all possible codon choices (Wilks *et al.*, 1989), or with moderately degenerate primers, in which only one or two of the most frequently used codons for each amino acid are represented (Cooper *et al.*, 1991). Despite these precedents, the PCR primers used in the present study did not amplify the correct target sequences. The most important region of the primer, where absolute homology with the target sequence is probably essential, is the 3' end of the primer. Sommer and Tautz (1989) suggest that the three 3' nucleotides should match exactly with the target sequence before efficient priming will occur, although sometimes a mismatch at the third nucleotide from the 3' end can be tolerated. For the primers ML1 and ML2, only the last two nucleotides at the 3' end were non-redundant positions, and for the third position from the 3' end not all possible nucleotides were used in the primer pool. It is possible that the wrong codon choices for the primer sequences prevented efficient annealing of the 3' end of the primers to the urease gene. The sequence that was amplified (ML12) may have had better homology with the 3' end of the primers, perhaps mismatches occurred at less important sites. Sequence data showed that a different primer species from the ML1 and ML2 primer pools was used for the amplification of each of the two PCR products that were cloned and sequenced (pML12.4 and pML12.7). For the ML1 primer region, three nucleotides were different between the two sequenced PCR products, with one difference at the third codon from the 3' end. For primer ML2, one difference, at the third nucleotide from the 3' end, was observed. Therefore, a mismatch at the third nucleotide from the 3' end of the primers was not sufficient to prevent amplification; however, further deleterious mismatches may have prevented the amplification of the urease-subunit gene. The long annealing time (2 minutes) required for amplification may indicate that the match between the primers and the template sequence was poor. Usually only a few seconds are required for annealing of primers (Innes and Gelfand, 1991). The ML1 and ML2 primers were 12-fold and 32-fold degenerate, respectively. The resulting lower concentration of each given primer sequence may account for the long annealing time required. An alternative explanation is that one or both of the primers had very poor homology with

the template sequence, therefore, primer/template complexes occurred infrequently and were highly unstable. Under these conditions a low annealing temperature and a long annealing time would improve the probability of the polymerase extending the primer/template substrate.

The hybridization data (Results 5.3) indicate that ML1 had poor homology with the urease-subunit gene, or any other *S. pombe* sequence. Both primers hybridized strongly to the amplified product (ML12), but ML1 did not hybridize significantly to any *S. pombe* genomic DNA. ML2 hybridized quite strongly to an unidentified sequence that did not correspond to the amplified sequence, perhaps the unidentified sequence was the urease gene. The amount of genomic DNA used in the hybridization experiments was 30 µg. The amount of DNA in one haploid cell is $1.5 \times 10^{-14}$ g (Bostock, 1970). Therefore, 30 µg of *S. pombe* genomic DNA corresponds to about $2 \times 10^{9}$ copies of a single-copy target sequence. The amount of ML12 used in the hybridizations was 10 ng, which corresponds to about $1 \times 10^{10}$ copies of target sequence. Hybridization of the primers to a homologous single-copy sequence should have been about one-fifth as intense as hybridization to the ML12 sequence and, therefore, should have been easily visible. The $T_m$ range for the ML1 and ML2 primer pools were 56 to 62 °C and 56 to 66 °C, respectively (calculated using the method of Itakura *et al.*, 1984 as described in Sambrook *et al.*, 1989). Hybridization of ML1 and ML2 was carried out at 41.5 °C. As a general guide, mismatched nucleotides reduce the $T_m$ by about 1 to 1.5 °C for each 1% of mismatch (Sambrook *et al.*, 1989). For a 20 nucleotide sequence this corresponds to 5 to 7.5 °C for each mismatch. Therefore, with the conditions used, up to three or four mismatches could have been tolerated. ML1 must have very poor homology with any sequence in *S. pombe* for no significant degree of hybridization to have occurred. The N-terminal amino acid sequence was determined for two separate preparations of purified *S. pombe* urease (Results 3.5.6) and for the purified N-terminal tryptic peptide T43. The sequence had significant identity with the N-terminal sequence of jack bean urease. Therefore, it is almost certain that the amino acid sequence back-translated to make ML1 corresponded to *S. pombe* urease.

The most likely explanation for the poor hybridization of the ML1 primer to *S. pombe* genomic DNA is that the incorrect codon choices were made for a number of the amino acids. Some of the codons considered to be less frequently used in *S. pombe* may be those used in the urease-subunit gene. *S. pombe* urease activity is constitutive, being neither induced nor repressed (Discussion 2.1). The urease enzyme is present at low concentration in *S. pombe* cells (Discussion 3.7), and appears to be very stable in crude cell-free extracts, even without protease inhibitors (Discussion 3.5).

Therefore, the urease-subunit gene may be quite poorly transcribed. Degenerate codons in *S. pombe* are not used at an equal frequency. Furthermore, codons that are commonly found in genes with a low level of expression are often the ones rarely used in highly expressed genes (Russell, 1989; Sharp *et al.*, 1988). The most frequently used codons for both high and low expressed genes were included in the primer sequences for most redundant positions; however, a few of the codons that are used quite frequently in poorly expressed genes were not included because they would have caused ambiguity in the sequence and increased the degeneracy. The arginine codon AGA, the leucine codon CTT (also frequently used in highly expressed genes) were avoided in ML1 and could have resulted in four mismatched nucleotide positions, assuming all other codon choices were correct. For primer ML2, no leucine or arginine codons were present, but the proline codon CCA (used twice), the serine codon TCA, and the glycine codon GGA, as well as other less frequently used codons, were not included. It is possible that if the alternative codons were used, better hybridization, and perhaps the correct PCR product, would have been obtained.

## 6. TRANSFORMATION OF *S. POMBE*

### 6.1 Improvement of the lithium chloride procedure for transformation of *S. pombe*

A method which uses lithium chloride to prepare competent *S. pombe* cells has been described by Bröker (1987). This method did not give efficient transformation of the *S. pombe* strains used in the present study (Results 6.2); however, a dramatic increase (>100 fold) in the transformation frequency was observed when heat denatured carrier DNA was included in transformations (Results 6.2). Schiestl and Gietz (1989) have investigated the effect denatured carrier DNA has on the frequency of transformation of *S. cerevisiae* when lithium acetate is used to yield competent cells. In the present study (*S. pombe* transformation) and in the study of Schiestl and Gietz (*S. cerevisiae* transformation) the *S. cerevisiae* URA3 and LEU2 genes were the vector marker genes used for selection of transformants. Also, 2-µm based plasmids were included in the plasmids tested in both studies. The observations of the present study agree closely with those of Schiestl and Gietz. Boiled, sonicated carrier DNA dramatically increases the transformation frequency of *S. cerevisiae*. The carrier DNA needs to be over 2 kb long to increase the transformation efficiency and the optimum size may be about 7 kb. Similarly, for *S. pombe* the transformation frequency was increased by sonicated, denatured carrier DNA (2 - 15 kb) but not by very small fragments of DNA. Carrier DNA does not improve the efficiency of transformation of *S. cerevisiae* spheroplasts and Schiestl and Gietz suggest that this may reflect an

inherent difference in the mechanisms of DNA uptake between the lithium acetate and spheroplast transformation methods. Similarly, carrier DNA may not improve the transformation efficiency of *S. pombe* protoplasts (Per Sunnerhagen, personal communication). Minor improvements in the frequency of *S. pombe* transformation were obtained by using the following modifications: (a) YES medium, rather than YPD, for growth of cells before transformation, and (b) growing the culture for a shorter time before transformation. Little effect was noted when the heat shock duration or the length of lithium chloride treatment were altered (Results 6.2). Schiestl and Gietz (1989) observed comparatively little effect on the transformation efficiency of *S. cerevisiae* when the duration of the heat shock was altered, but the use of younger less dense cultures did improve transformation. The similarities between *S. pombe* and *S. cerevisiae* transformation described above may indicate that the mechanism of DNA uptake is conserved between these two distantly related (Russell and Nurse, 1986) organisms and between the lithium acetate and lithium chloride methods of competent cell preparation.

The transformation of *S. pombe* was significantly improved (4 to 23-fold) by resuspending the transformed cells in 0.9% NaCl before plating onto selective media (Results 6.2). Treatment of cells with lithium ions may cause pores to form in the cell wall (Brzobohaty and Kovac, 1986; Hong, 1987). Disruption of the cell wall may make the cells osmotically fragile. Sorbitol (1.2 M) is used to stabilize osmotically fragile protoplasts (Beach and Nurse, 1981). TE containing sorbitol gave only slightly better transformation than water or TE without sorbitol (Results 5.2). Therefore, the improvement in transformation caused by resuspending cells in NaCl may not have been due to osmotic stabilizing of the cells.

## 6.2 Strain dependence of transformation

The ability to transform *S. pombe* using the lithium chloride procedure, and the frequency of transformation, were highly strain dependent (Results 6.2). No such strain dependency has been observed for the transformation of *S. cerevisiae* using the lithium acetate procedure (Schiestl and Gietz, 1989). Non-transformable strains were common amongst the *S. pombe* strains used in the present study, eg. two out of five spore clones from the same cross could not be transformed with the test plasmid (pFL20). Furthermore, strains not transformable with pFL20 also appeared to revert at low frequency to *ura4*$^+$. Strains not transformable with pDB262 were also observed and, where tested, were not transformable with pFL20, although no reversion to *leu1*$^+$ was noted. No relationship was observed between the inability to

transform/*ura4* reversion and any of the *ure*, *leu1*, or *ura4* alleles. Some of the non-transformable strains had no parents in common; however, the segregation patterns hint at the presence of a genetic factor that inhibits transformation and promotes apparent reversion of *ura4* auxotrophy. The reversion of *ura4* is curious because the *ura4* allele used in this study (*ura4 D-18* ) is supposed to be a 1.8 kb deletion of the entire *ura4* gene (Grimm *et al.*, 1988) and, therefore, it should be impossible for it to revert. Perhaps suppression, caused by mutation at another site, rather than reversion was responsible for the observed growth of the *ura4* mutants. The *ura4 D-18* strain was not specifically tested for the 1.8 kb deletion in this study and, therefore, may not have had the *D-18* allele.

## 7. ISOLATION OF GENES COMPLEMENTING THE *URE* MUTATIONS

### 7.1 Complementation of *ure*$^-$ strains with a *S. pombe* gene bank

Three different clones which complemented *ure*$^-$ mutants were isolated from a *S. pombe* gene bank. The clones restored urease activity to the *ure1*, *ure3*, or *ure4* mutant strains, demonstrated by growth on hypoxanthine and a positive indicator plate reaction. Each clone specifically complemented only one of the three *ure* mutants. This specificity, as well as the absolute requirement for each of the four *ure* genes for urease activity, indicates that each *ure* gene has a separate and essential function in producing active urease. Furthermore, the specificity of complementation by each of the gene bank clones argues against any of them being a general suppressor of the *ure*$^-$ phenotype. Possible functions of the *ure* genes have been discussed in a previous section (Discussion 2 - 2.4). Further work is necessary to confirm that the cloned genes are the wild-type alleles of the *ure* genes. The cloned genes must be shown to integrate at the chromosomal locus of the *ure* genes via homologous recombination.

The three clones which complement the *ure1*, *ure3*, and *ure4*, mutants were isolated at a frequency of between one per 3,500 and one per 5,000, yet no clones of the *ure2* gene were found in 70,000 transformants screened. About 60% of the clones in the *S. pombe* gene bank contained inserts greater than 5 kb (Results 6.3.1). The size of the *S. pombe* genome is about 14,000 kb (Moreno *et al.*, 1991). Therefore, no more than 5,000 clones (length of the genome ÷ length of inserts ÷ proportion of clones containing inserts = 14,000 ÷ 5 ÷ 0.6) would be required for one genome-equivalent of *S. pombe* DNA, assuming the gene bank is perfectly representative. The three complementing clones were isolated at close to the theoretical maximum expected

frequency. The exact probability that any given sequence will be amongst a given number of gene bank clones can be calculated from the following equation:

$$N = \frac{\ln(1-P)}{\ln(1-f)}$$ (Sambrook *et al.*, 1989)

where $P$ is the desired probability, $f$ is the fractional portion of the genome in a single recombinant, and $N$ is the necessary number of recombinants. To achieve a 99% probability of all sequences having been screened in a *S. pombe* gene bank with 5 kb insert size and 60% of clones containing inserts, 21,500 clones need to be screened. Amplification of the library may cause non-randomness of the gene bank and more clones would need to be screened. The gene bank used in this study was amplified by plating clones on solid media, rather than growing in liquid culture, to reduce over-representation of faster growing clones. There are two possible explanations to account for no *ure2* clones being isolated: (a) the *ure2* gene was poorly represented, or absent, from the gene bank, and (b) the *ure2* mutation cannot be complemented by a wild type *ure2* gene on a plasmid. Possible reasons for poor representation of a gene in a gene bank may include the following: (a) the gene product is toxic to *E. coli* cells so cells containing the cloned gene die or grow slowly, (b) the gene product may have a DNA sequence or conformation that is unstable in *E. coli*, eg. the sequence may undergo frequent recombination, (c) the DNA sequence may not be efficiently replicated in *E. coli*, (d) the gene of interest may have a hypersensitive restriction enzyme site within its sequence and, therefore, will be frequently cut when the genomic DNA is partially digested in preparation for gene bank construction. Possible reasons that could prevent complementation of a mutation by the cloned wild-type allele may include the following: (a) the gene may be toxic in *S. pombe* when expressed from a plasmid; the higher copy number or lack of proper transcription/translation controls may result in toxic levels of product, (b) the mutant allele may be dominant and unable to be complemented by a wild-type gene product, (c) the gene product may not be correctly processed or compartmentalized and, therefore, may not function correctly, (d) factors essential for expression of the gene, for example transcription enhancers/regulators, may not be appropriately postioned in relation to the cloned gene. Also, the correct DNA conformation may not be reproduced in the cloned gene and could affect transcription. None of the above factors has been discounted for the *S. pombe ure2* gene.

The five clones which complemented *ure1-1* were identical. This was unexpected because in a good gene bank independent clones are expected to be different, each

containing various amounts of DNA flanking the complementing gene. The identical clones isolated in the present study may represent a single plasmid species that had a growth/survival advantage over other clones and, therefore, was amplified to a greater extent. Alternatively, the identical *S. pombe* genomic DNA segment present in the clones may have had independent origins. Perhaps this fragment was commonly produced in the *Sau*3A partial digest used to produce the DNA for the gene bank and was independently cloned several times. This could occur if specific sites flanking the gene had much higher sensitivity to cutting by *Sau*3A.

### 7.2 Hybridization of the PCR primers to the *ure*$^-$-complementing clones

The *ure*$^-$- complementing clones (pT1-1, pT3-1, and pT4-1) were hybridized to the PCR primers ML1 and ML2. These primers correspond to the predicted likely nucleotide sequences of the *S. pombe* urease-subunit gene, back-translated from the amino acid sequence of the enzyme and with codon preferences taken into account. No hybridization was observed between any of the clones and either of the primers, despite a low hybridization and washing stringency (15 - 25 $^{o}$C below the calculated $T_m$ of the primer/DNA complex) being used. These results are difficult to interpret because the primers also did not hybridize to *S. pombe* total genomic DNA. Lack of hybridization could indicate that the complementing clones do not correspond to the gene for the urease subunit, or that the primers were just too divergent from the nucleotide sequence of the gene to allow hybridization under the conditions used.

## SUMMARY AND CONCLUSIONS

Several published methods were modified to aid the the studies into *Schizosaccharomyces pombe* urease. Selection of urease mutants by their inability to use hypoxanthine as a sole nitrogen source (Kinghorn and Fluri, 1984) was not effective when amino acid and nucleotide supplements were added to the solid media. Two indicator plates were developed in the present study which permitted the rapid identification of urease mutants and which were not affected by amino acid and nucleotide supplements. These media may also provide a more rapid and sensitive way to identify urease activity in other yeast species on solid media.

The lithium chloride method for *S. pombe* transformation was modified to improve the transformation frequency by as much as 100-fold. Two important factors required for a high transformation efficiency were (a) using denatured carrier DNA (size approximately 2 - 15 kb) and (b) resuspending cells in 0.9% sodium chloride after transformation. Transformation by this method was strain dependent.

*S. pombe* urease was purified and characterized. Like most bacterial, fungal and plant ureases, it is intracellular and isozymes are probably not present. *S. pombe* urease has a single subunit, like plant and other fungal ureases and unlike bacterial ureases. Urease subunit size is not conserved between plant, fungal, and *S. pombe* ($M_r$ 102,000) enzymes and neither is the subunit stoichiometry, a dimer for *S. pombe*, a hexamer for jack bean and *Aspergillus nidulans*. The native molecular weight for urease from *S. pombe* ($M_r$ 212,000), *A. nidulans*, bacteria and jack bean (the trimeric form) is between about $M_r$ 200,000 and $M_r$ 295,000.

The N-terminal sequence of *S. pombe* urease is conserved in jack bean and bacterial ureases. Further sequence data should allow the extent of homology, conserved domains and evolutionary relationships to be determined.

The kinetic characteristics of *S. pombe* urease ($K_m$ 1.03 mM; pH optimum 7.5 - 8.5; specific activity 709 U/mg) are similar to the urease from another fungus, *A. nidulans*. The low intracellular concentration and $K_m$ of the enzyme may indicate that the major function of *S. pombe* urease, like *A. nidulans* urease, is for purine degradation.

The four complementation groups previously described for *S. pombe* urease mutants (Kinghorn and Fluri, 1984) have been confirmed in the present study. Each *ure* gene has been mapped to the following approximate genetic locations: *ure1* on the left arm of chromosome III, 32 cM from *fur1* and 50 cM from *ade6*; *ure2* on the right arm of chromosome I, 69 cM from *ura2* and 100 cM from *ade4*; *ure3* on the right arm of chromosome I, 31 cM from *ade4* and 91 cM from *ura2*; *ure4* on the left arm of chromosome I, 100 cM from *lys1*.

Three different clones, each of which specifically complemented one of the *ure1*, *ure3*, or *ure4* mutants, were isolated from a *S. pombe* gene bank. Study of these urease clones should aid the functional characterization of the of the *ure* genes. A clone complementing the *ure2* mutant was not isolated, and it is likely that either the appropriate clone was not present in the gene bank, or that the *ure2* mutant could not be complemented in this way.

*S. pombe* is a good candidate for the isolation of urease genes and their transfer to wine yeast. Three of the four genes required for urease activity have been isolated in the present study and no obstacles hindering the isolation of the remaining gene are foreseen.

## FUTURE WORK

The three clones which complement the *ure1*, *ure3*, and *ure4* mutants are assumed to encode the respective *ure* genes because urease activity is specifically restored; however, it is necessary to confirm the identity of the cloned genes by showing that they integrate at the chromosomal locus of the *ure* genes by homologous recombination.

The *ure2* mutant was not complemented. Before further attempts are made to isolate the wild-type *ure2* gene, the *ure2* mutation must be checked for dominance over the wild-type allele in a diploid $ure2^{-}/ure2^{+}$ heterozygote. If the $ure2^{-}$ allele is dominant, the mutant allele could be isolated from a *S. pombe* $ure2^{-}$ gene bank by transforming wild-type *S. pombe* and selecting for urease deficiency. The isolated mutant gene could then be used to recover the wild-type gene from a gene bank by hybridization. If the $ure2^{-}$ allele is recessive, another wild-type gene bank should be screened by complementation of the *ure2* mutant. It may be necessary to use a low copy number vector if the *ure2* product is toxic.

The minimum sequence required for complementation should be determined for each of the isolated genes. Introns, and transcription start and stop sites could be identified by sequence comparison to cDNA clones or mRNA amplified by PCR, and by primer extension studies. Transcription regulatory controls are assumed to be absent because of the non-regulated nature of urease activity, however, this should be investigated by Northern hybridization analyses.

Once the cloned genes have been characterized, work can begin on the transfer and integration of the genes into *S. cerevisiae* (the usual wine yeast). If introns are present it may be necessary to use DNA copies of the mRNA (cDNA or PCR amplified mRNA) because introns are often not processed correctly in *S. cerevisiae*. Also, it may be necessary to clone the genes under the control of a promoter which is more efficiently expressed in *S. cerevisiae* than the natural *S. pombe* promoter.

Further tetrad analyses are required to more accurately map the *ure* genes. Convenient and informative marker genes for linkage analyses could include the following: *ade10* - should map midway between *ure1* and *fur1*, about 16 cM from each; *tps19* - should map between *ure2* and *ure3*, approximately 20 cM from each. The *ure4* gene was tentatively mapped 100 cM from *lys1*, on the left arm of chromosome I. The only other genes which have been mapped close to this region are *aro5* and *swi1*, which are about 55 cM and 62 cM (respectively) from the *lys1* gene, on the left arm of chromosome I. The next closest gene group includes the *his6* gene, which is nearly 230 cM from *lys1*, on the left arm.

The function of each of the *ure* genes should be identified. Nucleotide sequence data should reveal which gene encodes the enzyme subunit. Sequence data may also identify other possible functions for these genes. A nickel-specific transport gene (*hoxN*) from *Alcaligenes eutrophus* has been cloned and sequenced (Eitinger and Friedrich, 1991). Several bacterial genes associated with nickel incorporation have been sequenced (Mulrooney and Hausinger, 1990). Sequence similarity may indicate a similar role for a *ure* gene. Other motifs, such as transmembrane structures, which can be predicted from sequence data could also indicate transport roles. Apart from sequence analyses, many biochemical and physiological tests need to be done. An obvious starting point would be to test wild-type *S. pombe* urease for the presence of a nickel cofactor. If present, as expected, all mutants should be examined for the production of inactive apourease, lacking a nickel cofactor. The absence of a nickel cofactor could indicate the mutant is unable to transport, synthesize or incorporate a nickel cofactor into urease.

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