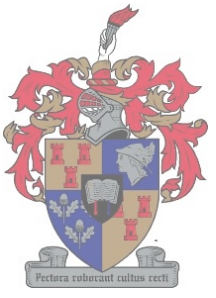
CLONING AND EXPRESSION OF THE *LACTOBACILLUS FERMENTUM* ACID UREASE GENE IN *SACCHAROMYCES CEREVISIAE*



Johanna Jacoba Visser

Thesis presented in partial fulfillment of the requirements for the Masters degree in Agricultural Science at the University of Stellenbosch

September 1999

Supervisor: Professor H.J.J. van Vuuren

DECLARATION

I the undersigned hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

8 : 11 : 1999

Johanna Jacoba Visser Date

SUMMARY

Arginine is one of the main amino acids present in grape must and is degraded by the wine yeast *Saccharomyces cerevisiae* to ornithine, ammonia and carbon dioxide. Urea is formed as an intermediate product and is secreted into the grape must, resulting in high concentrations of urea in fermenting grape must. Ethanol, produced during fermentation, reacts with the urea during long term storage and forms ethyl carbamate (urethane). Urethane is a carcinogenic and mutagenic substance representing a potential hazard to human health and therefore has to be addressed by the wine and related industries.

The aim of this study was to develop a wine yeast strain that could prevent the formation of urethane by degrading the urea produced during wine fermentation. The urease enzyme (not produced by *S. cerevisiae*) can degrade urea to ammonia and carbon dioxide. When using an acid urease, this reaction can take place at the low pH conditions associated with wine fermentation. The lactic acid bacterium *Lactobacillus fermentum* was chosen as a source of the genes that encode the three structural subunits of the acid urease. The bacterial genes are found in an operon, whereas three open reading frames (ORF) separated by linker sequences encode the eukaryotic enzyme from jack bean. Expression cassettes containing an ORF comprised of the three bacterial genes, as well as the linker sequences present in the jack bean urease gene were therefore constructed for expression in *S. cerevisiae*.

The production and activity of the recombinant protein were tested by expressing it first in a urease-positive strain of *Schizosaccharomyces pombe* that should provide the essential accessory proteins for its own urease, as well as for the recombinant protein. These accessory proteins are responsible for incorporating the nickel ions into the urease and are therefore essential for an active urease enzyme. The transcription of the recombinant gene was confirmed by northern blot analysis and the activity of the recombinant protein was tested under different pH conditions. However, the protein proved to be unstable, making it extremely difficult to quantify the activity.

OPSOMMING

Arginien is die hoof aminosuur teenwoordig in druiwemos en word deur die wyngis *Saccharomyces cerevisiae* na ornitien, ammoniak en koolstofdioksied afgebreek. Ureum word as 'n byproduk tydens hierdie reaksie gevorm en in die druiwemos uitgeskei, wat lei tot hoë konsentrasies ureum in die gistende druiwemos. Etanol wat gedurende die fermentasie geproduseer word, reageer met die ureum gedurende lang opbergingsperiodes en vorm etielkarbamaat. Etielkarbamaat is 'n karsinogeniese en mutageniese verbinding wat 'n potensiële gevaar vir menslike gesondheid inhou en dus deur die wyn- en verwante industrieë aangespreek moet word.

Die doel van hierdie studie was om 'n wyngis te ontwikkel wat die vorming van etielkarbamaat kan voorkom deur die ureum wat tydens wyn fermentasie geproduseer word, af te breek. Die urease ensiem (wat nie deur *S. cerevisiae* geproduseer word nie) breek ureum af na ammoniak en koolstofdioksied. Indien 'n suur urease gebruik word, kan hierdie reaksie by die lae pH kondisies wat met wynfermentasie geassosieer word, plaasvind. Die melksuurbakterium *Lactobacillus fermentum* is gebruik as bron van die gene wat die drie strukturele subeenhede van die suur urease ensiem kodeer. Die bakteriese gene word as 'n operon uitgedruk, terwyl die drie homoloë gene in die "jack bean" deur verbindingsfragmente geskei word. Uitdrukkingskasette wat 'n oopleesraam bestaande uit die drie bakteriese gene, sowel as die verbindingsfragmente van die "jack bean" urease gene bevat, is vir uitdrukking in *S. cerevisiae* gekonstrueer.

Die produksie en aktiwiteit van die rekombinante proteïen is eers in 'n urease-positiewe ras van *Schizosaccharomyces pombe* getoets wat die noodsaaklike hulpproteïene vir sy eie urease, sowel as vir die rekombinante proteïen, kan verskaf. Hierdie hulpproteïene is verantwoordelik vir die inbouing van die nikkel-ione in die urease en is dus noodsaaklik vir ensiemaktiwiteit. Transkripsie van die rekombinante proteïen is deur northernklad analise bevestig en die aktiwiteit van die rekombinante proteïen is onder verskillende pH toestande getoets. Die proteïen was egter onstabiel en gevolglik was dit uiters moeilik om die aktiwiteit te kwantifiseer.

BIOGRAPHICAL SKETCH

Ancha Visser was born on August 26, 1972 and raised in Stellenbosch in the Western Cape. In 1990 she matriculated from the Stellenbosch High School and enrolled for a B. Sc. (Agric.) Food Science degree at the University of Stellenbosch in 1991. This degree was obtained in 1994 and in the next year she completed her Hons. B. Sc. (Agric.) (Microbiology) degree at the same university. In 1996 Ms. Visser enrolled for a M. Sc. (Agric.) in Microbiology at the University of Stellenbosch under the supervision of Prof. H.J.J. van Vuuren.

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CHAPTER ONE

CHARACTERISTICS OF THE UREASE ENZYME

INTRODUCTION

Urea and urease are landmark molecules in the history of chemistry and biochemistry. In 1799, urea was isolated from urine and in 1828 the German chemist, Friedrich Wöhler (1800 - 1882), accidentally produced crystals of urea by heating ammonium cyanate. The fact that he produced an organic compound from an inorganic compound was very hard for Wöhler to accept, because organic beings were believed to consist of a strange and wonderful matter harbouring the essence of life. Almost fifty years later, in 1876, Pasteur found a "soluble material in putrefying urine that causes the same fermentation as that done by a microscopic organism". The word "enzyme" came in use in 1878 and "urease" was named in 1889. Jack bean urease was the first enzyme ever crystallised (Sumner, 1926). This historic event was described in the following way by the author: "After work both by myself and in collaboration with Dr. V. A. Graham and Dr. C. V. Noback that extends over a period of a little less than nine years, I discovered on the 29th of April a means of obtaining from the jack bean a new protein which crystallises beautifully and whose solutions possess, to an extraordinary degree, the ability to decompose urea into ammonium carbonate." For this achievement, J. B. Sumner won the Nobel Prize in 1926. This paper also played an important role in supporting the growing theory that enzymes are proteins. Almost fifty years later it was discovered that the jack bean urease contains a nickel ion, thus urease was also the first nickel metalloenzyme ever reported (Dixon *et al.*, 1975). This nickel ion is essential for an active enzyme, as will be discussed later.

Urea can be degraded with or without the help of enzymes. The non-enzymatic degradation of urea in aqueous media is very slow ($t_{½}$= 3.6 years at 38°C) (Andrews *et al.*, 1984). The sole products of this reaction are cyanic acid and ammonia. The enzyme urease (urea amidohydrolase: EC 3.5.1.5) is responsible for the enzymatic degradation of urea. Urease catalyses the hydrolysis of urea and the products of this reaction are ammonia and carbamate. Carbamate decomposes spontaneously to yield another molecule of ammonia and carbonic acid. These reactions lead to an increase in pH.

$$\underset{\text{urea}}{H_2N\text{-}CO\text{-}NH_2} + H_2O \xrightarrow{\text{urease}} NH_3 + \underset{\text{carbamate}}{H_2N\text{-}COOH}$$

$$H_2N\text{-}COOH + H_2O \longrightarrow NH_3 + H_2CO_3$$

Another pathway for urea degradation is its hydrolysis to NH_3 and CO_2 by the urea carboxylase and allophanate hydrolase system. This reaction is ATP-dependent, avidin-sensitive and needs the co-factors Mg^{2+} and K^+. Organisms can have either one or both enzyme systems. The yeasts *Saccharomyces cerevisiae* (Whitney and Cooper, 1972) and *Candida utilis* (Roon and Levenberg, 1968) and the green algae *Clorella,* only have the carboxylase and allophanate hydrolase system.

GENERAL METABOLISM OF NITROGEN

To understand the origin and occurrence of urea in nature, the general metabolism of nitrogen as shown in Figure 1 will be discussed. Nitrogen is essential and needs to be acquired by all living organisms since it is a component of important molecules such as DNA, proteins and co-enzymes. Air contains 79% free nitrogen, but this is only of nutritional importance to the few bacteria and algae that can fix the nitrogen gas by reducing it to NH_3 (Zubay, 1993). Besides ammonia, the two other major forms of fixed nitrogen are proteins and polynucleotides that are released into the environment after the decay of dead plants, animals or microorganisms (Mobley and Hausinger, 1989). Proteins and polynucleotide molecules are degraded or hydrolysed to their amino acid and nucleotide monomers, from which the guanidino group of arginine and the ring nitrogens of purines are released as urea and further hydrolysed by the enzyme urease. Urease is produced as an intracellular enzyme of microorganisms or a cell-free enzyme derived from plant and microbial cells of aquatic systems or soil. The cell-free enzyme is very stable because of presumed interactions with soil components.

Soil bacteria of the genus *Nitrosomonas* oxidize the ammonia released after the hydrolysis of urea by urease, to nitrite (NO_2^-) to obtain energy (Zubay, 1993). Nitrifying bacteria such as *Nitrobacter* have to oxidize nitrite to nitrate (NO_3^-) in order to yield energy. The nitrate is reduced by plants and microorganisms and incorporated into amino acids, thus completing a cycle. Nitrate can also be reduced partly to ammonia and partly to N_2. The latter is lost to the atmosphere and can only be recaptured by nitrogen-fixing bacteria and algae. Microorganisms that can fix nitrogen are designated as diazotrophs and they use a cluster of proteins that constitutes the nitrogenase system for this purpose (Zubay, 1993).

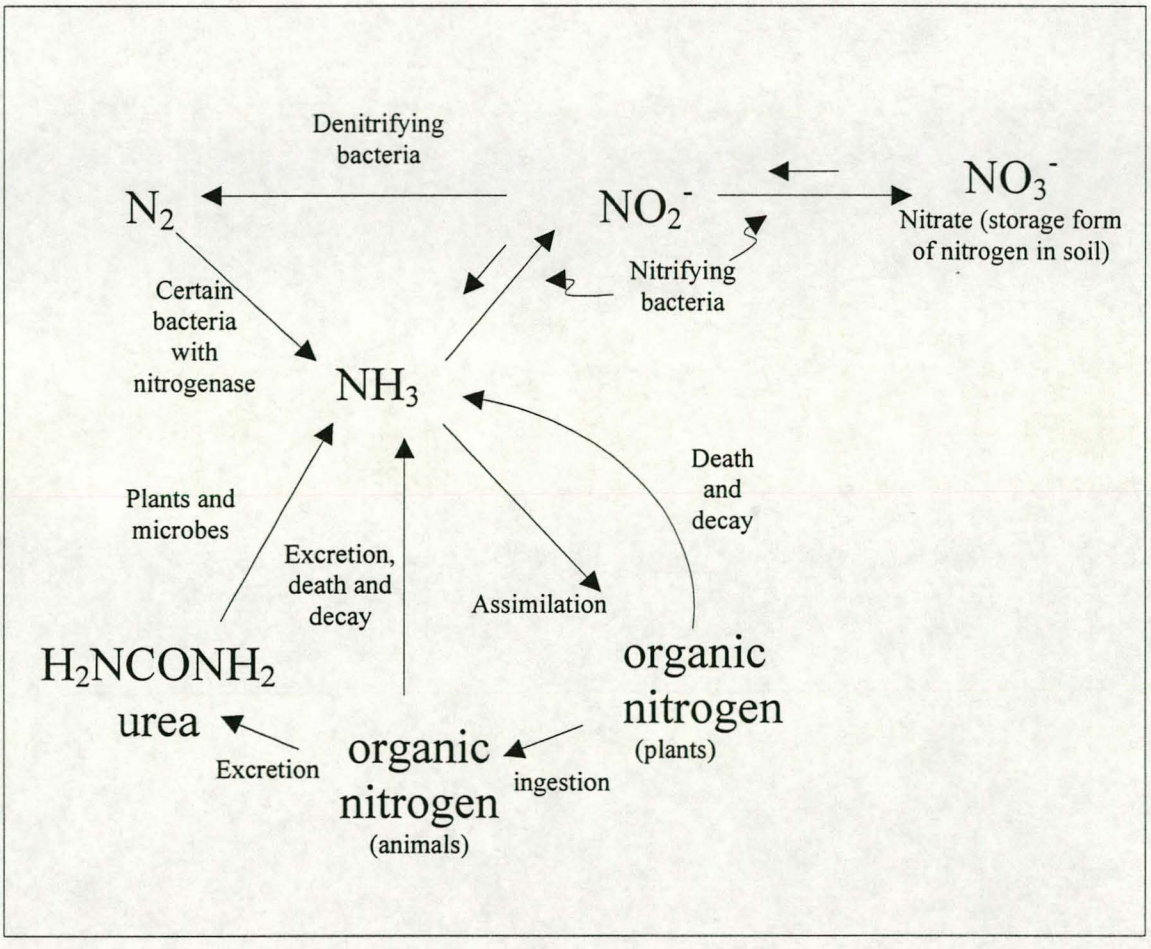

Figure 1. The nitrogen cycle (Bohinski, 1987).

Animals, birds and reptiles directly digest the proteins present in the food they ingest to supply in their nitrogen needs. The proteins are degraded to amino acids in their

digestive tract and then deaminated, resulting in the formation of ammonia. When this process takes place in mammals other than primates, the excess ammonia is converted to urea. This happens in the liver *via* the Krebs-Henseleit cycle (arginine, ornithine or urea cycle) shown in Figure 2 (Zubay, 1993). The purpose of forming urea from ammonia is to convert a toxic substance to a non-toxic one that is released into the environment. Excess ammonia produced during amino acid deamination in humans and other primates, birds and terrestrial reptiles is converted to uric acid and excreted (Zubay, 1993). The conversion of ammonia to uric acid involves the *de novo* pathway of purine biosynthesis.

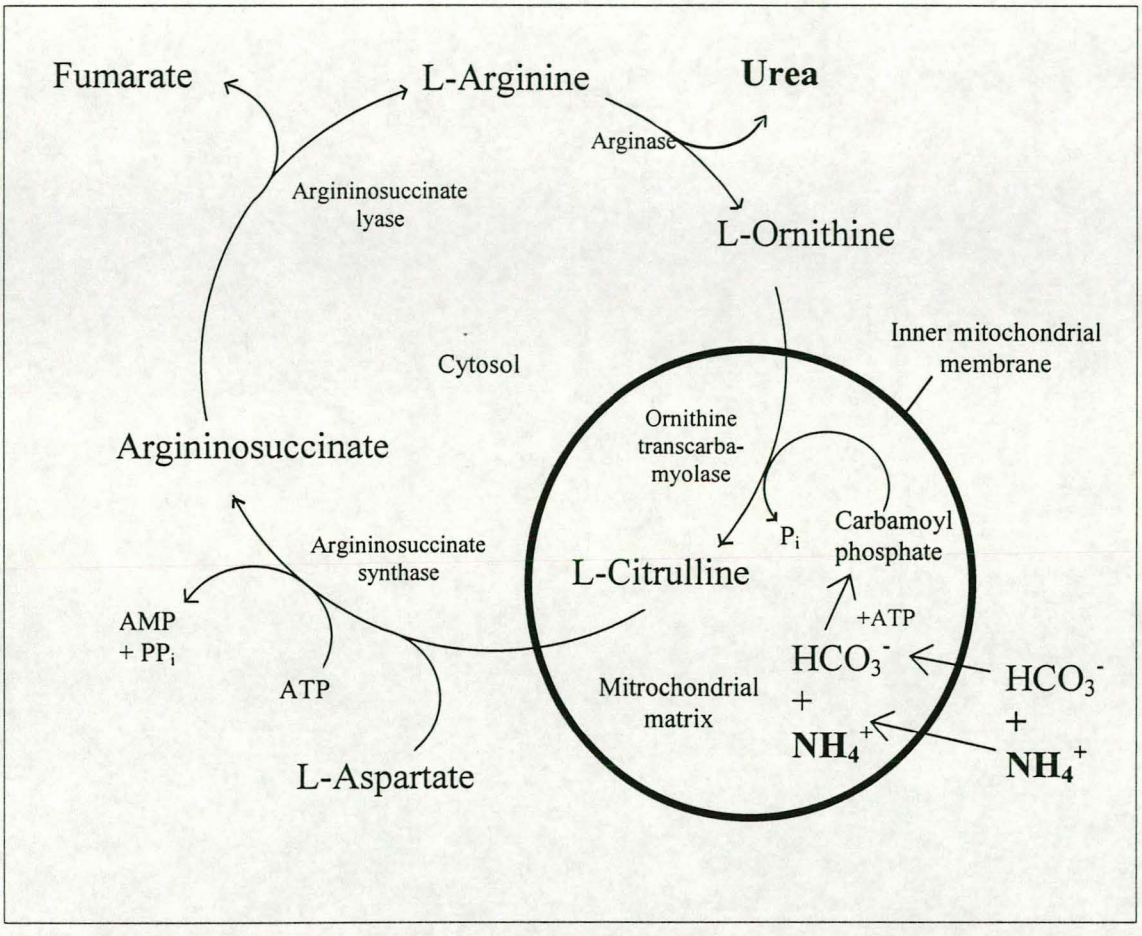

Figure 2. The Krebs-Henseleit cycle (Zubay, 1993).

For some organisms, ammonia is not toxic and is the major nitrogenous end product for some of the simpler aquatic and marine animal forms such as protozoans and nematodes,

as well as some terrestrial microorganisms. The ammonia can be reassimilated by the organisms but usually much more ammonia is liberated than is needed for biosynthesis. The excess ammonia diffuses to the surrounding water or escapes to the atmosphere. Unlike animals, where ammonia is in excess and is converted to urea (the non-toxic waste), plants have to conserve nitrogen and use their urease enzyme to recycle urea nitrogen to ammonia.

UREASE PRODUCERS

Ureases are synthesised by eukaryotes and prokaryotes. They can be found in almost all plants and especially in the seeds of some members of the families Fabaceae and Cucurbitaceae, as well as all tissues of soybean (*Glycine max*) (Polacco and Holland, 1993). Urease activity is also observed among eukaryotic microorganisms including some yeasts. It was shown that ascomycetous yeast are urease negative, with the exception of the genus *Schizosaccharomyces* (Booth and Vishniac, 1987). Basidiomycetous yeast, for example *Rhodosporidium paludigenum* (Phillips *et al.*, 1990), are usually urease positive. Ureases are also produced by fungi, e.g. *Aspergillus nidulans* (Creaser and Porter, 1985; Smith *et al.*, 1993) and *Ustilago violacea* (Baird and Garber, 1981).

Urease activity is found in a wide range of bacterial divisions, but within any division not all the species have urease activity. In 1947, the enzyme was already known to be present in more than 200 species of bacteria and the list has increased tremendously since then. Because of medical implications, the urease producers among the prokaryotic microorganisms are the most widely studied. Some microorganisms in this group are responsible for the development of certain human and animal pathological states (Mobley and Hausinger, 1989). Other prokaryotic urease-producing bacteria are found in the rumen of animals and some can be found in soil and water (Hausinger, 1993).

THE FUNCTIONS OF UREASES

Why do organisms produce the enzyme urease and how do they employ it?

NUTRITION FACTOR

From the discussion on the general metabolism of nitrogen, it is clear that ureases of soil and aquatic bacteria supply the cells with ammonia as a nitrogen source for growth (Hausinger, 1993). Fungal ureases are also involved in the transformation of environmental nitrogen sources to provide ammonia for cell growth. An exception is the urease of *Aspergillus tamarii*, which accounts for 8.5% of the total soluble protein and it was suggested to play a role as a storage protein.

Bacteria that inhabit the rumen of animals also produce urease for the production of ammonia (Mobley and Hausinger, 1989). This is an example of symbiosis: the animal produces high quantities of urea of which a substantial amount diffuses into the rumen where the ureolytic bacteria utilise it as a nitrogen source. This reaction produces ammonia, which is the major source of nitrogen for ruminal bacteria. The ruminant uses the subsequent increase in microbial population as a nutrient. The cycling of nitrogen in ruminants is illustrated in Figure 3.

Urea hydrolysis also occurs in the intestinal tract of monogastric species such as humans, mice, pigs, rats and rabbits. Approximately 20% of the urea produced in the human liver is transferred from the bloodstream to the intestinal tract and then hydrolysed by urease. Ureolytic strains most commonly found in humans include *Eubacterium aerofaciens, Eubacterium lentum* and *Peptostreptococcus productus* (Suzuki *et al.*, 1979).

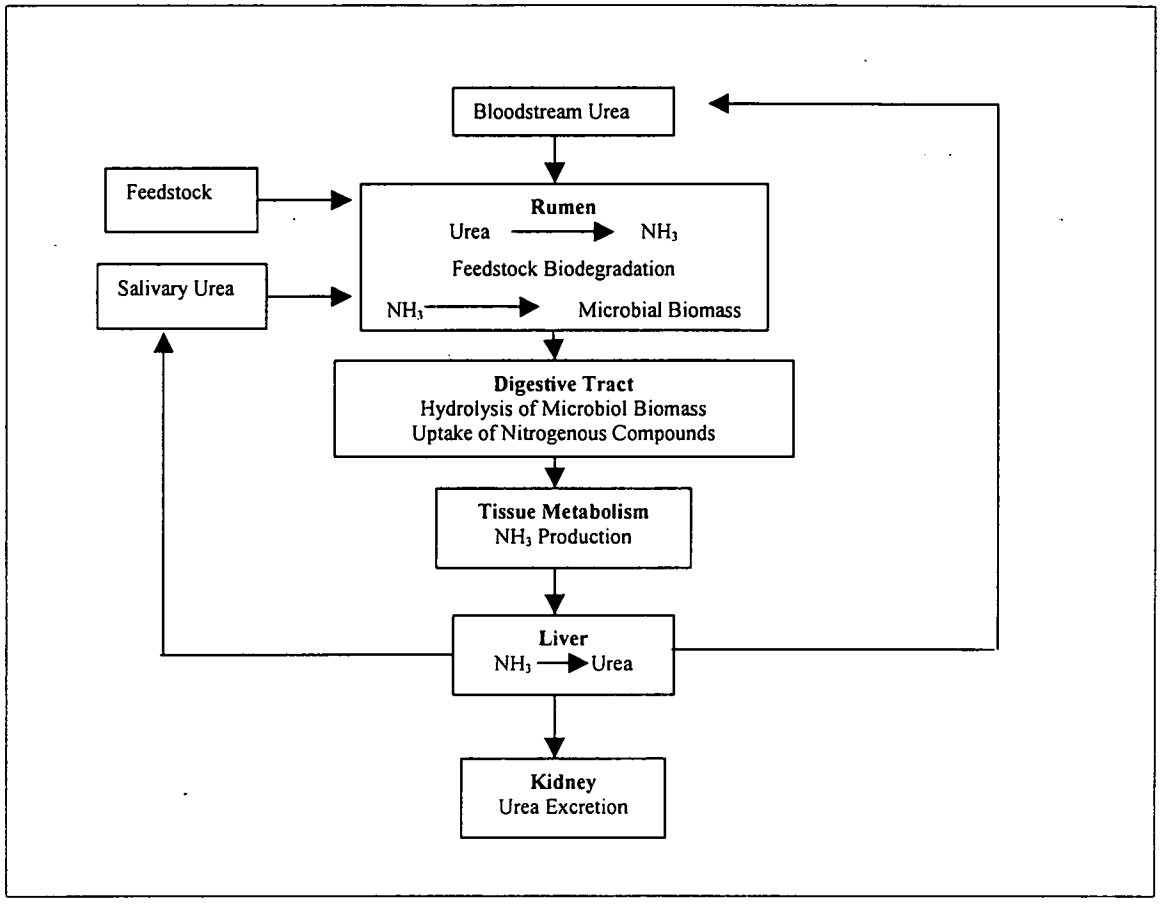

Figure 3. Nitrogen cycling in ruminants (Mobley and Hausinger, 1989).

THE ROLE OF UREASE IN PLANT CELLS

As previously discussed, plants have to conserve nitrogen and the ureolytic activity is necessary to recycle urea nitrogen. The soybean plant contains two urease isozymes: the ubiquitous urease, which is made in all tissues, and the embryo-specific urease, which is confined to the developing embryo (Polacco and Holland, 1993). Mutant plants lacking the ubiquitous urease exhibit the following abnormalities: necrotic leaf tips, accumulation of urea in leaves or seeds and retarded germination. None of these abnormalities occur with mutants lacking the embryo-specific urease. It has been suggested that the embryo-specific urease is responsible for seed chemical defence. The seed urease can cause either hepatic coma by subversion of the urea cycle or peptic ulceration by localised

increases in NH_4^+ and OH^- ions in the intestines of the vertebrate after ingestion of the soybean seed.

PATHOGENESIS

Bacterial ureases are directly associated with the formation of infection stones in humans. Enteric bacteria such as *Proteus mirabilis*, *Pseudomonas*, *Klebsiella* and *Staphylococcus* ssp., produce ureases that catalyse the hydrolysis of urea to carbonic acid and ammonia in the urinary tract of humans. This results in a pH increase that promotes precipitation of calcium or magnesium ions in urine and leads to the formation of urinary or renal stones composed of carbonate-apatite [$Ca_{10}(PO_4CO_3OH)_6(OH)$] or struvite ($MgNH_4PO_4.6H_2O$) (Mobley and Hausinger, 1989). It was found that obstructions in the urinary tract of patients with catheters were of the same composition as infection stones. The cause of this encrustation is urease-producing bacteria such as *P. mirabilis, Proteus vulgaris*, *Providencia stuartii*, *Morganella morganii* and *Providencia rettgeri* that colonise in the urinary tract of such patients.

The urease of *P. mirabilis* contributes to pathogenesis by causing pyelonephritis, an inflammation of the kidney and its pelvis, in humans (McLean *et al.*, 1985). The ammonia produced by the reaction of the enzyme is responsible for significant tissue damage, inflammation and cell invasion. The *Corynebacterium renale* urease is responsible for a similar inflammation in cattle. Another illness caused by ammonia produced by microbial ureases is hepatic coma (Mobley and Hausinger, 1989). This occurs when nitrogenous compounds from intestinal sources that have not been metabolised by the liver, intoxicate the brain.

Bordetella bronchiseptica is the aetiological agent of a respiratory disease of domestic animals (Monack and Falkow, 1993). This bacterium produces a potent urease of which the role during infection of the respiratory tract of the mammalian host is unknown. When testing the ability of an urease-mutant strain to colonise the respiratory tract of a mammal, it was shown that the urease did not enhance the ability of the bacteria to colonise. McMillan *et al.* (1998) suggested that the urease of *B. bronchiseptica* might be

one of the proteins that the bacterium expresses in response to phagolysosomal attack, enabling the bacteria to survive intracellularly.

The urease of *Helicobacter (Campylobacter) pylori* is by far the most studied urease. This bacterium causes gastritis and peptic ulceration in humans and its urease enzyme is essential for the virulence of the bacteria (Hu and Mobley, 1990; Dunn *et al.*, 1990). In the absence of urea, *H. pylori* is very sensitive to low pH conditions, thus the urease enzyme is critical for the colonisation by this bacteria in the acidic environment of the human gastric mucosa. It is postulated that the urease of *H. pylori* hydrolyses the urea to ammonia which neutralises the low pH of the stomach and thus enables the bacteria to survive and colonise (Eaton *et al.*, 1991). Furthermore, ammonium hydroxide generated by urea hydrolysis contributes significantly to histological damage (Mobley *et al.*, 1995b). The ammonium ion itself is not toxic, but the damage is done by the hydroxide ions generated by the equilibration of ammonia with water. An additional damaging effect of ammonia generated by the activity of the urease enzyme, is its interference with the normal hydrogen ion back-diffusion across the gastric mucosa that results in cytotoxicity to the underlying epithelium.

The treatment of the above-mentioned illnesses with urease inhibitors has been unsuccessful because half or more of the patients experienced side effects. For example, the commonly used inhibitor acetohydroxamic acid depresses bone marrow biosynthesis, inhibits DNA synthesis and is teratogenic when used in high doses.

ACID TOLERANCE

Bacterial ureases also play an important role in the survival of the microorganism in low pH environments. The survival of bacteria depends on their ability to tolerate environmental changes such as temperature, pH, osmolarity and nutrient availability. By making the right physiological adaption, bacteria can survive non-life-threatening or gradual environmental changes. The gram negative bacterium, *Yersinia enterocolitica*, is a good example of a bacterium with the ability to adapt: this organism can survive as a saprophyte at temperatures ranging from 4 to 28°C or can be free-living in environments

where essential nutrients tend to be scarce (Young *et al.*, 1996). It is also an enteric pathogen that survives at 37°C when it lives as a parasite in its mammalian host. Besides the change in temperature, Y. *enterocolitica* also experiences rapid changes in pH when going from a free-living environment to a host environment where the pH of the host stomach can be as low as 2. Another acidic environment that bacteria may encounter during the course of infection is phagosomes of polymorphonuclear leucocytes and macrophages (De Koning-Ward and Robins-Browne, 1997). It was shown that *Y. enterocolitica* and *M. morganii* can survive in extreme acidic conditions when urea was available (Young *et al.*, 1996). The acid tolerance of these two bacterial species requires a cytoplasmic urease that is activated 780-fold by low pH conditions (to be discussed in later sections). A hypothetical model that describes how urease is involved in acid tolerance of these bacteria is shown in Figure 4.

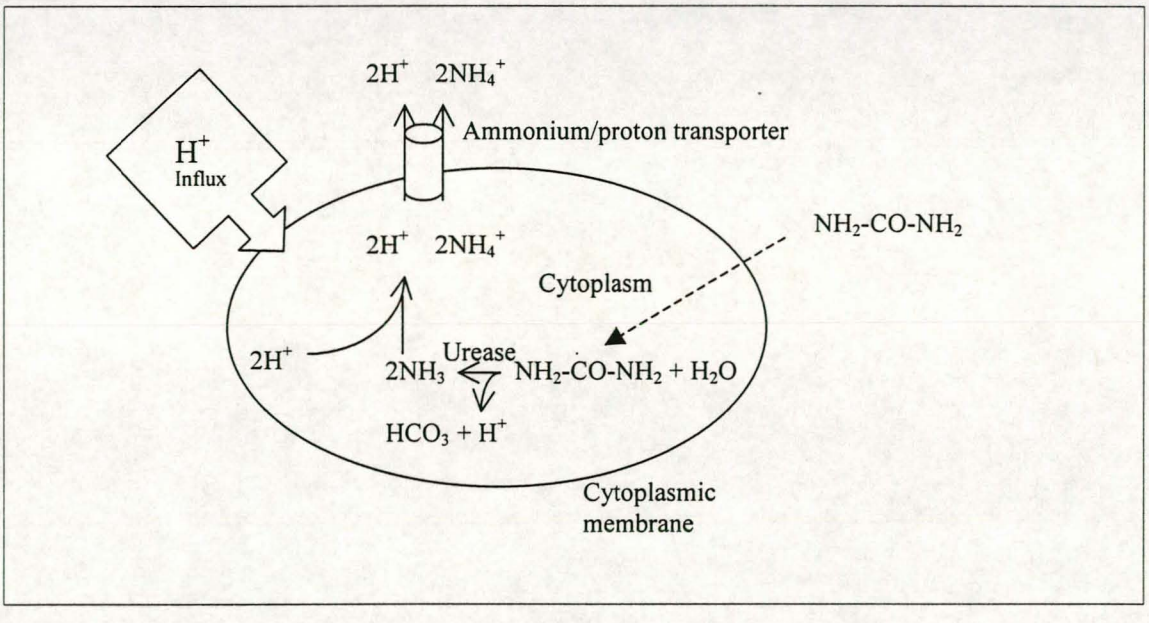

Figure 4. A model for urease-dependent acid tolerance (Young *et al.*, 1996).

Under low pH conditions, there is an influx of protons to the cytoplasm that results in an increase in urease activity (Young *et al.*, 1996). Urea enters the cytoplasm by diffusion and is hydrolysed by urease. The ammonia produced in this reaction spontaneously protonates to ammonium and neutralises 1 mol of protons per mol of urea hydrolysed. Ammonium that exits the cytoplasm via a symport system results in a proton efflux. The

energy to drive this system comes from the ammonium ion gradient generated when the urease-produced ammonia spontaneously converts to ammonium. This ammonium-driven proton transport increases the cell's capacity to oppose proton influx. Under normal conditions, this system could be reversed so that ammonium can be imported as a nitrogen source. The ureases of *Y. enterocolitica* and *M. morganii* are bifunctional in the sense that it has high activity under low pH conditions and low activity under neutral pH conditions when utilising urea as a nitrogen source.

The mechanism by which *H. pylori* protects itself against acidic environments is different from *Y. enterocolitica* and *M. morganii.* For the latter two bacteria, the urease is located in the cytoplasm; and this location is essential because the enzyme is labile below pH 5.5. Furthermore, the enzyme activity is regulated according to the need of the cell or the environmental pH, and the protective effect of ammonia does not extend beyond the cell in which it is produced. In contrast, the urease of *H. pylori* is normally located in the cytoplasm, but when it is released from dying cells it will remain active and associates with the outer surface of viable bacteria cells (Phadnis *et al.*, 1996). The urease has an optimum pH of 8, but is still partially active under low-pH conditions. It thus appears that both the cytoplasmic and surface-located ureases of *H. pylori* contribute to acid tolerance.

BACTERIAL UREASE AND DENTAL HEALTH

Dental caries is the result of dissolution of the tooth enamel through glycolytic acidification by plaque bacteria. Caries develop when there is a shift in dental plaque microbial composition away from a healthy or basic microflora. Urea is present as a component of saliva and cervical fluids in the human mouth (Chen *et al.*, 1996). An oral bacterium, *Streptococcus salivarius*, produces a urease that can hydrolyse the urea. The ammonia produced elevates the pH of the mouth and thus neutralises the plaque acids formed by glycolytic acidification. In this way, the urease of *S. salivarius* may inhibit the initiation and progression of dental caries.

PHYSICAL CHARACTERISTICS OF THE UREASE ENZYME

ENZYME LOCATION

Jeffries (1964) demonstrated that the urease enzyme of 22 species of bacteria were associated with the soluble extracts of disrupted cells. The same result was found for cell fraction studies done on *Klebsiella aerogenes*, *Ureoplasma urealyticum, P. stuartii* and *P. mirabilis* (Mobley and Hausinger, 1989). Urease antibodies confirmed the cytoplasmic localisation of *K. aerogenes* (Mulrooney *et al.*, 1989) and *U. urealyticum* (Myles *et al.*, 1991). Somewhat contradictory results were obtained with electron-microscopic histochemical studies on *Staphylococcus* and *P. mirabilis,* showing a membrane bound urease for the former and a urease associated with the periplasm and outer membrane for the latter. However, this technique detects ammonia and not urease, therefore urease-generated ammonia produced intracellularly may simply exit the cell and extracellularly react with the detection compound that is unable to enter the cell (Mobley *et al.*, 1995b). As previously discussed, the urease of viable *H. pylori* cells is located in the cytoplasm, while the urease of dead *H. pylori* cells is released and adheres to the cell surface of viable cells, protecting these cells in low pH conditions (Phadnis *et al.*, 1996). The jack bean urease was also shown to be cytoplasmic (Faye *et al.*, 1986). Booth and Vishniac (1987) suggested that all basidiomycetous yeast and some ascomycetous yeast produce extracellular ureases. However, in a recent study done on the urease of the ascomycetous fission yeast *Schizosaccharomyces pombe,* it was shown that the enzyme is intracellular and not membrane associated or extracellular (Lubbers *et al.*, 1996).

TEMPERATURE AND pH STABILITY

The optimum pH for microbial urease activity is usually near neutral. There are, however, a few microorganisms that produce ureases with their highest activity at low pH levels. This phenomenon was first reported by Moreau and co-workers (Moreau *et al.*, 1976) for the lactic acid bacterium *Lactobacillus fermentum*. In 1979, Suzuki and co-workers tested 560 bacterial strains found in the contents of the intestinal tract of humans

(Suzuki *et al.*, 1979). They reported that only three out of 52 strains of *L. fermentum* were urease producers with the optimum pH for urease activity at pH 4. Takebe and Kobashi (1988) isolated and identified *L. fermentum* from the cecum contents of rats and determined an optimal pH at 2.4. They named this enzyme with a low optimal pH an "acid urease" and all the other ureases with optimal pH near neutral, "neutral ureases". In 1989, Kakimoto *et al.* (1989a) identified the following microorganisms as acid urease producers: *Streptococcus mitior, S. salivarius, S. faecalis, S. faecium, S. avium, S. gallinarum, Lactobacillus reuteri, L. fermentum, L. animalis, L. salivarius, L. ruminis, L. viridescens, L. vacinostercus, L. acidophilus, L. amylovonis, L. arispatus, L. gasseri* as well as strains from the genera *Escherichia, Staphylococcus, Morganella* and *Bifidobacterium*. De Koning-Ward and Robins-Browne (1997) added another non-acidophilic bacterium, *Y. enterocolitica,* to this list. The urease from this bacterium has an optimum pH between 3.5 and 4.5. The urease from *L. reuteri* is most active at pH 2 and 65°C, but stable between pH 3 and 8, below 50°C (Kakimoto *et al.*, 1989b). The same properties were found for the urease from *L. fermentum* (Kakimoto *et al.*, 1990). For the acid urease from *S. mitior,* the optimum pH is 4.5 with maximum enzyme activity between 60 and 70°C.

The urease of the fission yeast *S. pombe* is active from pH 4 to 10 with maximum activity at pH 8.2 (Lubbers *et al.*, 1996). Other fungal ureases also fall into the same optimum pH range of 8.0 to 8.5 with the exception of the enzyme of *U. violacea* which has a pH optimum of 7 (Baird and Garber, 1981). The jack bean urease has a pH optimum of 7.0 to 7.75 (Mobley and Hausinger, 1989). No eukaryotic acid urease-producing organism has yet been reported.

UREASE INHIBITORS

The study of urease inhibitors is important because of its pharmacological and agricultural value. Hydroxamic acids are well-known inhibitors of urease (Blakeley and Zerner, 1984). The mechanism of inhibition is that of a slow, tight-binding competitive inhibitor (Todd and Hausinger, 1989) that competes with the substrate to bind to the enzyme. Acetohydroxamic acid is the derivative synthesised to use as a urease inhibitor

for plant and bacterial ureases. Hydroxyurea is both a substrate and an inhibitor for microbial ureases (Mobley and Hausinger, 1989) and when it is added to urease, rapid enzyme inhibition is followed by a slow recovery of activity as hydroxyurea is hydrolysed. Phosphoroamide compounds, e.g. phenylphosphorodiamidate, amido derivatives of phosphoric and thiophosphoric acids and N-acyl derivatives of phosphoric triamides are also effective inhibitors. The tetrahedral geometry of the phosphoroamide compound may mimic an intermediate state in enzymatic catalysis, thus acting as transition state analogs. Fully protonated phosphoric acid is a competitive inhibitor of urease. This is an important fact to consider when doing kinetic analysis in phosphate buffers at low pH. Thiols should also be excluded from urease assay buffers because it was shown that it competitively inhibits jack bean urease (Andrews *et al.*, 1984). Boric acid, boronic acids and fluoride are also inhibitors (Mobley *et al.*, 1995b). Metal ions such as Hg^{2+} and Ag^{+}, and in a lesser degree, Cu^{2+} are potent inhibitors of the ureases of *S. mitior* (Yamazaki *et al.*, 1990), *L. reuteri* (Kakimoto *et al.*, 1989b) and *L. fermentum* (Kakimoto *et al.*, 1990).

Ureases are also inactivated by oxidation (Lister, 1956; Mobley and Hausinger, 1989). Microbial ureases are, in general, quite stable in the presence of low concentrations of EDTA and thiol protectants when they are not subjected to pH extremes or high temperatures (Mobley and Hausinger, 1989). These are important considerations for purification and long term storage of the enzyme.

KINETIC PROPERTIES

K_M VALUES OF UREASES

Ureases presumably exhibit Michaelis-Menten-type kinetic behaviour, since no evidence of allosteric behaviour has been detected (Mobley *et al.*, 1995b). When urea is used as a substrate, ureases have K_m values from 0.1 to >100 mM. There is a correlation between K_m values of specific ureases and the ecological niche of the host organism. For example, *H. pylori* inhabits the gastric mucosal lining where low concentrations of urea

(1.7 to 3.4 mM) is present. The urease of *H. pylori* has one of the lowest K_m values of all ureases (0.17mM) and this low kinetic constant enables the urease to function under close to saturation conditions despite the low substrate concentration (Hu and Mobley, 1990). In contrast, microorganisms of the urinary tract are exposed to high urea concentrations and their enzymes show larger K_m values. Different strains of the same species sometimes possess different K_m values; the K_m values of the strains of *P. mirabilis* range from 13 to 60 mM. The K_m values of the acid ureases of *L. fermentum* and *S. mitior* are 17 and 2 mM respectively (Takebe and Kobashi, 1988; Yamazaki *et al.*, 1990).

Specific Activities

When determining specific activities of ureases, it is important that the protein should be completely purified and not assayed in a buffer containing any urease inhibitors. It is also important that only specific activities assayed under standard conditions will be compared, i.e. at pH 7.0 and 38°C. Under these conditions, homogeneous jack bean urease has a specific activity of approximately 3,500 µmol/min (U) per mg (Andrews *et al.*, 1984). The highest specific activity has been reported for the *U. urealyticum* urease with the values ranging from 33 530 to 180 000 U/min/mg (Mobley *et al.*, 1995b). Other bacterial ureases generally possess specific activities of 1 000 to 5 500 U/mg. The acid urease producers all have depressed specific activities, e.g. *L. reuteri*, *L. fermentum* and *S. mitior* have activities of 350 U/mg (Kakimoto *et al.*, 1989b), 304 U/mg (Kakimoto *et al.*, 1990) and 458 U/mg (Yamazaki *et al.*, 1990), respectively.

STRUCTURAL PROPERTIES

Molecular Weight of the Native Enzyme

The urease protein consists of subunits of which the size, number and properties differ between different organisms. Bacterial ureases are generally 200 to 250 kDa in size. Exceptions are a 125 kDa urease isolated from a mixed ruminal population and the purified ureases from *Selenomonas ruminantium* and *H. pylori* which possess molecular weights of 360 kDa (Mahadevan *et al.*, 1977) and 550 kDa (Hu and Mobley, 1990),

respectively. The molecular weights of native enzymes are usually measured by gel filtration chromatography. With this method, the enzyme tends to aggregate with contaminants or interact with the resin, leading to molecular weight values higher than the actual value (Jones and Mobley, 1988). This aggregation can be minimised by including 0.1 M NaCl in the buffers. Molecular weights can also be determined by using native gel electrophoretic methods.

The native size of eukaryotic ureases is in general somewhat bigger than the bacterial ureases. Jack bean urease is 590 kDa (Andrews *et al.*, 1984) and the urease from the basidomycetous yeast *R. paludigenum* is 569 kDa (Phillips *et al.*, 1990). However, some eukaryotic microorganisms have ureases similar in size to the bacterial ones. The *S. pombe* urease is 212 kDa (Lubbers *et al.*, 1996) which is similar to the size of ureases from other ascomycetes, e.g. the *A. nidulans* urease of 249 kDa (Creaser and Porter, 1985) and the *A. niger* urease of 250 kDa (Smith *et al.*, 1993).

SUBUNIT MOLECULAR WEIGHTS AND STOICHIOMETRY

Most bacterial ureases are heteropolymeric enzymes consisting of three different subunits. For example, the ureases of some of the members of the family *Enterobacteriaceae* such as *K. aerogenes and P. mirabilis,* consist of subunits with molecular masses of approximately 70, 11 and 9 kDa, designated α, β and γ, respectively (Todd and Hausinger, 1987; Jones and Mobley, 1988; Mobley and Hausinger, 1989). The same is true for many other bacteria including *Lactobacillus* and *Staphylococcus* spp., *P. stuartii, M. morganii, U. urealyticum, Y. enterocolitica* and *Bacillus* sp. strain TB-90 (Kakimoto *et al.*, 1989b, 1990; Christians *et al.*, 1991; Meada *et al.*, 1994; Thirkell *et al.*, 1989). The stoichiometry of the subunits for most of these microorganisms is listed in Table 1.

Helicobacter species have two subunits of about 61 to 66 kDa and 26 to 31 kDa with an estimated 1:1 stoichiometry (Dun *et al.*, 1990, 1991; Hu and Mobley, 1990). The estimated size of the urease is 535 to 650 kDa, therefore the assumption is that the native enzyme has six copies of each of the subunits (Hu and Mobley, 1990; Dun *et al.*, 1991).

In contrast, a single type of urease subunit seems to be a conserved feature for eukaryotes.

Table 1. Structural properties of ureases from prokaryotes and eukaryotes.

Source	Subunit M_r	Stoichiometry	Reference
Eukaryotes			
Jack bean	96 600 (α)	α_6	Takishima *et al.*, 1988
Soy bean	Ubiquitous urease: 91 000 (α)	α_3	Pollaco and Holland, 1993
	Embryo urease: 91 000 (α)	α_6	
Schizosaccharomyces pombe	102 000 (α)	α_2	Lubbers *et al.*, 1996
Aspergillus niger	83 000 (α)	NR	Smith *et al.*,1993
Aspergillus nudilans	40 000 (α)	α_6	Creaser and Porter, 1985
Ustilago violacea	80 000 (α)	NR	Baird and Garber, 1981
Prokaryotes			
Helicobacter pylori	62 000(α), 30 000(β)	$(\alpha\beta)_6$	Dunn *et al.*, 1991
Klebsiella aerogenes	72 000(α), 11 000(β), 9 000(γ)	$(\alpha\beta\gamma)_3$	Jabri *et al.*, 1995
Lactobacillus fermentum	67 000(α), 16 800(β), 8600(γ)	$(\alpha\beta_2\gamma)_2$	Kakimoto *et al.*, 1990
Lactobacillus reuteri	68 000(α), 16 100(β), 8 800(γ)	$(\alpha\beta_2\gamma)_2$	Kakimoto *et al.*, 1989b
Providencia stuartii	73 000(α), 10 000(β), 8 000(γ)	$\alpha_2\beta_4\gamma_4$	Mulrooney *et al.*, 1988
Selenomonas ruminantium	70 000(α), 8 000(β), 8 000(γ)	NR	Todd and Hausinger, 1987
Ureaplasma urealyticum	72 000(α), 14 000(β), 11 000(γ)	$\alpha_2\beta_2\gamma_2$	Thirkell *et al.*, 1989
Streptococcus mitior	66 000(α), 15 600(β), 8 600(γ)	$\alpha_2\beta_4\gamma_4$	Yamazaki *et al.*, 1990
Staphylococcus xylosus	64 000(α), 17 800(β),16300(γ)	NR	Christians *et al.*, 1991
Proteus mirabilis	73 000(α), 10 000(β), 8 000(γ)	$(\alpha\beta_2\gamma_2)_2$	Jones and Mobley, 1988
Bacillus sp. strain TB-90	61 000(α), 12 000(β), 11 000(γ)	NR	Maeda *et al.*, 1994

NR = Not reported

The jack bean urease, which is a homohexamer, has a native molecular weight of 590 kDa and a single subunit of 91 kDa (Andrews *et al.*, 1984; Takishima *et al.*, 1988). The native urease of *S. pombe* is 212 kDa with a subunit size of 102 kDa; thus the urease could be a dimer (Lubbers *et al.*, 1996). Other eukaryotic ureases have subunit sizes varying from 40 kDa for *A. nidulans* (Creaser and Porter, 1985), 72 kDa for

R. paludigenum (Phillips *et al.*, 1990), 80 kDa for *U. violacea* (Baird and Garber, 1981) and 83 kDa for *A. niger* (Smith *et al.*, 1993). There are also differences in the number of subunits of eukaryotic ureases, e.g. *A. niger* is a trimer (Smith *et al.*, 1993), the jack bean enzyme a hexamer, and the *R. paludigenum* urease may be an octamer (Phillips *et al.*, 1990).

The number of different subunits is determined by denaturing polyacrylamide gel electrophoresis. With this method, small subunits are poorly resolved from the dye front on gels containing less than 10% polyacrylamide. The urease from *S. ruminantium* was initially reported to contain a single subunit, but the three subunits of the enzyme became visible when the gel concentration was increased (Todd and Hausinger, 1987). To estimate the subunit stoichiometry, the intensity of stained subunit bands was scanned. However, this method may lead to incorrect values, because the assumption is made that equivalent dye binding per unit mass by each peptide band takes place.

STRUCTURAL DOMAINS

The third landmark in the history of the study of urease (after being the first enzyme to be crystallised and the first nickel metalloenzyme to be discovered), was the determination of the X-ray crystal structure of the enzyme from *K. aerogenes* by Jabri and co-workers (Jabri *et al.* 1995). This structure revealed many interesting facts about the molecular geometry at the active site. As shown in Figure 5, the three structural subunits of the urease of *K. aerogenes* are tightly associated as a trimer of trimers $[(\alpha\beta\gamma)_3]$ and not the previously proposed $(\alpha\beta_2\gamma_2)_2$ structure (Todd and Hausinger, 1987).

To form the trimer, each α subunit packs between the two symmetry-related α subunits and contacts two β subunits and two γ subunits to form the sides of the triangle. Each γ subunit interacts with two α subunits and tightly with two other γ subunits. Approximately 10% of the α, β and γ surfaces are buried to form the αβγ-unit and 23% or the αβγ-unit surface is buried when the trimer of αβγ-units is formed. The αβγ-unit, shown in Figure 6, is a T-shaped molecule with dimensions 75 by 80 by 80 Å.

Figure 5. A ribbon diagram of the $(\alpha\beta\gamma)_3$ urease trimer of *K. aerogenes*. The one $\alpha\beta\gamma$-unit is violet, the second is white and the third one is coloured according to the different subunits (α in red, β in orange and γ in yellow). The overall shape of the trimer is triangular with dimensions of 110 by 110 by 80 Å (Jabri *et al.*, 1995).

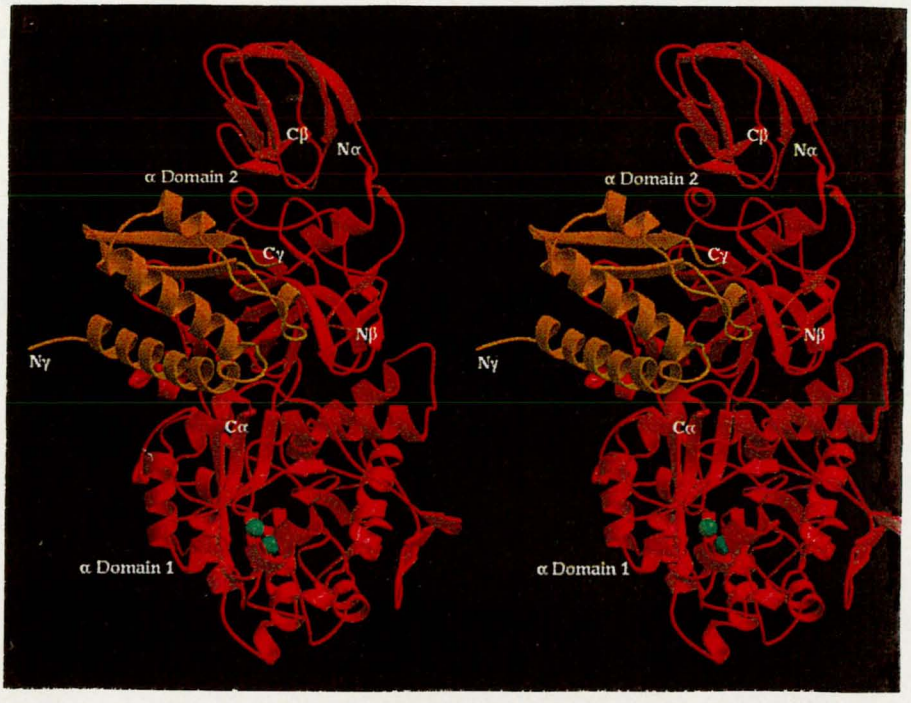

Figure 6. A stereogram of a $\alpha\beta\gamma$-unit displaying the secondary structures of the urease enzyme. The subunits α (red), β (orange) and γ (yellow) are indicated, with the two nickel atoms (green) located at the carboxyl termini of the strands in the $(\alpha\beta)_8$ barrel of the α subunit (Jabri *et al.*, 1995).

There are four structural domains in each αβγ-unit: two in the α chain and one in each of the β and γ chains (Figure 7). The α-subunit has a $(\alpha\beta)_8$ barrel domain which has an elliptical shape with a long axis of about 20 Å. The active site that contains a dinuclear metallocenter, is located in this $(\alpha\beta)_8$ barrel, with a helical excursion (H2-H4 in Figure 7)

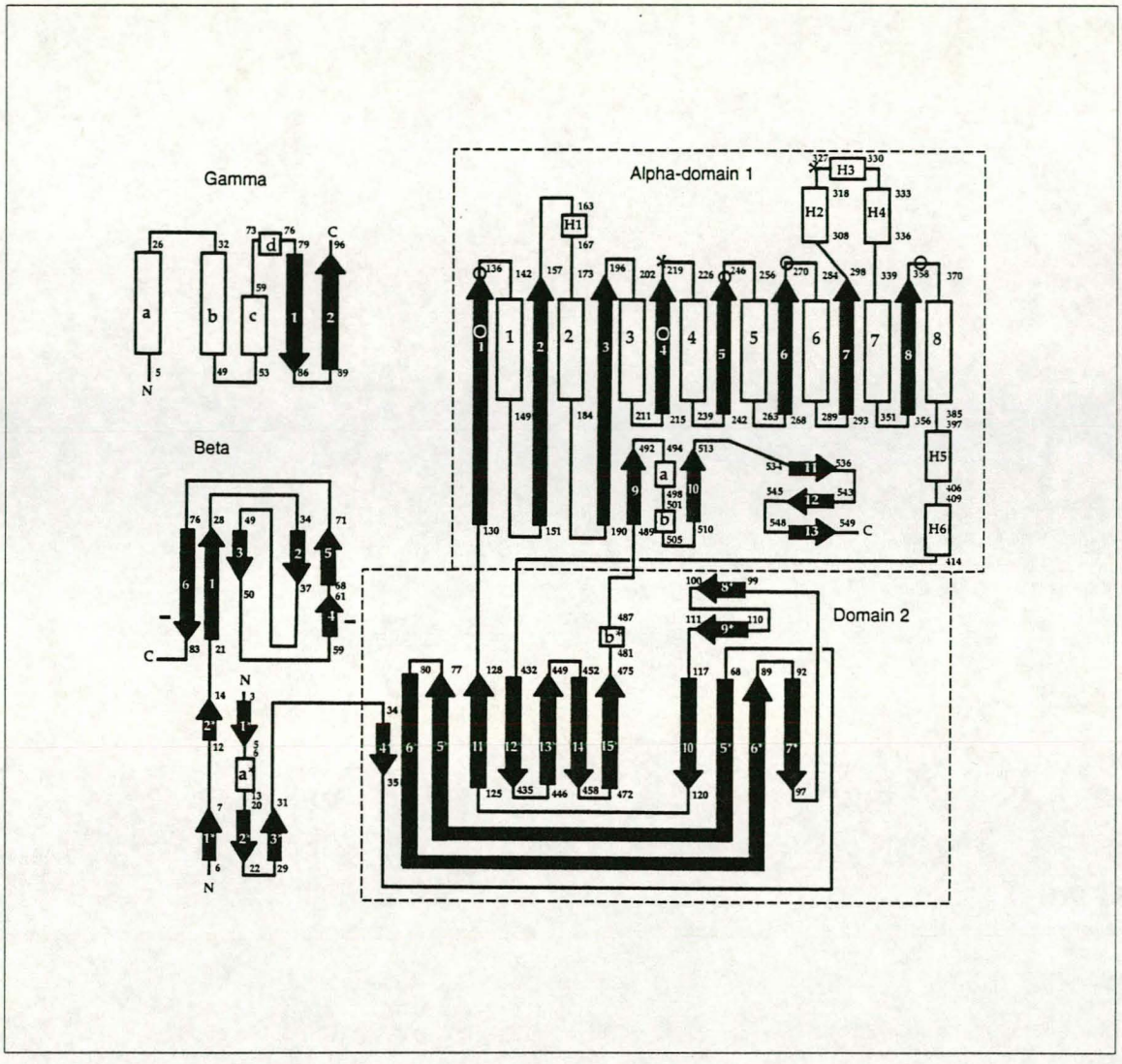

Figure 7. Topology diagrams of the urease subunit showing the four structural domains in one (αβγ)-unit. The helices (white rectangles) and strands (black arrows) are numbered in the order in which they appear in the individual domains. The helices and strands in the α subunit are numbered separately with H1-H6 indicating the $(\alpha\beta)_8$ barrel excursions. The circles in domain 1 of the α subunit represents the positions of nickel ligands ($His^{\alpha134}$, $His^{\alpha136}$, $Lys^{\alpha217}$, $His^{\alpha246}$, $His^{\alpha272}$, $Asp^{\alpha360}$) and the asterisks (*) shows the residues implicated in binding ($His^{\alpha219}$) and catalysis ($His^{\alpha320}$). In the β subunit the hyphen (-) indicates that strand 4 hydrogen bonds to strand 6. The beginning and end residue numbers for each element are given (Jabri *et al.*, 1995).

between strand 7 and helix 7 forming a "flap" across the active site. Apart from the $(\alpha\beta)_8$ barrel, there is also a primarily β domain in the α subunit. The structural domain in the β subunit is a rare left-handed jellyroll and the γ subunit has a novel αβ domain. Jabri and co-workers (1995) proposed that the function of the β subunit is to stabilise the trimer by associating with domain 2 of its own α subunit and domain 1 of a symmetry related α subunit. The γ subunit facilitates trimer formation through association with the α subunit and the symmetry related γ subunits.

THE NICKEL CENTER

Nickel was found in all the ureases investigated and is required for an urease enzyme to be active (Dixon *et al.*, 1979). When grown on medium depleted of nickel ions, *K. aerogenes* cells produced an inactive urease (Lee *et al.*, 1990). The inactive protein resembles the native enzyme except for being free of metal ions. Many enzymes contain metal ions that act in the same way as coenzymes, conferring on the enzyme a property it would not possess in the absence of the ion. The metal ions are usually held by coordinate-covalent bonds from amino acid side chains, but sometimes they are bound by prosthetic groups like heme. Examples of other nickel metalloenzymes include hydrogenases, methyl-coenzyme M reductases and carbon monoxide dehydrogenases. Urease is the only known nickel metalloenzyme that catalyse a hydrolysis rather than a redox reaction (Jabri and Karplus, 1996).

Urease inhibitor studies showed that there are two nickel ions per catalytic unit and that nickel is at the active site and does not play a structural role (Hausinger, 1993). This was shown for the jack bean (Dixon *et al.*, 1975) and for bacterial *K. aerogenes* (Todd and Hausinger, 1991) ureases. Some microorganisms, like *Bacillus pasteurii* (Christians and Kaltwasser, 1986), *Brevibacterium ammoniagenes* (Nakano *et al.*, 1984), *H. pylori* (Hawtin *et al.*, 1991) and *Staphylococcus xylosus* (Cristians *et al.*, 1991), were reported to have a lower nickel content. In some cases, the nickel content is as low as that expected for proteins containing a mononickel center. However, it is possible that insufficient nickel ions were present in the growth medium that caused incomplete loading of the protein with the metal ion.

The urease crystal structure of *K. aerogenes* showed that the nickel ions in the active site are 3.5 Å apart (Jabri *et al.*, 1995). Nickel-1 (Ni-1) is coordinated by three ligands: $His^{\alpha 246}$ through the Nδ atom, $His^{\alpha 272}$ through Nε and $Lys^{\alpha 217*}$ of which the ε-amino residue were modified in a reaction with carbon dioxide resulting in a carbamate group. The carbamate bridges the two nickel ions. This reaction between the lysine-carbon dioxide and metal ion (nickel) is synergistic because the metal ion stabilises the carbamate and the carbamate binds to, and holds the metal in the active site (Lippard, 1995). Nickel-2 (Ni-2) is coordinated by five ligands: $His^{\alpha 134}$ and $His^{\alpha 136}$ both through Nε, $Asp^{\alpha 136}$ through Oδ1, Wat-1, and again $Lys^{\alpha 217*}$ through Oθ2. Figure 8 shows the bi-nickel center and different ligands.

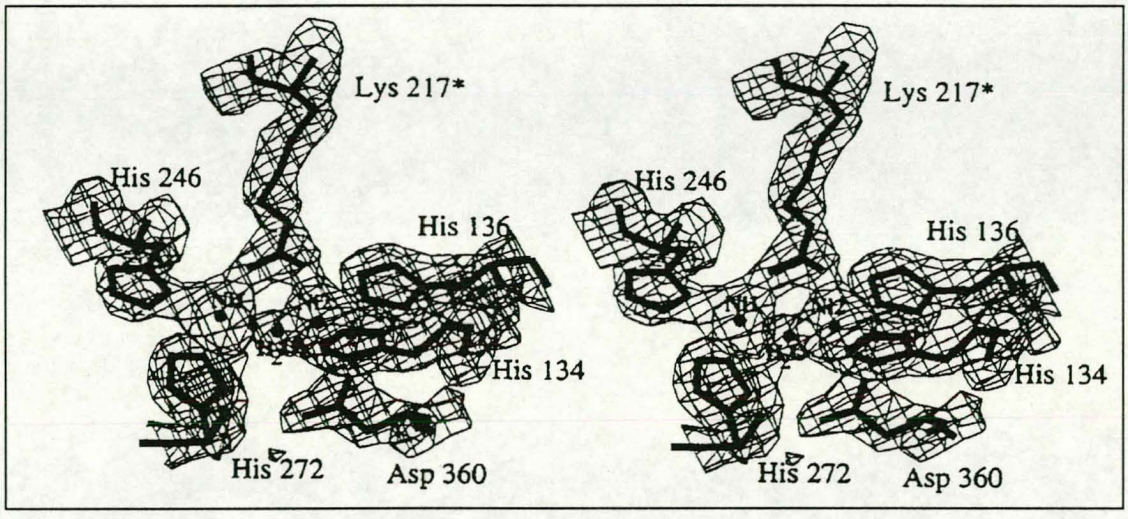

Figure 8. Stereogram of the bi-nickel center and different ligands (Jabri *et al.*, 1995).

MODEL FOR MECHANISM OF UREA CATALYSIS

The geometry of Ni-1 is pseudo-tetrahedral when including the empty site shown in Figure 9. This "empty site" is partially occupied, most likely by a water molecule, in the crystal structure and may be responsible for binding the urea molecule during catalysis (Lippard, 1995). A potential urea-binding pocket is located in the active site, adjacent to the water molecule that coordinates the second nickel ion (discussed above). This pocket is lined by several acidic and basic side chains, for example $His^{\alpha 219}$, that could participate

in the catalysis. A narrow channel leads from the enzyme surface to the pocket but is blocked by a side chain of $Cys^{\alpha 319}$. The H_2-H_4 flap forms one wall of this channel and because this flap is highly mobile, it can easily open to allow access to the active site.

Figure 9 is a diagram proposing a possible mechanism for the chemistry at the urease active site. This model proposes that when urea binds to Ni-1, its oxygen atom is close to the protonated ε nitrogen of $His^{\alpha 219}$ (A^H in Figure 9) located in the pocket lining. This

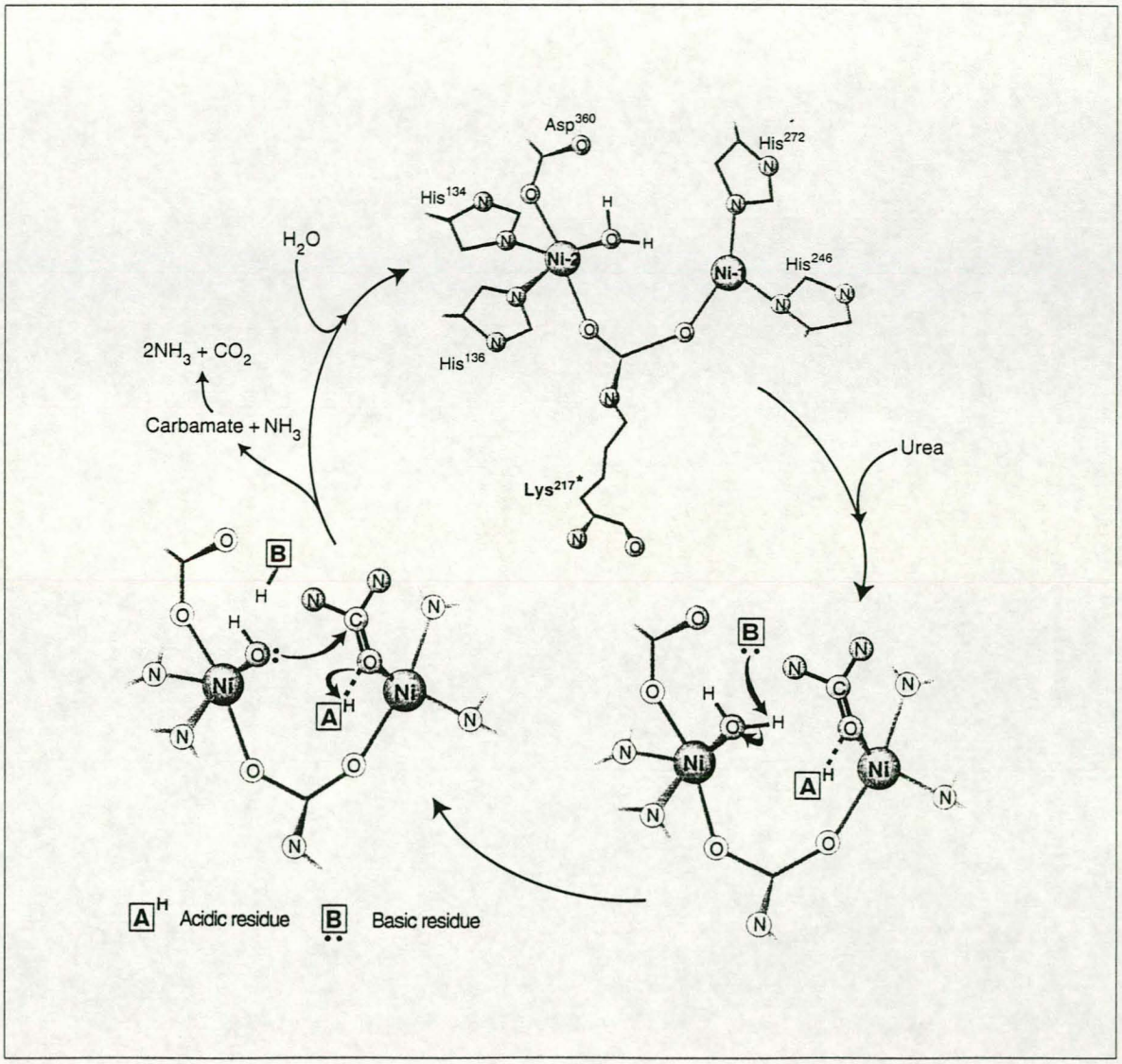

Figure 9. Possible mechanism for the chemistry at the active site of urease (Lippard, 1995).

might help the nickel (II) centre in polarising the carbonyl group of urea for nucleophylic attack. Furthermore, either an oxygen atom of $Asp^{\alpha 360}$ or a nitrogen atom of $His^{\alpha 320}$ can act as a base (B in Figure 9) to remove a proton from the coordinated water molecule to generate the nucleophile - a hydroxide ion (Lippard, 1995).

GENETICS

GENE CLUSTER LOCI

For most urealytic organisms, the urease gene cluster is located on the chromosomal DNA. Examples of chromosomally encoded ureases are genes of the bacterium *P. mirabilis* (Jones and Mobley, 1988) and the nine urease genes of *Bacillus* sp. strain TB-90 (Maeda *et al.*, 1994). An interesting observation was that some strains of *Escherichia coli* carry plasmid encoded ureases (Collins and Falkow, 1988). This was first suspected after observing that a low percentage of the *E. coli* strains isolated are urease positive and the urease phenotype is very unstable. The urease gene cluster of *P. stuartii* is also plasmid encoded and the transfer of this gene cluster to *E. coli* by a 140 MDa conjugative plasmid was demonstrated (Grant *et al.*, 1981; Mobley *et al.*, 1995b). The ureases of *S. faecium* isolated from sheep rumen (Mobley and Hausinger, 1989) and *Salmonella cubana* (Mobley *et al.*, 1995b) are also plasmid encoded.

THE UREASE GENES

The urease gene clusters of all organisms studied thus far contain structural and accessory genes. The structural genes are usually designated *ureA, ureB* and *ureC* and encode the alpha, beta and gamma subunits of the enzyme, respectively. The number of accessory genes differs for each organism, e.g. *K. aerogenes* has only four accessory genes i.e. *ureD*, *ureE*, *ureF* and *ureG,* whereas *Bacillus* sp. TB-90 has two additional ones called *ureH* and *ureI.*

Genetic organisation

The genetic organisation of the urease operons of five bacterial species is shown in Figure 10. A few general features can be distinguished. First, urea inducible gene

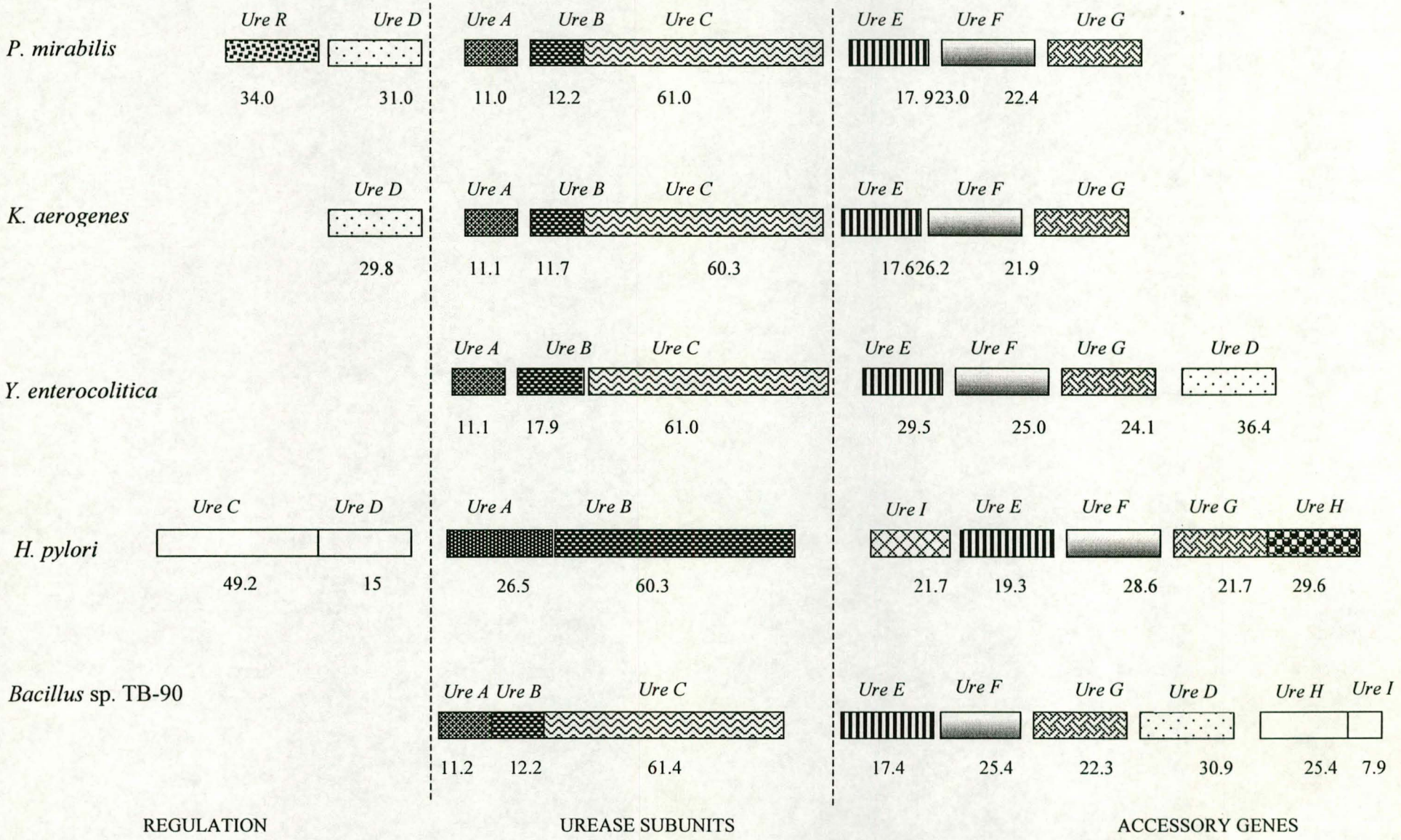

Figure 10. Genetic organisation of the urease operons of *P. mirabilis* (Isalnd and Mobley, 1995), *K. aerogenes* (Park *et al*., 1994), *Y. enterocolitica* (Mobley *et al*., 1995b), *H. pylori* (Cussac *et al*., 1992) and *Bacillus* sp. TB-90 (Maeda *et al*., 1994). Open boxes represent genes that are unique to a certain operon. The numbers beneath the boxes show the predicted size of the corresponding polypeptides in kDa.

clusters contain and begin with the *ureR* regulatory gene (Mobley *et al.*, 1995b). This gene is transcribed in the opposite direction to the ORF's of the rest of the gene cluster. Second, the structural subunits are always aligned from the smallest to the largest subunit. Third, the accessory genes *ureE, F and G* are always contiguous, and fourth, *ureD* and its *Helicobacter* homologue *ureH* can either precede *ureA* or follow *ureG*.

The minimal number of genes required to form an active urease appears to be seven, i.e. three structural subunits genes (*ureABC*) and four accessory genes (*ureDEFG*). This corresponds to a 6.6 kb fragment in *Y. enterocolitica* (De Koning-Ward *et al.*, 1994) and a 7.6 kb fragment in *P. mirabilis* (Jones and Mobley, 1989) that is required to retain urease activity when expressed in a heterologous host. There are some additional genes such as *ureI* of *H. pylori* and *ureH* of *Bacillus* sp. strain TB-90 that are not essential for an active urease. The two ORF's in *H. pylori*, *ureC* and *ureD* show no significant homology to the genes in other bacteria designated by the same names. De Koning-Ward *et al.* (1994) suggested that these two genes should receive new names and that *ureH* should be renamed as *ureD*, while *ureA* and *ureB* should be changed to *ureAB* and *ureC* respectively.

Transcriptional organisation

There are only a few reports on the transcriptional regulation of bacterial urease clusters. Most of these reports are nucleotide sequence analyses identifying putative promoter sequences based on homology to RNA polymerase recognition sites. Other work included determining mRNA transcript lengths with northern blots, or *lacZ* fusions to various gene fragments to identify the location of regulated promoters. For *Y. enterocolitica,* a putative promoter sequence was found upstream of *ureA* and another one within *ureA* (De Koning-Ward *et al.*, 1994). Downstream of *ureD* a *rho*-independent terminator sequence was found. The *P. mirabilis* urease cluster shows the *ureR* gene 400 bp upstream of and transcribed in the opposite direction than the other seven genes (Jones and Mobley, 1989; Island and Mobley, 1995). A promoter located between *ureR* and *ureD* seems to be urea-regulated and UreR-dependent. This promoter also controls the expression of *ureA*. In *Klebsiella* species (Mulrooney and Hausinger, 1990; Collins *et*

al., 1993), a nitrogen-regulated promoter was located upstream of *ureD*, while a *rho*-independent transcription terminator signal was found downstream of *ureG*.

STRUCTURAL GENES

The comparison of subunit type, size and stoichiometries in different organisms shown in Table 1, gives the impression that ureases are not highly conserved among the different species. This is strongly contradicted when examining the amino acid sequences of ureases. Jabri and Karplus (1996) compared the sequences of the structural proteins of 15 ureases, including those from jack bean and *L. fermentum,* and found 189 identical residues (24%).

Urease producing organisms can be divided in three groups when comparing the way their structural genes are organised: *Helicobacter* species form the one group and their ureases are composed of two distinct subunits encoded by two adjacent genes (Mobley *et al.*, 1995b). All ureases from non-*Helicobacter* bacterial species are composed of three distinct subunits encoded by three contiguous genes. As discussed earlier, eukaryotic ureases, i.e. those belonging to jack bean, soybean and *S. pombe,* are composed of one distinct subunit encoded by one gene. Despite these differences in subunit composition, there is a high level of homology between the structural genes. In Figure 11 it is shown that the α, β and γ subunits of *K. aerogenes* encoded by the *ureA*, *ureB* and *ureC* genes, correspond to residues 1 - 101, 132 - 237 and 271 - 840 of the jack bean sequence. In a similar way, the two-subunit urease sequence of *H. pylori* corresponds to two stretches of the jack bean urease.

There are differences in the spacing between the conserved subunit-encoding regions amongst the gene clusters of different organisms (Mobley *et al.*, 1995b), and the *Rhizobium meliloti* urease genes are even interrupted by other genes. When using the sequence from jack bean as a query sequence in the program NCBI TBLASTN to identify other ureases and related proteins, it was shown that ureases share no significant sequence similarities with other proteins (Jabri and Karplus, 1996).

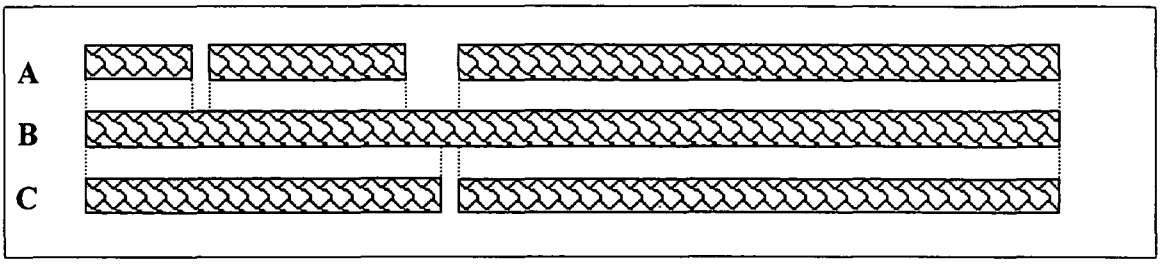

Figure 11. Sequence similarities amongst three typical urease proteins. Bar B represents jack bean urease. Bar A is representative of the urease enzymes of *K. aerogenes* (Mulrooney and Hausinger, 1990), *P. mirabilis* (Jones and Mobley, 1989), *P. vulgaris* (Mörsdorf and Kaltwasser, 1990) and *U. urealyticum* (Mobley *et al.*, 1995b) and shows that the three subunits in these enzymes are related to portions of the jack bean urease. DNA sequence analyses of *H. pylori* urease genes (Mobley *et al.*, 1995b) in C, indicate that the two-subunit enzyme is also related to the jack bean protein.

ACCESSORY GENES

Expression of only the structural genes encoding the urease subunits has been shown for several bacteria (Jones and Mobley, 1988; Mulrooney *et al.*, 1988; Mulrooney and Hausinger, 1990; Lee *et al.*, 1990, 1992), soybean (Polacco and Holland, 1993), *A. nidulans* (Mackay and Pateman, 1980) and *S. pombe* (Fluri and Kinghorn, 1985) to be not sufficient for the production of catalytic active ureases. Lee *et al.* (1990) found that it was also impossible to activate purified *K. aerogenes* urease apo-enzyme by simple addition of nickel (Ni) ions. However, when the apo-urease was synthesised by *K. aerogenes* cultures growing in Ni-free medium, the enzyme showed activity when nickel ions were added. This indicated that an additional cellular factor was required for the incorporation of the metal ion.

Function of accessory genes

The experiments described above led to the realisation that three or more accessory genes encoding the accessory proteins are required for co- and (or) post-translational modifications of the apo-urease to produce an active urease. Four urease genes have been reported for *A. nidulans* (Mackay and Pateman, 1980) and *S. pombe* (Fluri and Kinghorn, 1985). Both these microorganisms have only one type of subunit (Lubbers *et al.*, 1996; Creaser and Porter, 1985) and therefore need only one gene to encode an

urease. For *A. nidulans,* one of the "extra" genes appears to encode an urea active transport protein while the other may be involved in the synthesis or incorporation of the essential nickel cofactor (Mackay and Pateman, 1980; Lubbers *et al.*, 1996). The function of the extra *S. pombe* genes is unknown, but one or two may also be involved in nickel incorporation. In soybean it is the two loci called *Eu2* and *Eu3* that encode proteins associated with urease maturation (Polacco and Holland, 1993).

Relative to eukaryotes, much more is known about the function of the accessory genes from bacteria such as *K. aerogenes* (Park *et al.,* 1994; Moncrief and Hausinger, 1997). The *UreA, -B* and *-C* genes of this microorganism encode the structural proteins that are required for the assembly of the catalytically inactive enzyme also known as the Apo. A series of UreD-urease (D-Apo) (Park *et al.*, 1994), UreD-UreF-urease (DF-Apo) (Moncrief and Hausinger, 1996) and UreD-UreF-UreG-urease (DFG-Apo) (Park and Hausinger, 1995b) apoprotein complexes have been identified. The accessory proteins in these complexes are thought to modify the properties of Apo. The following paragraphs will discuss these complexes, the studies done on them and their possible functions.

Park *et al.* (1994) isolated D-Apo complexes with the formula $(\alpha\beta\gamma)_3UreD_n$ where n = 1, 2 or 3. They could partially activate these complexes (*in vitro*) by addition of nickel ions; the higher the value of 'n', the bigger the activation. They also found that UreD dissociates from the Apo during activation. From these results, they postulated that ureD is a molecular chaperone that stabilises the urease apoprotein conformation so that it is competent for nickel incorporation.

Somewhat contradictory to this hypothesis, Park and Hausinger (1995a) showed that purified *K. aerogenes* urease apoprotein can be activated *in vitro* and in the absence of any accessory proteins, by providing CO_2 in addition to nickel ions. They proposed that for metallocenter assembly and apoprotein activation, CO_2 has to bind to a deprotonated side chain of the urease apoprotein, forming a ligand which facilitates productive nickel binding. Tree months later, this was proven correct when Jabri *et al.* (1995) published the crystal structure of the *K. aerogenes* urease, showing a carbamylated lysine. In a

situation where low CO_2 concentrations are prevailing, a high percentage of apoprotein lacks bound CO_2. Park and Hausinger (1995a) suggested that under such conditions, the function of ureD is that of a molecular chaperone that controls the sequence of metallocenter assembly and activation of the apoprotein so that the CO_2 binds before the nickel ion, and no non-productive binding of nickel to apoprotein (that lacks CO_2) will occur.

Thus, *in vitro* activation of the urease apoprotein and the UreD-urease apoprotein was possible in the presence of CO_2 and Ni^{2+} ions. However, *in vivo* cell this was not sufficient, presumably because the prevailing CO_2 concentrations in the cell were not high enough. It was shown that a functional UreD, UreF and UreG are required for *in vivo* activation, and complexes that include these three accessory proteins were reported (Park and Hausinger, 1995b). These UreD-UreF-UreG-apoprotein complexes, as shown in Figure 12, were considered to be the key cellular urease activation machinery.

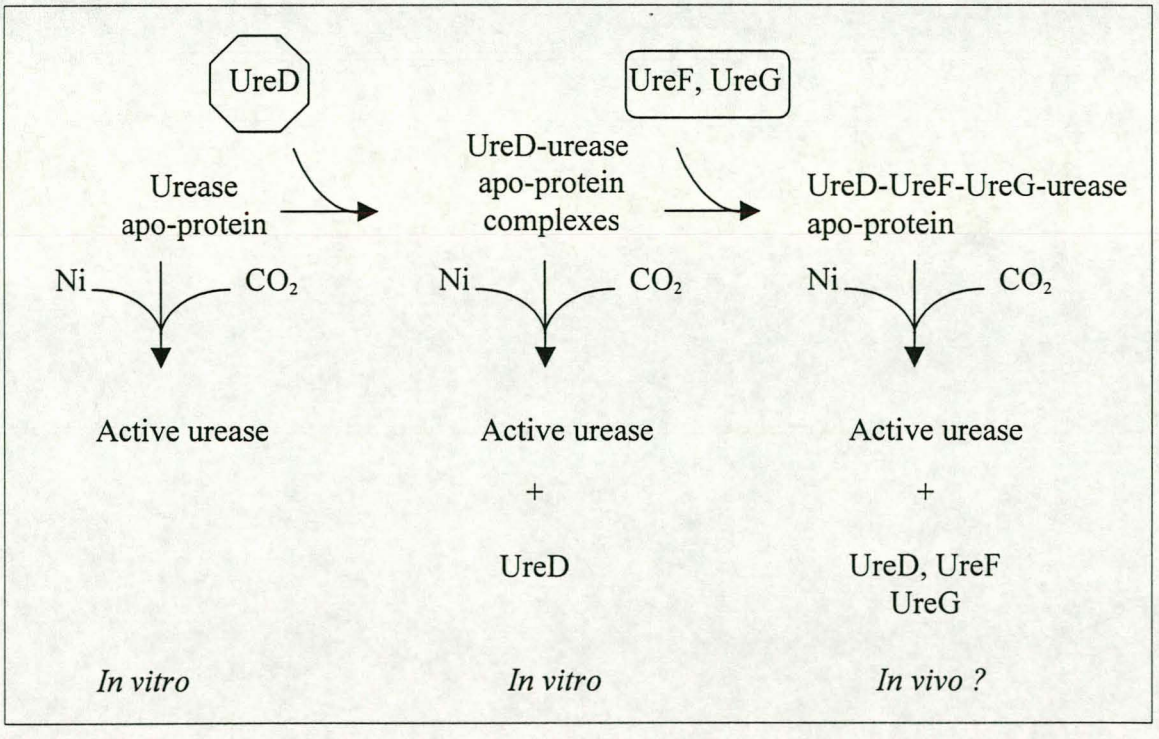

Figure 12. Model showing possible ways of *in vivo* and *in vitro* activation processes for urease activation. Although Apo and D-Apo complexes can be activated *in vitro* in the presence of Ni^{2+} and CO_2, functional *ureF* and *ureG* genes are required for *in vivo* activation. Park and Hausinger (1995b) proposed that the DFG-Apo complex is the enzyme species competent for *in vivo* activation.

Since the incubation of the Apo and D-Apo proteins in the presence of only Ni^{2+} ions (no CO_2) leads to the formation of inactive proteins (Park and Hausinger, 1995b), Park and Hausinger (1996) decided to study this inactivation or inhibitory effect in order to unravel the functions of UreD, -F and -G in the DFG-Apo complexes. They examined the kinetics and stoichiometry of nickel binding in the inhibitory reactions and the ability of other metal ions to inhibit Ni^{2+}- and CO_2-promoted urease activation. They demonstrated for the first time that other metals could bind to Apo and D-Apo, but that only manganese-substituted urease possessed catalytic activity. The reversibility of metal ion binding was also characterised.

The results and conclusions of the study on urease metal ion interactions are shown in Figure 13. In this figure, Apo-CO_2 denotes the carbamylated protein while the other states represents various nickel-containing or metal substituted urease species. Park and Hausinger (1996) showed the production of three products from the reaction between the urease apoprotein and nickel ions: an active carbamylated species (Apo-CO_2-Ni^A), an inactive carbamalyted species (Apo-CO_2-Ni^I) and an inactive non-carbamylated enzyme (Apo-Ni). Apo-Ni is generated when urease apoprotein is exposed to nickel ion in the absence of bicarbonate. The nickel ions can be removed from the Apo-Ni and Apo-CO_2-Ni^I species by treatment with EDTA, restoring the ability of the protein to be activated. However, the nickel ion cannot be removed from the Apo-CO_2-Ni^A species. Three metal-substituted (other than nickel) urease species can also be generated: Apo-M, Apo-CO_2-M (metal ion associated with apoprotein) and Apo-CO_2-M′ (metal ion bound or "locked in" to apoprotein). EDTA treatment can restore the Apo-M and Apo-CO_2-M species to activation competence but the Apo-CO_2-M′ is similar to the Apo-CO_2-Ni^A where the metal ion is inaccessible to the chelator.

It was now clear that only nickel (and to a small extent manganese) ions as the metallocenter of the urease enzyme, could confer an active enzyme. The next question was how does the cell ensure that only nickel ions are incorporated. Park and Hausinger (1996) suggested that the accessory proteins must play an important role in this regard. They suggested that the interactions of nickel and other metal ions with UreD-urease

apoprotein follows the same pattern as shown in Figure 13, except that metal ion binding is accompanied by UreD dissociation. In the absence of CO_2, an active species is produced when nickel ions bind to D-Apo. Furthermore, UreD appears to reduce the rate of apoprotein interaction with nickel ions (and other metal ions). If the rate constant of reactions where inactive proteins are formed, were reduced to a greater

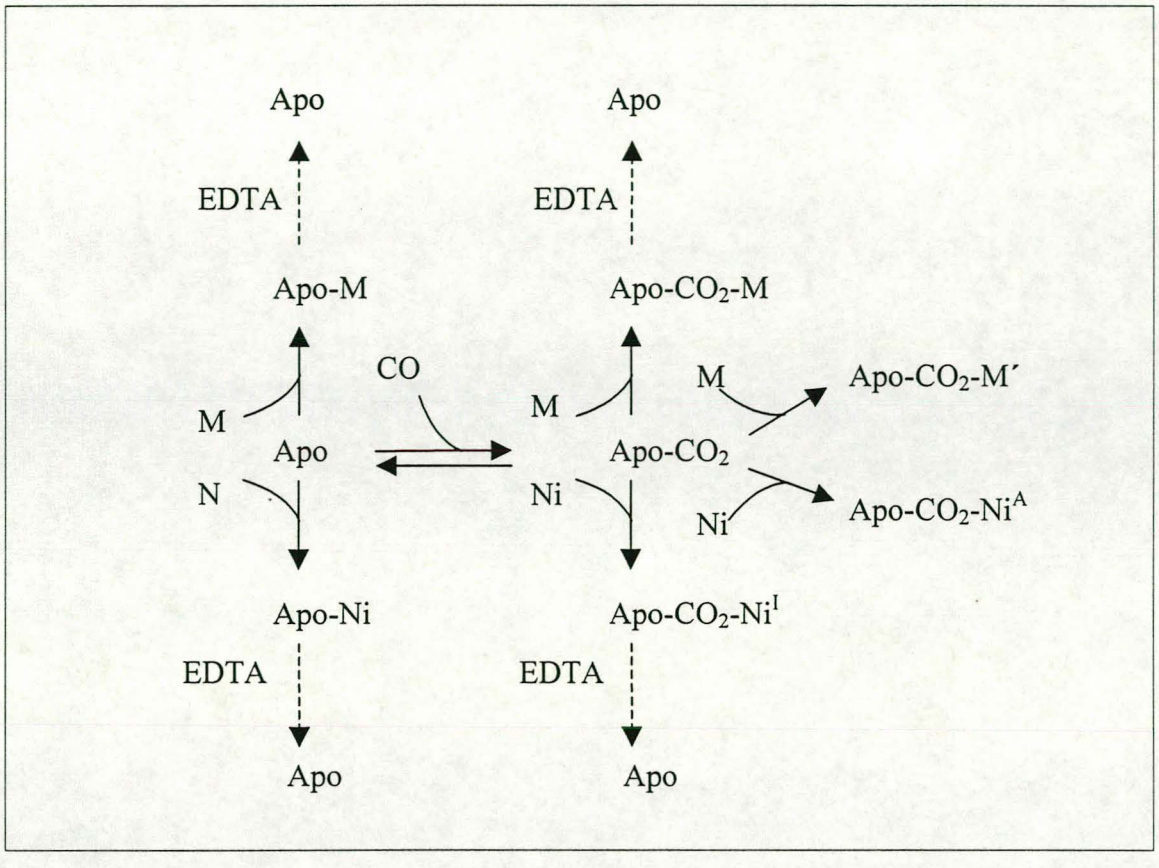

Figure 13. Model showing interactions between the urease apoprotein (Apo) and metal ions (Park and Hausinger, 1996). The carbamylated urease apoprotein is indicated as Apo-CO_2. An active enzyme (Apo-CO_2-Ni^A) is the result of productive binding of nickel ions to Apo-CO_2. Inactive Ni-containing species (Apo-Ni and Apo-CO_2-Ni^I) are formed by non-productive binding of nickel ions to Apo and Apo-CO_2. Other metal ions also bind to Apo to form Apo-M, and to Apo-CO_2 to form Apo-CO_2-M and Apo-CO_2-M'. EDTA can remove the metal ions from four inactive species (Apo-Ni, Apo-CO_2-Ni^I, Apo-M and Apo-CO_2-M) but the chelator is unable to remove nickel ions from Apo-CO_2-Ni^A or other metal ions from the Apo-CO_2-M' species.

extent than the reduction in rate constants of reactions where active proteins are formed, it would result in an increase in the amount of enzyme activated compared to the production of urease apoprotein. This function of UreD still needs to be confirmed.

Park and Hausinger (1996) also suggested that the functions of the UreD-UreF-UreG-urease apoprotein may include ensuring that only nickel ions bind to the urease apoprotein or reversing the binding of inappropriate metal ions or non-productively bound nickel ions. Moncrief and Hausinger (1996) identified the complex called UreD-UreF-urease apoprotein (DF-Apo), studied the activation properties and compared it with the Apo and D-Apo. From these studies they concluded that when UreF binds to the UreD-urease apoprotein, it modulates the activation process by eliminating the binding of nickel ions to noncarbamylated protein. This confirms the earlier findings of Park and Hausinger (1995a) discussed previously.

The UreE gene product of some bacteria, for example *P. mirabilis* and *K. aerogenes*, contains a large number of histidine residues (Park and Hausinger, 1995b; Sriwanthana *et al.*, 1994) at the C-terminus, which enable the protein to bind approximately six nickel ions per dimer. De Koning-Ward *et al.* (1994) suggested that these nickel ions are then transferred to the apo-urease. However, there are some exceptions: the UreE protein of *H. pylori* shows high homology with the UreE proteins of other bacteria, but it lacks this histidine tail (Mobley *et al.*, 1995a).

UreG is the most conserved accessory protein and also the only one that exhibits clear sequence homology to other proteins. The UreG of *K. aerogenes* is related to HypB, a guanosine 5'-triphosphate-binding protein required for incorporation of nickel into hydrogenase (Lutz *et al.*, 1991; Maier *et al.*, 1993). The UreG proteins of *Y. enterocolitica*, *P. mirabilis*, *K. aerogenes, H. pylori* and *E. coli* all contain an ATP-binding motif (GPVGSGKT) also known as a P-loop motif. It was postulated that this region might provide the energy required for activation (Lee *et al.*, 1990). Although ureG contains a P-loop motif usually found in nucleotide-binding proteins, it was shown (Moncrief and Hausinger, 1997) that purified ureG does not bind or hydrolyse ATP- or GTP. However, the P-loop was essential because cells containing ureG proteins with mutated P-loop motifs were unable to form the UreD-UreF-UreG-urease apoprotein. It was therefore suggested that ureG should combine with other accessory proteins and the

apoprotein in an appropriate complex before the ureG P-loop can provide the energy for urease activation.

The urease gene cluster of *H. pylori* contains nine urease genes (Cussac *et al.*, 1992) with *UreA* and *ureB* encoding the structural polypeptides of the enzyme. Along with *ureA* and *ureB*, *ureF* and *ureH* were shown to be essential for urease expression in *E. coli*. *UreC*, *ureD* and *ureI* are not essential for urease expression, but they do belong to the urease gene cluster. When deleting the *ureC* and *ureD* genes, a higher urease activity was observed suggesting that *ureC* and *ureD* may have a regulatory role.

Accessory genes and nickel transport

The uptake of nickel ions is a prerequisite for organisms catalysing nickel-dependent reactions. Ureolytic organisms need nickel in the cytoplasm, because nearly all ureases are situated in the cytoplasm (discussed previously). These organisms usually inhabit environments in which free divalent cations such as Ni^{2+} are bound by cellular components including histidine- and cysteine-rich proteins, negatively charged glycoproteins, or nucleic acids. These organisms must therefore possess a high-affinity system in order to scavenge the nickel ions from the macromolecules (Mobley *et al.*, 1995a). Nickel uptake is mediated by non-specific Mg^{2+}transport systems and by high affinity systems specific for the transport of nickel (Hausinger, 1993). Nickel is an essential trace element, but when supplied at elevated concentrations, nickel acts as a toxic transient metal. Organisms requiring this metal ion therefore need an uptake system with a high affinity but low capacity for nickel.

In *Alcaligenes eutrophus,* an integral membrane protein with seven transmembrane helices, called HoxN, is involved in high-affinity nickel transport (Wolfram *et al.*, 1995). When the gene encoding this protein was introduced into *E. coli,* it resulted in a functional nickel permease that increased the cytoplasmic content of nickel, especially in nickel-limiting conditions. It was also shown that HoxN increased the urease activity under nickel-limiting conditions. In *H. pylori* the protein that facilitates high affinity nickel transport is called NixA and this protein has a 41% identity to HoxN (Mobley *et al.*, 1995a).

Two accessory genes in the urease operon of thermophilic *Bacillus* sp. strain TB-90, *ureH* and *ureI*, are required for urease activity under nickel limiting conditions. These two genes have no homologs among the urease clusters of other organisms (Maeda *et al*,. 1994). A role in nickel uptake has been postulated for UreH because it shows significant similarity to HoxN of *A. eutrophus* (23% identity) and its hydropathy profile indicates that it may be a transmembrane protein. This suggests that *ureH* and *ureI* of *Bacillus* TB-90 may form a multicomponent high-affinity nickel transporter.

While *hoxN* and the *ureH* are part of their respective metalloenzyme gene clusters, *nixA* differs in that it is separated from the other *H. pylori* urease genes with at least 5 kb (Mobley *et al*., 1995a). Hendricks and Mobley (1997) identified a novel ABC transport system in *H. pylori* that enhances the synthesis of an active urease and may be responsible (together with n*ixA*) for transport of nickel into *H. pylori*.

EVOLUTION OF UREASE GENES

The high level of homology that exist between the urease sequences of all organisms suggests that all ureases are related and share common ancestral genes, because of. Sequences very similar to the intron splice acceptor consensus sequences, were found in the DNA between the *ureA* and *ureB* ORF's of *P. mirabilis* (Mobley *et al*., 1995b). This suggested that the origin of *ureA* and *ureB* might have resulted from an intron splice site that is not functional in prokaryotes. The *ureB* and *ureC* ORF's overlap with one nucleotide. This feature is compatible with either an eukaryote-to-prokaryote or prokaryote-to-eukaryote horizontal gene transfer and only a small modification in nucleotide sequence is necessary to result in a disruption or a fusion of *ureB* and *ureC*.

The relatedness of the urease clusters of a few species were determined by constructing dendograms on the basis of amino acid sequences (De Koning-Ward *et al.*, 1994). The results, seen in Figure 14, showed that the ureases from *P. mirabilis*, *P. vulgaris* and *E. coli* are highly conserved (greater than 88% homology between the polypeptides).

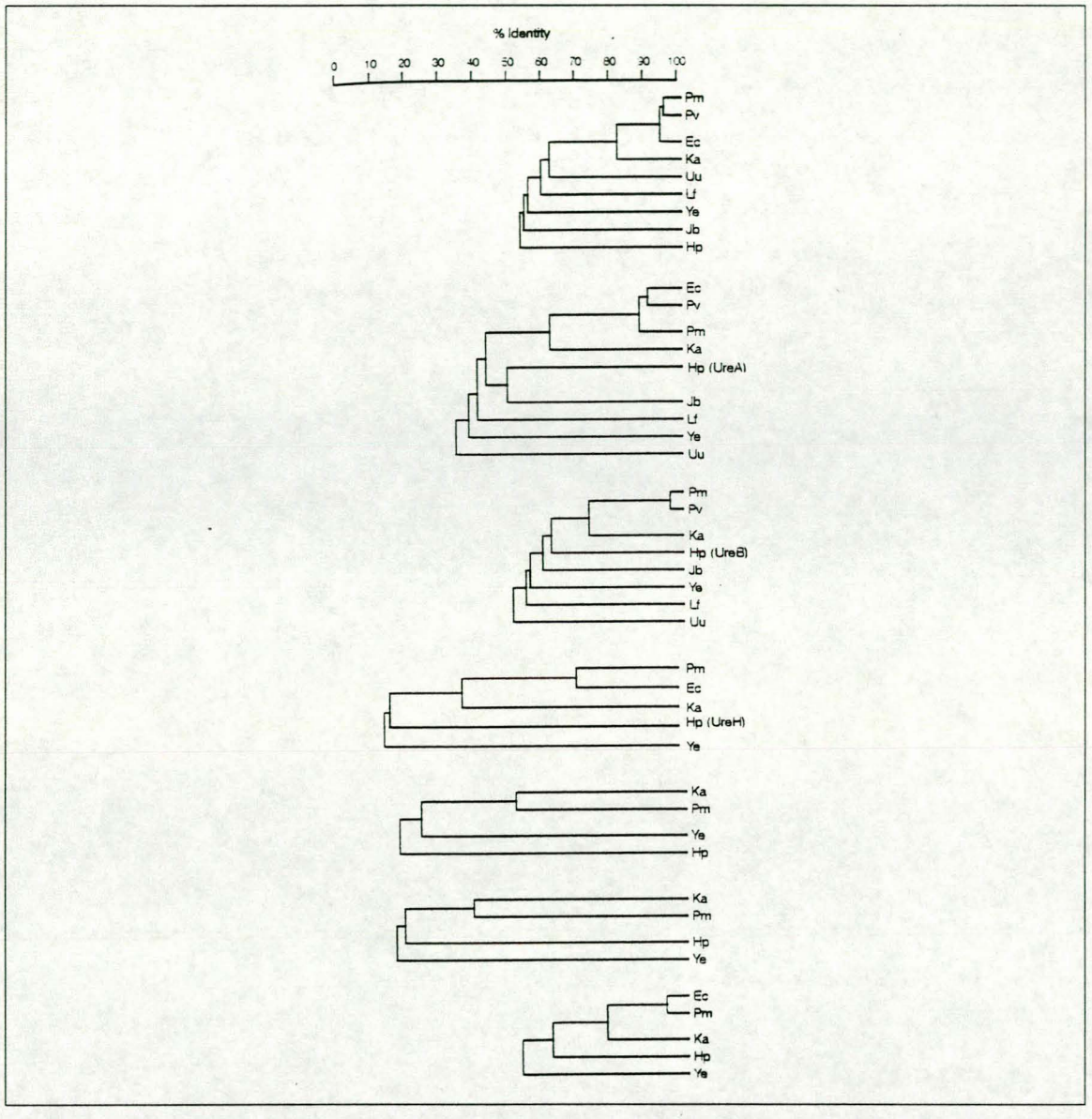

Figure 14. Dendograms of the Ure-encoding regions of nine organisms, constructed by comparing the percentage homology of the amino acid sequences. The letters A to G represents different ORF's that have been compared. The scale shows the percentage degree of relatedness where one organism joins another group of organisms. The abbreviations: Pm. *P. mirabilis*, Pv. *P. vulgaris*, Ec. *E. coli*, Ka. *K. aerogenes*, Hp. *H. pylori*, Lf. *L. fermentum*, Uu. *U. urealyticum*, Ye. *Y. enterocolitica*, Jb. Jack bean (De Koning-Ward *et al.*, 1994).

The *Y. enterocolitica* complex showed little homology to the ureases of other members of the Enterobacteriaceae family, suggesting that the *ure* gene complex of *Y. enterocolitica* branched off from the other Enterobacteriaceae long ago or that the genes were acquired *en masse* by *Y. enterocolitica* from another organism. The same branching patterns are observed for each of the different polypeptides (De Koning-Ward *et al.*, 1994), suggesting that the structural and accessory genes of bacterial ureases evolved together as a unit. There is no clue yet as to the nature of a common ancestor from which the *ure* genes of these organisms could have evolved.

REGULATION OF UREASE SYNTHESIS

The regulation of bacterial ureases usually follows specific patterns. For those bacteria that utilise the urease enzyme for liberation of nitrogen as a nutrition factor, urease synthesis is repressed in the presence of ammonia or any other rich nitrogen source. In nitrogen starvation or nitrogen-limiting conditions, the synthesis of urease is derepressed. Urease synthesis can also be induced by urea, as seen with uropathogens. Other bacteria produce ureases constitutively and the synthesis is unaffected by any nitrogen source. The need for the high levels of urease produced by these organisms is still unclear. The different stages of cellular differentiation or the pH can also regulate urease production. In the following paragraphs the different regulatory mechanisms will be discussed in more detail.

CONSTITUTIVE EXPRESSION

M. morganii is unique from other uropathogens in the sense that the expression of urease is constitutive (Rosenstein *et al.*, 1981; Hu *et al.*, 1990). The cyanobacterium *Anabaena cylindrica* synthesizes urease constitutively independent of the nitrogen source or the amount of urea present (Mackerras and Smith, 1986). At high concentrations of urea (10 mM), the amount of ammonia formed was in excess of the bacterium's biosynthetic requirements (incorporation by glutamine synthethase) and cell death was the result. The activity of this enzyme was, however, nickel-dependent. *E. coli* strains with chromosomally encoded ureases have constitutive urease expression (D'Orazio and

Collins, 1993) in contrast with plasmid encoded ureases that are induced in the presence of urea. The yeast *S. pombe* (Kinghorn and Fluri, 1984; Fluri and Kinghorn 1985, Lubbers *et al.*, 1996) *R. paludigenum* (Phillips *et al.*, 1990) and *Neurospora crassa* (Mackay and Pateman, 1982) also showed constitutive expression similar to other filamentous fungi.

The urease subunits of plants are large and form multimers, suggesting that the enzyme may be under allosteric control (Polacco and Holland, 1993). There are, however, no known effectors of plant ureases and *in vivo* they show uncontrolled hydrolysis of urea to such an extent that the soybean callus is killed in the presence of more than 25 mM urea due to excessive ammonia production. The reason for this constitutive control may perhaps be that plants, having evolved under chronic nitrogen-limited conditions, do not have mechanisms to deal with nitrogen excess. There have been a few reports of possible induction of the urease enzyme by urea in barley, rice and potatoes, but Polacco and Holland (1993) explained this as being the induction of the urease in commensal bacteria present in the plant tissue. Finally, some soil bacteria, e.g. *B. pasteurii* and *Sporosarcina urea*, are also constitutive urease producers (Mobley and Hausinger, 1989).

INDUCTION BY UREA

The uropathogenic bacterial species *P. mirabilis* is a common cause of urinary tract infection. The urease of this bacterium and that of *Providencia* species are 5 to 25-fold induced by urea (Rosenstein *et al.*, 1981; Jones and Mobley, 1987; Nicholson *et al.*, 1993). Clones of the urease gene cluster of *P. mirabilis* containing *ureD, A, B, C, E, F* and *G* showed a basal level of urease expression (Nicholson *et al.*, 1993). However, when the clones contained sequences upstream of *ureD*, induction by urea occurred. The reason for induction was found to be a gene 400 bp upstream of *ureD,* designated *ureR*. This gene has an ORF of 882 nucleotides and the predicted size of the polypeptide is 33 415 Da. The gene is orientated in the opposite direction to the other genes in the urease cluster. The polypeptide sequence shows a helix-turn-helix DNA-binding motif followed 30 residues downstream by an AraC family signature. Members of the AraC family are DNA-binding regulating proteins with a common C-terminal and are

responsible for the positive control of genes involved in virulence and carbon catabolism. This and other experimental work showed that UreR is a positive regulator for expression of the urease structural and accessory genes in the presence of urea (Island and Mobley, 1995). A strong urea-regulated promoter was identified upstream of *ureD*. Contrary to this, the putative promoter sequences upstream of *ureA* and *ureF* are not regulated by *ureR* and urea. *UreD,* together with *ureA* (and perhaps also *ureB* and *C*) are expressed as an operon and in this way UreR regulates the expression of all the genes.

The expression of plasmid encoded ureases of some *E. coli* strains (D'Orazio and Collins, 1993), *S. cubana* and *P. stuarti* (Grant *et al.*, 1981) is induced by urea. The *E. coli* strain with the urea-inducible urease possesses a regulatory system similar to that of *P. mirabilis* (D'Orazio and Collins, 1995). Transcription of *ureD* is initiated at a urea-dependent promoter *(ureDp*). Expression from *ureDp* requires the *ureR* product and UreR is positively auto-regulating itself. The intergenic region of *ureR* and *ureD* contains the divergent promoters *ureDp* and *ureRp*. Transcription from these two promoters requires the effector molecule urea and the activator protein UreR. The UreR-binding site is a 135 bp fragment situated in the middle of the *ureD-ureR* intergenic region (D'Orazio *et al.*, 1996).

The lactic acid bacterium *L. fermentum* showed a four-fold increase in the production of urease when 0.5% urea was added to the growth medium (Kakimoto *et al.*, 1990) while the addition of Ni^{2+} and Mn^{2+} also had a synergistically stimulating effect.

NITROGEN REGULATION

The ureases of some enteric bacteria are produced at high levels when the cells experience nitrogen starvation. In the same bacteria, urease synthesis is repressed in the presence of ammonia or other nitrogen-rich compounds (including urea) that release ammonia. It was earlier suggested that the main factor regulating many enzymes related to nitrogen metabolism was glutamine synthetase (Friedrich and Magasanik, 1977), but combined genetic and biochemical analysis showed that the activity of enzymes involved in nitrogen metabolism are regulated by the global nitrogen regulatory system

(Magasanik, 1993). However, glutamine synthetase still plays an important role in this system.

When cells grow in an excess of ammonia, they synthesise glutamate by the reductive amination of α-ketoglutarate. A portion of the glutamate is then converted to glutamine in a reaction catalysed by glutamine synthetase (GS) (Magasanik, 1993). When cells are growing in scarce nitrogen conditions, glutamate is synthesised by a two-stage process. First, glutamate and ammonia are converted to glutamine by means of GS and then glutamine reacts with α-ketoglutarate and NADPH in a reaction catalysed by glutamate synthetase. This second reaction produces two molecules of glutamate, thus the overall effect of the two reactions is the synthesis of one molecule of glutamate from α-ketoglutarate, ammonia, NADPH and ATP. From this it is clear that glutamine is an essential intermediate of all cellular nitrogen compounds in cells that utilise these reactions. This implies that these pathways require very high activity of GS and the synthesis of GS should therefore be linked to nitrogen regulation and that the signal for nitrogen excess or deprivation is the intracellular concentration of glutamine (Reitzer *et al.*, 1987).

The gene encoding GS is designated *glnA* and is a member of the *glnALG* operon. *GlnG* (*ntrC*) and *glnL* (*ntrB*) encode the structural genes for the nitrogen regulators I and II (NR_I and NR_{II}). These regulators are responsible for the regulation of this and other operons in response to the availability of nitrogen. Transcription at the *glnALG* operon can be initiated at three promoters: *glnAp1* and *glnAp2* upstream of *glnA,* and *glnLp* located between *glnA* and *glnLG*. In cells growing in nitrogen excess conditions, transcription is initiated at *glnAp1* and *glnLp* by the common σ^{70}-RNA polymerase. Binding sites for the response regulator NR_I overlaps these promoters and NR_I represses the synthesis of GS and its own synthesis, resulting in a very low intracellular concentration of NR_I.

In cells grown in nitrogen limiting conditions, NR_{II} phosphorylates NR_I and therefore activates transcription of the *glnAp2* promoter. This promoter and many other promoters

regulating nitrogen genes, have a unique consensus sequence CTGG-PyPuPyPu....TTGCA and is dependent on an alternative sigma factor, σ^{54}(NTRA). The transcription activation at *glnAp2* leads to an accumulation of GS (encoded by *glnA*) and NR_I (encoded by *glnG*). The intracellular increase in phosphorylated NR_I enables the cell to activate transcription of other nitrogen-related genes equipped with promoters dependent on σ^{54}-RNA polymerase.

Collins and co-workers (1993) studied the nitrogen regulated urease locus of *Klebsiella pneumoniae*. They found that the transcription of *ureD* and *ureA* were controlled by a 312 bp fragment that includes the start of the *ureD* ORF. The regions upstream of the translational start of *ureD* contained sequences representing both σ^{54} - and σ^{70} -binding sites, but none were identified for interaction with NR_I. Furthermore, it was shown that a high level of transcription from the *ureD* promoter required an additional factor designated NAC. NAC is a member of the LysR-family of positive transcriptional regulators that acts at some σ^{70}-promoters of nitrogen controlled genes, including urease and those required for histidine utilisation (*hut*). This couples the σ^{70}-promoters of some nitrogen-controlled genes to the nitrogen regulatory system. The transcription of NAC is from a σ^{54}-promoter and activated by NR_I.

A few examples of other microorganisms with ureases controlled by the nitrogen regulatory system, are *A. nidulans* (Mackay and Pateman, 1982), *K. aerogenes* (Mulrooney *et al.*, 1989) and *Bacillus subtilis* (Mobley *et al.*, 1995b). The regulation of *H. pylori* urease seems to be nitrogen-regulated since sequences upstream of *ureD* and *ureA* are similar to the σ^{54}-binding site (Cussac *et al.*, 1992) and optimal activity of a recombinant *H. pylori* urease in *E. coli* was achieved in cultures grown under nitrogen limiting conditions. However, the concentration of exogenous nickel is very important, since Hu and Mobley (1993) found that some amino acids like cysteine and histidine found in rich media, acts as chelating factors of Ni^{2+} ions. The amino acid - Ni^{2+}complex cannot enter the cell and thus the low urease activity can be the result of nickel depletion and not only the effect of nitrogen regulation.

REGULATION BY pH

An oral cavity is the natural habitat of *S. salivarius* and these bacteria play an important role in inhibiting the formation of dental caries by means of ureolysis. The urease enzyme of this bacterium shows no sign of being induced by urea or repressed by ammonia (Sissons *et al.*, 1990), but the synthesis of the enzyme is enhanced at low pH levels (Sissons *et al.*, 1992). Expression is also modulated by glucose availability and growth rate (Chen and Burne, 1996). It was also shown that differential expression was controlled at least in part at the transcriptional level. The production of the urease of *H. pylori*, which is the virulence factor of this bacterium, is stimulated at low external pH conditions (Scott *et al.*, 1998). The activity of its urease protects the bacteria against the gastric acidity by increasing the periplasmic pH and membrane potential.

Y. enterocolitica is a gastrointestinal pathogen which survives buffered acidic conditions in the stomach where the pH is as low as 1.5, as long as urea is available (Young *et al.*, 1996). The DNA sequence of the urease cluster of this bacterium showed no *ureR* or σ^{54} promoter sequence upstream of *ureA* (De Koning-Ward and Robins-Browne, 1994). Instead, this enzyme is activated 780-fold by low pH conditions (Young *et al.*, 1996). The same was observed for *M. morganii* and DNA sequence analysis showed that these two organisms have a region of high similarity near the active site which is distinct from most other ureases, but is also present in the urease of *L. fermentum.* This is of particular interest since the *L. fermentum* urease is also known for having a low pH optimum. There is also evidence of pH regulation of urease levels in *K. pneumoniae, C. renale* and some *Staphyloccus* strains (Sissons *et al.*, 1990).

DEVELOPMENTAL CONTROL

There is some dispute among researchers about the question of urease regulation in *P. mirabilis* during swarming. Some researchers believe that urease activity is constitutive for the swarming (long) cells, whereas very low activity was observed in the nonswarmer (short) cells of this bacterium (Falkinham and Hoffman, 1984). Jin and Murray (1987) argued that urease is constitutively produced in both swarming and

nonswarming cells and that the activity of both types can be increased tremendously by exposure to urea. Finally, Alison *et al.* (1992) showed that differentiation into swarm cells results in substantial increases in urease activity, while reduced levels of urease were found with mutants defective in swarming.

GROWTH PHASE CONTROL

Although Young and co-workers (1996) showed that the synthesis of the urease of *Y. enterocolitica* is induced by low pH conditions, De Koning-Ward and Robins-Browne (1997) showed that urease is also regulated by the growth phase of the bacterial cells. Maximal activity was found during stationary phase of growth. This coincides with the phase when *Y. enterocolitica* shows maximal resistance to acidic pH conditions (De Koning-Ward and Robins-Browne, 1995).

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CHAPTER TWO

CLONING AND EXPRESSION OF THE *LACTOBACILLUS FERMENTUM* ACID UREASE GENE IN *SACCHAROMYCES CEREVISIAE*

INTRODUCTION

URETHANE, A POTENTIAL CARCINOGEN

Urethane (ethyl carbamate) has been used as an intermediate in the synthetic preparation and modification of resins and as a solubilizer for pesticides, fumigants and cosmetics. It is also known for its narcotic action and was used for many years as an anaesthetic (Mirvish, 1968). Urethane is a versatile carcinogen after conversion to a metabolite, probably vinyl carbamate, which can induce cancer in various organisms. Its mutagenicity is variable: strong in some organisms, and no effect in others (Sotomayor and Collins, 1990). In mammals, urethane is clastogenic in somatic cells and affects diverse tissues such as the liver, bone marrow and lungs. After ingestion, urethane is rapidly and evenly distributed in the mammalian body and more than 90% is degraded to CO_2, H_2O, and NH_3 and excreted within 24 hours. The rate of elimination from plasma varies according to the species and age of the animal (Sotomayor and Collins, 1990). It was shown to be only weakly mutagenic in a few bacterial and fungal species. It is important for this study to note that urethane is not mutagenic to *S. pombe,* but weakly mutagenic for some strains of *S. cerevisiae*.

It was shown that ethanol inhibits the localisation of urethane in mouse tissues after oral administration of these two compounds (Waddel *et al.*, 1987; Yamamoto *et al.*, 1988). When the ethanol levels in blood are higher than 0.15%, urethane was only found in the lumen of the stomach and intestines and was absent in all the other tissues where it usually employs its carcinogenic action. This has important implications for human mutagenic and carcinogenic risk assessment, because it suggests a smaller risk than was previously thought. However, other investigators speculated that such an acute administration of ethanol might only postpone the metabolism of urethane (Schlatter and Lutz, 1990).

URETHANE IN WINE AND RELATED BEVERAGES

Urethane occurs naturally in fermented beverages such as beer and sake and fermented foods such as olives and yoghurt, while no urethane has been detected in unfermented food (Ough, 1976a). It was also detected in cheese, tea, soy sauces and bread, with the levels of urethane in toasted bread six times higher than in the fresh counterpart (Canas *et al*, 1989). With normal dietary habits, excluding alcoholic intake, an estimated daily urethane intake for adults is 20 ng/kg body weight (Schlatter and Lutz, 1990) of which bread is assumed to be the main source. However, when a daily diet includes as little as 7 ml stone-fruit brandy it can lead to a ten-fold increase in urethane intake. This increase is due to the urethane content of stone-fruit brandy that ranges from 200 to 20 000 ng/g.

After the Liquor Control Board of Ontario, Canada, discovered large quantities (up to 13 400 μg/L) of urethane in sherries, dessert wines and distilled spirits from various countries in 1985, Canada introduced regulatory limits for various types of alcoholic beverages. According to these regulations, urethane concentrations may not exceed the following levels: 30 μg/L in natural wine (<14% ethanol), 100 μg/L in fortified wines (>14% alcohol), 150 μg/L in distilled products, and 400 μg/L in stone fruit brandies. In 1988, the USA set voluntary goals trying to limit the levels of urethane to 15 μg/L in natural wines and 60 μg/L in fortified wines. It has been shown that most young wines do not contain measurable levels (<10 μg/L) of urethane, but the precursors are present and can generate a considerable amount of urethane when the wine is stored at elevated temperatures (Ough *et al.*, 1988a).

Precursors of urethane. Many studies have been done to determine the origin of urethane in wine. Lofroth and Gejvall (1971) reported that the reaction between ethanol and diethylpyrocarbonate (DEPC), an antimicrobial agent in alcoholic beverages, is the most important origin of urethane. However, Ough (1976a, b) detected urethane in wine that was not treated with DEPC and suggested that the obvious source of urethane was the hydrolysis of yeast carbamyl phosphate by ethanol. Carbamyl phosphate is produced in the yeast from ATP, CO_2 and ammonia (Ough, 1976b). The reaction, catalysed by

carbamyl phosphate synthase, is necessary for arginine synthesis and enhanced levels of carbamyl phosphate are produced when a high intracellular concentration of ammonia prevails. A subsequent list of possible precursors also includes N-carbamyl compounds such as urea, citrulline, allantoin and β-ureido propionic acid (Ough *et al.*, 1988a, b).

One way in which citrulline and carbamyl phosphate can end up in the fermenting grape must, is by degradation of L-arginine in the arginine deaminase metabolic pathway (Liu *et al.*, 1994). This pathway consists of three enzymatic reactions (Figure 1) and is performed by lactic acid bacteria such as *Oenococcus oeni* and *Lactobacillus buchneri*. The authors suggested that lactic acid bacteria that produce the enzymes in this pathway would have an advantage over other bacteria since the degradation of arginine serves as an additional energy source. These bacteria will tend to outgrow the rest and result in the production of high quantities of carbamyl phosphate if spontaneous malolactic fermentation is allowed.

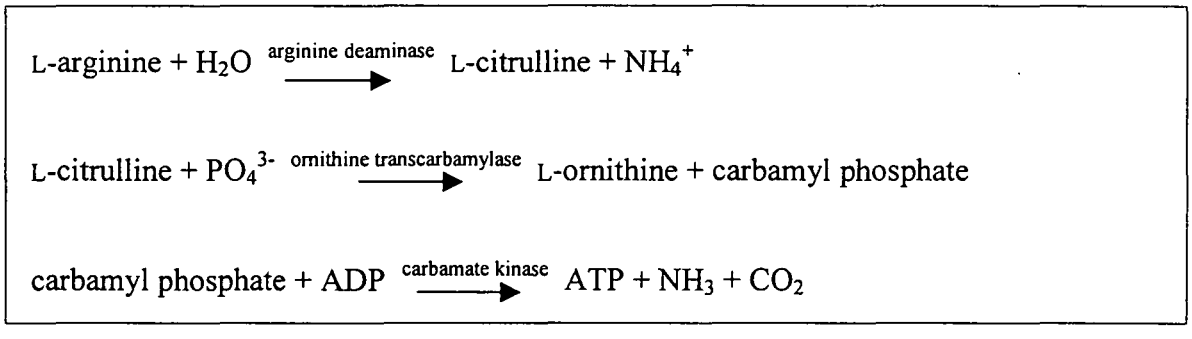


Figure 1. The enzymatic reactions in the arginine deaminase pathway in lactic acid bacteria.

The most important precursor in sake wine is believed to be the urea produced during the metabolism of arginine by the yeast *S. cerevisiae* (Kitamoto *et al.*, 1991). There are two schools of thought with regard to the most important precursor in wine: one is that, similar to sake, urea is the most important precursor in wine (Ough *et al.*, 1988b; Ough, 1991), whereas others believe that other precursors are also present and the effect of urea production by the wine yeast is of little significance (Tegmo-Larsson and Henick-Kling, 1990a, b). Tegmo-Larsson and Henick-Kling (1990b) showed that the same levels of urethane would develop in non-fermented and fermented grape juice when the ethanol

levels of the two juices are equalised by adding ethanol to the unfermented juice. This suggested that yeast growth and fermentation have no influence on the urethane concentration, except for the increase in alcohol content. However, the five grape varieties tested are known to have low amino acid concentrations and different results may be obtained if grape juice with a higher nitrogen concentration was used. In favour of the first argument, it was shown (Monteiro *et al.*, 1989) that radioactively labelled arginine (guanido-^{14}C) added to fermenting grape juice produced radioactive urea presumably *via* the catabolic activity of the arginase enzyme of the wine yeast.

Other factors determining urethane production. It is known that wine can show a considerable increase in urethane during storage (Stevens and Ough, 1993). Furthermore, an increase in storage temperature results in a dramatic increase in urethane concentration and storage temperatures above 24°C should therefore be avoided. It is also possible that the urethane found in wine may be the result of the breakdown of inexpensive polyurethane materials used in wine production or from the use of urethane as a solubilizer for pesticides in vineyards (Ingledew *et al.*, 1987). Experiments done by Riffkin *et al.* (1989) indicated that the urethane precursor in whiskey distillation may be a copper-peptide or -protein complex.

PREVENTING URETHANE FORMATION IN WINE

VITICULTURE PRACTICES

It has been shown that highly fertilised vines will deliver wines with a higher urethane content after heat-induced ageing than unfertilised vines (Ough *et al.*, 1989). In general, grapevines have a very low nitrogen requirement relative to other crops. When grapes are grown on soils previously used for vegetables, it can result in high nitrogen levels in the juice and therefore increased urethane in the wine. It is therefore the responsibility of the grower to modify viticulture procedures if the nitrogen levels, in particular arginine, rise over the limit of 1 000 mg/L.

The spraying of urea shortly prior to the harvest to remove leaves, is also not recommended since urea that sticks to the grape berries will be present in the grape juice and therefore contribute to the urea levels in wine (Augustyn, 1997).

AMINO ACID COMPOSITION AND UREA CONCENTRATION IN GRAPE JUICE

Urea was widely used as a nutrient supplement for yeast during wine fermentations to avoid so-called stuck fermentation. Since it became known that urea forms urethane in the presence of ethanol, the application of urea as yeast nutrient has been prohibited. Other yeast nutrients such as casamino acids, diammonium phosphate, glycine and yeast extract were also tested (Ingledew *et al.,* 1987), but none proved to be responsible for the production of urethane or urethane precursors.

The amount of urethane that can be formed during fermentation is dependent on the amino acid composition of the grape must. The amino acid composition in grape juice is dependent on the grape cultivar (Castor, 1953), the maturity of the grape and vineyard fertilisation (Ough *et al.*, 1989). If high quantities of L-arginine are present, it can produce excess urea by the action of the arginase enzyme in the cytosol. This excess urea will be excreted and react non-enzymatically with ethanol to form urethane. It would therefore be ideal if winemakers could determine the nitrogen status of the grape juice before considering the addition of yeast nutrients to prevent over-supplementing.

YEAST STRAINS

Ough *et al.* (1991) showed that the yeast strain used in a fermentation can have an influence on the rate of uptake of certain amino acids, as well as the secretion of urea. When different strains were evaluated under standard conditions for grape juice fermentation, Ough *et al.* (1991) found that Prise de Mousse secretes little urea, whereas Montrachet and Eperney 2 secrete large amounts. Yeast will also secrete more urea at higher fermentation temperatures. All yeasts secrete urea, but the extent to which they re-absorb the urea differs (An and Ough, 1993). The nitrogen content of the must has an influence on the re-absorption and this process is suppressed especially in conditions of high ammonia concentration. The Montrachet yeast strain will re-absorb the urea to

metabolise it only when alanine, glutamine and arginine are completely metabolised. It is therefore important to inoculate grape must with a low-urea producing strain of wine yeast when the juice has a high arginine content.

DISRUPTION OF THE ARGINASE GENE

In *S. cerevisiae,* urea is formed during the breakdown of L-arginine by the arginase enzyme (Middelhoven, 1964) encoded by the *CAR1* gene (Sumrada and Cooper, 1982). Kitamoto and co-workers disrupted the two copies of this gene in the sake yeast to minimise the production of urea (Kitamoto *et al.*, 1991, Suizu *et al.*, 1990). The resulting mutant was used to brew sake that contained no urea or urethane.

REMOVAL OF UREA WITH THE UREASE ENZYME

Urea is considered by many as the main precursor of urethane in wine and sake, therefore the use of the urease enzyme to limit the urea levels in alcoholic beverages has been considered for many years. However, the pH conditions in wine and sake is acidic and most known ureases are inactivated at these pH levels. The Japanese sake brewers were inspired in their search for an acid urease producer and isolated the lactic acid bacterium *L. fermentum* from the ileum-caecum contents of rats (Suzuki *et al.*, 1979). Dead cells of *L. fermentum* (Yoshizawa and Takahashi, 1988), as well as a crude cell extract (Kobashi *et al.*, 1988), were tested for urea degradation in sake wine. The addition of 5 mg (40 IU/mg protein) of acid urease from crude cell extracts to unrefined Japanese sake containing 20% ethanol and 35 ppm urea, reduced the urea level to undetectable levels (1 ppm in 2 days at 15°C and in 15 h at 30°C).

This remarkable result was closely followed by Ough and Trioli (1988) who tested the effect of dead cells of *L. fermentum* on the urea levels in wine. It was found that higher concentrations of the enzyme were required to show the same effect in wine as in sake. This was due to the lower pH of wine (it was shown that the enzyme was much more effective at pH 4 than at pH 3) and the urease inhibitors present in wine (discussed below). Ough and Trioli (1988) also established that citrulline was not removed by the

urease and could still contribute to the potential production of urethane although most of the urea was degraded. Urease activity against six other known urethane precursors, namely N-carbamyl arginine, N-carbamyl aspartate, N-carbamyl glutamate, N-carbamyl asparagine, N-carbamyl alanine and N-carbamyl isobutyrate was also tested, but the enzyme was unable to reduce the levels of any of these compounds.

Several compounds were identified as inhibitors of urease activity. Significant inhibition was shown by L-malic acid, L-lactic acid, D-lactic acid, pyruvic acid, α-ketoglutaric acid and acetic acid. Of these, L-malic acid was the most important inhibitor. L-Malic acid is naturally present in the grape berry and in wine that has not undergone malolactic fermentation. The L-malic acid concentration can reach 10 mg/L and even higher in colder wine regions. The urease enzyme should therefore only be added after the completion of malolactic fermentation to ensure enzymatic activity.

Compounds that inhibit the acid urease to a minor degree were Cu^{2+}, Fe^{3+}, Ca^{2+}, PO_4^{3-}, phenolic compounds, SO_2 and ethanol (Trioli and Ough, 1989). Fluoride was reported to be an irreversible inhibitor of the acid urease (Famuyiwa and Ough, 1991). Fluoride levels can vary between 0.1 and 5.5 mg/L in wine; the primary source is "cryolite" (sodium aluminium fluoride), an insecticide used to control leafrollers on the vine.

The urease enzyme was also used in reducing urea levels in a sherry base (Kodama and Yotsuzuka, 1996). Sherry undergoes a baking step at 30 - 60°C for 5 to 6 weeks after fermentation and fortification; sherry base refers to the wine before the baking step. Although the levels of urethane formed during the baking process was drastically reduced by the urease enzyme, it did not lead to a complete prevention of urethane production since urethane precursors other than urea were still present.

HETEROLOGOUS EXPRESSION OF THE *L. FERMENTUM* UREASE ENZYME IN WINE YEAST

Although the use of the urease enzyme in the form of dead *L. fermentum* cells was effective in reducing the levels of urea, and therefore urethane, it is an adverse practise to add bacterial cells to a food product. Purified enzyme can be added to the wine but this is

a very expensive practise. An alternative approach was to develop wine yeast that could prevent the formation of urethane by degrading the urea produced during fermentation. Successful expression of a heterologous urease in the wine yeast *S. cerevisiae*, which does not contain a native urease enzyme, could enable the yeast to degrade urea to ammonia and carbon dioxide. If a suitable acid urease could be expressed, this reaction can take place at the low pH conditions associated with wine fermentation.

In this study, the lactic acid bacterium *L. fermentum* was chosen as a source of acid urease genes. The following sections describe the construction of various expression cassettes containing the three different structural genes encoding the subunits of the enzyme. The transcription and biological activity of the recombinant protein were evaluated by expressing it in a urease-positive strain of *S. pombe* that contains a native urease enzyme. Unlike *S. cerevisiae*, cells of *S. pombe* should provide the essential accessory proteins for the correct folding and activity of the recombinant protein. Transcription and translation of the recombinant gene were demonstrated, albeit at a low level. However, the protein proved to be unstable, making it difficult to quantify its activity.

IDENTIFICATION OF AN ACID UREASE PRODUCING YEAST

INTRODUCTION

A large number of urease genes have been cloned and sequenced, mostly from prokaryotes such as *H. pylori* (Labigne *et al.*, 1991), *K. aerogenes* (Lee *et al.*, 1992), *P. mirabilis* (Jones and Mobley, 1989) and *L. fermentum* (Suzuki *et al.*, 1992). Only a few eukaryotic urease genes have been cloned, including those of *S. pombe* (Tange and Niwa, 1997), jack bean (Riddles *et al.*, 1992) and the pathogenic fungus *Coccidiodes immitis* (Yu *et al.*, 1997). As previously discussed, an acid urease enzyme shows optimum activity at low pH levels, in contrast to the neutral ureases that are inactive and irreversibly denatured in such conditions. Of all the prokaryotic urease producers, only a few *Streptococcus* and *Lactobacillus* species and some strains of *Escherichia*, *Staphylococcus*, *Morganella, Bifidobacterium* and *Yersinia* are acid urease producers. No eukaryotic acid urease producer has yet been reported.

In this study, we specifically required an acid urease that will still be active in the low pH conditions associated with wine. The first objective of this study was therefore to identify an acid urease gene, preferably from another yeast to ensure functional expression in *S. cerevisiae.* Expression of the genes from a genetically remote organism in a yeast system may result in a different codon preference and therefore poor expression. Furthermore, the bacterial urease gene consists of three open reading frames (ORF's) arranged contiguously in an operon, while the eukaryotic genes consist of only one ORF.

Ten known urease producing yeast strains (Kreger-van Rij, 1984) were selected to screen for acid urease production. The urease producing capabilities of these strains were tested with Cristensen's agar (Christensen, 1946) and the Rapid Urea broth Test (RUT) for yeast (Roberts *et al.*, 1978). These two assays are basically the same, except that the first is done on solid media and the second in liquid broth; the latter shows results within four hours whereas the agar slant tubes can take up to five days. In both cases the media contain urea and the pH indicator phenol red that turns the

media from light orange to deep pink when the pH increase upon the hydrolysis of urea to ammonia.

MATERIALS AND METHODS

Strains and media. The following yeast strains were used in the assay and were obtained from the Centraalbureau voor Schimmelculture (CBS), the Council of Scientific and Industrial Research (CSIR) and the Northern Regional Research Laboratory (NRRL): *Cryptococcus albidus* var. albidus (CSIR Y 0073), *Cryptococcus curvatus* (CBS 0570 T), *Rhodosporidium toruloides* (CBS 0014), *Rhodotorula graminis* (CBS 2826 T), *Sterigmatomyces halophilus* (NRRL YB 4619 T), *Tremella fuciformis* (CBS 6970 T), *Tremella mesenterica* (CBS 6973), *Yarrowia lipolytica* (CSIR Y 0513), *Yarrowia lipolytica* (CBS 2073) and *Trichosporon pullulans* (CBS 2535). The yeast strains were maintained on YM agar slant tubes (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose, 2% agar) at room temperature, except for *R. toruloides* and the *Tremella* strains that were kept at 30°C.

***In vivo* urease activity assays.** The yeast strains were streaked onto Christensen's agar slant tubes (0.1% peptone, 0.1% glucose, 0.5% NaCl, 0.2% KH_2PO_4, 0.0012 % phenol red, 2% agar pH adjusted to 6.8 and 2% filter sterilised urea added after autoclaving), incubated at 25°C. Cultures were observed daily for up to 5 days and reactions were recorded as positive after the straw-coloured media turned deep pink. The *in vivo* urease activities were also determined by the Rapid Urease Test (RUT) as described by Roberts *et al.* (1978). The composition of this liquid media was similar to Christensen's agar, except for the omission of the agar, but the results were obtained after incubation for only 4 h at 37°C.

RESULTS AND DISCUSSION

Although all necessary precautions (Ng *et al.*, 1997a; b) were taken to ensure accuracy and reproducibility unexpected results were obtained (Table 1). According to Kreger-van Rij (1984) all the yeast strains tested are urease producers, but this could not be confirmed with our results. Different conditions were subsequently used to establish the optimum age of the preculture before transferring the yeast to the test agar or broth. Precultures were also grown in liquid media instead of on agar slants to obtain

better activity. After several attempts, only five yeasts showed positive results with the Christensen agar assay. The RUT test indicated eight urease producing yeasts, but a positive result could only be observed after extended incubation (longer than the prescribed 4 h).

Table 1. Urease activity of various urease positive yeast strains on Christensen agar and RUT tests media.

Yeast strain	RUT (inoculated from 8 h old colonies)	RUT (inoculated from 12 h old cultures)	RUT (inoculated from 72 h old cultures)
C. albidus	-	-	w (48 h)
C. curvatus	-	-	s (12 h)
R. toruloides	-	-	w (48 h)
S. halophilus	-	-	-
R. graminis	-	w (48 h)	w (48h)
T. fuciformis	ND	w (48 h)	-
T. mesenterica	-	-	s (12 h)
Y. lypolitica (CBS)	w (48 h)	-	-
Y. lypolitica (CSIR)	-	-	-
T. pullulans	w (12 h)	-	s (12 h)
	Christensen agar (inoculated from media)	**Christensen agar (inoculated from colonies)**	
C. albidus	-	+	
C. curvatus	-	-	
R. toruloides	-	+	
S. halophilus	-	-	
R. graminis	+	+	
T. fuciformis	-	-	
T. mesenterica	-	-	
Y. lypolitica (CBS)	+	+	
Y. lypolitica (CSIR)	+	+	
T. pullulans	-	-	

(-) no pink colour developed within the prescribed time; (+) positive result within the prescribed time, (w) weak positive reaction, (the extended time that was required for a positive reaction indicated in brackets); (s) strong positive result (the extended time required for a positive result indicated in brackets); ND = not determined.

It is important to note that false negatives can develop when the endogenous metabolism of an organism produces an excess of hydrogen ions, while false positives can arise where urease activity is not the only or major alkalising factor. Furthermore, the pH of the agar was at 6.8 and the broth was at 5.5, thus this assay was only valid for activity at near neutral conditions. As previously discussed, almost all ureases are

intracellular enzymes and the cytosolic environment where it functions will therefore always be neutral, except for some lactic acid bacteria where it can be somewhat acidic.

Several other assay methods for urease activity in prokaryotes are available, including a plate assay to distinguish between acid and neutral urease producers amongst lactic acid bacteria (Kakimoto *et al.*, 1989). However, a test assay for urease activity at low pH values in eukaryotic systems has not yet been developed. The lack of accuracy of the methods described above necessitated the development of an *in vitro* assay to test the urease activity under specific pH conditions.

DEVELOPMENT OF *IN VITRO* UREASE ACTIVITY ASSAY

INTRODUCTION

The results obtained with the Christensen agar and RUT tests indicated that the yeast *R. graminis* was the best urease producer and this yeast was therefore chosen for the development of an *in vitro* assay. The yeast cells were grown in medium containing urea as sole nitrogen source, or a mixture of L-asparagine and urea to determine if the urease enzyme is only produced under specific nitrogen conditions. A clear cell extract was prepared and incubated with either citrate buffer (pH 3) or potassium phosphate buffer (pH 7) containing urea.

During incubation, urease present in the cell extract reacts with the urea to form ammonia. The supernatant of the culture was tested for the presence of ammonia by using the urea/ammonia test kit of Boehringer Mannheim. In this assay, the ammonia produced during urea hydrolysis reacts with 2-oxoglutarate in the presence of glutamate dehydrogenase (GDH) and reduced nicotinamide adenine dinucleotide (NADH). This reaction forms L-glutamate, while NADH is oxidised to NAD^+:

$$\text{Urea} + H_2O \xrightarrow{\text{urease}} 2NH_3 + CO_2$$

$$\text{2- oxoglutarate} + \text{NADH} + NH_4^+ \xrightarrow{\text{GDH}} \text{L-glutamate} + NAD^+ + H_2O$$

The amount of NADH oxidised in the second reaction is stoichiometric to the amount of ammonia produced in the first reaction and can be determined by spectrophotometric analysis by absorbency (OD) at 340 nm.

MATERIALS AND METHODS

Whole cell extracts. Yeast cells were grown in 10 ml YNB media (0.17% Yeast Nitrogen Base [YNB] without amino acids and $(NH_4)_2SO_4$,, 1% glucose) and either 0.3% urea + 0.2% L-asparagine [Medium 1] or 0.5% urea [Medium 2]. Cells were harvested at OD_{600} = 0.8, washed and resuspended in 200 µl lysis buffer (50 mM NaCl, 50 mM citrate-phosphate buffer (pH 3), 10 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol). Cell walls were disrupted with glass beads and the cell debris was removed by centrifugation.

***In vitro* urease activity assay.** The clear cellular extract (50 µl) was added to 50 µmol urea and citrate-phosphate buffer (pH 3) or potassium-phosphate buffer (pH 7) to a final volume of 1 ml and incubated for 1 h at 37°C. This was followed by deproteinising and precipitation with 10% Trichloroacetic acid (TCA). Twenty microliters of cell extract was added to 1 ml of the reaction mixture (provided in test kit) and incubated for 5 min. at 25°C. Upon the addition of 20 µl of glutamate dehydrogenase (GDH), the NH_3 produced during the incubation step reacts with the 2-oxoglutarate and NADH. The level of NADH was determined spectrophotometrically at OD_{340}, but the incubation time after GDH addition was increased to 40 min. The jack bean urease provided in the urease assay kit was used as a positive control for determining urease activity at pH 7. This urease was not expected to be active at pH 3 since it is a neutral urease with an optimum activity at pH 7 - 7.75 (Mobley and Hausinger, 1989).

RESULTS AND DISCUSSION

Our results showed (Table 2) that all the samples contained NH_3 that was produced after the hydrolysis of urea during the incubation step at 37°C. No difference was found between the cells grown in Medium 1 (urea + L-asparagine) and those grown in Medium 2 (only urea); only Medium 2 was therefore used in further studies.

Table 2. Urease activity obtained in citrate-phosphate buffers (pH 3.0 and 7.0) for cell free extracts of *R. graminis* and the jack bean urease.

	OD_{340}		
	A. Before addition of GDH	**B. 40 min after addition of GDH**	**ΔOD_{340} (A - B)**
Citrate-phosphate buffer pH 3			
R. graminis cell extract	5.49	3.27	2.22
Jack bean urease	4.35	2.12	2.41
Citrate-phosphate buffer pH 7			
R. graminis cell extract	5.54	4.16	1.38
Jack bean urease	4.96	3.32	1.64

Because the amount of NADH oxidised to NAD^+ (ΔOD_{340}) is stoichiometric to the amount of NH_3 produced, one can assume that a higher ΔOD_{340} indicates a higher urease activity. For both the jack bean and the *R. graminis* urease, the results seemed to indicate a higher urease activity at pH 3 than at pH 7. This result is highly unlikely since the jack bean urease has a neutral pH optimum (Mobley and Hausinger, 1989). Furthermore, it is unusual to find that there is only a small difference in the activity levels between the crude cell free extract of *R. graminis* and the purified jack bean urease. To evaluate the accuracy of the assay, cell free extracts of *S. cerevisiae* were included as a negative control for urease activity at both pH levels. The results showed a false positive activity (data not shown) similar to that of *R. graminis*. We concluded that the specific conditions used in this assay were not suitable for the reliable determination of urease activities in yeast cells, and the results were therefore disregarded.

FURTHER EFFORTS IN DEVELOPING AN *IN VITRO* ASSAY

The urea/ammonia kit used to determine the urease activity in the above-mentioned assay required neutralisation of acidic samples before conducting the assay. We therefore repeated the previous assay on a larger scale to allow for a neutralising step.

The procedures were similar to that described above, with the following exceptions: cells were grown in 100 ml Medium 2 and 500 µl clear cell free extract was used in the reaction mixture of which the final volume was 10 ml. After 1 ml of the reaction mixture was precipitated with 4 ml TCA, the supernatant was neutralised with 10 M KOH and 100 µl of the cell free extract was added to the reaction mixture.

RESULTS AND DISCUSSION

As shown in Table 3, the urease of *R. graminis* showed stronger activity at pH 7 than at pH 3. The jack bean urease showed very weak activity at both pH levels; since it is a purified enzyme, we concluded that the enzyme has lost its activity (Table 3). We also considered the fact that some studies showed an inhibitory effect on urease activity when phosphate, Tris or boric acid buffers were used. An alternative approach was therefore taken using a fresh sample of jack bean urease, as well as a freshly prepared citrate buffer at pH 3 and pH 7. The modified assay worked well for the jack bean urease, but no activity for the *R. graminis* urease was detected in this buffer (data not shown).

Table 3. Urease activity obtained in citrate-phosphate buffers (pH 3.0 and 7.0) for cell free extracts of *R. graminis* and the jack bean urease.

	OD_{340} A. Before GDH addition	OD_{340} B. 40 min. after GDH addition	ΔOD_{340} (A - B)
Citrate-phosphate buffer pH 3			
R. graminis cell extract	4.13	2.85	1.28
Jack bean	4.17	3.19	0.98
Citrate-phosphate buffer pH 7			
R. graminis cell extract	4.22	0.45	3.77
Jack bean	3.94	3.11	0.83

At this stage of the project, it was decided that an alternative approach should be followed. Instead of identifying a yeast producing an acid urease, we decided to clone the acid urease genes from the lactic acid bacterium *L. fermentum* and express them in a yeast system.

ISOLATION, CLONING AND EXPRESSION OF THE UREASE GENES OF *L. FERMENTUM*

INTRODUCTION

The urease enzyme of *L. fermentum* is encoded by three contiguous open reading frames, alpha (1721 bp), beta (374 bp) and gamma (302 bp), in the urease operon (Mobley *et al.*, 1995). This differs from eukaryotes where there is a single ORF encoding the enzyme (Figure 1).

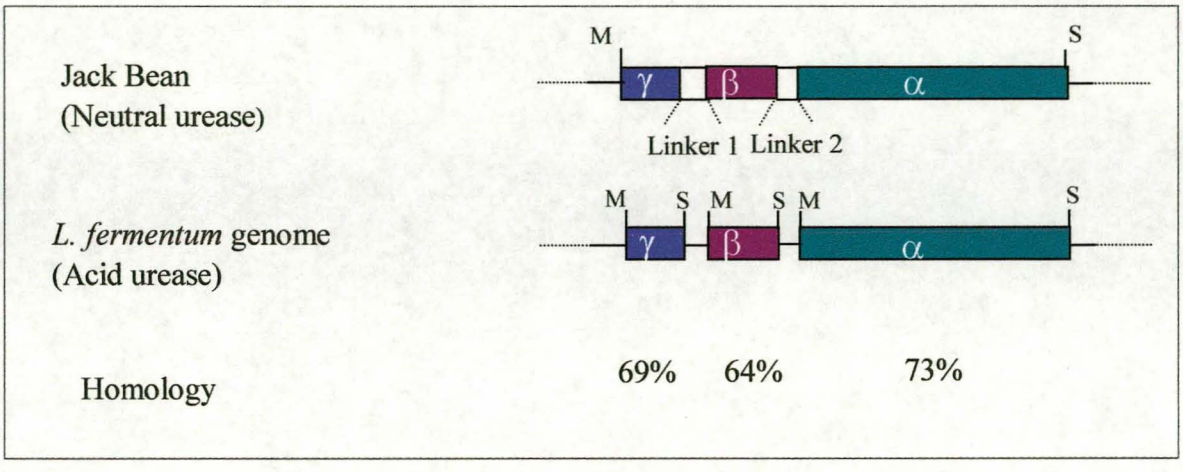


Figure 1. Schematic representation of the structural genes of the jack bean and *L. fermentum* urease showing the strong homology between the two species. M = ATG encoding methionine; S = Stop codon.

Since the ultimate goal of this study was to express the *L. fermentum* enzyme in an eukaryotic yeast system, a single ORF was created by combining the three ORF's from the bacterial urease. This was achieved by replacing the stop codons of the γ and β subunits with suitable restriction enzyme sites, whereas the stop codon of the α subunit was kept intact. This construct, consisting of the structural urease genes of *L. fermentum,* was cloned under the control of the strong, constitutive *PGK1* promoter derived from the yeast *S. cerevisiae*.

When comparing the urease gene sequences of *L. fermentum* with the only eukaryotic urease gene sequence known at the time, i.e. jack bean, a very high degree of homology was observed (Figure 1). The only major difference occurred in the two linker sequences of jack bean (78 and 33 bp, respectively) that separate the three

stretches that are homologous to the γ, β and α subunits of *L. fermentum*. A second construct was therefore designed to carry a combination of the structural genes (γ, β and α) of *L. fermentum* and the linker sequences of jack bean urease. The purpose of these linker sequences is to mimic the eukaryotic model and to create adequate space for the recombinant protein subunits to fold in the correct three-dimensional structure.

The urea that is produced as an intermediate when *S. cerevisiae* degrades allantoin and arginine, is a denaturing agent and therefore the yeast secretes excess urea into the grape must. This takes place via a facilitated diffusion system as soon as the urea reaches a specific concentration inside the cell. It is also in the grape must where the urea reacts with the ethanol to form ethyl carbamate. It would therefore be advantageous to not only provide the yeast with a urease enzyme, but also to enable the yeast to secrete the urease into the grape must where the urea concentration needs to be regulated. Two additional vectors were therefore constructed to produce a fusion of the MFα-secretion factor to the urease ORF.

It has been shown that to obtain urease activity from recombinant urease genes in a host organism, one needs to express the structural genes as well as the accessory genes (Jones and Mobley, 1988; Mulrooney *et al.*, 1988; Lee *et al.*, 1992). These accessory genes have very specific functions in the incorporation of a nickel ion into the apoprotein, as discussed in previous sections. Although we intended to express the recombinant urease in *S. cerevisiae,* preliminary assays were performed in *S. pombe* strain 603. This strain produces its own urease (specific activity: 700 - 800 μmol urea.min^{-1}.mg protein^{-1} [Lubbers *et al.*, 1996]) and will therefore contain the accessory genes to incorporate nickel ions into its native enzyme, and probably also into the recombinant enzyme. An increased urease activity in the transformed *S. pombe* strain would indicate expression of the recombinant genes from the constitutive *PGK1* promoter. It is interesting to note that, when expressing the urease genes of *Helicobacter pylori* in *E. coli*, Hu and Mobley (1993) found that the limiting factor for high levels of urease activity was the synthesis of the structural genes and not the expression of accessory genes.

Transcription of the different constructs was evaluated in both *S. pombe* and *S. cerevisiae* by northern blot analysis, whereas translation of the recombinant protein was evaluated by native and denaturing SDS protein gels and enzyme activity assays.

MATERIALS AND METHODS

Strains and media. The acid urease producing strain of *L. fermentum,* JCM 5869 (Kakimoto *et al.*, 1990), was used as source of the acid urease genes. *Escherichia coli* strain JM109 (*end*A1, *rec*A1, *gyr*A96, *thi*, *hsdR*17, (r_k^-,m_k^+), relA1 supE44, λ^-, Δ(*lac - proAB*), [F', *traD*36, *proA*$^+$*B*$^+$, *lacI*^{q}ZΔM15]) was used for plasmid transformation and propagation and these cells were grown in LB medium (0.5% yeast extract, 1% NaCl, 1% tryptone) supplemented with ampicillin (200 mg/l). Plasmids containing the various constructs were transformed into *S. pombe* strain 603 (972 *leu*1-32, *me*$^-$ LH-67 h$^-$) (Osothsilp and Subden, 1986) and *S. cerevisiae* strain Y294 (MAT α *leu 2-3, 112 ura 3-52 , trp 1-289, his 3*). Yeast transformants were selected and maintained on SC^{-leu} plates (0.17% yeast nitrogen base without amino acids and ammonium sulphate [Difco laboratories, Detroit, MI], 0.5% $(NH_4)_2SO_4$, 2% glucose, 1.5% agar, and supplemented with amino acids as required).

Isolation of chromosomal DNA and manipulations. Total chromosomal DNA was isolated from *L. fermentum* (Dellaglio *et al.*, 1973) and used for the amplification of the urease genes with the polymerase chain reaction (PCR). The PCR primers (Figure 2) were based on the DNA sequence of the urease genes of *L. fermentum* (GenBank accession number D10605). The DNA sequence of the cloned fragment in pAV16 was determined by automated cycle sequencing with the DigDye terminator cycle sequencing kit (Perkin Elmer, USA) and confirmed by the method of Sanger *et al.* (1977) using the T7-sequencing kit (Amersham Pharmacia Biotech Ltd, UK).

Construction of the γβα-cassette (pAV16). After obtaining PCR products for all three subunits, the cloning strategy involved the step-wise cloning of the γ, β and α ORF's into the cloning vector pSP73 (Promega Corporation, USA). Cloning of the γ-ORF into the *Xho*I, *Pst*I sites of pSP73 led to plasmid p9 (Table 4). The β-ORF was cloned into the *Pst*I, *Sal*I sites of p9, resulting in plasmid p94. Finally, the α-ORF

was cloned into the *Sal*I site of p94 and the final product was designated p9412. The γβα-fragment was isolated from this vector with *Xho*I restriction digests and ligated into the episomal expression vector pHVX2 (Volschenk *et al.*, 1997) to yield plasmid pAV16 (Figure 3). Standard techniques (Sambrook *et al.*, 1989) were used for plasmid isolation, restriction enzyme analysis and agarose gel electrophoresis. All the clones were confirmed with restriction enzyme analysis and DNA sequencing.

*Xho*I
JV1 (γ) : 5' AAA **CTC GAG** A ATG AGG CTC ACA AAG CGT GA 3'

*Pst*I
JV2 (γ): 5' AAA **CTG CAG** GCG AAT TGG GTC AGT AAC AG 3'

*Pst*I
JV3B (β): 5' AAA **CTG CAG** ATG GTT CCT GGA GAA TAT AA 3'

*Sal*I
JV4 (β): 5' AAA **GTC GAC** CTT TTC TTC AGG ATA ACT CT 3'

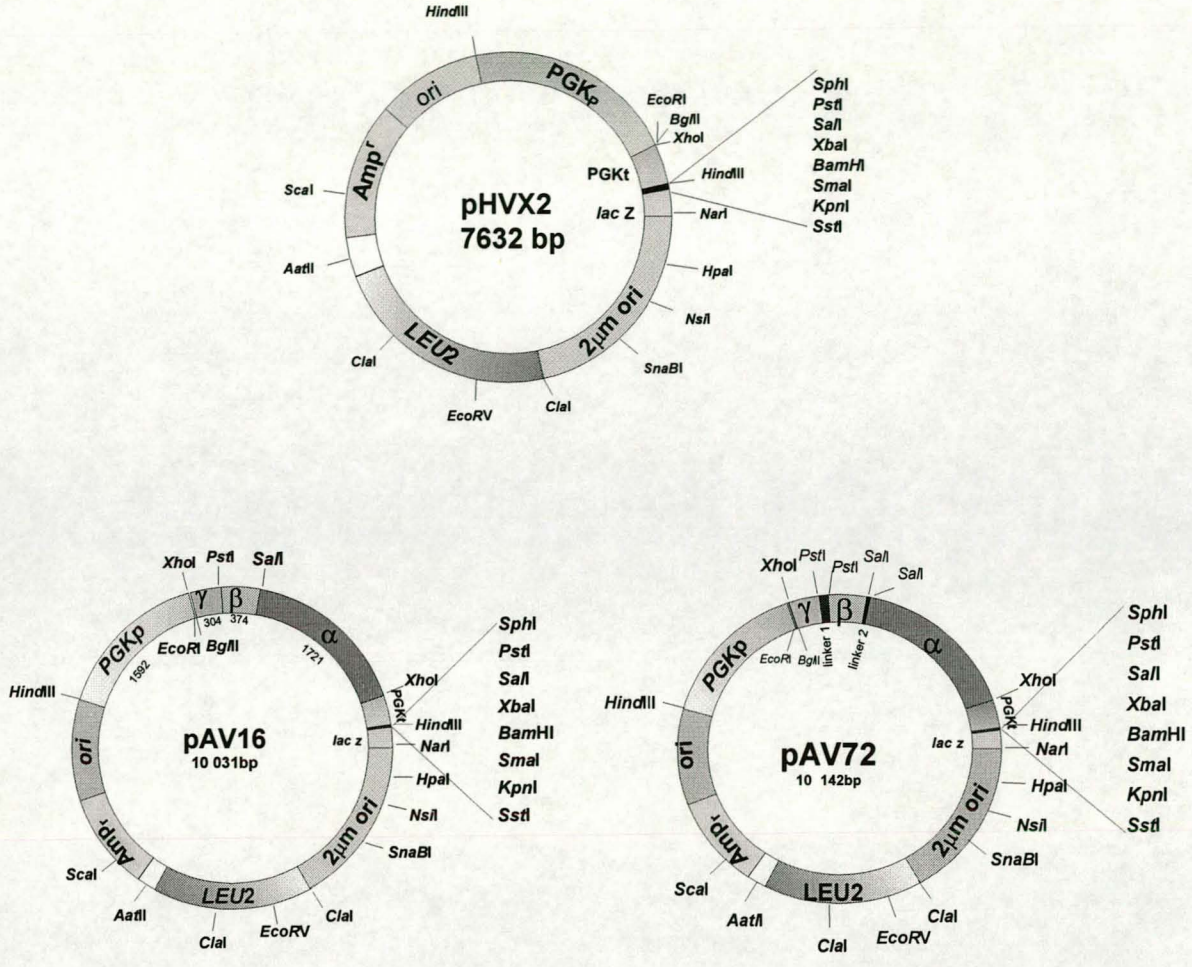
*Sal*I
JV5 (α): 5' AAA **GTC GAC** ATG AGT TTT GAT ATG GAT CA 3'

*Sal*I *Xho*I
JV6 (α): 5' AAA **GTC GAC** AA **CTC GAG** TTA ATA TAG GTA GTA GCG TTG AG 3'

Figure 2. Primers used to isolate and amplify the alpha (α), beta (β) and gamma (γ) ORF's of *L. fermentum*. JV1 and JV2 are the 5' and 3' primers for γ, JV3B and JV4 for β, and JV5 and JV6 for α. The underlined sequences indicate the restriction enzyme sites that were included in the primers to serve as cloning sites.

Subcloning of linkers (pAV72). Linker sequences were manufactured synthetically based on the DNA sequence of the jack bean urease (Riddles *et al.*, 1991). The restriction enzyme sites *Sal*I and *Pst*I were destroyed in pSP73 using the Klenow 3' → 5' exonuclease activity, resulting in pJV400 (Table 4). The γβα-fragment was isolated from p9412 (described above) and cloned into the *Xho*I site of pJV400 to create pJV401. Linker 2 was inserted between β and α into the *Sal*I site of pJV401, forming pJV40117. Finally, linker 1 was cloned into the *Pst*I site between γ and β to create pJV401175. The orientation of the linkers was confirmed with PCR using linker 1

and primer JV4 to confirm the orientation of linker 1, and linker 2 and primer JV3B for linker 2. The γ-linker1-β-linker2-α-fragment was isolated from pJV401175 and cloned into expression vector pHVX2, resulting in pAV72.



*Pst*I

Linker 1 5' AAA **CTG CAG** AAT GGA GAG CTC CAA GAA GCA TTA TTC GGT TCC TTG CTT CCA GTA CCT TCA CTC GAC AAG TTT GCT GAG ACA **CTG CAG** AAA 3'

*Sal*I

Linker 2 5' AAA **GTC GAC** GCA AGT GAA GGA TTT ACC AAG GAA GAC **GTC GAC** AAA 3'

Figure 3. The yeast episomal plasmid pHVX2, containing the *PGK* promoter and terminator sequences, was used for expression of the structural urease genes of *L. fermentum* in pAV16 and pAV72. The sequences of the jack bean linkers that were cloned between the structural genes in pAV72 are also shown.

Table 4. List of plasmids constructed for cloning and expression of the urease subunits.

Vector	Description	Reference
pSP73	Cloning vector with multiple cloning site	Promega
p9	pSP73 with γ-ORF into *Xho*I, *Pst*I site	this work
p94	p9 with β-ORF in *Pst*I, *Sal*I site	this work
p9412	p94 with α-ORF in *Sal*I site	this work
pJV400	pSP73 with *Sal*I and *Pst*I sites destroyed	this work
pJV401	pJV400 with γβα-fragment, from p9412 in *Xho*I site	this work
pJV40117	pJV401 with linker 2 in *Sal*I site	this work
pJV401175	pJV40117 with linker 1 in *Pst*I site	this work
pHVX2	Expression vector with *PGK* promoter and terminator signals	Volschenk *et al.*, 1997
pAV16	pHVX2 with γβα-fragment from p9412 in *Xho*I site	this work
pAV72	pHVX2 with γ-linker1-β-linker2-α-fragment from pJV401175 in *Xho*I site	this work
pPR2	Yeast centromeric plasmid containing MFα secretion signal	van Rensburg *et al.*, 1995
pAM100	pHVX2 with MFα secretion signal in *Eco*RI, *Xho*I site	this work
pAM69	pAM100 with γ-linker1-β-linker2-α-fragment from pJV401175 in *Xho*I site	this work
pAM120	pAM100 with γβα-fragment, from pAV16, in *Xho*I site	this work

Subcloning of the MFα-secretion signal (pAM120 and pAM69). The MFα secretion signal was isolated and amplified from pPR2 (van Rensburg *et al.*, 1995) by PCR with primers MHMFA1 and MHMFA2 (Figure 4). Plasmid pAM69 was constructed by subcloning the γ-linker1-β-linker2-α-fragment from p401175 into the *Xho*I site of pAM100. The pAM120 plasmid was constructed by subcloning the γβα-fragment from pAV16 into the *Xho*I site of pAM100 (Figure 5).

*Eco*RI

MHMFA1 5' - GGG GGG **GAA TTC** AAG AAT GAG ATT TCC TTC - 3'

*Xho*I

MHMFA2 5' - GGG GGG **CTC GAG** CCA AGC TTC AGC CTC TCT - 3'

Figure 4. Primers designed for isolation and amplification of the MFα secretion signal from pPR2.

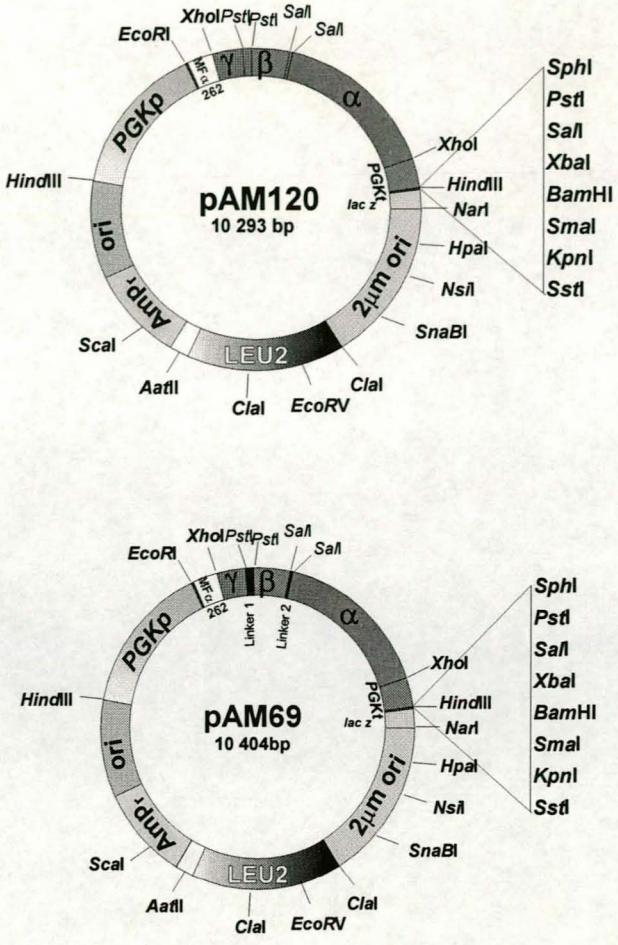


Figure 5. Plasmids pAM120 and pAM69 are similar to pAV16 and pAV72, except for the addition of the MFα secretion signal.

Transformation of yeast. The newly constructed plasmids pAV16 and pAV72, as well as the expression vector pHVX2 in which the subunit fragments were cloned, were transformed into *S. pombe* 603 (Okazaki *et al.*, 1990) and *S. cerevisiae* Y294 (Hill *et al.*, 1991).

RNA isolation and northern blot analysis. Total RNA was isolated (Ausubel *et al.*, 1987) from *S. pombe* and *S. cerevisiae* cells transformed with pHVX2, pAV16 or pAV72. A total of 10 µg RNA of each sample was loaded onto a 1% agarose gel

containing formaldehyde. The sample preparation, gel electrophoresis and transfer to the Hybond-N nylon membrane were done according to Ausubel *et al.* (1987). The PCR-amplified α and γ fragments were labelled with [α^{32}P]dATP (Feinberg and Vogelstein, 1984) using the Random Primed DNA labelling kit (Boehringer Mannheim, Germany), and used individually as probes to detect the mRNA transcribed from the recombinant genes. The RNA molecular weight marker I of Boehringer Mannheim was used to determine the sizes of the transcripts.

Crude cell extract preparation. The *L. fermentum* strain JCM 5869 was grown in 1 ℓ MRS (Biolab) supplemented with 0.005% $NiCl_2.6H_2O$ and 0.1% urea, and the pH adjusted to 5. Strains of *S. pombe* 603 transformed with pHVX2, pAV16 or pAV72 were grown in 1 ℓ SC^{-leu} until late exponential phase (OD_{600} of 1.00). Cells were harvested, washed in 10 ml 0.9% NaCl and resuspended in 8 ml 10 mM phosphate buffer (pH 7) containing 10 mM EDTA and 10 mM β-mercapto-ethanol. Crude cell extracts of the yeast strains were obtained by breaking the cells with micro glass beads (12 pulses of 30 seconds, with 1 min. on ice between pulses), followed by centrifugation to remove the cell debris. Crude cell extracts of *L. fermentum* were obtained with 15 min. sonication (pulses of 30 seconds with 30 seconds on ice between pulses) followed by centrifugation. All steps were performed at 4°C and 50% glycerol (v/v) were added to the cell extracts.

Polyacrylamide gels. Equal amounts of total protein extract were loaded on denaturing or non-denaturing polyacrylamide gels (Ausubel *et al.*, 1987). The Kaleidoscope prestained standard of Bio Rad Laboratories were used as a protein molecular weight marker and the urease enzyme from the Boehringer Mannheim urea/ammonia kit was used as a positive control. Immobilised urease activity present in the non-denaturing gels was detected as described by Blatter *et al.* (1967) and Fishbein (1969). With the method of Blatter, the non-denaturing gel was submerged in a pH 4 or pH 7 buffer after electrophoresis. The buffers contained 10 % urea, 10 mM NiCl and the pH indicator phenol red (0.012 %) for the pH 7 buffer or 0.012 % bromo-cresol green for the pH 4 buffer. For the Fishbein method, the urease stain solution contained 0.01 M citrate buffer at pH 6, 0.08 M urea, 0.04 % p-Nitro blue

tetrazolium and 2 mM dithiothreitol. The gel was equilibrated in citrate buffer (pH 6) for 2 hours after electrophoresis, followed by staining for 20 min.

***In vitro* urease assays.** Total protein extracts from the different wild type and transformed yeast strains and *L. fermentum* were prepared in the same way as for the enzyme activity assays. Assays were performed in 0.1 M sodium acetate buffer (pH 2, 3, 4, 5 or 6) or 0.02 M phosphate buffer (pH 7), with all buffers containing 50 mM urea, 10 mM EDTA and 10 mM β-mercapto-ethanol. Assays were initiated by pipetting 5 µl of the cell extract into 200 µl of each buffer, pre-warmed to 37°C. References were prepared by adding 5 µl of ddH_2O to the various buffers and all reaction mixtures were incubated for 2 h at 37°C. The jack bean urease from the ammonia/urea kit (Boehringer Mannheim, Germany) was used as a positive control by incubating 5 µl of the purified enzyme (100 U/mg) with the different buffers. All reactions were terminated by addition of 500 µl phenol-nitroprusside followed by 500 µl alkaline hypochlorite (Sigma Diagnostics). After incubation for 30 min. at room temperature, the absorbance was determined at 635 nm and the amount of ammonia produced was calculated.

The protein concentration in the crude cell extracts was determined with the Bio-Rad protein assay (Bio-Rad). A protein standard curve was obtained with a dilution series of bovine serum albumin in 10 mM phosphate buffer (pH 7) containing 10 mM EDTA and 10 mM β-mercapto-ethanol. The amount of NH_3 produced was determined from standard curves of NH_4Cl in the various buffers.

RESULTS AND DISCUSSION

Cloning the *L. fermentum* urease genes. The polymerase chain reaction was used to isolate and amplify the different subunit genes. The primers (Figure 2) were designed in such a way that the stop codon of both the gamma (γ) and the beta (β) subunit genes were omitted while the stop codon of alpha (α) was retained. Each primer was also designed to include a restriction enzyme site to provide cloning sites for the linkers and to enable the linking of γ with β, and β with α. The two codons representing the *Pst*I site between the γ and the β subunits encode the amino acids leucine and

glutamine, while the codons representing the *Sal*I site between the α and β subunits encode valine and aspartic acid. The expression cassette created in this way was subcloned under the strong and constitutive *PGK1* promoter and terminator couple (Figure 3).

The sequence of the γβα-fragment in pAV16 was determined by automated cycle sequencing and confirmed in triplicate by Sanger's ddNTP method (Sanger *et al.*, 1977). We confirmed that the γβα-fragment was in reading frame with the *PGK1* promoter, that the stop codon of the γ fragment was replaced by the *Pst*I restriction enzyme site and that the *Sal*I restriction enzyme site replaced the stop codon between the β and α fragments. Furthermore, DNA sequence analysis showed that the highly conserved active site of the enzyme, composed of $His^{\alpha 134}$, $His^{\alpha 136}$, $Lys^{\alpha 217}$, $His^{\alpha 246}$, $His^{\alpha 272}$ and $Asp^{\alpha 360}$, was still intact. However, all the clones showed three amino acid changes when compared to the GenBank sequence of *L. fermentum*: Amino acid 301 of the γβα-fragment codes for threonine instead of leucine; glutamic acid replaced aspartic acid at amino acid 561 and threonine replaced alanine at amino acid 712. This implies that an aliphatic side chain group was changed to a hydroxyl group at amino acid 301 and 561, while two acidic amino acids were exchanged at amino acid 712.

Introduction of recombinant genes into yeast. The yeast *S. pombe* 603 is a urease positive strain and was used as a host that should provide the necessary auxiliary proteins required for nickel incorporation in the recombinant urease. Since all the structural sections of the recombinant gene originates from *L. fermentum,* we expected to see an acidic pH optimum for the recombinant enzyme. Furthermore, expression of the recombinant protein under control of the strong, constitutive *PGK1* promoter should result in an enhanced activity that would allow us to distinguish between the native and recombinant enzymes in *S. pombe*. This would also allow us to evaluate the expression of the recombinant *L. fermentum* urease genes before work on the cloning of the accessory urease genes was undertaken. Although *S. cerevisiae* strain Y294 lacks the necessary auxiliary proteins, we transformed this strain with the

relevant plasmids to determine if no other system in the yeast exists that could incorporate the nickel ions and result in an active recombinant urease enzyme.

Transformants of all three plasmids were obtained for both yeast strains. The growth of the transformants was satisfactory after a single transfer to fresh media, but additional transfers resulted in very poor growth. This may indicate that the production of the recombinant product imposes stress onto the host or that the product has a toxic effect.

Transcription and translation of recombinant urease genes. Transcription of the different constructs was evaluated in both the *S. pombe* and *S. cerevisiae* strains by northern blot analysis. Total RNA was isolated from *S. pombe* and *S. cerevisiae* cells transformed with pHVX2, pAV16 or pAV72 and probed with either [αP^{32}} labelled α or γ PCR products.

Transcription of the $\gamma\beta\alpha$-fragment from plasmids pAV16 and pAV72 was obtained in both yeast strains. As shown in Figure 6, the level of transcription in *S. cerevisiae* was higher than in *S. pombe*. This may be due to the fact that the *PGK1* promoter that regulates the transcription of the recombinant genes, was derived from *S. cerevisiae* and may not be recognised as well by the transcription machinery of *S. pombe*.

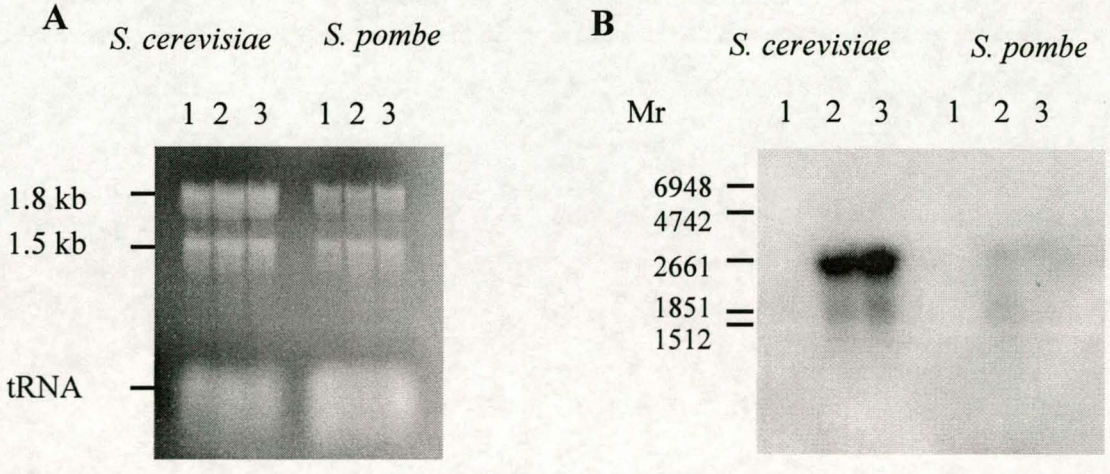


Figure 6. Agarose-formaldehyde gel electrophoresis (A) and northern blot (B) of mRNA transcripts from recombinant $\alpha\beta\gamma$ genes expressed in *S. cerevisiae* and *S. pombe*. Lanes 1, 2 and 3 represent pHVX2, pAV16 and pAV72, respectively.

Translation of the recombinant DNA was examined by loading total protein extracts of pHVX2 (control plasmid), pAV16 and pAV72 transformants onto denaturing polyacrylamide gels, followed by Coomassie staining to visualise the overexpressed recombinant urease. Cell free extracts were also separated on native polyacrylamied gels (non-denaturing conditions) after which the gel was submerged in a buffer containing urea and a pH indicator. The gel was then observed for the development of a coloured band (blue in pH 4, red in pH 7 for the Blatter method or transparent on a black background for the Fishbein method) that would indicate an active urease that converted urea to ammonia, resulting in the colour change. The *S. cerevisiae* pAV16 and pAV72 transformants showed an additional peptide on the denaturing acrylamide gels, but the concentration was very low (data not shown). This low level of translation may be due to the difference in codon usage between *L. fermentum* and *S. cerevisiae*. The absence of visible expression of the recombinant protein in *S. pombe* may be due to the low transcription level as previously mentioned. In the non-denaturing gels using the method of Blatter *et al.* (1967), only the purified urease enzyme showed coloured bands in the neutral pH buffers. No activity bands were observed for *L. fermentum* or any of the transformants, probably due to a low urease content of the protein extracts. The Fisbein method (1969) gave unsatisfactory results.

Urease activity assays. Urease enzyme activity assays were performed on pAV16 and pAV72 yeast transformants. The indophenol assay (Weatherburn, 1967) where ammonia reacts with alkaline hypochlorite and phenol to form indophenol in the presence of the catalyst sodium nitroprusside ($Na_2Fe(CN)_3NO.2H_2O$), was employed. The concentration of ammonia is directly proportional to the absorbance of indophenol, which can be measured spectrophotometrically. The preparation of cell free extracts from the various transformed and untransformed yeast strains was done similar to the protein gel analysis. However, the media were supplemented with urea and nickel, and the pH of the bacterial media was adjusted to 5 to provide the acidic conditions required for the production of the bacterial enzyme.

No significant results were obtained with the *S. cerevisiae* extracts, probably due to the absence of auxiliary proteins. The *S. pombe* extracts showed enhanced urease activity for pAV16 and pAV72 transformants at pH 7 (Figure 7). However, the recombinant urease was highly unstable and quickly degraded, making the quantification of the enzyme activity extremely difficult.

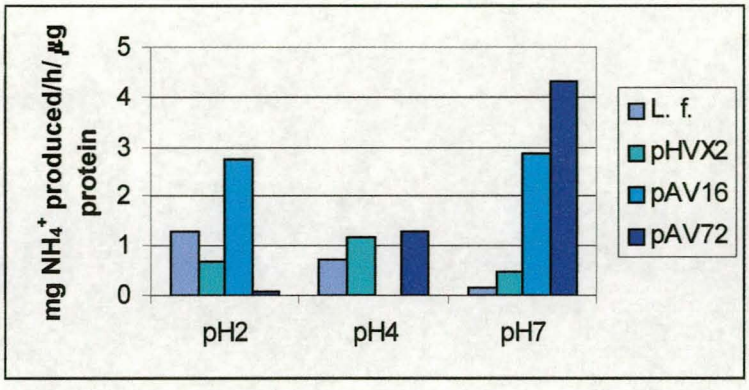


Figure 7. *In vitro* urease activity assays showing the amount of NH_3 that is produced by the cell free extracts of *L. fermentum* (L.f.), *S. pombe* 603 transformed with pHVX2, pAV16 or pAV72. The assays were performed at the different pH values indicated on the graph.

CONCLUDING REMARKS

We have constructed a novel recombinant urease gene by fusing the α, β and γ subunits of the *L. fermentum* urease operon (pAV16). In addition, we have inserted jack bean urease linker sequences between the α and β, as well as the β and γ subunits (pAV72). Both constructs were successfully expressed under control of the *S. cerevisiae PGK1* promoter and terminator signals in the yeasts *S. cerevisiae* and *S. pombe*. The level of transcription in *S. cerevisiae* was found to be much higher than in *S. pombe*, probably due to the fact that the *PGK1* promoter and terminator signals were derived from *S. cerevisiae*.

Translation of the plasmid-born recombinant protein was determined with polyacrylamide gel electrophoresis on total cell extracts from the recombinant yeasts, followed by Coomassie staining to visualise the protein (Data not shown). Only the *S. cerevisiae* pAV16 and pAV72 transformants showed a peptide of the expected size, but at very low concentrations. An attempt to confirm the nature of the peptide with monoclonal antibodies derived from the jack bean urease enzyme was unsuccessful.

Total protein extracts were obtained from the yeast transformants and compared with the extracts from the untransformed yeast and *L. fermentum* strains for their ability to convert urea to ammonia at different pH levels. No significant results were obtained for the *S. cerevisiae* extracts, but the *S. pombe* extracts showed enhanced urease activity for pAV16 and pAV72 transformants at pH 7. However, the recombinant protein was highly unstable and was quickly degraded, which made the enzyme activity extremely difficult to quantify. Protein stability and activity may be improved by using a yeast host strain that is protease-deficient or by using purified recombinant protein in the enzyme assay.

The absence of recombinant urease activity in transformed *S. cerevisiae* cells is probably due to the lack of the essential auxiliary proteins present only in urease producing species such as *S. pombe*. Without these proteins, the organism is unable to assemble the various subunits into an active urease. Therefore, the accessory genes of

L. fermentum will have to be cloned and expressed in addition to the structural urease genes to enable *S. cerevisiae* to express an active urease.

Results presented above confirmed that the fusion between the prokaryotic structural genes and the eukaryotic linker sequences resulted in a recombinant protein that could be expressed in *S. cerevisiae* and *S. pombe*. This type of fusion can provide valuable information about urease gene and protein evolution and the necessary components to ensure optimal activity at low pH levels. However, it is clear that this direct approach is not sufficient to transform a non-urease producer to a urease producer. As with many other similar projects, this testifies that expression of only the structural urease gene is not a quick solution for solving problems concerning the production of ethyl carbamate in the food and beverage industry. Furthermore, it emphasises the fact that the human race underestimates the complexity of the creation.

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