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STAPHYLOCOCCUS LEEI: METABOLIC PROFILE; ANTIMICROBIAL SUSCEPTIBILITY; PURIFICATION OF MICROBIAL UREASE; CLONING, SEQUENCING AND EXPRESSION OF THE UREASE GENE IN *E. COLI*; DEVELOPMENT OF ELISA AND PCR METHODS TO DETERMINE THE INCIDENCE OF INFECTION

by

Ming Jin

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy,

The City University of New York

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ABSTRACT

Staphylococcus leei: Metabolic Profile; Antimicrobial Susceptibility; Purification of Microbial Urease; Cloning, Sequencing and Expression of the Urease Gene in *E. coli*; Development of ELISA and PCR methods to Determine the Incidence of Infection

by

Ming Jin

Adviser: Professor David H. Calhoun

A gram-positive coccoid strain (*Staphylococcus leei*) was isolated from biopsy material obtained from patients in Korea suffering from gastritis (Lee *et al.*, 1997). It was found that this organism binds to porcine gastric antrum and to porcine gastric mucin. Oral administration of the coccoid organism to neonatal piglets resulted in severe growth retardation, and coccoid organisms were detected and cultivated from the antral mucin of these piglets. These observations suggest a hypothesis that the coccoid organisms isolated from Korean gastric patients may be human pathogens.

This organism is a novel stomach adapted *Staphylococcus* species most closely related to *S. cohnii, S. xylosus,* or *S. saprophyticus* based on fatty acid composition, metabolism characterization, and 16S rRNA sequence. Nutritional requirements tests show that the *S. leei* can grow well in rich media, LB, BM and blood agar plates; the amino acids leucine, isoleucine, methionine, proline, and valine are required for the growth of *S. leei*; the vitamins pantothenic acid, thiamin, and nicotinic acid are also essential for the growth of *S. leei*. Antibiotic resistance profile of *S. leei* shows that it is resistant to ampicillin, nalidixic acid, penicillin, sulfisoxazole, and tetracycline.

S. leei has high urease activity and it is similar in respect of low K_m value and acid resistance to the urease of the stomach adapted pathogen, *Helicobacter pylori*. The urease of *H. pylori* is a virulence factor essential for colonization of the stomach, and is the basis for a widely used diagnostic ELISA. The urease of *S. leei* was purified using the BioCAD HPLC Workstation. Q-Sepharose and Poros HP2, Sephacryl-S300, and Hydroxyapatite columns were applied to purify the urease. Urease was purified 98 fold, as indicated by SDS-PAGE, to a specific activity of 731 units/mg. The urease is composed of three subunits, alpha (65 kDa), beta (20 kDa), and gamma (12 kDa). 16 mg of purified urease was obtained from 100 liters of cells lysate. Purified urease was used to detect antibody by ELISA and no cross-reaction was found between *S. leei* and *H. pylori*.

The urease gene of *S. leei* was cloned using PCR method. A PCR product of 2.4 kb containing the *ureA*, *ureB*, and *ureC* genes which code for the urease gamma, beta, and alpha subunits respectively was obtained using primers based on conserved DNA sequence regions comparing several different species. The PCR product was cloned to TOPO vector and the sequence of the urease gene of *S. leei* was determined. The urease gene was constructed in pET23a vector, and low level of urease protein was expressed in BL21 (DE3) cell induced by IPTG. The urease gene of *S. leei* was used to design two sets of primers based on the low homologous regions compared to the urease gene of *H. pylori*. When using the two sets of primers, 210 and 415 bp of PCR products were obtained with genomic DNA of *S. leei*, but not with genomic DNA of *H. pylori*. The two sets of primers are good for using to distinguish *S. leei* from *H. pylori*.

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TABLE OF CONTENTS

ABBREVIATIONS

BSA	bovine serum albumin
CIP	calf intestinal phosphatase
DTT	dithiothreitol
EDTA	ethylenediamine tetra acetic acid
ELISA	enzyme linked immunosorbant assay
IgG	Immunoglobulin G
IPTG	Isopropyl β-D-Thiogalacto-Pyranoside
HPLC	high performance liquid chromatography
kb	kilo basepair
kDa	kilo Dolton
mS	milli Siemen
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
psi	pounds per square inch
SDS	sodium dodecyl sulfate
rRNA	ribosomal RNA

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1. INTRODUCTION

Potentially Pathogenic Gastric Coccus

In 1997 a coccoid organism, which has high urease activity, was obtained from patients suffering from gastritis in Korea (Lee *et al.*, 1997). This organism was isolated and described from cultivated biopsy material. Preliminary taxonomic classification of this microorganism showed similarity to *Staphylococcus cohnii* based on fatty acid analysis using the MIDI/Hewlett Packard microbial identification system, and also showed similarity to *Staphylococcus xylosus* based on metabolic characterization using the Biolog Identification System. The prototype isolate, designated *Staphylococcus leei*, was tested for binding ability to various gastrointestinal mucosal tissues from a 4-day-old piglet. It was found that the cocoid binds to porcine gastric antrum and to porcine gastric mucin but not fundus, duodenum, or small intestine tissue. Oral administration of the coccoid organism to neonatal piglets resulted in severe growth retardation, and coccoid organisms were detected and cultivated from the antral mucin of these piglets (Lee *et al.*, 1997). The above observations suggest that the coccoid organisms isolated from these gastric patients may be human pathogens in gastric disorders.

A large proportion of a variety of gastric disorders is due to infection by *Helicobacter pylori* (Marshall, 1994). *H. pylori* known previously as *Campylobacter pylori*, is a spiral gram-negative bacterium with significant urease activity. The urease of *H. pylori* contributes to the virulence of *H. pylori*.

Eradication *of H. pylori* does not always correlate with clinical recovery (Treiber and Lambert, 1998), and there is still discussion among clinicians regarding causes other than *H. pylori* for gastric disorders (Koshy and Pitchumoni, 1998). Reports continue to

appear in the clinical literature of instances of weak associations between *H. pylori* infection peptic ulcers, and cirrhosis (Tsai, 1998). Some patients suffering from gastric disorders are culture negative and antibody negative for *H. pylori*, and these conditions are often attributed to other causes such as the habitual use of certain drugs (Marshall, 1994). In one report (Hansing *et al.*, 1992) uncharacterized rods and coccal organism were proposed as the causative agent for gastric inflammation in 8.5% of patients that were biopsied. In another report (Bode *et al.*, 1988) observed both the well-characterized spiral shaped *H. pylori* and a different coccoid form in a gastric biopsy, and they proposed that *H. pylori* could assume a coccoid morphology. In these two reports these coccoid forms could not be cultivated and for this reason no further characterization was possible. These reports show that *H. pylori* may not be the only organism causing human gastric disorder.

Antimicrobial treatment is used as one of the approaches in treating gastric disorder. It was reported (Blaser, 1987) that *H. pylori* is susceptible to erythromycin, tetracycline, penicillin, ampicillin, cefoxitin, ciprofloxacin, gentamicin, cephalothin, clindamycin, kanamycin, amoxicillin, clarithromycin, and metronidazole, but is resistant to nalidixic acid, sulfonamides, vancomycin, and trimethoprim. The standard antimicrobial agents used for *H. pylori* typically are amoxicillin, clarithromycin, and metronidazole, along with proton pump inhibitors or ranitidine-bismuth citrate. Clinical evidence has also shown that these antibiotics only have limited effectiveness on some patients. Treatment failures among patients infected with *H. pylori* occur routinely at various rates depending upon the treatment therapy and patient demographic group, and this failure is commonly attributed to patients non-compliance with the recommended

therapy and other factors. This treatment failure could be due to misdiagnosis as an *H. pylori* infection. This further shows that *H. pylori* may not be the sole organism for causing human gastric disorder.

Recent reports indicate that *Staphylococci* can be directly isolated by cultivating biopsies taken from the hypochlorhydric human stomachs in Finland (Vakevainen *et al.*, 2001). *Staphylococcus* was isolated from stomachs using PCR method in Sweden (Monstein *et al.*, 2000). These recent reports support the hypothesis that the human stomach can be colonized with *Staphylococci.*

Characterization of Urease

Urease (urea amidohydrolase, EC 3.5.1.5) catalyzes the hydrolysis of urea to ammonia and carbamate, which spontaneously decomposes to yield carbon dioxide and a second molecule of ammonia (Mobley *et al.*, 1995). Ureases have been isolated from various bacteria, fungi, and higher plants. The best-investigated urease protein is that from jack bean (*Canavalia ensiformis*) which was the first enzyme to be crystallized (Sumner, 1926) and found to contain a functionally important nickel molecule (Blakeley and Zerner, 1984). The protein structure and gene sequences of ureases from many kinds of sources have been analyzed. The ureases from non-*Helicobacter* bacterial species are composed of three distinct subunits encoded by three contiguous genes, (*ureA, ureB* and *ureC).* The ureases from *Helicobacter* species are composed of two subunits encoded by two adjacent genes (*ureA* and *ureB);* the urease of jack bean is made up of only one distinct subunit encoded by one gene (Mobley *et al.*, 1995). The genetic fusion of the *ureA* and *ureB* genes of non-*Helicobacter* bacterial species was hypothesized, and this would result in the appropriate sized *Helicobacter* urease subunit gene. Although urease

from different species may be composed of one, two, three distinct types of subunits, the ureases are related closely by amino acid sequence homology.

There are many bacterial species with strong ureases (Mobley and Hausinger, 1989, and Mobley *et al.*, 1995), and several of these species can cause human disease. Urease activity plays a central role in the pathogenesis elicited by a number of bacterial species as seen in *Proteus mirabilis* (McLean *et al.*, 1988), and *Staphylococcus saprophyticus* (Gatermann *et al.*, 1989 and Osterberg *et al.*, 1990), which cause urinary tract infections.

Urease of *H. pylori* and *S. leei*

The ureases of *H. pylori* species are composed of two subunits of approximately 26.5 (α subunit) and 60.3 (β subunit) kDa. Urease is a virulence factor in gastric ulceration caused by *H. pylori* (Marshall and Warren, 1984). In the absence of urea, *H. pylori* is sensitive to acidic pH (Goodwin *et al.*, 1986). The urease protects this gastric organism from gastric acid by neutralizing the acid with the ammonia generated through urea hydrolysis (Ferrero and Lee, 1991). The K_m values for the urease of various *Helicobacter species* are reported as 0.2-0.5 mM (Dunn *et al.*, 1990, Evans *et al.*, 1991, Gootz *et al.*, 1994, and Hu and Mobley, 1990), which is low relative to microorganisms found in the urinary tract (Mobley *et al.*, 1995). This has been interpreted to suggest that a correlation exists between the K_m and an organism's ecological niche. *H. pylori* colonizes the gastric mucosal lining and depends upon the concentration of urea supplied by diffusion from the serum (Eaton *et al.*, 1991). The urease of *H. pylori* was shown to be readily released from the cell upon exposure to water, 0.15 M NaCl, or nondenaturing detergents (Dunn *et al.*, 1990).

The urease of *S. leei* was purified using conventional chromatography and then characterized (Lee and Calhoun, 1997). This urease was found to be composed of two subunits of 21 and 65 kDa. The molecular weight is about 260 kDa for the native enzyme and a hexameric holoenzyme containing three subunits each of the 65 and 21 kDa. The K_m value of *S. leei* urease is 0.56 mM of urea. It is more closely related to the K_m value of 0.2-0.5 mM reported for the urease of various *Helicobacter species* (Dunn *et al.*, 1990, Evans *et al.*, 1991, Gootz *et al.*, 1994, and Hu and Mobley, 1990) than to the K_m values 9.5 mM of *S. saprophyticus* (Schafer and Kaltwasser, 1994). After exposure to pH 1.3, *S. leei* urease retained 75% of original activity (Lee and Calhoun, 1997), thus indicating that this urease is well suited to function in an acidic environment.

The properties of the urease of *S. leei*, including K_m value, and acid resistance are not typical of previously described *Staphylococcus* species. These properties are more similar to those of the urease of the known stomach pathogen, *H. pylori*, and provide further support for the pathogenic potential for the coccoid isolate.

Antibodies in Ulcer Patients for Urease of *H. pylori* and *S. leei*

Patients with active gastritis due to *H. pylori* show significantly elevated immunoglobulin G and A titers to the urease compared with preinfection levels. ELISA assay methods have been developed using the purified urease of *H. pylori* as antigen to measure these immune responses (Perez-Perez and Dunn, 1989). The serum from patients in the New York City metropolitan area with symptoms of stomach ulcers were tested with the urease purified from *S. leei* (Coico and Calhoun, unpublished). All serum samples were first tested by Quest Diagnostics (Teterboro, New Jersey) for antibodies against the urease of *H. pylori*. A commercial ELISA kit (BioWhittaker, Cat. No. 30-

678U) revealed samples that tested positive for patients 1-20 and negative for patients 21-40 (Table 1.1) for *H. pylori* specific antibodies. Serum reactivity against the urease purified from the coccus was also tested by ELISA. Goat anti-human Ig-horseradish peroxidase was used to detect human Ig bound to the urease and absorbance was measured with a BioRAD ELISA reader. The results indicate that 1/20 patients (5%) who are positive for *H. pylori* react with the urease purified from the coccus. In contrast, 5/20 patients (20%) who are negative for *H. pylori* react with the urease purified from the coccus. Although the sample size of this study is small these findings are noteworthy because it represents the first evidence of infection by this type of coccoid organism outside of Korea. Since the diagnostic ELISA kit used to detect infection by *H. pylori* uses the purified urease of *H. pylori* as the antigen, it is evident that there is little or no immunological cross-reactivity between the urease of the coccus and that of *H. pylori*. Therefore, there is a need for a coccus specific diagnostic system.

The Adhesin for Gastric Mucin from *H. pylori* and *S. leei*

Adhesins are glycoproteins, which usually recognize carbohydrates on eukaryotic cells, and they are bacterial colonization factors. *H. pylori* has adhesions that are believed to participate in pathogenesis, including at least two protein hemagglutinins, lipopolysaccharides, and outer membrane proteins that recognize several receptors, including gastric mucin.

The adhesin of *S. leei* was purified by conventional chromatography (Lee and Calhoun, unpublished). *S. leei* has an adhesin specific for gastric mucin. The chemical composition of the acid washed pellets of cell wall fragments was as follows (molar percentages are shown in parentheses): muramic acid (2.3%), galactosamine (0.8%),

glucosamine (5.4%), glutamic acid (10.6%), serine (7.1%), glycine (47.9%), threonine (0.1%), alanine (22.9%), phenylalanine (0.1%) and lysine (11.0%). A close examination of the composition indicates that the adhesin contains all of the constituents of peptidoglycan, which is made up of repeating units consisting of muramic acid, glucosamine, alanine, glutamic acid, lysine and glycine in molar ration of 1:1:2:1:1:5. However, the adhesin contains serine and galactosamine, and possibly threonine and phenylalanine, which are not normally present in peptidoglycan, and there are significant differences in molar ratios of common amino acids and amino sugars. Judging from the inability of Coomassie blue, which stains positively charged amino acids, to stain adhesin, the E-amino group of the lysine in the adhesin is not free as in the case of peptidoglycan.

Objective of This Study

Since *S. leei* was isolated in 1997, the initial determination of phylogenic status of this organism, and the characterization of its urease and adhesin were studied, but many aspects of its metabolism and physiology, including its specific growth requirements are still largely unknown. I tested the nutritional requirements for the growth of *S. leei* (amino acid and vitamins) on different nutrient media (rich media and M9 salt with different supplements).

Although the phylogenic status of *S. leei* has been already tested based on fatty acid composition, and biochemical markers, it is known that *S. leei* is related to *S. cohnii*, and *S. xylosus* respectively (Lee *et al.*, 1997), but it needs further characterization based on comparing 16S rRNA sequence. The sequence of ribosomal RNA permits the determination of phylogenic status. It is useful because sequence changes for this gene

occur more slowly than for other genes. The phylogenic status of *S. leei* was assayed based on the 16S rRNA sequence analysis.

In the diagnostic laboratory, pathogenic *Staphylococci* are distinguished from nonpathogenic strains by their production of intracellular coagulases that clot plasma. This is a method used to distinguish pathogenic *staphylococcal* strains from nonpathogenic strains. *S. leei* is a newly discovered possible gastric pathogen which has been associated with gastritis, and peptic ulcers. In order to know the pathogenic status of *S. leei*, I assayed the extracellular coagulases using rabbit serum.

Because of the failure of some antibiotic treatment to *H. pylori*, the comparison of the profile of antibiotic susceptibility between *S. leei* and *H. pylori* can give us some insight into the relationship between these organisms and the cause of the disease. And the antibiotic susceptibility profile has not been tested yet for the organism. I tested the profile of antibiotic susceptibility of *S. leei* to 37 antimicrobial agents representing the different families of antibiotics, of which most were previously used to test antibiotic susceptibility for *H. pylori*.

The characterizations of *S. leei* included nutrient requirement, phylogenic status based on 16S rRNA sequence, coagulase examination, and antibiotic resistance profile were presented in Chapter 2.

S. leei is the prototype isolate of a group of strains isolated from biopsy material obtained from patients in Korea with gastric disorders. It has a number of properties indicating that it is a human pathogen, such as presence of high urease activity and adhesin for gastric mucin. More data are needed, however, to establish the incidence of

Staphylococci leei in human gastric patients is necessary to elucidate possible pathogenicity.

The methods most commonly used for detection of gastric disorder caused by *H. pylori* are (1) detection of urease activity in biopsy samples and in the stomach using CLOtest and 13C-urea breath test respectively; (2) detection of urease antibody to the urease of *H. pylori* antigen using ELISA; and (3) PCR method with specific primers derived from *H. pylori* urease gene. Among these methods, detection of urease activity using CLO test or C13-urea breath test are not effective for distinguish the infection of *S. leei* from *H. pylori* because *S. leei* has urease levels comparable to those of *H. pylori*. The ELISA and PCR methods are good for distinguishing *S. leei* from *H. pylori* and detecting *S. leei* infection.

In order to detect specific urease antibody using ELISA in the sera of gastric patients that is specific for the urease produced from *S. leei*, large amounts of urease should be obtained. A new purification protocol for the PerSeptive Biosystems HPLC Workstation was devised and this method was used to purified 100 liter of cell lysate of *S. leei*. This study is described in Chapter 3.

In order to detect the infection of *S. leei* using PCR method, and in order to understand the relationship of the urease of *S. leei* with other species, especially *H. pylori* and *S. xylosus*, it is necessary to know the DNA sequence of *S. leei* urease. The urease gene is first found using PCR method and cloned to pCR 2.1-TOPO vector (Invitrogen). Then the urease *ureABC* genes of *S. leei* is sequenced. This sequence was used to design the PCR primers to detect the incidence of infection of *S. leei*. The sequence was also used to compare with other urease genes. *S. leei* urease is the antigen used in the ELISA

to detect specific antibodies for the infection of *S. leei*. The current purification method for urease depends on the growth and cell lysis (using Dynomill) of large quantities (100 liters) of the potentially pathogenic *S. leei* by a subcontractor using appropriate biohazard containment methods at considerable expense. Only 16 mg of urease was purified from the lysis of 100 liters of cells (see urease purification section). In order to make this enzyme available in large quantities for purification I constructed an *E. coli* expression vector (pET23a) that can produce the *S. leei* urease at high levels. This *E. coli* expression system can also be used safely and locally with smaller quantities of cells. The *E. coli* cells are easy to lyse to obtain cell extracts for purification of *S. leei* urease protein. The cloning, sequencing, and expression of the urease gene of *S. leei* in *E. coli* is described in Chapter 4.

After I purified the *S. leei* urease protein and elucidated the urease gene sequence of *S. leei*, I devised two tests that reliably distinguish *S. leei* from *H. pylori*. One is an ELISA that uses the purified urease of *S. leei* as an antigen. There is an FDA approved ELISA for *H. pylori* using the purified urease of *H. pylori* as an antigen. In the preliminary experiments I tested the samples, in collaboration with scientists at Enteric Products, Inc.

The second method to reliably distinguish *S. leei* from *H. pylori* and to detect *S. leei* infection is PCR. I cloned and sequenced the *ureABC* genes of *S. leei* and used this sequence to prepare two sets of PCR primers derived from DNA segments of low homology that produce DNA fragments of 210 and 415. A widely used set of PCR primers (Lage *et al.*, 1995) derived from the *ureC* gene of *H. pylori* produces a PCR fragment of 294 bp. By using these three sets of primers separately on *S. leei* and *H.*

pylori DNA, I determined that they are specific and do not cross-react with DNA from the heterologous host.

A method was derived to obtain useful quantities of DNA from the stomach adapted *Staphylococci* that can be used to screen biopsy samples by PCR. *S. leei* has an outer integument that is particularly difficult to disrupt and the protocol used to disrupt *H. pylori* in biopsy samples does not disrupt *S. leei*. I tested three isolation methods and test enzymes specific for the cell wall, including lysostaphin, mutanolysin, lysozyme, alone and in combination. I also evaluate the CLOtest, a urease activity test for *H. pylori*, for *S. leei*. All of the methods developed to determine the incidence of infection of *S. leei* are presented in Chapter 5.

Table 1.1 ELISA Testing Immune Response of Anonymous Ulcer Patients from New York Metro Area to Urease *Staphylococcus leei* (Coico and Calhoun, unpublished)

H. Pylori Positive	Titer*					*H. Pylori* Negative	Titer*				
Patient No.	1/10	1/20	1/40	1/80	1/160	Patient No.	1/10	1/20	1/40	1/80	1/160
1	0	0	0	0	0	21	0	0	0	0	0
2	0	0	0	0	0	22	0	0	0	0	0
3	0	0	0	0	0	23	0	0	0	0	0
4	0	0	0	0	0	24	0	0	0	0	0
5	0	0	0	0	0	25	0	0	0	0	0
6	0	0	0	0	0	26	+	+	+	+	+
7	0	0	0	0	0	27	+	+	+	+	+
8	0	0	0	0	0	28	0	0	0	0	0
9	0	0	0	0	0	29	0	0	0	0	0
10	0	0	0	0	0	30	0	0	0	0	0
11	0	0	0	0	0	31	0	0	0	0	0
12	0	0	0	0	0	32	0	0	0	0	0
13	0	0	0	0	0	33	+	+	+	+	+
14	0	0	0	0	0	34	+	+	+	+	+
15	+	+	+	+	+/-	35	+	+	+	+/-	0
16	0	0	0	0	0	36	0	0	0	0	0
17	0	0	0	0	0	37	0	0	0	0	0
18	0	0	0	0	0	38	0	0	0	0	0
19	0	0	0	0	0	39	0	0	0	0	0
20	0	0	0	0	0	40	0	0	0	0	0

*Normal human serum controls were negative. 0, negative; + positive; +/-, intermediate.

CHAPTER 2. CHARACTERIZATION OF *S. LEEI*

Materials and Methods

1. Determine the Nutritional Requirements of *S. leei*

All stock solutions (100 x) of each single nutrient used in this study were from Sigma and Cal Biochemistry Company, and all of them are soluble in water at the concentrations specified in Table 2.1 (Holliday, 1956).

To determine the optimal media for growth, *S. leei* was streaked onto different agar growth media to isolate single colonies. Three types of media were used. The first was LB agar (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, 1 ml of 1M NaOH in 1 liter H_2O, pH 7.2); the second was BM agar (Christians *et al.*, 1991) (5 g of peptone, 2.5 g of yeast extract, 2.5 g of NaCl in 1 liter H_2O, pH 7.2); and the third was blood agar (Tryptic Soy Agar with 5% sheep blood, B21239X, Fisher Scientific). All plates were incubated at 37^0C for 3 days after which the colony size was measured. M9 agar with different nutrient supplements (bases, vitamins, 1% casamino acid, vitamins with bases, and vitamins with 1% casamino acid) were prepared. *S. leei* was streaked onto each of the media for isolation of single colonies. The size of colonies on each plate was measured after three days of incubation at 37^0C.

In order to test the single amino acid requirement, *S. leei* was streaked onto 19 plates for isolation of single colony. Each plate contained M9 salt with all nutrients except missing one amino acid. The size of one colony on each plate was measured after two days of incubation at 37^0C.

In order to test for vitamin requirements, *S. leei* was streaked onto 12 agar media for isolation of single colony. Each media contained M9 salt, 1% casamino acid (Cat.

No.0320-15-5, Difco Laboratory), and all vitamins except one missing vitamin. The size of one colony on each plate was measured after three days of incubation at 37^0C.

2. Determine the Phylogenic Status of *S. leei* by 16S rRNA Sequence

S. leei identifications assigned were based on 16S rRNA gene sequence similarity. The 16S rRNA gene of *S. leei* was PCR amplified from genomic DNA isolated from *S. leei* colonies (MIDI Labs, Wilmington, DE). Primers used for the amplification correspond to *E. coli* positions of 5 and 1540. Amplification products were purified from excess primers and dNTPs using Microcon 100 (Amicon) molecular weight cut-off membranes and then checked for quality and quantity by running a portion of the products on an agarose gel. Cycle sequencing of the 16S rRNA amplification products was carried out using AmpliTaq FS DNA polymerase and dRhodamine dye terminators. Excess dye-labeled terminators were removed from the sequencing reaction using a Sephadex G-50 spin column. The products were collected by centrifugation, dried under vacuum and frozen at –20 °C until ready to load. Samples were resuspended in a solution of formamide/blue dextran/ EDTA and denatured prior to loading. The samples were electrophoresed on an ABI Prism 377 DNA Sequencer. Data was analyzed using PE/Applied Biosystems DNA editing and assembly software. The top ten alignment matches are presented in a percent genetic distance format. In this format a low percent indicates a close match.

3. Determination of Antibiotic Susceptibility Profile of *S. leei*.

Diffusion Discs. Twenty-one discs of different antibiotic were used for this test. Listed in Table 2.3 is the amount of antibiotic on each disc. Sixteen discs were from Becton Dickinson and Company, amoxicillin/k clavulanate, cefoxitin, cephalothin,

erythromycin, furazolizone, kanamycin, nalidixic acid, nitrofurantoin, penicillin, piperacillin, rifampin, sulfisoxazole, tetracycline, trimethoprim, trimethoprim/sulfamethozole, and vancomycin. Four discs were from Difco Laboratories, ampicillin, ciprofloxacin, clindamycin, and gentamicin. The last disc, clarithromycin was prepared using a blank paper disc from Becton Dickinson and Company and with the antibiotic tablet from PREVPAC (TAP Pharmaceuticals, Deerfield, IL). One tablet of 500 mg was dissolved in 50 ml of water, and then 10 times diluted to the concentration of 1 μg/μl. 15 μl of the diluted antibiotic was added to one blank paper disc, and the amount of this disc was the 15 μg.

Discs diffusion test. A 0.05 ml of overnight culture (five times diluted, about 1 x 10^7 cells) of *S. leei* was first inoculated onto Mueller Hinton agar in 100 mm Petri dishs (BBL Cat. No. 221177, Becton Dickinson and Company, Cockeysville, MD), and then 19 antibiotic discs were placed individually onto the bacterial lawn using Sensi-Disc dispenser (for the discs of Becton Dickinson and Company), or DIFCO DISPENS-O-DISC dispenser (for the discs of Difco Laboratories), and gently pressed down onto the agar. No more than six discs were applied to each plate. The plates were incubated at 37^0C for 18 hours. The diameter of zones of inhibition surrounding the discs were measured.

Breakpoint susceptibility test. MicroScan Dried Gram Positive Pos Breakpoint Combo Panel Type 12 (No. B1017-154, DADE BEHRING Inc, West Sacramento, CA) was used. This panel contains 29 different antimicrobial agents listed in Table 2.3, and is designed for use in determining antimicrobial agent susceptibility and/or identification to the species level of rapidly growing aerobic and facultative gram-positive cocci. This

work was conducted at the Microbiology Laboratory at the Mount Sinai Hospital in New York City. The manufacture's procedure was followed. The panel was incubated at 37^0C for 18 hours, and then read on MicroScan instrumentation.

Organism identification. The Pos Breakpoint Combo Panel Type 12 from DATA BEHRING Company also was used for the identification of unknown test organisms based on the MicroScan Dried Gram Positive Biotype Codebook. This codebook has been generated from MicroScan's own database. The Gram Positive Codebook provides for computer analysis of 27 tests for Streptococcaceae and 18 tests for Micrococcaceae. These tests are translated into a 9 or 6 digit biotype number respectively. Refer to the codebook for the method of recording results. The codebook lists the organism identification and the relative probabilities. All possibilities are printed in the order of the highest probability up to a cumulative total of 99.9%.

4. Determine the Pathogenicity of *S. leei* by Testing for Coagulase Production

The method described by Smibert and Krieg (1981) was used for this test. First 0.5 ml of rabbit plasma (Cat. No. 212125, Difco Laboratories, Detroit MI) was added to test tube, using a sterile bacteriological loop, thoroughly emulsify 1 loopful of colony of *S. leei* on LB agar into test tube of plasma, mixed gently. Same procedure was used for *Staphylococcus aureus* (positive control) and *Staphylococcus epidimermidis* (negative control). All three test tubes were incubated at 37^0C for up to 4 hours. The formation of clots was examined hourly until a clot is evident or until 4 hours have elapsed, if no clot has formed within 4 hours, the test tube were reinsulated and examined after 24 hour. Solid or loose clots suspended in the plasma indicates a positive result. Granular or ropy formations are inconclusive.

Results and Discussion

1. Nutritional Requirements of *S. leei*

The amino acids, vitamins, purine, and pyrimidine for optimal growth of *S. leei* were determined. In the previous work (Lee *et al.*, 1997, and Lee and Calhoun, 1997) *S. leei* had been cultivated at 37^{0}C on blood agar plates containing 7% sheep blood under microaerophilic conditions (10% CO_2, 5% O_2, and 85% N_2). These growth conditions were based on the untested assumption that the nutritional requirements might be similar to the other known stomach pathogen, *H. pylori*. Initially, growth was tested using routine laboratory media, including LB, BM and blood agar, and good growth was seen on all three plates (Figure 2.1). The growth of *S. leei* was tested on M9 minimal agar with different supplements. After three days of incubation at 37^{0}C, no colonies developed on M9 agar with bases or M9 agar with 1% casamino acid; the colony size of *S. leei* on M9 agar with vitamins, with vitamin and bases, and with vitamins and 1% casamino acid were 0.8, 1.5, and 3.5 mm respectively (Figure 2.1). These results indicate that growth of *S. leei* requires vitamins, and is stimulated by bases and casamino acids.

M9 minimal medium agar was prepared containing 19 amino acids, 11 vitamins, and 4 bases, and good growth was seen after two days of culture at 37^{0}C. Well-isolated single colonies were present when any single amino acid was absent. However reduced colony size was apparent when proline, methionine, leucine, isoleucine, and valine were absent (Figure 2.2). The requirement for leucine, isoleucine and valine suggests that one or more of the three share a bifunctional enzyme pathway for the synthesis of these amino acids and therefore have a growth limiting rate. The partial proline requirement indicates that a partial deficiency in one of the three enzymes that convert glutamate to

proline will affect the growth of the culture. The partial methionine requirement in the growth experiment implicates one of the five enzymes in the conversion of aspartate-β-semialdehyde to methionine. The presence of both small and large colony sizes on plates lacking either proline, leucine, isoleucine, and valine suggests that mutant genetic variants (revertants) are present in the population that possess greater biosynthetic capacity, and these form the larger colonies. This phenomenon is not unusual, and is reminiscent of wild type isolates of *Lactobacillus casei* that require 12 amino acids and 4 vitamins. Morisita *et al.*, (1974) were able to isolate single step mutants with respect to seven of the 12 amino acids and three of the 4 vitamins. Observations of this type led to the definition of cryptic genes (Hall *et al.*, 1983) Cryptic genes are phenotypiclly silent DNA sequences not normally expressed during the life cycle of an individual. They may however, be activated in a few individuals within a large population by mutation, recombination, insertion elements, or other genetic mechanism thereby affecting the growth of the culture.

For the single vitamin requirement test, each plate contained M9 salt, 1% casamino acid, and all vitamins, and were incubated at 37^0C for three days, and good growth was seen. Growth was also observed when any single vitamin was missing, however reduced colony size was apparent when thiamine, nicotinic acid or pantothenic acid were absent (Figure 2.3). This result indicates that especially thiamin, and also nicotinic acid and pantothenic acid, are required for optimal growth of *S. leei*.

2. Phylogenic Status of *S. leei* by 16S rRNA Sequence Determination

The *S. leei* 16S rRNA gene sequence analysis was performed using a PE Applied Biosystem MicroSeq™ and analyzed using microbial analysis software and database

(MIDI LABS, Willmington, DE). The top ten alignment matches are presented in a percent genetic distance format. In this format a low percent indicates a close match. The 16S rRNA sequence of *S. leei* is most closely matched to *S. saprophyticus* with a sequence difference of only 0.03%, followed by *S. xylosus* (0.26%) and *S. cohnii urealyticus*. (0.78%) (Table 2.2). The taxonomic classification of *S. leei* based on 16S rRNA sequence is similar to the previously taxonomic classification based on fatty acid analysis and biochemical marker, which indicate *S. leei* is closely related to *S. cohnii* and *S. xylosus* respectively (Lee and Calhoun, 1997). The 16S rRNA sequence difference of *S. leei* and *H. pylori* is 23.3%, and the gram-positive *S. leei* is clearly not closely related to the gram-negative *H. pylori*. *S. leei* appears to be a new species adapted to growth in human stomach, and is a member of the DNA homology group that contains *S. saprophyticus, S. xylosus, and S. cohnii* which are closely related to each other based on nucleic acid hybridization analyses (Kloos, 1980).

There is extensive literature on the presence of large numbers of *Staphylococci* with very high urease present in the stomach of various ruminants, including cows, goats, sheep, deer, and others (Laukova and Koniarova, 1995; and Laukova, 1997). This high urease activity containing *Staphylococci* protects these animals from urea poisoning, a common hazard for ruminants (Sundberg *et al.*, 1980; and Caldow and Wain, 1991). It is well established that these *Staphylococci* are transferred to meats, sausages, mild cheese, vegetables, and other foods consumed by humans (Meugnier *et al.*, 1996; and Perreten *et al.*, 1998). The relationship of the *S. leei* group of organisms to the *Staphylococci* in animals is not known, but it may be significant that three of the most commonly reported bacterial infection in animals are *S. cohnii*, *S. xylosus*, and *S. saprophyticus*, the same

three species most closely related to *S. leei*. If these human and animal stomach adapted *Staphylococci* are related, this would provide mechanisms for infection and would indicate the reservoir for infection in humans.

3. Antibiotic Susceptibility of *S. leei*

A total of 37 antibiotics were tested on *S. leei*. 21 of them were tested using discs from Becton Dickinson and Company, and Difco Laboratory; 29 of them were tested using Pos Breakpoint Combo Panel from DATA BEHRING Company; 13 of them were tested using both methods. The susceptibility of *S. leei* to the antibiotics on the discs is expressed in terms of zones of inhibition diameter, and to antibiotic on the Pos Breakpoint Combo Panel in the term of breakpoint (μg/ml). The susceptibility of *S. leei* to antibiotic using Pos Breakpoint Combo Panel is determined by comparing the MIC of *staphylococci* to the attainable urine level of the antimicrobial agents. The interpretive criteria of breakpoint recommended by National Committee for Clinical Laboratory Standards (NCCLS) is listed in the manufacturer's manual. The susceptibility of *S. leei* to antibiotic using the discs from Becton Dickinson and Company or Difco Laboratory is determined by zone diameter interpretive chart listed in the manufacturer's manual, which is adapted from NCCLS document.

The result of the breakpoint susceptibility test with the Pos Breakpoint Combo Panel is expressed in the breakpoint (μg/ml), and the result of disc diffusion test is expressed in the zone of inhibition diameter (mm); both are listed in Table 2.4. The interpretive criteria of breakpoint and zone diameter interpretive standards (mm) for *Staphylococci* are also listed in the Table 2.4. The susceptibility of *S. leei* to each antibiotic is determined by comparing to the interpretive standards. The result shows that

S. leei is susceptible to all antibiotics except ampicillin, nalidixic acid, penicillin, sulfisoxazole, and tetracycline.

The susceptibility of *S. leei* to these antibiotics was compared to *H. pylori* to these antibiotics except for ampicillin/sulbactan, cefazolin, cefdinir, ceftriaxone, clindamycin, grepafloxacin, imipenem, levofloxacin, norfloxacin, oxacillin, piperacillin, sulfonamides, synercid, ticarcillin/K clavlante, trimethoprim/sulfamethoxazole and trovafloxacin. The available data for the susceptibility of *H. pylori* to antibiotics was obtained from previous reports (Blaser, 1987, McNutly and Dent, 1988, and Goodwin, 1997). The comparison is shown in Table 2.5. *S. leei* and *H. pylori* both show resistant to nalidixic acid, and both show susceptible to amoxicillin/K clavulanate, cefotaxime, cefoxitin, cefuroxime, cephalothin, chloramphenicol, ciprofloxacin, clarithrimycin, erythromycin, furazolidone, gentamicin, kanamycin, nitrofurantoin, ofloxacin, rifampin. However *S. leei* is susceptible to vancomycin and trimethoprim, but *H. pylori* is resistant to them; *S. leei* is resistant to ampicillin, penicillin, and tetracycline, but *H. pylori* is susceptible to them.

Most used for *H. pylori* are amoxicillin and clarithromycin. The two antimicrobial agents are also good to use for treatment of the potential pathogenesis of *S. leei* because *S. leei* is susceptible to the both antimicrobial agents. *S. leei* is susceptible to vancomycin and trimethoprim, but the *H. pylori* is not; this suggests that the two antimicrobial agents can be used not only for the treatment of *S. leei* but also to distinguishing infection with *S. leei* and *H. pylori*. *S. leei* is resistant to ampicillin, penicillin, and tetracycline, but the *H. pylori* is susceptible to them; this possibly explains the failure of treatment for presumed *H. pylori* infection to cure some patients.

The organism identification of *S. leei* using the Pos Breakpoint Combo Panel

shows that *S. leei* 99.9% close to *S. saprophyticus*. This result further confirms that the phylogenic test based on the 16S rRNA sequence, which shows that *S. leei* is closely matched to *S. saprophyticus* with a sequence difference of only 0.03%.

4. Coagulase Test of *S. leei*

After 1 hour incubation at 37^0C, loose clots were formed in the tube of *S. aureus*; after 4 hours incubation at 37^0C, loose clots were formed in the tube of *S. leei*; after 24 hours incubation at 37^0C, loose clots were not seen in the tube of *S. epidermidis*. This shows that the *S. leei* is coagulase positive, and it is a pathogen strain.

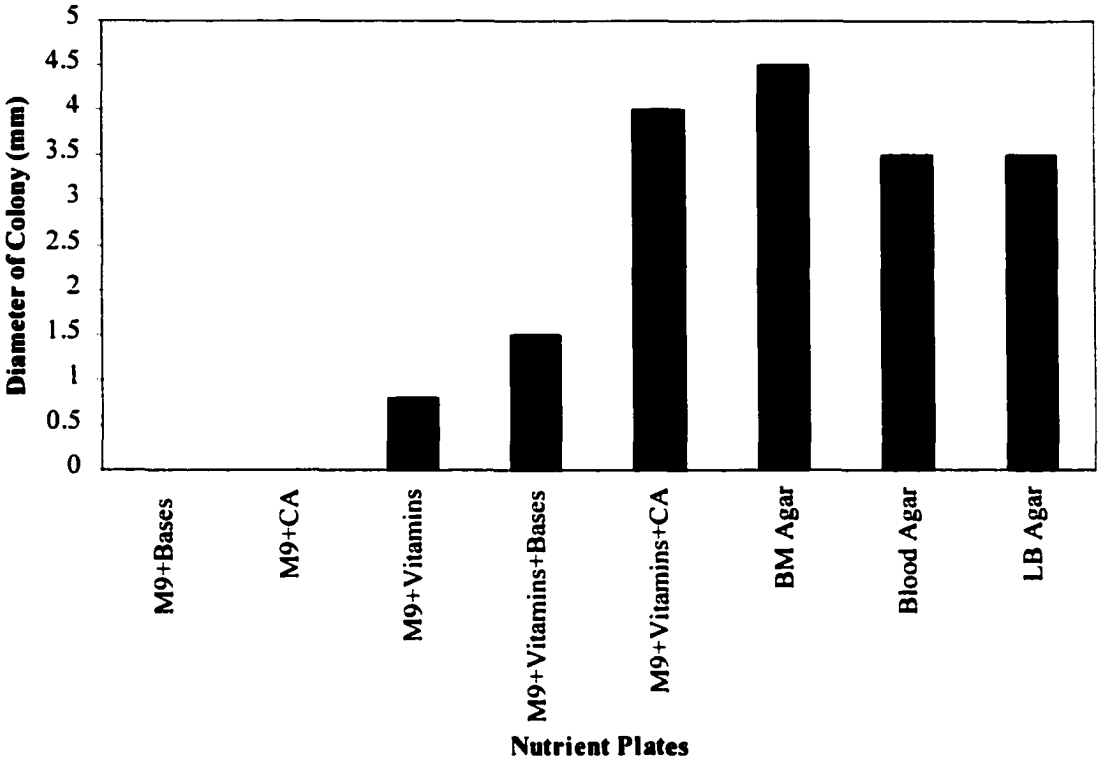


Figure 2.1. *Staphylococcus leei* growth on rich media and M9 media with different supplements. The diameter of colony on each plate was measured after three days of incubation at 37^0C.

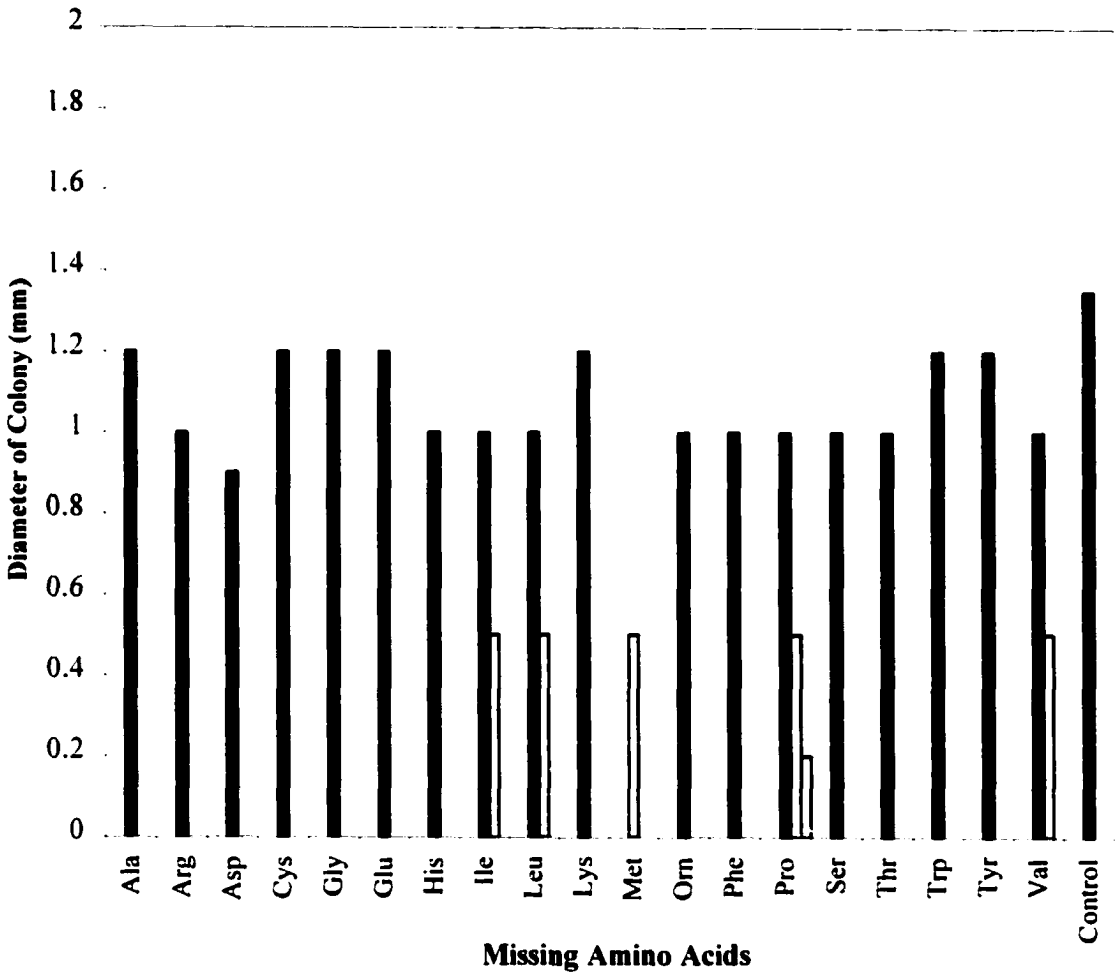


Figure 2.2. *Staphylococcus leei* growth on the plates of M9 supplied with all nutrients only missing one amino acid, the diameter of the colony on each plate was measured after two days of incubation at 37^0C.

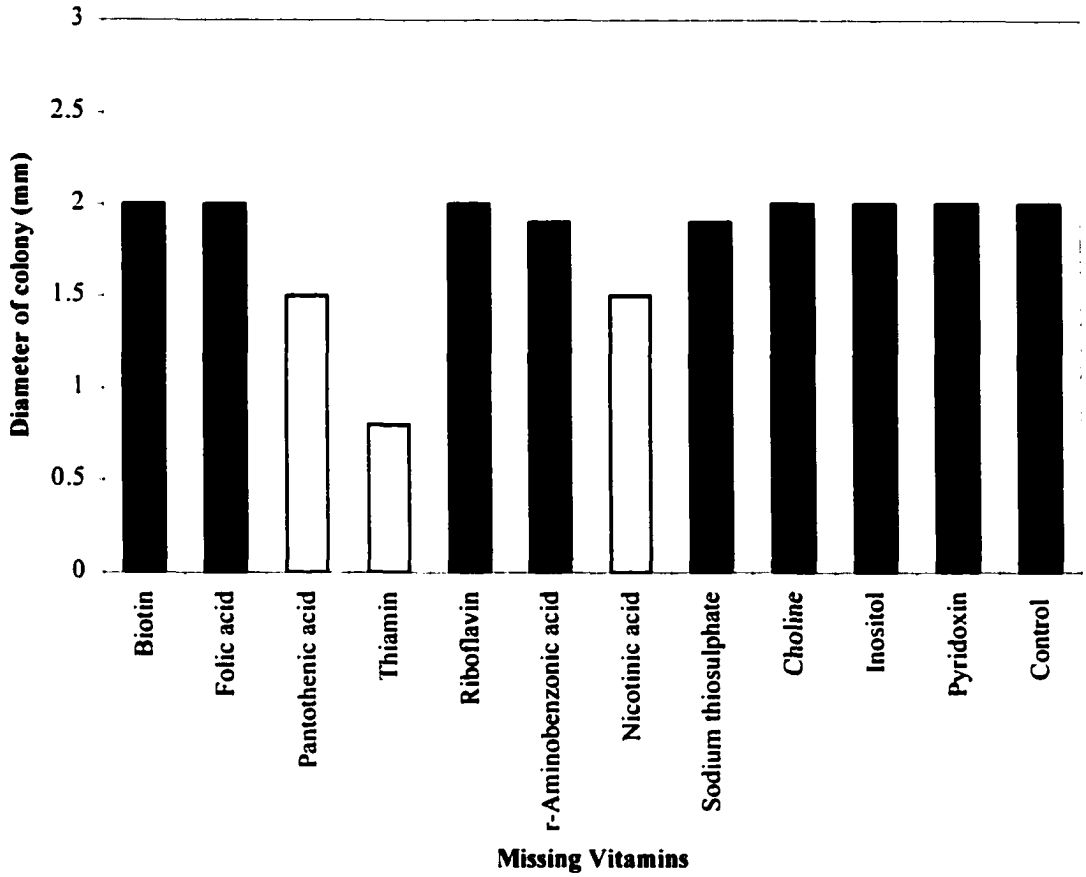


Figure 3.3. *Staphylococcus leei* growth on the plates of M9 supplied with 1% casamino acid, and all vitamins only missing one vitamin. The diameter of the colony of each plate was measured after three days of incubation at 37^0C.

Table 2.1. Stock Solutions of Individual Nutritional Supplements (100 x)

Nutrients	Concentration (mg/ml)	To Dissolve	Source (Cat. No.)
A. Bases			
Adenine	5	heat	Calbiochem (1152)
Cytosine	5		Calbiochem (2510)
Guanine	5	HCl	Calbiochem (3700)
Hypoxasnthine	5	heat	Sigma (H9377)
Thymine	5	heat	Calbiochem (6090)
Uracil	5	heat	Calbiochem (6630)
B. Vitamins			
Biotin*	0.001	heat	Sigma (B4501)
Choline	1		Sigma (C1879)
Folic acid	0.05	heat	Sigma (F7876)
Inositol	0.5		Sigma (I5125)
Nicotinic acid	0.05		Sigma (N4126)
P-Aminobenzoic *	0.05		Sigma (A9878)
Pantothenic acid *	0.05		Sigma (P2250)
Pyridoxin	0.05		Sigma (P5669)
Riboflavin	0.25	heat	Sigma (R4500)
Sodium thiosulphate	5		Sigma (S1648)
Thiamin	0.001		Sigma (T4625)
C. Amino Acids			
Alanine	10		Calbiochem (1250)
Arginine	10		Calbiochem (1820)
Aspartic acid **	10	KOH	Calbiochem (18905)
Cysteine	10		Calbiochem (2430)
Glutamic acid **	10	NaOH	Calbiochem (3510)
Glycine	10		Sigma (G7126)
Histidine	10		Calbiochem (3810)
Isoleucine	10		Calbiochem (4160)
Leucine	10		Calbiochem (4330)
Lysine	10		Calbiochem (4400)
Methiomine	10		Calbiochem (4500)
Ornithine	10		Calbiochem (4980)
Phenylalanine	10		Calbiochem (5202)
Proline	10		Sigma (P0380)
Serine	10		Calbiochem (5640)
Threonine	10		Calbiochem (6034)
Tryptophan **	10		Calbiochem (65405)
Tryosine **	10	HCl	Calbiochem (6570)
Valine	10		Calbiochem (6760)

Note: * Storage at 4^0C, ** Sterilized by filtration

Table 2.2. Genetic Relationship of *Staphylococcus leei* with Other Bacterial Species Based on 16S rRNA Sequence Analysis

Alignment: *Staphylococcus leei*

Difference (%)[1]	Primer[2]	Close match[3]
0.03 %	1548	*Staphylococcus saprophyticus saprophyticus*[4]
0.26 %	1548	*Staphylococcus xylosus*
0.78 %	1548	*Staphylococcus cohnii urealyticus*
0.90 %	1548	*Staphylococcus gallinarum*
0.97 %	1548	*Staphylococcus cohnii cohnii*
0.97 %	1548	*Staphylococcus arlettae*
0.97 %	1548	*Staphylococcus equorum*
1.33 %	1548	*Staphylococcus haemolyticus*
1.61 %	1548	*Staphylococcus pasteuri*
1.65 %	1548	*Staphylococcus warneri*
23.3 %	1548	*Helicobacter pylori*

Notes:

1. % Difference – Percent difference between the sample and the close match.
2. Prime – Primer used for PCR correspond to *E. coli* 16S r RNA positions
3. Close match – Close match to sample when aligned in a pairwise manner against the MicroWeq Database and GenBank Database
4. Closest matched species

Table 2.3. Antibiotic Used for the Test of Susceptibility of *Staphylococcus leei*

Antimicrobial Agents	Breakpoint Combo Panel		Zone Diffusion Disc	
	Abbr.	Conc. (μg/ml)	Abbr.	Amount (μg)
Amoxicillin/K Clavulanate	Aug	4/2	AmC-30	30
Ampicillin	Am	0.25, 2-8	AM-10	10
Ampicillin/Sulbactam	A/S	8/4-16/8		
Cefazolin	Cfz	8-16		
Cefdinir	Cdn	1-2		
Cefotaxime	Cft	8, 32		
Cefoxitin			FOX-30	30
Ceftriaxone	Cax	8, 32		
Cefuroxime	Crm	8-16		
Cephalothin	Cf	8-16	CF-30	30
Chloramphenicol	C	8-16		
Ciprofloxacin	Cp	1-2	CIP-5	5
Clarithromycin				15
Clindamycin	Cd	0.5, 2	CC-2	2
Erythromycin	E	0.25-0.5	E-15	15
Furazolizone			FX-100	100
Gentamicin	Gm	4-8	GM-10	10
Grepafloxacin	Grx	1-2		
Imipenem	Imp	4-8		
Kanamycin			K-30	30
Levofloxacin	Lvx	2-4		
Nalidixic acid			NA-30	30
Nitrofurantoin	Fd	32-64	F/M-300	300
Norfloxacin	Nxn	4-8		
Ofloxacin	Ofl	2-4		
Oxacillin	Ox	0.25, 1-2		
Penicillin	P	0.03, 0.12, 2, 8	P-10	10 units
Piperacillin			PIP-100	100
Rifampin	Rif	1-2	RA-5	5
Sulfisoxazole			G-0.25	250
Synercid	Syn	1-2		
Tetracycline	Te	4-8	TE-30	30
Ticarcillin/K Clavulanate	Tim	8/2		
Trimethoprim			TMP-5	5
Trimethoprim/Sulfamethoxazole	T/S	2/38		
Trovafloxacin	Tva	2-4		
Vancomycin	Va	4, 16	Va-30	30

Table 2.4. Antibiotic Susceptibility of *Staphylococcus leei*

Antimicrobial Agents	Breakpoint Combo Panel (μg/ml)			Zone Diffusion Disc (mm)		
	Breakpoint	Susceptible*	Result	Zone	Susceptible*	Result
Amoxicillin/K Clavulanate	≤4/2	≤4/2	S	30	≥20	S
Ampicillin	≤0.25	≤0.25	R	24	≥29	R
Ampicillin/Sulbactam	≤8/4	≤8/4	S			
Cefazolin	≤8	≤8	S			
Cefdinir	≤1	≤1	S			
Cefotaxime	≤8	≤8	S			
Cefoxitin				21	≥18	S
Ceftriaxone	≤8	≤8	S			
Cefuroxime	≤8	≤8	S			
Cephalothin	≤8	≤8	S	30	≥18	S
Chloramphenicol	≤8	≤8	S			
Ciprofloxacin	≤1	≤1	S	21	≥21	S
Clarithromycin				20	≥18	S
Clindamycin	≤0.5	≤0.5	S	19	≥19	S
Erythromycin	≤0.25	≤0.5	S	21	≥21	S
Furazolizone				18		S
Gentamicin	≤4	≤4	S	21	≥15	S
Grepafloxacin	≤1	≤1	S			
Imipenem	≤4	≤4	S			
Kanamycin				20	≥18	S
Levofloxacin	≤2	≤2	S			
Nalidixic acid				0	≥19	R
Nitrofurantoin	≤32	≤32		20	≥17	S
Norfloxacin	≤4	≤4				
Ofloxacin	≤2	≤2	S			
Oxacillin	≤0.25	≤0.25	S			
Penicillin	0.12	≤0.12	R	27	≥29	R
Piperacillin				25	≥18	S
Rifampin	≤1	≤1	S	28	≥20	S
sulfisoxazole				0	≥17	R
Synercid	≤1	≤1	S			
Tetracycline	>8	≤4	R	9	≥23	R
Ticarcillin/K Clavulanate	≤8	≤8/2	S			
Trimethoprim				22	≥16	S
Trimethoprim/Sulfamethoxazole	≤2/38	≤2/38	S			
Trovafloxacin	≤2	≤2	S			
Vancomycin	≤4	≤4	S	12	≥12	S

* The standards are obtained from the NCCLS

Table 2.5. Comparison of Antibiotic Sensitivity of *S. leei* and *H. pylori*

Antimicrobial Agents	*S. leei*	*H. pylori*
Amoxicillin/K Clavulanate	S	S
Ampicillin	R	S
Ampicillin/Sulbactan	S	
Cefazolin	S	
Cefdinir	S	
Cefotaxime	S	S
Cefoxitin	S	S
Ceftriaxone	S	
Cefuroxime	S	S
Cephalothin	S	S
Chloramphenicol	S	S
Ciprofloxacin	S	S
Clarithromycin	S	S
Clindamycin	S	
Erythromycin	S	S
Furazolizone	S	S
Gentamicin	S	S
Grepafloxacin	S	
Imipenem	S	
Kanamycin	S	S
Levofloxacin	S	
Nalidixic acid	R	R
Nitrofurantoin	S	S
Norfloxacin	S	
Ofloxacin	S	S
Oxacillin	S	
Penicillin	R	S
Piperacillin	S	
Rifampin	S	S
Sulfisoxazole	R	
Synercid	S	
Tetracycline	R	S
Ticarcillin/K Clavlanate	S	
Trimethoprim	S	R
Trimethoprim/Sulfamethoxazole	S	
Trovafloxacin	S	
Vancomycin	S	R

CHAPTER 3. UREASE PURIFICATION

Strategy of Experiment

The BioCAD Workstation was used for purification of urease of *S. leei*. This Workstation is widely used by biotechnology and pharmaceutical companies for "Good Manufacturing Processes" purifications required by the FDA and for many other diagnostics and therapeutics processes. The initial conventional chromatography method (Lee and Calhoun, 1997; Christinas *et al.;* 1991, and Schafer and Kaltwasser, 1994) for the purification of the urease of *S. leei* and the methods for purification of ureases in other bacteria were adapted to PerSeptive Biosystems BioCAD Perfusion HPLC Workstation. A standard chromatographic approach to devise a rapid and efficient purification strategy was used, and the purification scheme is shown in Figure 3.1.

Materials and Methods

Cell Growth and Lysis. *S. leei* was grown in LB media containing 10 g tryptone, 5 g yeast extract, 10 g NaCl per liter, pH 7.2 at 37^{0}C. Cell lysis was carried out in Bioprocessing Resource Center, Inc., at Pennsylvania State University, State College, PA. A 100 liter culture incubated in the reactor overnight at 37^{0}C produced an OD = 6.7 yielding about 2.7 g/L of wet cells. The cells were resuspended in 4000 ml of lysis buffer [20 mM Tris-HCl, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1 mM nickel sulfate, pH 7.8] and were lysed in a Dynomill for 15 min with 0.5 mm glass beads at 20^{0}C. The total cell lysate was 3.7 L.

Centrifugation and hollow fiber filtration. A portion of 700 ml lysate was centrifuged for 40 min at 18,000 rpm and the supernatant was collected and clarified using a 0.2 μm hollow fiber (Spectrum Cat. No. M12M-260-01N, Laguna Hills, CA),

designed for separating bacteria and large size molecules. After filtration, the filtrate was collected and first tested for contamination of *S. leei*. The filtrate was inoculated onto LB plate and YPD plates (20 g peptone, 10 g yeast extract, 20 g glucose, and 20 g agar per liter) and incubated at 37^0C and 32^0C for three days respectively. Then the filtrate was applied to 10 K Hollow Fiber (Spectrum Cat. No. M15S-260-01N, Laguna Hills, CA) to separate smaller size protein molecules (less than 10 kDa), and desalted using the diafiltration procedure with four volumes of 20 mM Tris-HCl buffer containing 1 mM EDTA, 0.1 mM NiSO4, pH 7.4 at 4^0C. The retentate was used as starting material for urease purification.

Chromatography purification of urease. A portion of 86 ml retentate derived from the 10K Hollow fiber filtration was applied at 4 ml/min to Q-Sepharose Fast Flow anion exchange chromatography column (20 mmD × 300mmL, 90 ml) (Amersham Pharmacia Biotech, Cat. No.17-0510-01) previously equilibrated with 20 mM Bis-Tris Propane buffer which contained 1 mM EDTA, 0.1 mM $NiSO_4$, pH 7.4 at 4^0C. This buffer was filtered using 0.45 μm HA membrane filter (Cat. No. HAWP04700, Millipore) before apply to the column. All buffers used following for this purification were filtered using this membrane. Urease was eluted using a linear gradient from 0 to 1 M KCl for 20-column volumes (1,800 ml). After assaying for urease activity, the fractions containing urease peak activities were pooled (306 ml). Ammonium sulfate was added to the pool to a final concentration of 1.7 M. The pool was then loaded at 15 ml/min to Poros HP2 hydrophobic interaction chromatography column (10 mmD × 190 mmL, 15 ml) (PerSeptive Biosystems, Cat. No. 1-4529-06. Framingham, MA) previously equilibrated with 20 mM Bis-Tris Propane buffer containing 1.7 M $(NH_4)_2SO_4$, 1 mM

EDTA, 0.1 mM $NiSO_4$, pH 7.4 at 4^0C. Urease was eluted in a linear gradient from 1.7 to 0 M of $(NH_4)_2SO_4$ for 20 column volumes (300 ml). Urease fractions obtained were pooled and concentrated to 3 ml using centrifugal filter device (Cat. No. UFV2BGC40, Millipore, Beford, MA). The pool of 3 ml (about 1 % column bed volume) was loaded to HiPrep Sephacryl S-300 column, (26 mmD x 600 mmL, 300 ml) (Amersham Pharmacia Biotech, Cat. No.17-1196-01). The separation range of this column is 10 to 1500 kDa. The column was equilibrated and eluted with 20 mM Bis-Tris Propane, 100 mM KCl, 1 mM EDTA and 0.1 mM $NiSO_4$, pH 7.0 at the rate of 1 ml/min. A HMW Gel filtration calibration kit which contained blue dextran 2000, thyroglobumin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), catalase (232 kDa) (Amersham Phamacia Biotheh, Cat. No. 17-0441-01, Piscataway, NJ) was used as calibrating standards. The fractions which contained the urease activity were pooled and concentrated to 3.5 ml, and then applied to Hydroxyapatite column (Cat No. 158-2000, BioRad, Hercules, CA). The column was previously equilibrated with 20 mM Bis-Tris Propane, pH 7.4 at 4^0C. The urease was eluted out with potassium phosphate buffer gradient from 0 to 100 mM, pH 7.0. The urease fractions were pooled and concentrated to 1.2 ml.

Urease activity assay. Urease activity was measured by quantitating the rate of ammonia release from urea. The ammonia released was monitored by the pH change with phenol red (phenol red assay) (Maeda *et al.*, 1993). A 10 µl sample was added to cuvette with 1 ml test solution containing 1 mM Tris-HCl, 0.2 M urea, 0.2 mM phenol red, pH 6.8, and mixed well. The absorbance increase (phenol red base form) was measured at wavelength of 560 nm within 1 min at room temperature on HITACHI spectrophotometer (Model U-3010, HITACHI, Tokyo, Japan). One unit of enzyme

activity is defined as the amount of enzyme required to convert one μmole of urea change in one minute at 37^0C.

Urease protein electrophoresis and staining. Denatured urease SDS-PAGE was performed with a linear gradient 4% to 20% polyacrylamide gel (Cat. No.161-2259, Bio-Rad, Hercules, CA,). Each sample was mixed with equal volume of sample buffer (Cat. No. 161-0737, Bio-Rad, Hercules, CA) containing 2.9 ml of deionized water, 1.0 ml of 0.5 M Tris-HCl, 2.0 ml of Glycerol, 1.6 ml of 10% SDS, 0.4 ml of β-mercaptoethanol, and 0.1 ml of 0.1% bromophenol blue. The sample with sample buffer was boiled for 5 minutes, and then loaded onto the gel. The electrode buffer (5x) contains 15 g of Tris base, 72 g of Glycine, and 5 g of SDS in 1 liter, pH 8.3. The gel was run at 200 volts for 40 minutes in electrophoresis chamber (Model SE250, Hoffer Scientific Instruments, San Francisco, CA). After electrophoresis, the gel was stained with Coomassie blue for 30 min on a shaking platform with staining solution (40% Methanol, 10% Acetic acid, and 0.1% Coomassie blue R-250), and then destained with destaining solution (40% Methanol, and 10% Acetic acid) on a shaking platform with several changes of destaining solution until the background was clear. The method for running native urease PAGE was similar to running the SDS PAGE, the difference was that the sample buffer and electrode buffer did not contain SDS, and 2-mercaptoethanol (Cat. No. 161-0738 and 161-0734, Bio-Rad, Hercules, CA), and the sample was not boiled. The *in situ* phenol red pH indicator staining method (Lee and Calhoun 1997, Shaik-M *et al.*, 1980) was used for native urease protein staining. After electrophoresis the gel was washed twice for 30 min with 20 mM sodium acetate buffer, pH 5.0. The gel was further equilibrated for 5 min in the solution containing 1 mM EDTA, 0.1 mM $NiSO_4$ and 1 mM phenol red, pH

3.0. The enzymatic reaction was initiated by subsequently transferring the gel into a phenol red staining solution containing 1 mM phenol red, 100 mM urea, 1 mM EDTA, 0.1 mM $NiSO_4$, pH 3.0. The areas on the gel containing urease activity were visible as increasingly dark red colored bands on a yellow background.

Results and Discussion

The cell lysate of *S. leei* was clarified by centrifugation and filtration using 0.2 μm hollow fiber to filter out any residual bacterial cells and cell debris. After this step, the filtrate was tested for contamination of *S. leei*, and no bacterial growth was found. This indicates that after the 0.2 μm hollow fiber filtration, the sample was safe to work with for the next purification steps. After desalting using diafiltration with 10 K hollow fiber, the conductivity of the cell lysate was reduced from 7.8 to 3.1 mS. At the low salt condition, the urease was bound to the Q-Sepharose column, and eluted off at conductivity of 50 mS (about 0.5 M KCl) in a small peak area of OD_{280} (Figure 3.2). Three large peaks of OD_{280} are seen before the urease, and there are also several OD_{280} peaks of after the urease. Even though there is no single peak of OD_{280} for the urease, the first Q-Sepharose column was effective for capturing the urease and separating large amount of contaminants. The 306 ml of sample eluted from the Q-Sepharose column was added ammonia sulfate to 1.7 M. The high salt sample was bound to the Poros HP2 column and was eluted off at conductivity of 100 mS (about 0.8 M ammonia sulfate). The urease was found in a single peak of OD_{280} (Figure 3.3). In Sephacryl S-300 column, the urease protein was eluted off the column first. This is because of the large size of the urease (Figure 3.4). In Hydroxyapatite column, the urease was eluted off at about 50 mM phophate buffer (Figure 3.5). A sample of each purification step was

saved, and the samples were run a 4-20% SDS-PAGE, followed by Coomassie blue staining. The final pool of Hydroxyapatite column showed three bands (three subunits of the urease, 65, 20 and 12 kDa) (Figure 3.6). The 20 kDa band on the SDS-PAGE gel was cut off and analyzed for the partial amino acids sequences via mass spectrum by Protein Center of Rockefeller University. The partial amino acid sequence is EPGDEKEVQLVEY. This sequence is compared with the amino acid sequence of beta subunit of *S. leei* urease (see urease sequencing section) and shown to be 100 % identical. This result confirms that the purified protein is urease of *S. leei*. Each sample pool was also run on a native PAGE and followed by *in situ* native stain. Each sample pool showed a red color on a yellow background (Figure 3.7). This also indicates that the purified protein after Hydroxyapatite column is the urease of *S. leei*. With the standard molecular weight standard curve analysis carried out on Sephacryl S300 column, the molecular weight of urease of *S. leei* is 480 kDa. The specific activity, purification fold, and yield after centrifugation, filtration and four-column purification protocol are 731, 98, and 18% respectively (Table 3.1). The total urease protein obtained from the 86 ml sample is 0.42 mg. This successful purification method was used for the total 3.7 liter of cell lysate from 100 liter cells culture, and the total obtained purified urease is 16 mg.

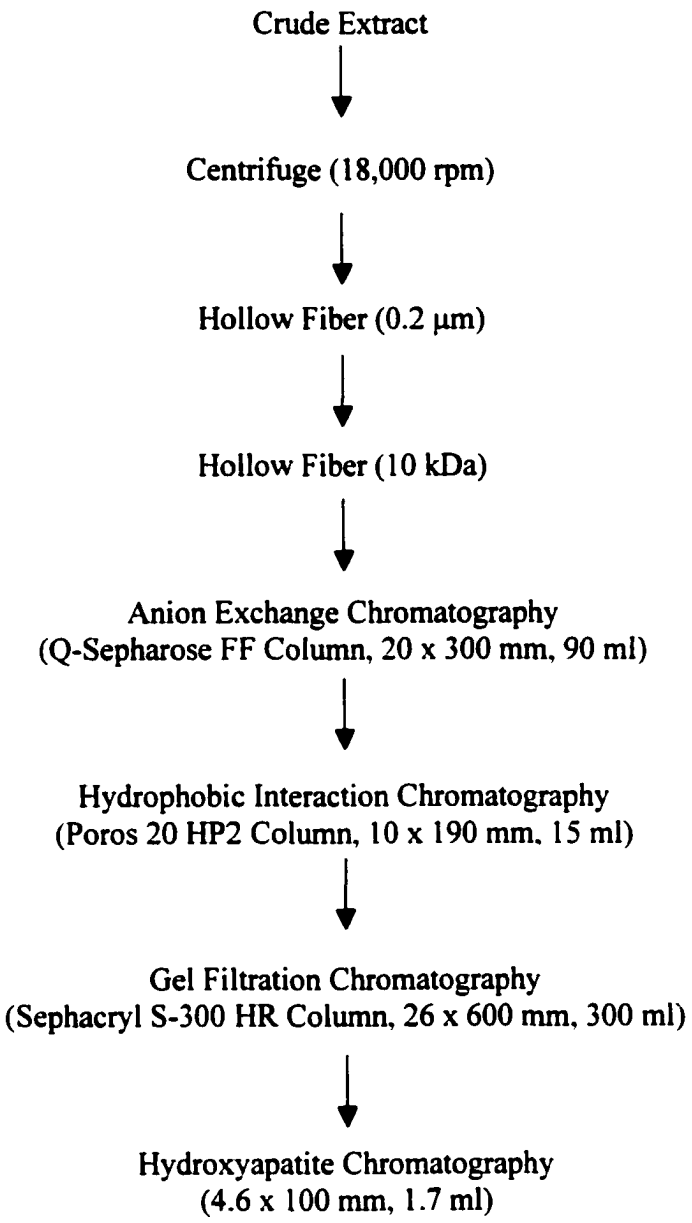


Figure 3.1 Scheme of Purification of Urease of *Staphylococcus leei*

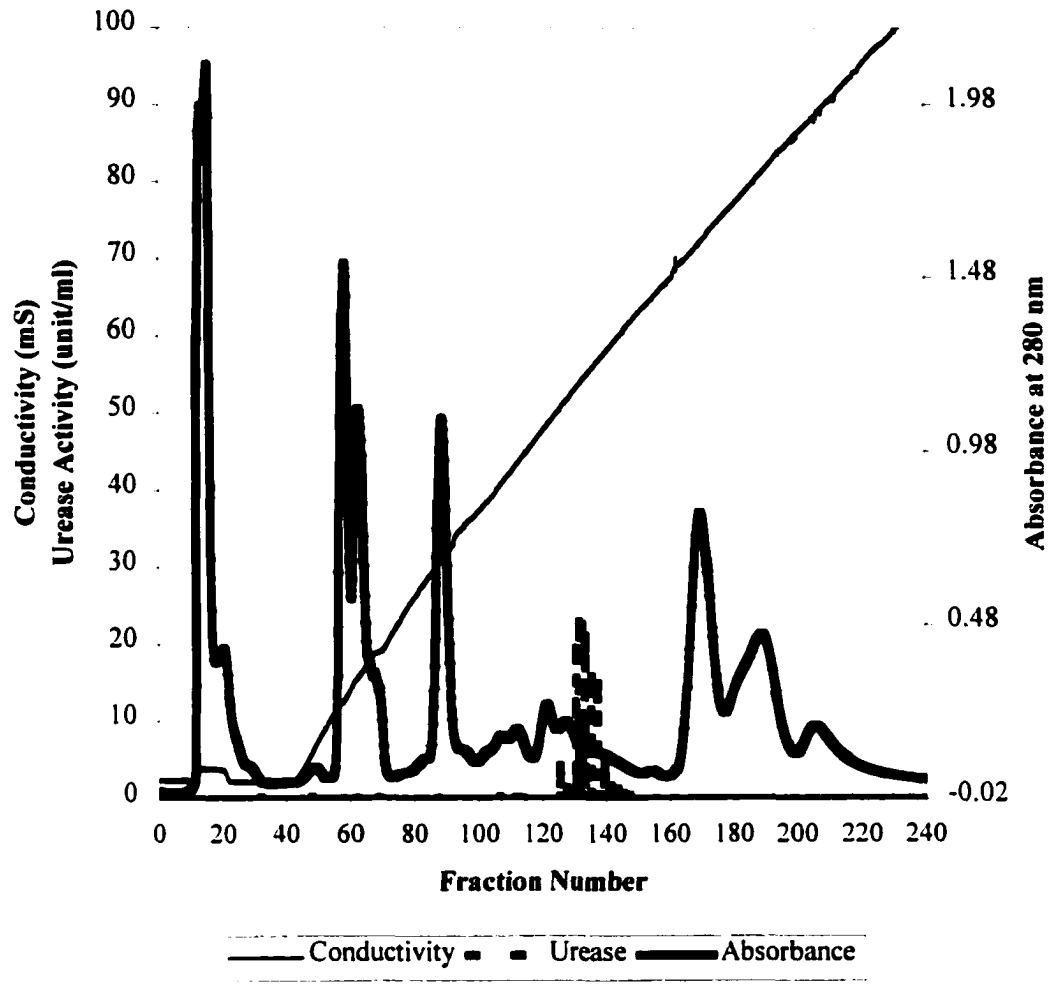


Figure 3.2. Profile of Q-Sephorose column for purification of urease of *S. leei.*

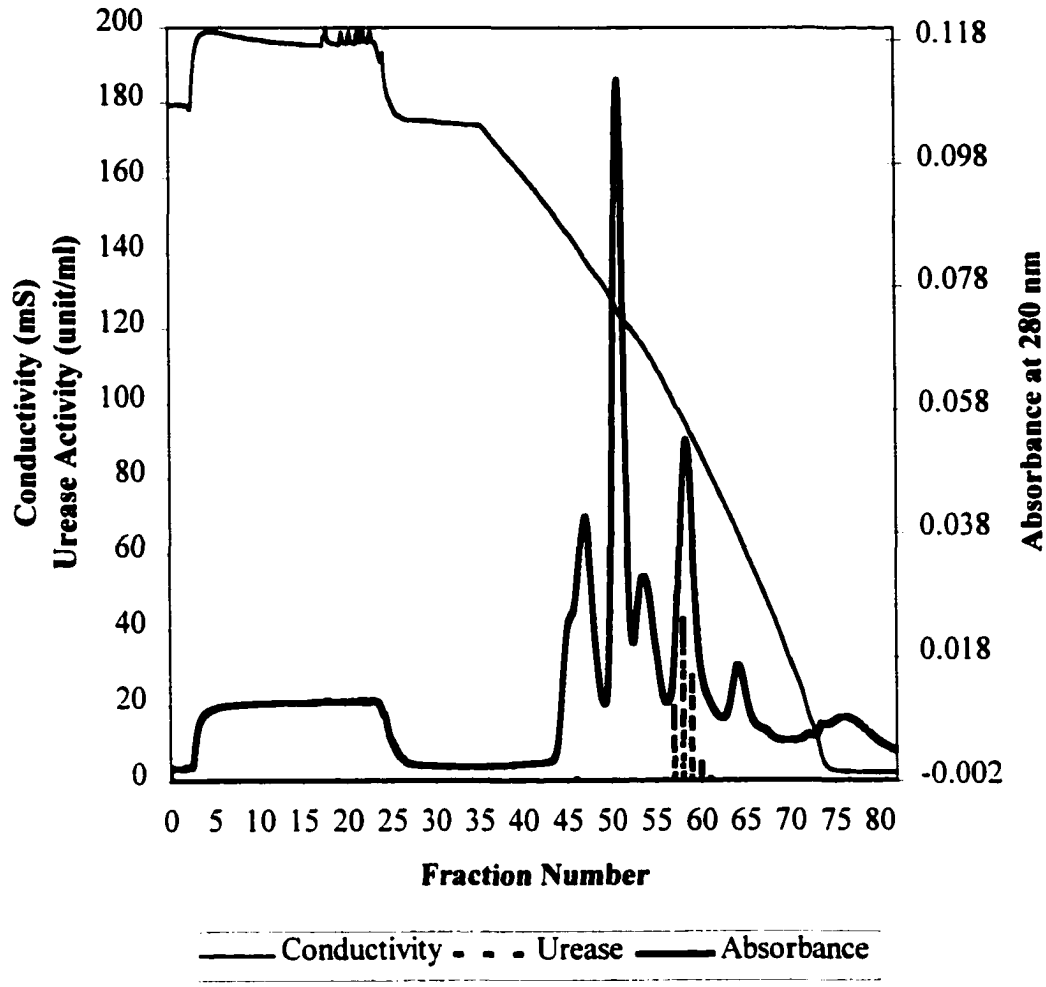


Figure 3.3. Profile of Poros HP2 column for purification of urease of *S. leei.*

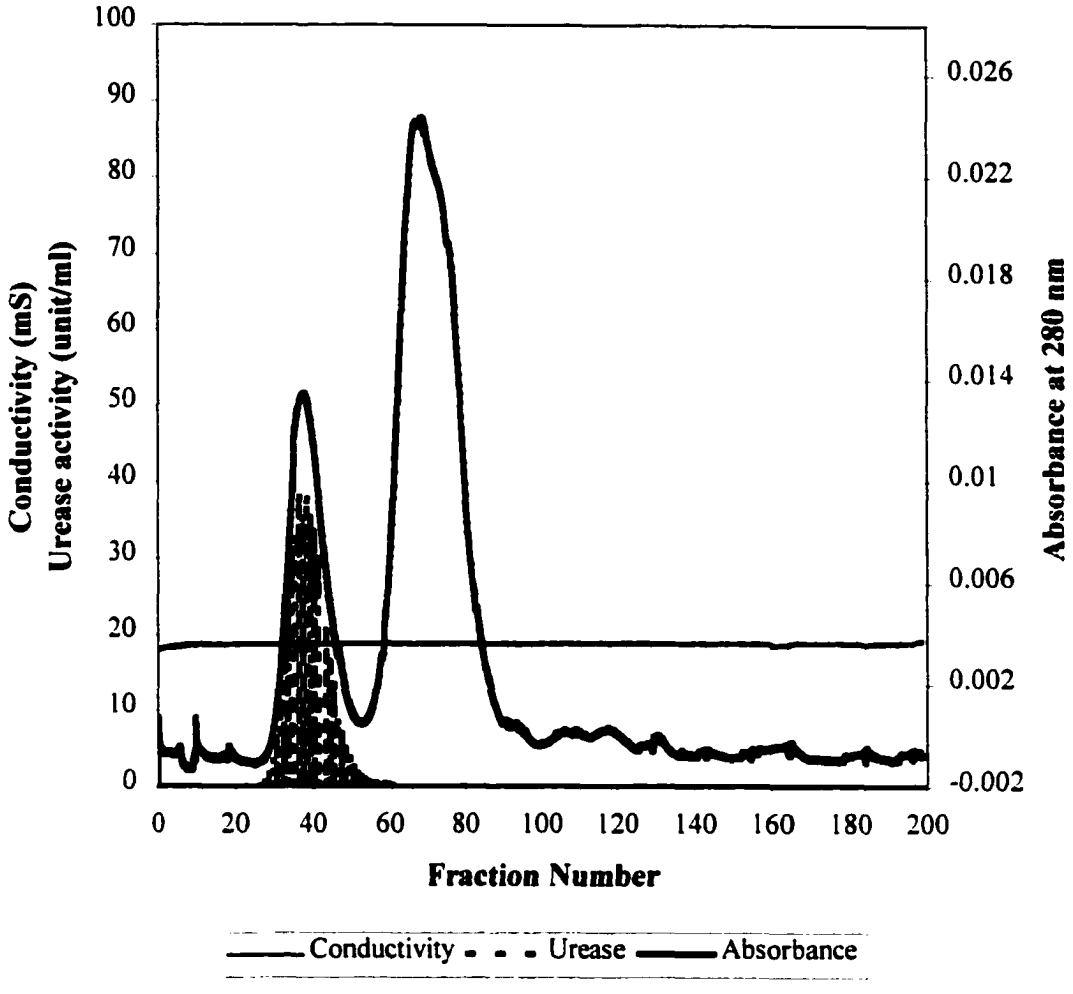


Figure 3.4. Profile of Sephacryl 300 column for purification of urease of *S. leei.*

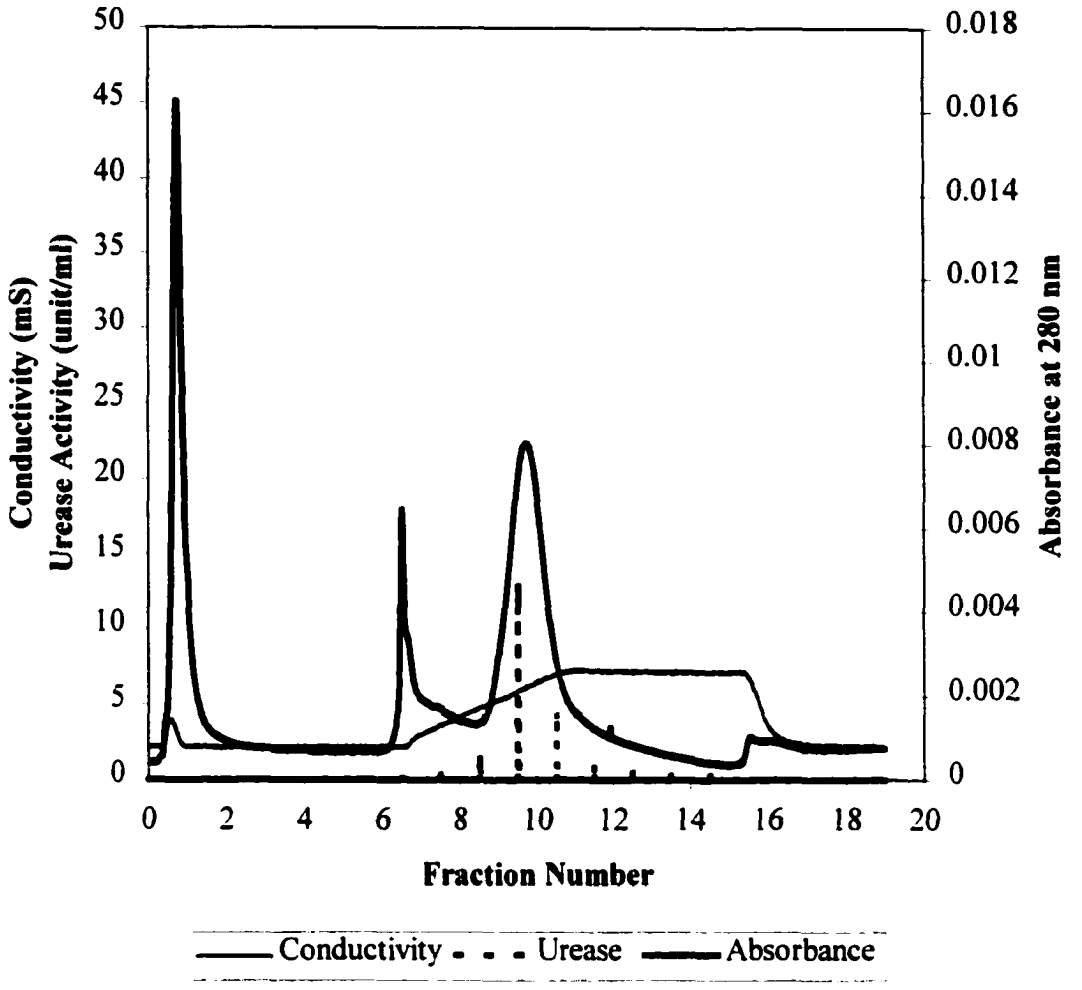


Figure 3.5. Profile of Hydroxyapatite column for purification of urease of *S. leei*

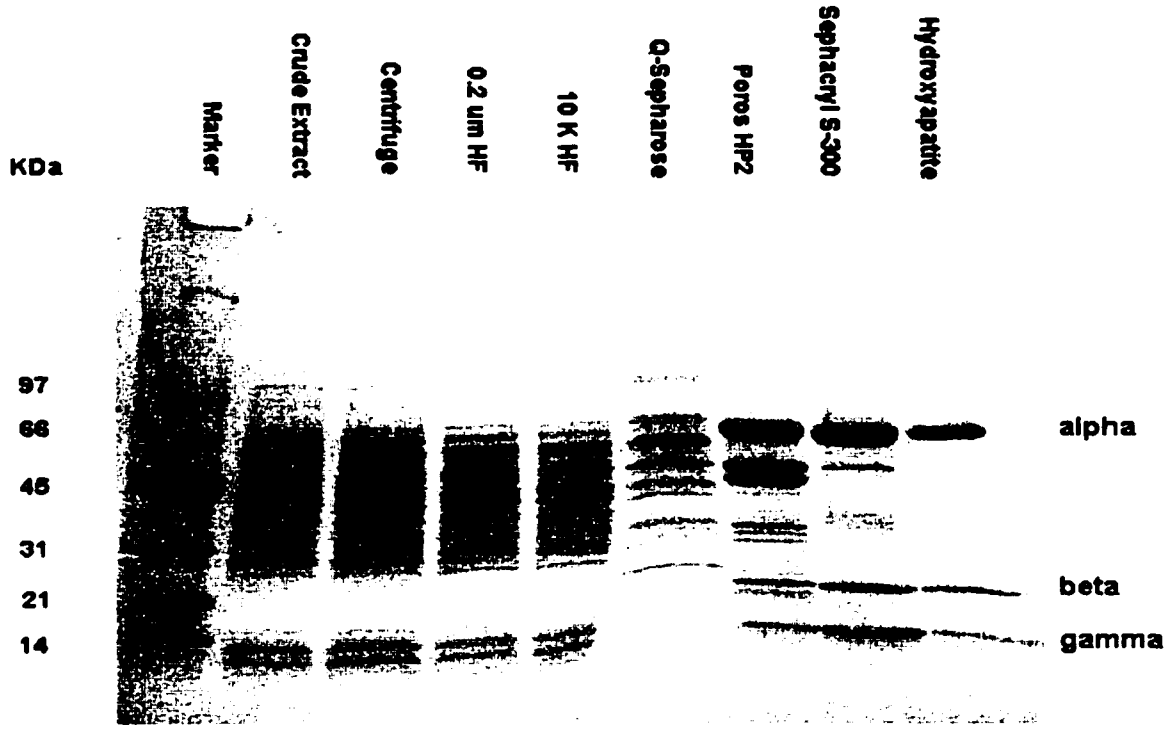


Figure 3.6. SDS PAGE gel for each step of urease purification, the sample from each step was run on the 4-20% SDS PAGE and then stained with coomassie blue. The lane after Hydroxyapatite column showed three bands, 65, 20, and 12 kDa, which are the three subunits of the urease of *S. leei*, alpha, beta, and gamma respectively.

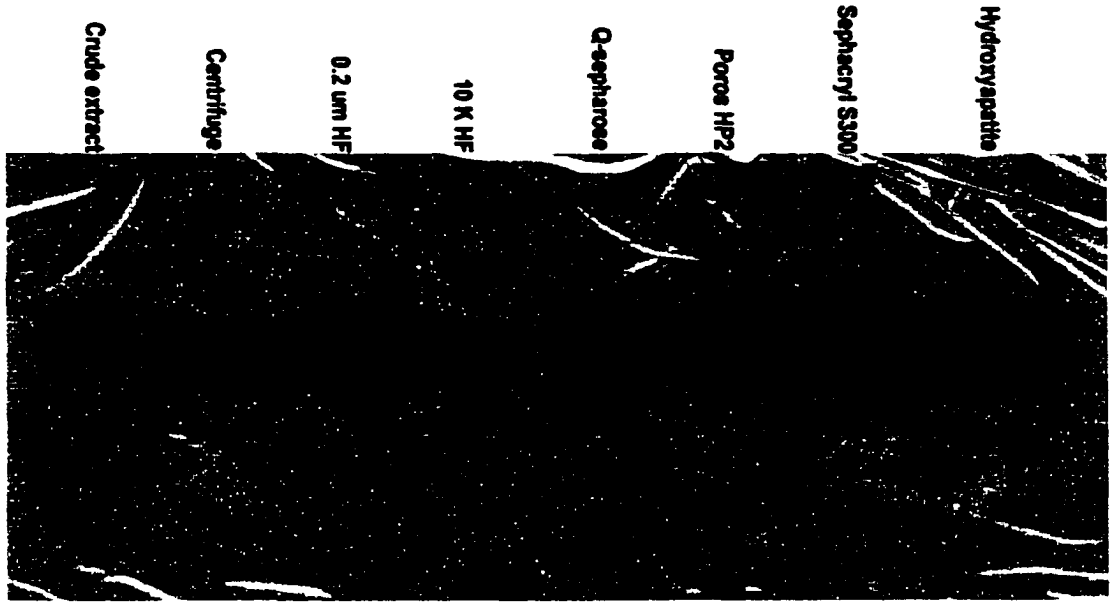


Figure 3.7. Native stain of the urease of *S. leei*. The sample of the each purification step was first run on the 4-20% PAGE without SDS and β-mercaptoethanol. Then the gel was stained with phenol red native staining. Each sample showed the urease activity with red color band on the yellow background.

Table 3.1. Purification of Urease of *Staphylococcus leei*

Step	Total Activity (unit)	Total Protein (mg)	Specific Activity (unit/mg)	Purification (fold)	Yield (%)	Volume (ml)
Crude Extract	1,704	228	7.5	1.0	100	86
Centrifuge	1,610	166	9.7	1.3	94.5	84
0.2 μm Hollow Fiber	1,753	166	11	1.4	103	82
10 K Hollow Fiber	1,990	149	13	1.8	117	90
Q-Sepharose Column	1,241	56	22	3.0	73	306
Poros HP2 Column	1,120	2.5	455	61	66	3.0
Sephacryl 300 Column	635	0.97	655	87	37	3.5
Hydroxyapatite	308	0.42	731	98	18	1.2

CHAPTER 4. UREASE GENE CLONING, SEQUENCING AND EXPRESSION OF *S. LEEI* IN *E. COLI*

Strategy of Experiment

The urease gene of *S. leei* was first obtained using PCR method. The urease gene is a family, and as a family they are all related to each other. As more and more urease genes are cloned and sequenced, we gather information to determine homology of urease genes of different species from the GenBank database. The primers for PCR were designed based on the conserved DNA sequence regions comparing several different species, and the genomic DNA of *S. leei* as a template for the PCR was isolated using genomic DNA isolation kit from Promega. The PCR products were cloned to pCR 2.1 TOPO vector from Invitrogen.

The sequence of the urease gene of *S. leei* in the pCR 2.1 TOPO vector was completed using 310 Genetics Analyzer from Applied Biosystems. The urease gene sequence of *S. leei* was then analyzed and compared with other urease genes from different species.

Plasmid pET23a (Novagen) was chosen as an expression vector to clone the *ure ABC* genes of *S. leei* as an *Eco*RI fragment to the *Eco*RI site of the plasmid. This plasmid contains a T7 promoter and expression is in BL21 (DE3) cell so that IPTG can be used to induce the T7 RNA polymerase.

Materials and Methods

Urease Gene Cloning

Isolation of genomic DNA of *S. leei*. 0.8 g of cell pellet of strain *S. leei* (from Bioprocessing Resource Center, Inc. at Pennsylvania State University, State College, PA)

was suspended in 1,920 µl of 50 mM EDTA, pH 8.0 and divided into four microtubes, each 480 µl. 60 µl of Lysozyme (10 mg/ml, Cat. No. L7651, Sigma, St. Louis, MO) and 60 µl of lysostaphin (1 mg/ml, Cat. No. L7386, Sigma, St. Louis, MO) were added to each microtube and incubated at 37^0C for 60 min. Then the genomic DNA was isolated using Wizard Genomic DNA isolation Kit (Cat. #A1120, Promega, Madison, WI) according to the manufacturer's instructions. The four tubes of DNA were pooled together in 1 ml volume. The concentration was 0.26 µg/µl, and the total genomic DNA was 260 µg.

Primers design. Because *S. leei* is closely related to *S. xylosus*, and since the complete sequence of urease *ureABC* gene of *S. xylosus* is known (Jose *et al.*, 1994, GenBank Accession No. X74600), we used it to design a pair of primers. One pair of primers was designed using the initial 28 base pairs and last 28 base pairs of *S. xylosus* urease *ureABC* gene. These two primers are named primer 1 and 2. With this pair of primers the completed urease *ureABC* sequence of *S. leei* was possible to obtain. The other pair of primers was designed based on the conserved amino acid and nucleotide sequences of urease from different bacterial species, *S. xylosus* (Jose *et al.*, 1994), *P. mirabilis* (Jones and Mobley, 1989), *K. aerogenes* (Mulrooney and Hausinger, 1990), and *Bacillus* sp strain TB-90 (Maeda *et al.*, 1994). The two regions of most homology were used as one pair of primer, named primer 3 and 4. These four primers were synthesized by Life Technology Company (Rockville, MD) (Table 4-1).

Producing PCR products. Two PCR reactions were performed, one was with primer 1 and 2, and the other was with primer 3 and 4. The genomic DNA of *S. leei* was the template for both PCR reactions. Each reaction mixture (50 µl) contained 5 µl of *S.*

leei genomic DNA (0.1μg/μl), 5 μl of 10 x PCR buffer, 1 μl of 50 mM dNTPs, 2 μl of 0.1 μM of primer each, and 2.5 units of Taq DNA polymerase. The mixture was first denatured at 94^{0}C for 30 seconds, then amplified by 30 cycles, each cycle comprising a 45 seconds denaturing step at 94^{0}C, a 45 seconds annealing step at 64^{0}C, and a 180 seconds elongation step at 72^{0}C. 10 μl of each PCR mixture was subjected to 1 % agarose gel electrophoresis for the detection of amplified DNA products.

Cloning PCR product. After Two PCR products were obtained (one is 2.4 kb with primer 1 and 2, the other is 1.3 kb with primer 3 and 4) (see result section), the next step was cloning them to pCR 2.1-TOPO vector in *E. coli* TOP10 cells using TOPO TA cloning kit (Cat. No. K4500-01, Invitrogen, Carlsbad, CA). The plasmids of 2.4 kb and 1.3 kb PCR products in pCR 2.1-TOPO vector were named pCC128 and pCC129 respectively. For each cloning, TOPO cloning reaction (6 μl) contained 2 μl of fresh PCR product, 1 μl of Salt Solution (1.2 M NaCl, 0.06 M $MgCl_2$), 1 μl of pCR 2.1-TOPO vector, and 2 μl of sterile water. The reaction was mixed gently and incubated for 5 minutes at room temperature. 2 μl of the TOPO cloning reaction was added into a vial of 50 μl of *E. coli* TOP10 competent cells and mixed gently, then placed on ice for 30 minutes. The cells were heated at 42^{0}C for 30 seconds without shaking, and then the tube was immediately transferred to ice. 250 μl of SOC medium (20 g tryptone, 5 g yeast extract, 0.5 g NaCl, 2.5 mM KCl, 10 mM $MgSO_4$, and 20 mM Glucose) was added to the tube in room temperature, and the tube was shaken (200 rpm) at 37^{0}C for 1 hour. 50 μl of the transformation reaction was plated to selective LB plates containing ampicillin (50 μg/ml) and pre-plated 40 μl of X-gal (40 mg/ml), and then the plates were incubated overnight at 37^{0}C. After overnight incubation, the positive clones were analyzed. 9

white colonies were taken from the plate of 2.4 kb PCR product cloning (328 white and 51 blue colonies), and 9 colonies were taken from the plate of 1.3 kb PCR product cloning (84 white and 296 blue colonies). The colonies taken as positive clone for both cloning were incubated overnight in 3 ml of LB medium containing 50 μg/ml ampicillin. The plasmids were isolated using Promega MiniPrep kit (Cat # A7141, Madison, WI), and analyzed by restriction analysis (digested with *Eco*RI). The digested plasmids were run on 1% agarose gel. Each clone was cultured for three generations on LB plates with 50 μg/ml ampicillin incubated at 37^0C for 24 hours.

Urease Gene Sequence

The plasmid pCC128 (contains the 2.4 kb PCR product) was used for sequencing the 2.4 kb PCR product. DNA sequencing was performed at the City College of New York RCMI DNA Center using the ABI prism BigDye terminator cycle sequencing ready reaction kit with AmpliTaq DNA polymerase and dye-labeled terminators (No. 4303152, Applied Biosystems, Foster City, CA). Both universal M13 forward and reverse primers (Cat. No. Q5391 and Q5401, Promega, Madison, WI) were used in the first sequencing assay (Table 4-1). The 2.4 kb DNA fragment was sequenced in both directions. The cycling sequence was performed in the tube containing 0.5 μg of pCC128 (2.4 kb PCR product / pCR 2.1-TOPO vector) DNA, 3.2 pmol primer, and 8 μl of terminator ready reaction mix, final volume was 20 μl. Total 25 cycles was run, and each cycle was 96^0C for 10 sec, 50^0C for 5 sec, 60^0C for 4 min. Then the sequencing was carried on 310 Genetics Analyzer (Applied Biosystems, Foster City, CA) and the sequence was analyzed with the 310 genetic analysis software. Base on the sequence information obtained from these DNA fragments, new sequence-specific primers (primer 5, 6, 7, 8) were synthesized

(Research Genetics, Huntsville, AL) (Table 4.1), and the procedure described above was repeated until the complete sequence of the 2,444 bp of PCR product was determined. The nucleotide sequence and the predicted amino acids sequence were analyzed and thus identified the gene product using Gene Runner (Hastings Software, Inc.). The database search for the sequence homologous to urease gene was done in BLAST of National Center for Biotechnology Information and ClustalW of European Bioinformatics Institute.

Urease Gene Expression

Construction of plasmid pCC130 (2.4 kb urease in pET23a). After we completed the sequence of 2.4 kb PCR product and identified it as the urease sequence of *S. leei* (see result section), pET23a vector (Novagen, Madison, WI) was used to construct a new plasmid, pCC130 for expression of *S. leei* urease gene. The plasmid pCC130 is shown in Figure 4.1. The 2.4 kb urease gene of *S. leei* in pCC128 was obtained by digestion with *Eco*RI, after purification of the urease gene using Genclean III kit (Cat. No. 1001-600, Bio 101, Inc. Vista, CA) following the manufacture's instruction; the purified urease gene was ligated to unique *Eco*RI site of pET23a vector. The ligation mixture (25 µl) contained 3 µl of (0.2 µg) of pET23a vector DNA, 7 µl (0.35 µg) of the purified 2.4 kb urease DNA, 2 µl (800 units) of T4 DNA ligase (Cat. No. M0202S, New England BioLabs, Beverly, MA), 2.5 µl of 10 x ligase buffer (50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 1 mM ATP, 10 mM DTT, and 50 µg/ml BSA), and 10 µl of water. The ligation mixture was put at 16^0C for 48 hours, and then was transformed to BL21 (DE3) cell (described below). The urease gene may be ligated to pET23a vector in two different

directions because of the one *Eco*RI site insertion. The direction of the urease insertion was tested below after transformation to BL21 (DE3) cells.

Preparation of competent cells of BL21 (DE3). BL21 (DE3) cells were first prepared to be electrocompetent cells. 500 ml of LB medium was inoculated with 10 ml of fresh overnight culture of BL21 (DE3). The Cells were grown at 37^0C with vigorous shaking till an OD_{600} of approximately 0.5. The culture was centrifuged at 4,000 g (5,000 rpm) at 4^0C for 15 min in a Sorvall RC 5C centrifuge with a SLA-3000 rotor (Sorvall, Newtown, CT). The supernatant was removed and the pellet was gently resuspended in 500 ml ice-cold of 10% glycerol (No. G33-500, Fisher scientific, Fair Lawn, NJ). The cells were centrifuged again as described above and resuspended in 250 ml of ice-cold of 10% glycerol. The cells were centrifuged for the third time as described above and resuspended at a final volume of 2 ml in ice-cold 10% glycerol, and aliquots of 40 µl each, and then stored at -70^0C.

Transformation of pCC130 to BL21 (DE3) cell. The cell suspension was slowly thawed at room temperature and then immediately placed on ice. Sterile cuvettes (No. 165-2086, BioRad, Hercules, CA) were placed on ice. In a cold (on ice) 1.5 ml eppendorf tube, 40 µl of the cell suspension was mixed with 2 µl of ligation mixture DNA (0.04 µg) of pCC130. The mixture was first placed on ice for 1 minute, and then transferred to the cold electroporation cuvette and shaken to the bottom. The cuvette was pushed into the chamber until the cuvette was seated between the contacts in the base of the chamber, and pulsed (Gene Pulse Controller Plus and Capacitance Extender II, BioRad, Hercules, CA) at 2.5 kV, 25 µF and 300 Ω. The cuvette was removed from the chamber and 1 ml of SOC medium was immediately added to the sample. The cells were

gently resuspended and transferred to a 1.5 ml microtube and incubated in a shaker (Model G25, New Brunswich Scientific, New Brunswich, NJ) at 37^0C and 200 rpm for one hour. The cells were plated on LB plate with 50 μg/ml of ampicillin and incubated at 37^0C overnight. The 16 positive clones of the colonies on the ampicillin selective LB plates were picked, and inoculated in 3 ml in LB media with ampicillin. The plasmids were isolated using Promega Miniprep kit and were used for analysis of the positive clone.

Identifying the positive clone. The plasmids from last step were digested with *Eco*RI and run on the 1% agarose gel for checking the size of inserted urease gene of *S. leei*. The insertion direction of urease gene of *S. leei* in pCC130 was identified by *Xba*I digestion. In the pCC130 vector, there is an *Xba*I site at the position 2720, which is in pET23a part. There is a unique *Xba*I site in the urease gene of *S. leei* at the position 498 of 2,444 bp. If the urease is inserted forward, the position of *Xba*I site of urease in the pCC130 vector is 2138. When the plasmid is digested with *Xba*I, we assume that we will obtain two DNA fragments, 5528 and 582 bp. If the urease is inserted backward, the position of *Xba*I site of the urease in the pCC130 vector is 690, then when the plasmid is digested with *Xba*I, two fragments of 4037 and 2073 bp fragment would be assumed to be obtained. Each plasmid of positive clone was digested with *Xba*I and then run 1% gel. The direction of insertion of urease to the pET23a vector was based on the DNA fragments on the agarose gel.

Urease gene expression induced by IPTG. The pCC130 with the forward direction of urease gene insertion was used for expression of urease protein. One single colony from a freshly streaked plate was inoculated into 2 ml LB medium containing

ampicillin (50 μg/ml), and incubated with shaking (200 rpm) at 37^{0}C until the OD_{600} at 0.6. The culture was stored at 4^{0}C overnight. After which, the cells were collected by centrifugation at 3,000 rpm, and resuspended in 2 ml fresh LB medium. 2 ml of cells was inoculated to 30 ml LB medium with 50 μg/ml ampicillin in a 125-ml flask. The culture was incubated with shaking (200 rpm) at 37^{0}C until OD_{600} of 0.6, and then the 14 ml of the cells were removed for the uninduced control and 1 ml of cells were used for titering as described in Plasmid Stability Test below. 0.15 ml of 40 mM IPTG were added to the remaining 15 ml of cells (final concentration of 0.4 mM). The uninduced control cells and IPTG-induced cells were incubated for 3 hours at 37^{0}C with shaking (200 rpm). The flasks of cells were placed on ice for 5 min and then the cells were harvested by centrifugation at 5,000 g for 5 min at 4^{0}C. The supernatant was saved, and the cell pellets were resuspended in 5 ml of cold 20 mM Tris-HCl, pH 8.0. The cells were centrifuged again as above, the supernatants were removed and the cells were stored as a frozen pellet at -70^{0}C.

Plasmid stability test. Before induction, the culture was tested to determine the fraction of cells that carry the target plasmid. 1 x 10^5 cells were plated onto four LB agar plates with the following differences: plate 1 contained both IPTG and ampicillin, plate 2 contained just IPTG, plate 3 contained just ampicillin, and plate 4 was LB medium only. The four plates were incubated overnight at 37^{0}C, and then the colonies on each plate were counted.

Lysis of cells and analysis of urease expression. Cells were first resuspended with 0.5 ml of Bis-Tris propane buffer containing 20 mM Bis-Tris propane, 1 mM EDTA, and 0.5 mM NiSO4, pH 7.4 at 4^{0}C, and then transferred to eppendorf tubes. The

cell solutions were lysed by a freeze-thaw method. The cells were put in freezing solution (dry ice with ethanol) for 2 min and then put in 37^0C water bath for 2 min, the freeze-thaw steps were repeated 10 times. The lysed cells were centrifuged at 14,000 rpm for 5 min. The supernatants were run a 4-20% SDS-PAGE, followed staining with Coomassie blue. The supernatants were also assayed for urease activity using phenol red method. In order to get the urease activity, each sample was first incubated with 1 mM $NiSO_4$ and 200 mM $NaHCO_3$ at 37^0C for 3 hours, and then each sample was measured for urease activity.

Results and Discussion

Two PCR products are obtained, one is 2.4 kb using primer 1 and 2, and the other is 1.3 kb using primer 3 and 4. Both products are seen on 1% agarose gel (Figure 4.2). Each PCR product is cloned to pCR 2.1-TOPO vector, named pCC128 (with 2.4 kb PCR product) and pCC129 (with 1.3 kb PCR product). After digestion analysis with *Eco*RI, they all show the vector 3.9 kb and PCR product 2.4 and 1.3 kb fragments on a 1% agarose gel (Figure 4.3). After three generations of cell cultures on LB plates with ampicillin for each clone, the same DNA fragments are shown on the 1% agarose gel. This indicates that the plasmids are stable.

The 2.4 PCR product was sequenced, the complete sequence is first done on BLAST with the GenBank database. BLAST result shows that the sequence is homologous with the urease gene from other species and this indicates that the cloned gene of the PCR product is the urease gene of *S. leei*. Analysis of the 2.4 kb DNA sequence reveals that two open reading frames that are designated *ureA*, *ureB*, and *ureC*. These genes are all transcribed in the same direction. *ureA* (1 to 303) encoded peptide of

100 amino acids of the 12 kDa, the UreA protein (gamma subunit); *ureB* (from 319 to 723) encoded peptide of 134 amino acid of 20 kDa, the UreB protein (beta subunit); *ureC* (from 730 to 2,444) encoded peptide of 571 amino acids of 65 kDa, the UreC protein (alpha subunit) (Figure 4.4). Since the complete urease gene of *S. leei* is obtained from the 2.4 kb PCR product, the 1.3 kb PCR product which is partial urease gene is not used for further study, The amino acid sequence and nucleotide sequence of *S. leei* were compared with other species urease gene, especially with *S. xylosus* and *H. pylori.* The alignment of urease amino acid sequence of *S. leei* with urease of *S. xylosus* and *H. pylori* is shown in Figure 4.5. The UreA, UreB, and UreC protein of *S. leei* and *S. xylosus* showed 98%, 91% and 97% amino acid identity, respectively. The *H. pylori* has only UreA and UreB, two subunits. The UreA and ureB protein of *S. leei* showed 48% of amino acid identity with UreA of *H. pylori*; the UreC protein of *S. leei* showed 57% of amino acid identity with UreB of *H. pylori.* The alignment of urease nucleotide sequence of *S. leei* with of *S. xylosus* and *H. pylori* is showed in Figure 4.6. The identity of urease nucleotide sequence of *S. leei* and *S. xylosus* is 82%. The nucleotide sequence identity (82%) is lower than the amino acids sequence identity (95%) between these two species. This is because most changes in nucleotide sequence happen at the third position in the genetic code. The identity of urease nucleotide sequence of *S. leei* and *H. pylori* is 60%. This percent is close to the identity of amino acids between these two species.

The pCC130 plasmid (urease 2.4 bp gene/ pET23a) was constructed. The positive clone (urease inserted to pET23a in forward direction) was transformed to *E. coli* BL21 (DE3) cell. The new strain of BL21 (DE3) cell with pCC130 is called CC704. CC704 was used for urease gene expression. The results of the plasmid stability test are that the

colony numbers on the LB plate 1 (IPTG + ampicillin), 2 (ampicillin), 3 (IPTG), and 4 (none) are 0, 99, 3, and 101 respectively. In a typical culture useful for producing target proteins, almost all cells will form colonies both on media without additives and on media containing only antibiotic; less than 2% of the cells will form colonies containing only IPTG; and less than 0.01% will form a colony on media containing both antibiotic and IPTG. The plasmid stability test result shows that the plasmid is stable, and no plasmid is lost and no mutant appears during culture. After 3 hours of inducing by IPTG, the cells are collected and lysed, and then run on SDS-PAGE; the electrophoresis result is in Figure 4.7. On the gel, there is no difference between the bands of the negative control (pET23a vector only) with induced or uninduced cells. If we compare the induced and uninduced for the CC704 (pCC130 in BL21-DE3), there are weak extra bands at the size 65 kDa and 20 kDa, which matches the position of purified urease alpha subunit (65 kDa) and beta subunit (20 kDa), this indicates that the urease is expressed at a low level. Unfortunately, no urease activity is detected in any sample. The 2.4 kb urease gene is the urease structural gene only, it does not contain the accessory protein, and this is the reason that there is no urease activity. Previously reported studies show that urease activity can be obtained *in vitro* with high concentrations of nickel sulfate (1 mM) and carbon dioxide (Park and Hausinger, 1995). This *in vitro* method was tried, but the urease still did not show activity. In order to express the active urease, further studies will have to be done. The urease gene, which contains the accessory protein, will have to be obtained by screening the genomic library of *S. leei*. The sequence of the urease structural gene is already known, and this sequence can be used to design a probe to

screen the genomic library, and then find the urease gene with the accessory protein. Once this is successful the urease protein expressed should have enzyme activity.

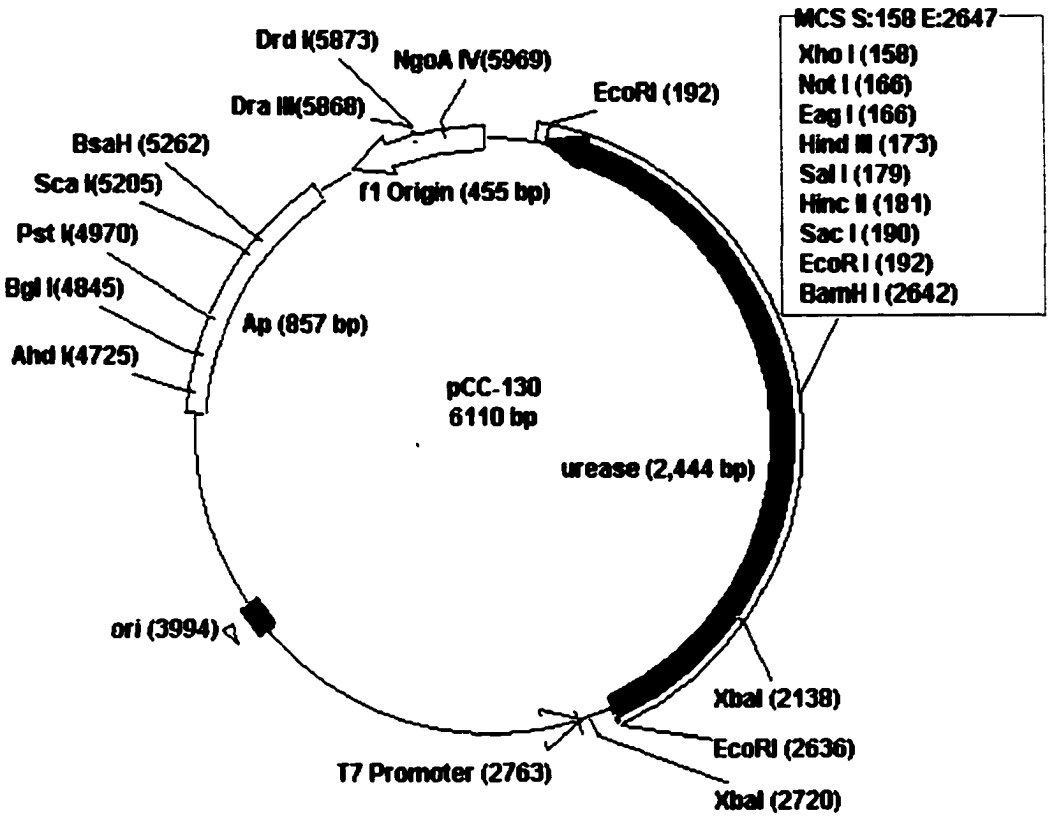


Figure 4.1. The map of plasmid pCC-130 (urease gene 2.4 kb of *S. leei* / pET23a), 2.4 kb of urease gene was inserted to the *Eco*R I site in the multiple cloning site of pET23a vector.

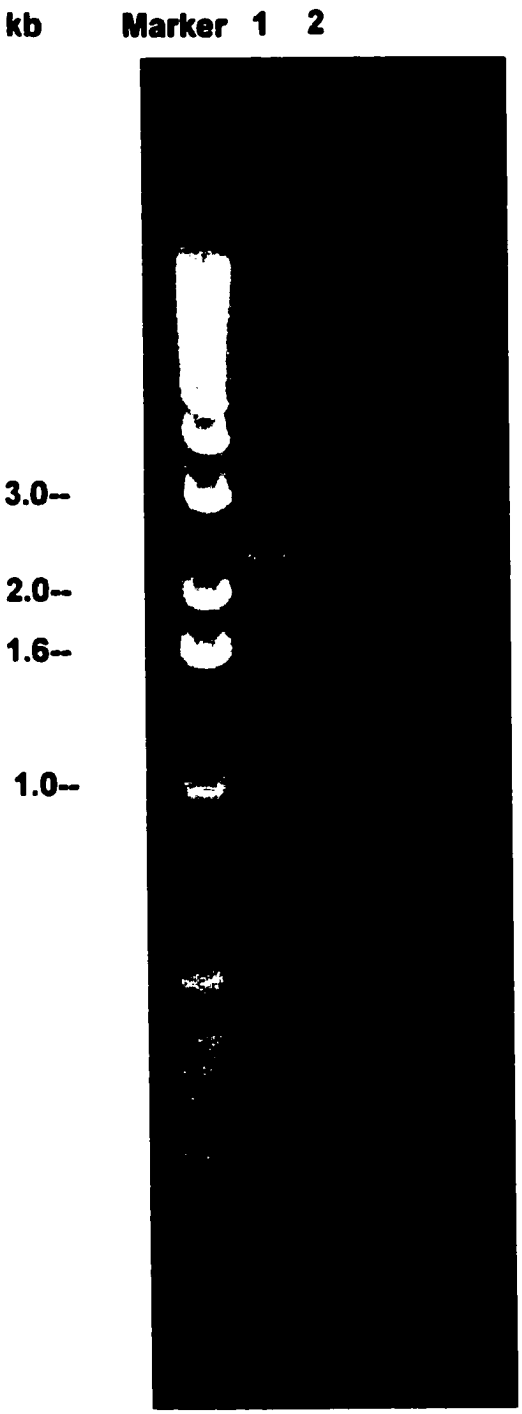


Figure 4.2. PCR amplification products of the *S. leei* urease gene from genomic DNA of *S. leei* with two sets of primers. Lane 1 is 2.4 kb PCR product, Lane 2 is 1.3 kb PCR product.

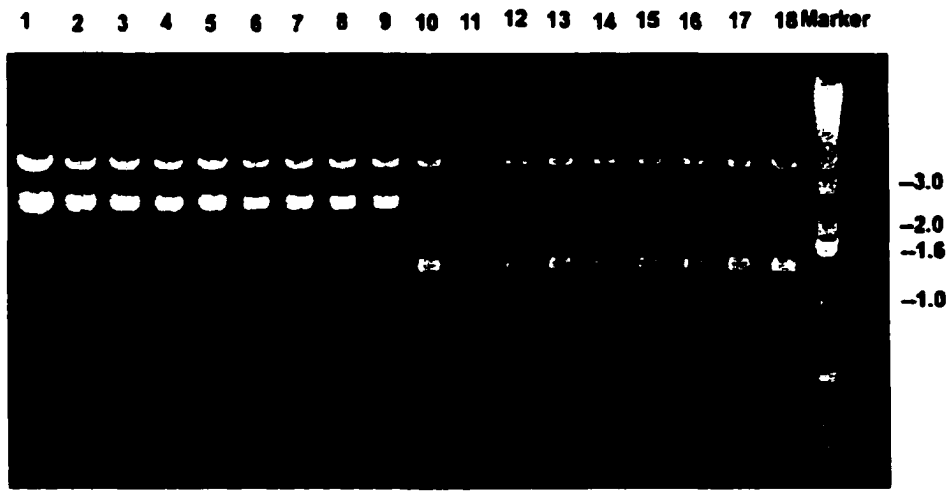


Figure 4.3. Analysis of cloning of urease gene from PCR products. The two PCR products cloned in pCR 2.1-TOPO vector were digested with *Eco*R I. Lane 1-9 are cloned 2.4 kb PCR product and 3.9 kb vector; lane 10-17 are the cloned 1.3 kb PCR product and 3.9 kb vector.

```
GTGCATTTTACACAACGTGAACAAGACAAACTGATGTTGGTCATCGCAGCAGATTTAGCT    60
 M  H  F  T  Q  R  E  Q  D  K  L  M  L  V  I  A  A  D  L  A
ureA
CGCAGACGTCAACAACGAGGTTTGAAATTAAATTATTCGGAAGCTGTAGCTATTATCAGT   120
 R  R  R  Q  Q  R  G  L  K  L  N  Y  S  E  A  V  A  I  I  S
TTCGAGTTACTCGAAGGCGCTAGAGACGGAAAAACAGTTGCCGAACTCATGAGCTATGGT   180
 F  E  L  L  E  G  A  R  D  G  K  T  V  A  E  L  M  S  Y  G
AAACAAATATTAAATGAAGATGACGTGATGGAAGGCGTTGCAGATATGTTAACTGAAATG   240
 K  Q  I  L  N  E  D  D  V  M  E  G  V  A  D  M  L  T  E  M
GAAATCGAAGCAACGTTCCCAGATGGTACGAAACTCATTACTGTCCATCACCCAATCGTT   300
 E  I  E  A  T  F  P  D  G  T  K  L  I  T  V  H  H  P  I  V
TAGAGGAGGATTAAAATTATGAAACCTGGTGAAATTATAGTCAAACGTACAGAAATCGAA   360
***              M  K  P  G  E  I  I  V  K  R  T  E  I  E
                 ureB
GTGAATCAAGGGCATAATGCAACAATCCTTAATGTTAAAAATACAGGCGATCGCCCTATC   420
 V  N  Q  G  H  N  A  T  I  L  N  V  K  N  T  G  D  R  P  I
CAAGTTGGTTCACACTATCACTTTTTCGAAGCTAACCCAGCGTTACAATTCGATCATGAA   480
 Q  V  G  S  H  Y  H  F  F  E  A  N  P  A  L  Q  F  D  H  E
AAAGCCTATGGCAAACGTCTAGATATACCAGCAGGCGCGGCTGTTCGTTTTGAACCTGGT   540
 K  A  Y  G  K  R  L  D  I  P  A  G  A  A  V  R  F  E  P  G
GATGAAAAAGAAGTACAACTCGTAGAATACAGTGGTAAACGTAAAATCTACGGTTTTCAT   600
 D  E  K  E  V  Q  L  V  E  Y  S  G  K  R  K  I  Y  G  F  H
GGTGACGTAAATGGTTCCATCGATGAGTCACGTGTTTATAAACTGGAAGATGATAGTACT   660
 G  D  V  N  G  S  I  D  E  S  R  V  Y  K  L  E  D  D  S  T
GCAACAGAAGTCATTGCAGAACAAGACAAAACGAGTGAAAATGCTAACAAAGGAAGAGGG   720
 A  T  E  V  I  A  E  Q  D  K  T  S  E  N  A  N  K  G  R  G
TAAAAAGTATGAGCTTTAAAATGACGCAATCCCAATATACAAGTCTCTATGGACCAACTG   780
***        M  S  F  K  M  T  Q  S  Q  Y  T  S  L  Y  G  P  T
           ureC
TTGGTGATTCTGTAAGATTAGGCGATACAAACTTATTTGCTCGCGTAGAACGTGATTACG   840
V  G  D  S  V  R  L  G  D  T  N  L  F  A  R  V  E  R  D  Y
CTACATATGGAGATGAAGCTGCATTCGGCGGTGGTAAGTCTATCCGTGATGGTATGGCAC   900
A  T  Y  G  D  E  A  A  F  G  G  G  K  S  I  R  D  G  M  A
AAAATCCAAATGTCACACGTGATGACAAGCAAGTTGCTGATTTAGTCATCACAAATGCGA   960
Q  N  P  N  V  T  R  D  D  K  Q  V  A  D  L  V  I  T  N  A
TGATTATTGATTACGATAAGATTGTCAAAGCCGACATCGGCGTTAAAAATGGCTATATCA  1020
M  I  I  D  Y  D  K  I  V  K  A  D  I  G  V  K  N  G  Y  I
TGAAAATTGGTAAAGCAGGCAATCCAGATATTATGGATAACGTAGATATTATTATTGGTG  1080
M  K  I  G  K  A  G  N  P  D  I  M  D  N  V  D  I  I  I  G
CTACAACAGACATTATTTCTGCTGAAGGTAAAATTGTAACTGCGGGTGGCATAGATACAC  1140
A  T  T  D  I  I  S  A  E  G  K  I  V  T  A  G  G  I  D  T
ATGTCCATTTCATTAACCCTGAACAATCGCAAGTTGCATTAGAAAGTGGTATCACAACAC  1200
H  V  H  F  I  N  P  E  Q  S  Q  V  A  L  E  S  G  I  T  T
ATATCGGTGGCGGTACAGGCGCGTCTGAGGGTACGAAGGCGACAACTGTCACACCTGGAC  1260
H  I  G  G  G  T  G  A  S  E  G  T  K  A  T  T  V  T  P  G
CATGGCACTTACATCGTATGTTACTCGCAGCAGAGTCATTACCGTTGAATATTGGCTTTA  1320
P  W  H  L  H  R  M  L  L  A  A  E  S  L  P  L  N  I  G  F
CTGGTAAAGGGCAAGCTGTAAACCACACAGCTTTAGTCGAACAAATTCACGCTGGTGCAA  1380
T  G  K  G  Q  A  V  N  H  T  A  L  V  E  Q  I  H  A  G  A
TCGGCTTAAAAGTGCATGAAGACTGGGGTGCAACGCCTTCAGCACTTGATCATGCTTTAC  1440
I  G  L  K  V  H  E  D  W  G  A  T  P  S  A  L  D  H  A  L
AAGTCGCAGATGACTATGACGTACAAATCGCGTTGCACGCAGATACGTTAAATGAAGCTG  1500
Q  V  A  D  D  Y  D  V  Q  I  A  L  H  A  D  T  L  N  E  A
GTTTCATGGAAGAAACAATGGCTGCAGTAAAAGATCGCGTATTGCATATGTATCATACGG  1560
G  F  M  E  E  T  M  A  A  V  K  D  R  V  L  H  M  Y  H  T
AAGGTGCTGGTGGTGGTCATGCGCCCGATTTAATTAAGTCTGCTGCGTATGCTAACATTT  1620
E  G  A  G  G  G  H  A  P  D  L  I  K  S  A  A  Y  A  N  I
```

```
TACCTTCTTCAACAAATCCGACGTTACCTTATACTGTAAATACAATCGATGAACATTTAG   1680
L  P  S  S  T  N  P  T  L  P  Y  T  V  N  T  I  D  E  H  L
ATATGGTTATGATTACCCACCATTTAAATGCATCTATCCCTGAAGATATTGCGTTTGCGG   1740
D  M  V  M  I  T  H  H  L  N  A  S  I  P  E  D  I  A  F  A
ATTCACGTATTCGTAAAGAAACCATTGCAGCTGAAGATGTACTTCAAGATATGGGCGTAT   1800
D  S  R  I  R  K  E  T  I  A  A  E  D  V  L  Q  D  M  G  V
TTAGTATGGTAAGTTCTGATTCACAAGCAATGGGACGTGTCGGAGAAGTAATTACCCGTA   1860
F  S  M  V  S  S  D  S  Q  A  M  G  R  V  G  E  V  I  T  R
CTTGGCAAGTTGCTCACCGTATGAAAGAACAACGTGGATTATTAGATGGCGACAGTGAAT   1920
T  W  Q  V  A  H  R  M  K  E  Q  R  G  L  L  D  G  D  S  E
ACAATGACAATAATCGTATTAAACGCTATATAGCAAAATATACAATTAACCCAGCCATTA   1980
Y  N  D  N  N  R  I  K  R  Y  I  A  K  Y  T  I  N  P  A  I
CACATGGTATTTCTGACTATGTAGGTTCAATTGATGAAGGTAAATTAGCCGACATCATTC   2040
T  H  G  I  S  D  Y  V  G  S  I  D  E  G  K  L  A  D  I  I
TTTGGGAACCAGCATTCTTCGGCGTTAAACCTGATGTCATCGTTAAAGGCGGGTTAATCA   2100
L  W  E  P  A  F  F  G  V  K  P  D  V  I  V  K  G  G  L  I
ACGCTGCAATCAACGGAGATGCGAATGGCTCTATCCCTACTTCAGAACCTTTAAAATATC   2160
N  A  A  I  N  G  D  A  N  G  S  I  P  T  S  E  P  L  K  Y
GCAAAATGTATGGTCAATTAGGTGGAAATCTACAAAGTACATCGATGACTTTTGTTTCTA   2220
R  K  M  Y  G  Q  L  G  G  N  L  Q  S  T  S  M  T  F  V  S
CAACTGCTTATGAAAACGATATTGGTAAACTTTTAGGCTTAAAACGCAAATTAAGACCTG   2280
T  T  A  Y  E  N  D  I  G  K  L  L  G  L  K  R  K  L  R  P
TGCACAATATCCGTAAATTAAGTAAAAAAGATATGAAAAACAATAATGCAACACCAGATT   2340
V  H  N  I  R  K  L  S  K  K  D  M  K  N  N  N  A  T  P  D
TAGACGTTGACCCACAAACATATGAAGTTTTTGTTGATGGAGAGAAAATTACAAGCGAAC   2400
L  D  V  D  P  Q  T  Y  E  V  F  V  D  G  E  K  I  T  S  E
CTGCTACAGAATTACCATTGACGCAACGCTATTTCTTATTCTAG                   2444
P  A  T  E  L  P  L  T  Q  R  Y  F  L  F  ***
```

Figure 4.4. Nucleotide sequence of the urease genes of *S. leei*. The deduced amino acid sequences are shown for *ureA* (1 through 303 bp), *ureB* (320 through 723 bp), *ureC* (730 through 2444 bp). The putative shine-Dalgarno sites for *ureB* and *ureC* are underline, and the stop codons for each gene are marked with stars. At the start codon, the gene of each subunit is represented in bold face.

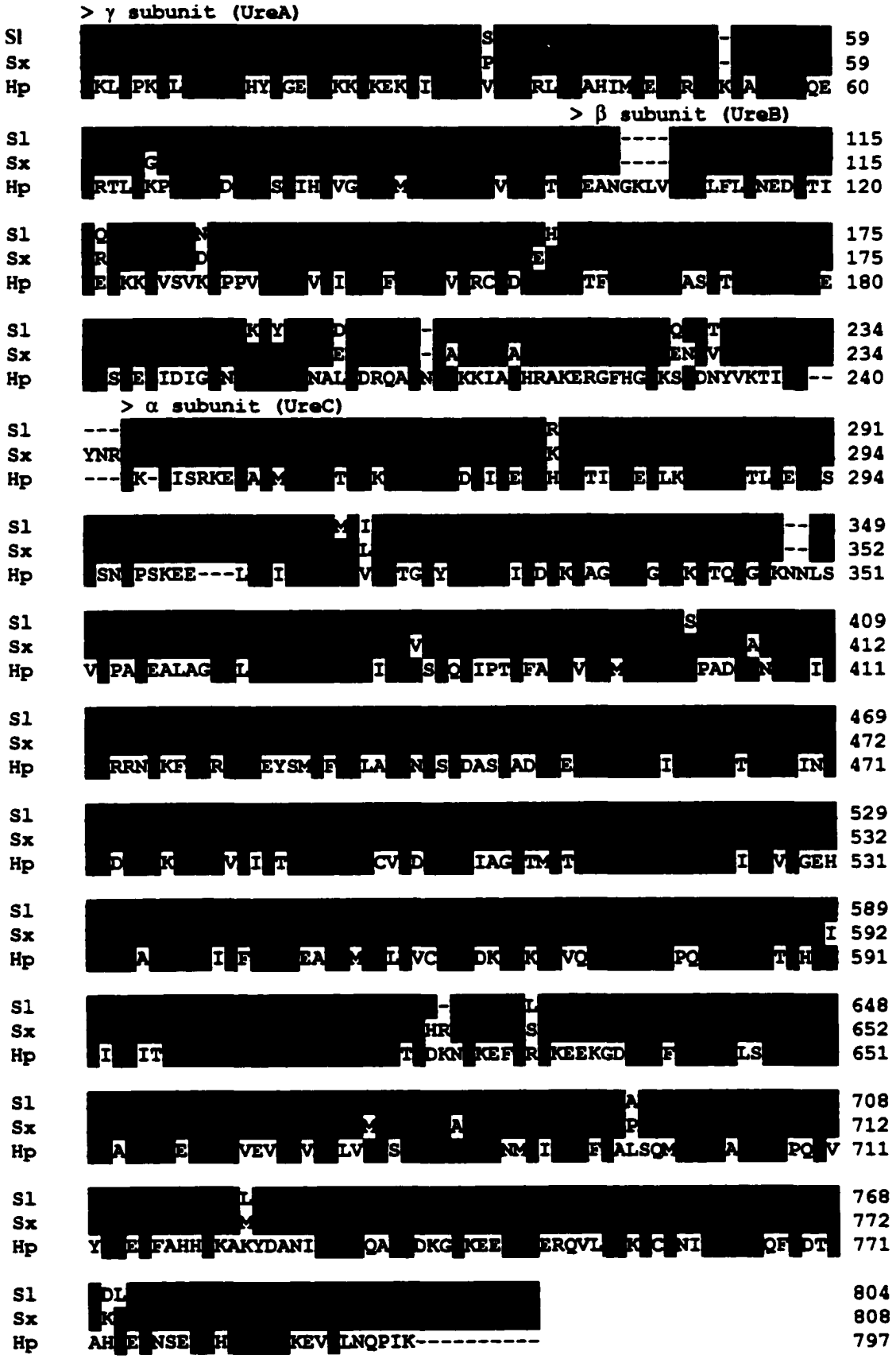
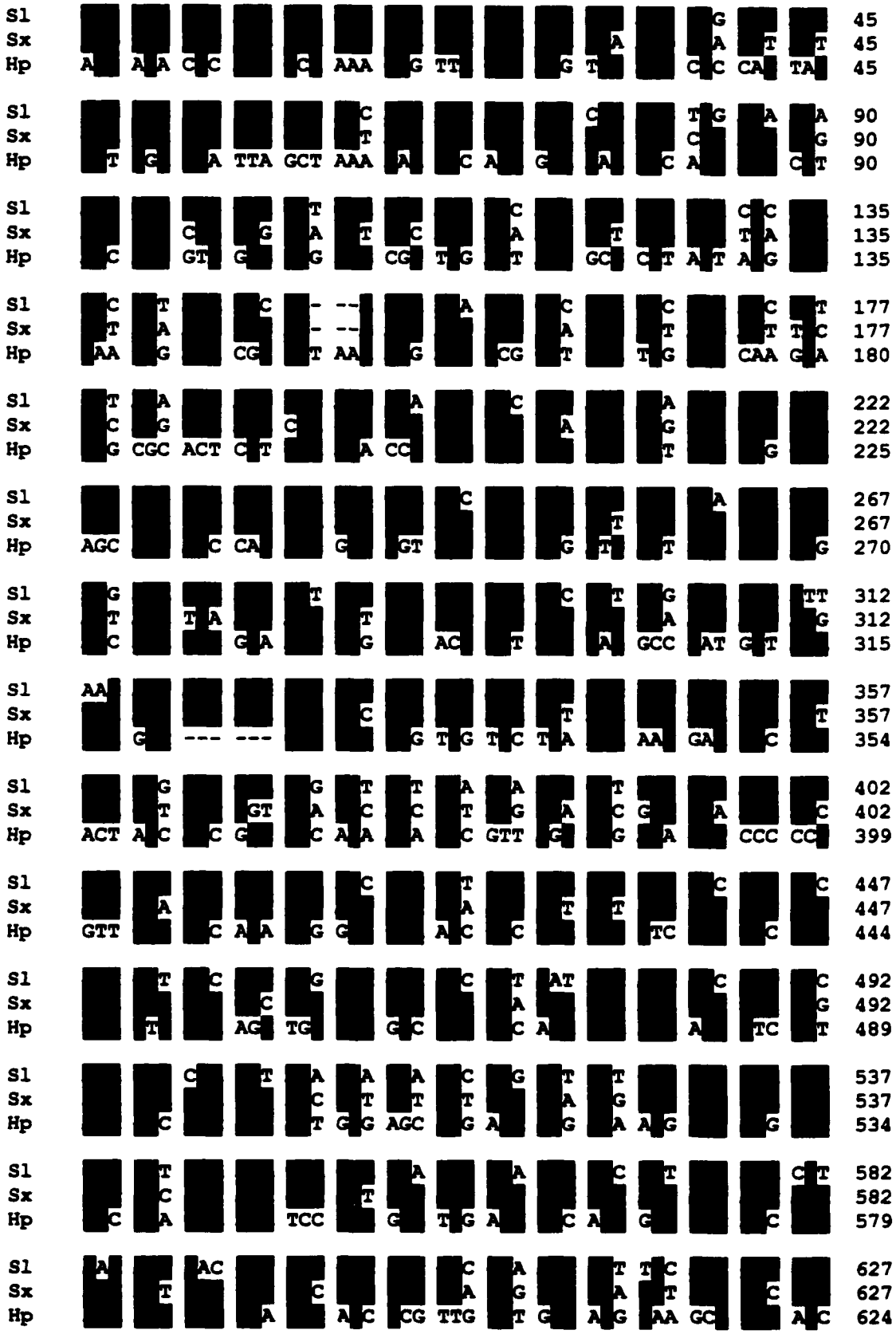
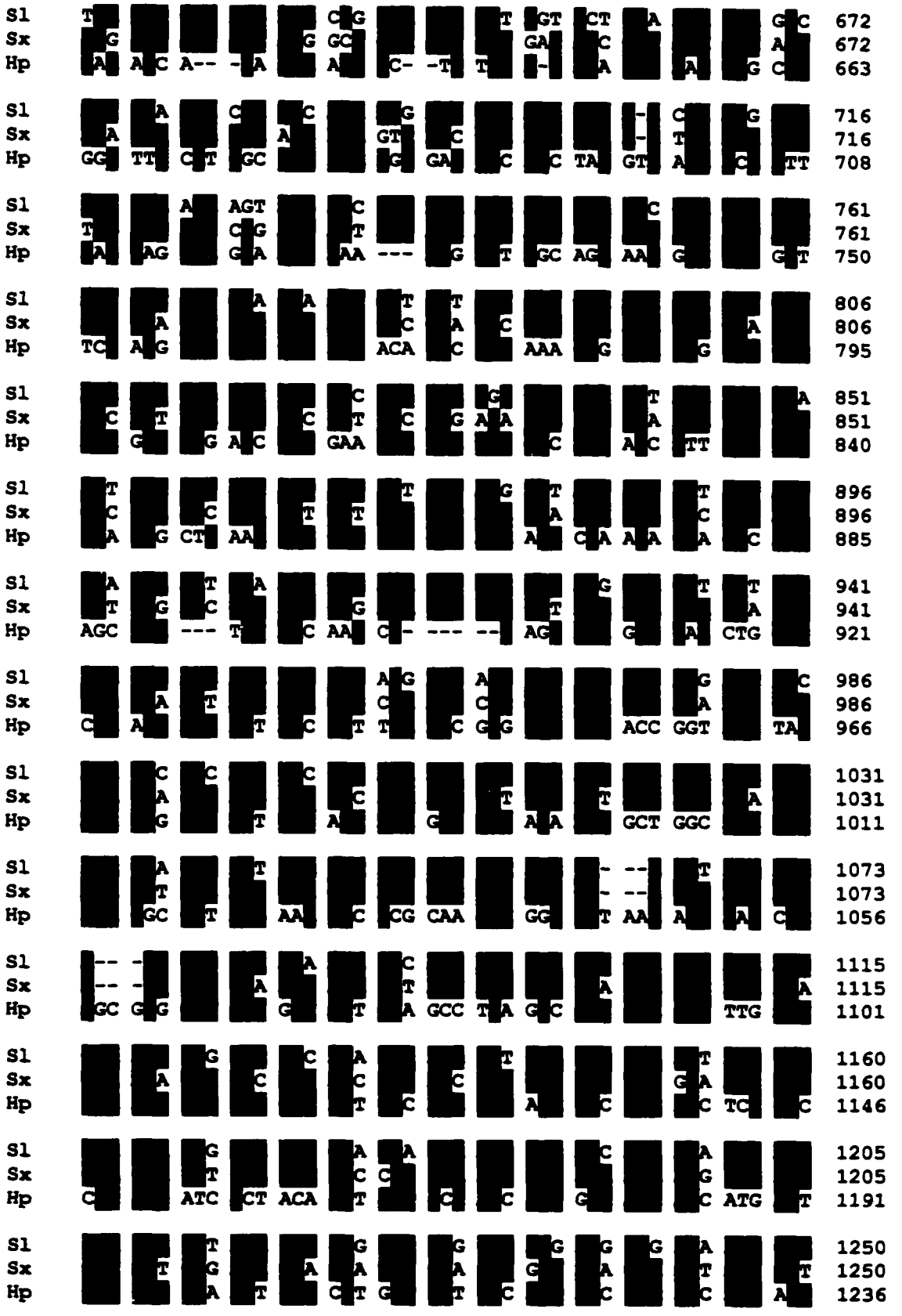
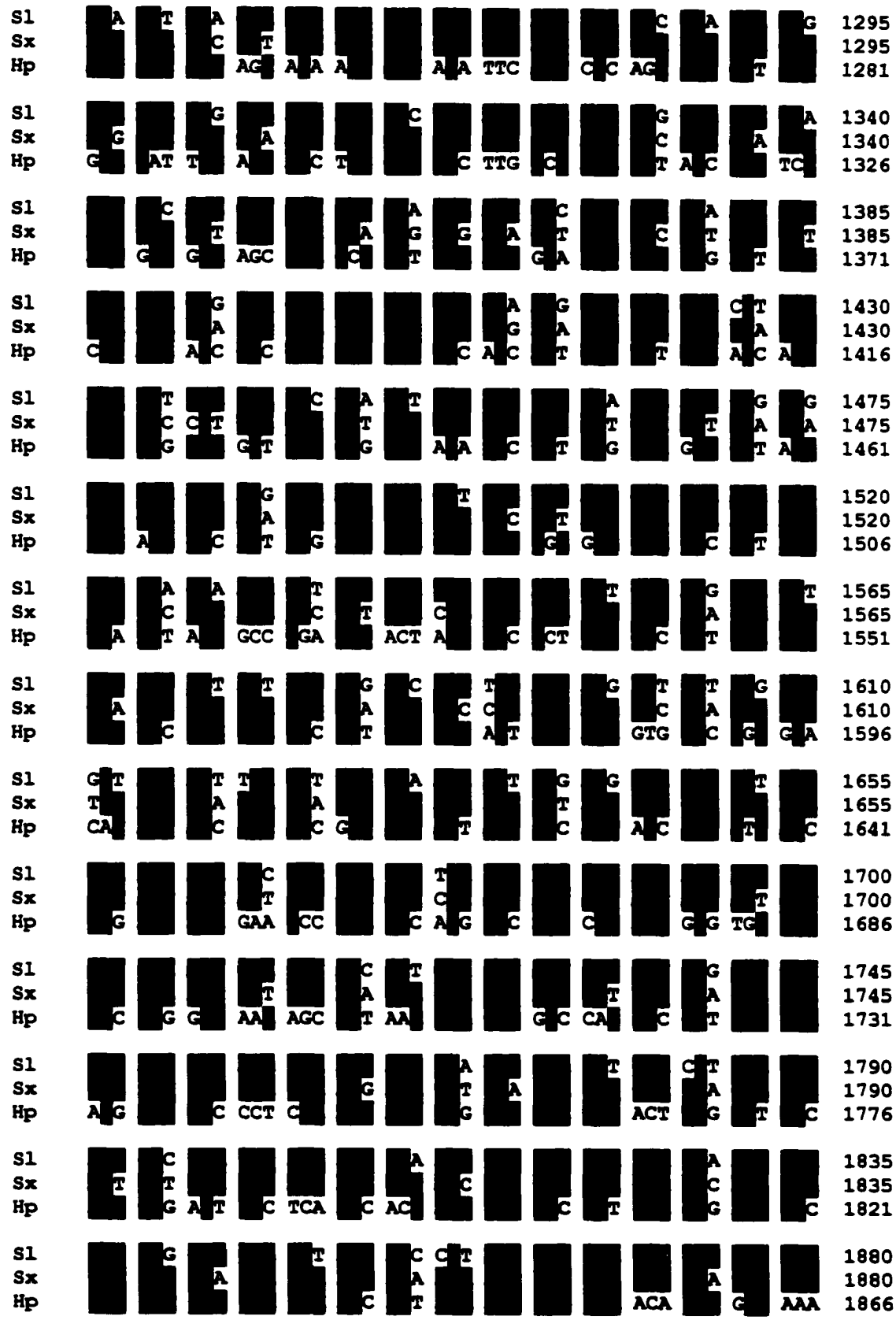


Figure 4.5. Alignment of deduced protein sequences of *S. leei* with sequence of *S. xylosus* (GenBank accession No. X74600) and *H. pylori* (GenBank accession No. M60398). Shaded residues indicate amino acids identical among the three sequences, and dished lines represent breaks introduced to optimize alignment. Sl is *S. leei*; Sx is *S. xylosus*; Hp is *H. pylori*. The ">" indicated the start position of each subunit of *S. leei, S. xylosus*, and *H. pylori*. Urease of *S. leei* and *S. xylosus* have three subunits, alpha, beta, and gamma. The urease of *H. pylori* has two subunits, alpha and beta. The alpha subunit of *H. pylori* is similar to alpha subunits of *S. leei and S. xylosus*; the beta subunit of *H. pylori* is similar to the sum of beta and gamma subunits of *S. leei and S. xylosus*.







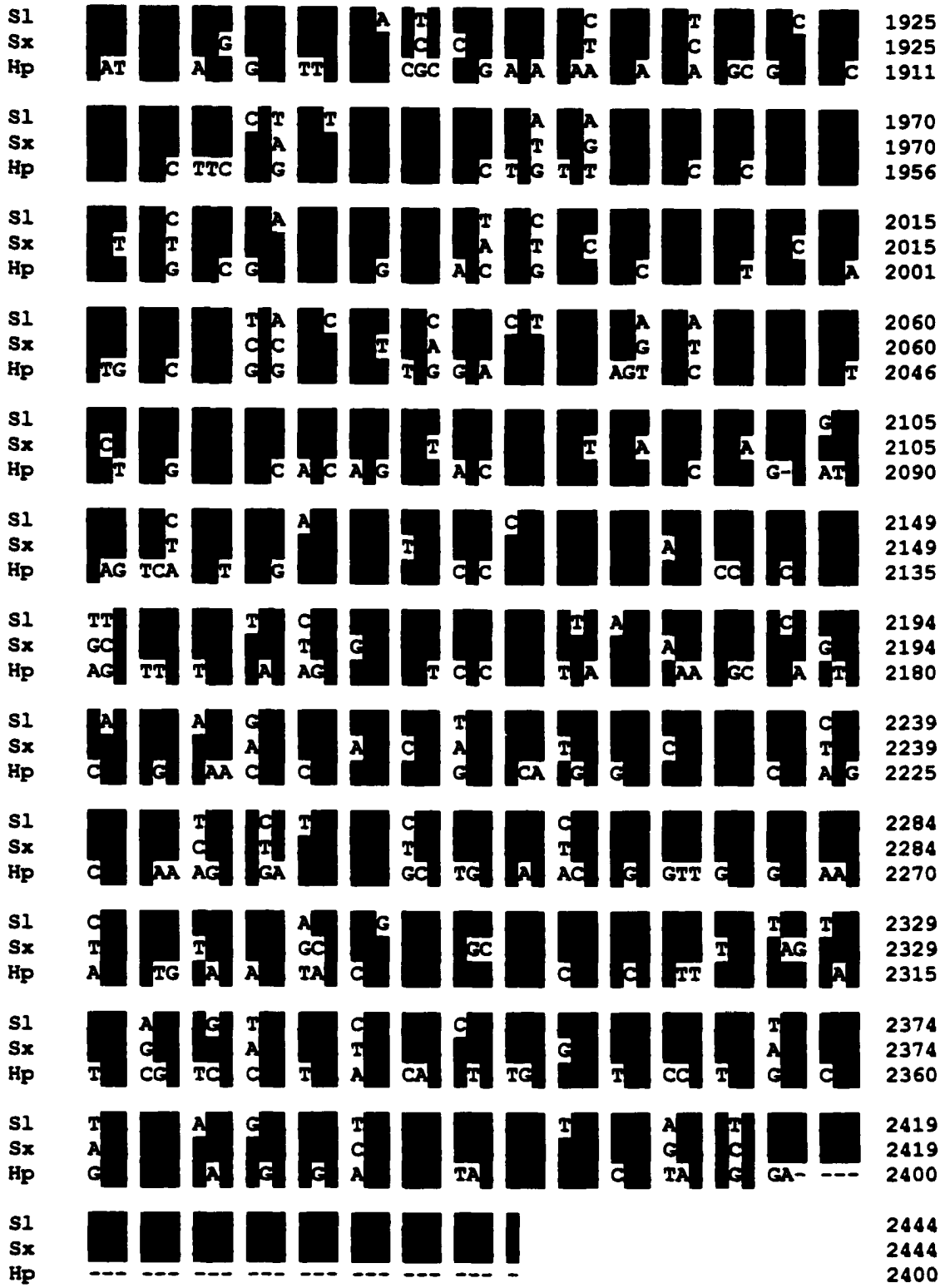


Figure 4.6. Alignment of urease DNA sequence of *S. leei* with sequences of *S. xylosus* (GenBank accession No. X74600), and *H. pylori* (GenBank accession No. M60398). Shaded sequences indicate nucleotide identical among the three species, and dished lines represent breaks introduced to optimize alignment. Sl is *S. leei*; Sx is *S. xylosus*; and Hp is *H. pylori*.

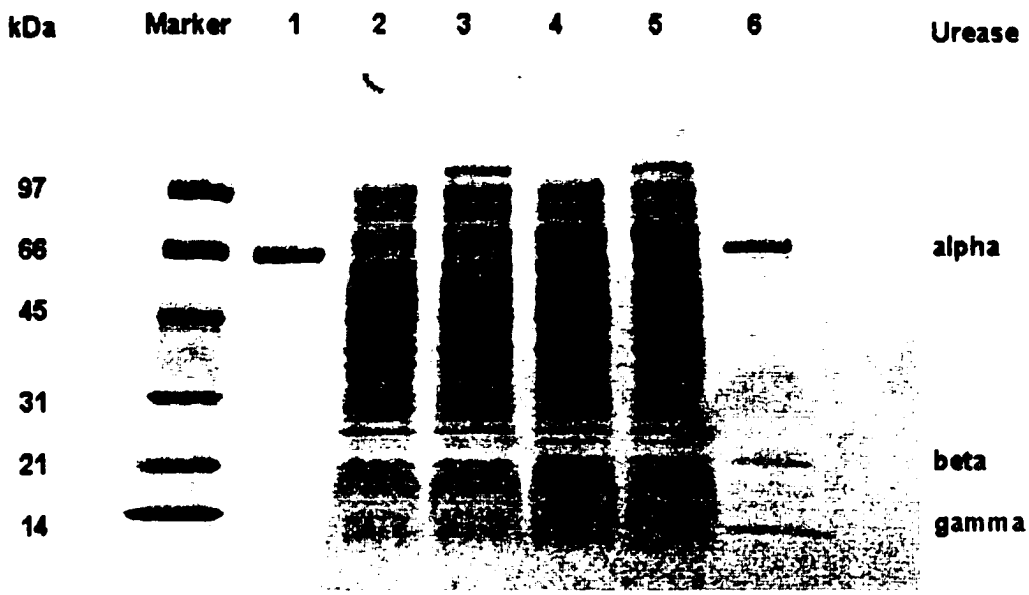


Figure 4.7. 4- 20% SDS-PAGE analysis of urease expression from *E. coli* BL21 (DE3) cells. Lane 1 BSA protein; lane 2, vector pET23a only without IPTG induced; lane 3, vector pET23a only induced with IPTG (0.4 mM) for three hours; lane 4, pCC130 without IPTG induced; lane 5, pCC130 induced with IPTG (0.4 mM) for three hours; and lane 6, Purified urease protein of *S. leei*.

Table 4.1. Primers Used in Cloning, Sequencing, and Expression of Urease Gene of *Staphylococcus leei*

Primer	Length	Sequence (5' to 3')	Used for
1	28	GTGCATTTTACACAACGTGAACAAGACA	PCR
2	27	CTAGAATAAGAAATAGCGTTGCGTCAA	PCR
3	28	GAAGCAACTTTCCCTGATGGTACTAAAT	PCR
4	28	CAGGTGCATGTCCGCCACCTGCGCCTTC	PCR
5	21	ATCGCGTTGCACGCAGATACG	sequence
6	21	TTCCCAAAGAATGATGTCGGC	sequence
7	21	TAGATATACCAGCAGGCGCGG	sequence
8	21	CTTCGTACCCTCAGACGCGCC	sequence
M13 forward	17	GTTTTCCCAGTCACGAC	sequence
M13 reverse	17	CAGGAAACAGCTATGAC	sequence

CHAPTER 5. DEVELOPMENT METHODS TO DETERMINE THE INCIDENCE OF INFECTION OF *S. LEE*

Materials and Methods

CLOtest

S. leei was inoculated to 0.5 ml LB media in 1.5 ml eppendorf tube, the tube was put in a 50-ml centrifuge tube for protection against contamination, and incubated at 37^0C overnight. The overnight culture (about 10^6 cells/μl) was diluted to different concentrations with LB media, 10^6, 10^5, 10^4, 10^3, 10^2, and 10^1 cells/μl. Then 10 μl sample (5 μl of different concentration *S. leei* with 5 μl LB media) was applied to CLOtest, each containing cells of *S. leei* at 5×10^6, 5×10^5, 5×10^4, 5×10^3, 5×10^2, and 5×10^1 respectively. Each dilution sample was inoculated to three LB agar after optimal dilution to check the cell number of each dilution.

CLOtests (No. 108660, Ballard Medical Products, Draper, UT) used for this test, were a gift from Dr. Robert Raffiniello (State University of New York HSCB). The label on each CLOtest was peeled to open the CLOtest plastic slide, so that the yellow gel could be seen, but the label was not removed. 10 μl sample with a different cell number was pushed into each yellow CLOtest gel using a pipette. The CLOtest label was re-sealed by pressing the label back on the plastic slide to cover the gel. Then each CLOtest was kept at 37^0C for 72 hours; the results observed were the color change around the specimen. If the specimen is positive, the color changes from yellow to orange or red in the gel.

Testing Antibody Specific for Ureases of *S. leei* and *H. pylori*

Serum samples from two ulcer patients with antibodies to *H. pylori* were tested by ELISA with the purified urease of *H. pylori* as antigen before and after the sera were pre-

incubated with either the purified urease of *H. pylori* or *S. leei*. This work was conducted with the scientists at Enteric Products, Inc.

PCR Method to Detect *S. leei* and *H. pylori*

Genomic DNA sample for PCR amplification. Genomic DNA of *H. pylori* was obtained from ATCC (Cat. No 435040), and genomic DNA of *S. leei* was isolated using Promega genomic DNA isolation kit following the manual procedure (Appendix 2).

Primers. The *ureABC* gene of *S. leei* was sequenced and the urease DNA sequences of *S. leei* was compared with *H. pylori urease* DNA sequence (GenBank accession No. M60398). The two sets of PCR primers were derived from DNA segments of low homology that produce DNA fragments of 210 and 415 bp. A widely used set of PCR primers derived from the *H. pylori ureC* gene produces a PCR DNA fragment of 294 bp (Lage *et al.*, 1995). The two sets of primers from urease gene of *S. leei* and one set of primer from urease gene of *H. pylori* were synthesized by Research Genetics. Primer sequences, and expected lengths of amplified DNA products are shown in Table 5.1.

PCR conditions and amplification. Reactions were performed in a 50 µl volume in a GeneAmp reaction tube (Part No. N801-0180, Perkin Elmer, Norwalk, CT). reaction mixtures contained 0.4 uM each primer, 0.2 uM each deoxynucleotide triphosphate (dNTP), and reaction buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM MgCl2, and 0.01% gelatin), 5 µl of 10 ng/µl of genomic DNA of *S. leei* and *H. pylori*, 0.5 µl of Taq DNA polymerase (2.5 units). All reagents were in the PCR Core Kit (No. 1 578 553, Roche, Indianapolis, IN). One drop of light mineral oil (Cat. No. M5904, Sigma, St. Louis, MO) was added to the each sample before the PCR amplification. The

mixture was first denatured at 94^0C for 10 min and cooled on ice. 35 PCR cycles were run for each sample, each cycle comprising a 2 min denaturing step at 94^0C, a 2 min annealing step at 55^0C, and a 2 min elongation step at 72^0C, performed on DNA Thermal Cycler (Model 9810, Perkin Elmer, Norwalk, CT). For each run, water replaced the DNA sample as a negative control. To avoid cross-contaminations, mixture preparation, sample handling, PCR amplification were done in separate rooms, and different pipets was used for handling samples. The PCR products were detected and analyzed after the PCR amplification. 10 μl amount of each PCR mixture was subjected to 2% agarose gel for detection of amplified DNA products.

Comparison of Three Isolation Methods of Genomic DNA of *S. leei*

The Genomic DNA of 1 ml overnight culture of *S. leei* was isolated using three methods, the first method used was Genomic DNA Isolation Kit from Promega Company with lyse enzymes (Appendix 1); the second method used was obtained from Dr. Robert Marrero with enzymes (Appendix 2); and the third method used was obtained from Dr. Robert Reffaniello (State University of New York HSCB), which he used to isolated genomic DNA of *H. pylori* with proteinase K (Appendix 3). The enzymes used were lysozyme (10 mg/ml, Cat. No. L2879), lysostaphin (1 mg/ml, Cat. No. L7386), and mutanolysin (500 units/ml, Cat. No. M9901); the three enzymes are from Sigma, St. Louis, MO.

The final sample volume obtained from the three methods was 100 μl. Each sample was 10 times serially diluted as follows: 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, and 10^7, and then 5 μl of each dilution sample was run for PCR using the method described above.

The 5 μl of each diluted DNA sample corresponded to lysed cell number of 5 x (10^7, 10^6, 10^5, 10^4, 10^3, 10^2, and 10^1).

Results and Discussion

CLOtest

The CLOtest result is shown in Figure 5.1. The light red color started to show up only after a 30 min incubation, then changed to full red within 1 hour, when 5 x 10^6 cells were applied to the CLOtest. The full red color showed up after 3 hours of incubation when the 5 x10^5 cells were applied to the CLOtest. The full red color showed up after 24 hours incubation when 5 x10^4 cells were applied to the CLOtest. No red color showed up after 72 hours of incubation, when the 5 x 10^3, 5 x 10^2, and 5 x 10^1 cell were applied to the CLOtest. The CLOtest test is positive when using the higher number of cells of *S. leei*. When the number of cells is lower than 5 x 10^3, the CLOtest is negative. The CLOtest positive of *S. leei* indicates that this simple test of the urease activity is not useful to distinguish between infections of *S. leei* and *H. pylori* since H. pylori gives a positive CLOtest. This confirms that the presence of *S. leei* would give a false positive result showing infection of *H. pylori*.

Testing Antibody Specific for Ureases of *S. leei* and *H. pylori*

Serum samples from two ulcer patients with antibodies to *H. pylori* were used in an ELISA with the purified urease of *H. pylori* as antigen before and after the sera were pre-incubated with either the purified urease of *H. pylori* or the purified urease of *S. leei*. The result is shown in Table 5.2. Since incubation of *H. pylori* positive sera with the urease of *S. leei* does not remove antibodies of the urease of *H. pylori*, the absorbance does not change before incubation and after incubation with the urease of *S. leei*, this

result suggests that the two ureases are distinct antigens, and it confirmed the previous ELISA test for 40 sera samples (Table 1.1). This ELISA method will be used to detect the large number of patient's sera to find incidence of infection of *S. leei* with the urease of *S. leei.*

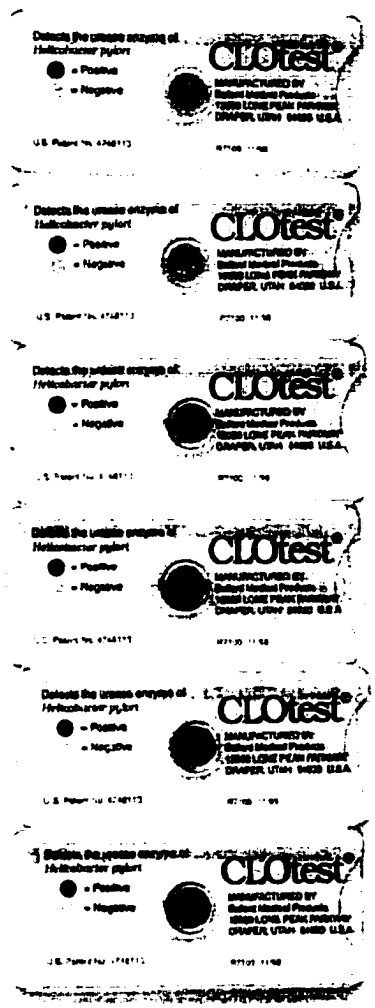
PCR Method to Detect *S. leei* and *H. pylori*

The three sets of primers, two derived from urease gene of *S. leei*, one from the ureC gene of *H. pylori* were used to run PCR with genomic DNA of *S. leei* and *H. pylori*. A negative control was run for each reaction with water instead of DNA sample. The results are shown in Figure 5.2. Using one set of primers (Sl1a and Sl1b) of *S. leei*, a PCR product of 415 bp was obtained with only genomic DNA of *S. leei*, and using another set of primers (Sl2a and Sl2b), a PCR product of 210 bp was obtained with only genomic DNA of *S. leei*. Using either of the two sets of primer, no PCR product was seen on the 2% agarose gel with genomic DNA of *H. pylori*. Using *H. pylori* primers of Hp1a and Hp1b, a 294 bp PCR product was obtained with only genomic DNA of *H. pylori*. This result indicates that no cross-reaction is happening. Either 415 or 210 bp PCR product of *S. leei* is easy to separate and see on the 2% agarose compared with the 294 bp of PCR product of *H. pylori*. The two sets of primers of *S. leei* are useful to detect the incidence of infection of *S. leei* with no cross-reaction with *H. pylori*.

Comparison of Three Isolation Methods of Genomic DNA of *S. leei*

The samples from each method, Promega Company, Dr. Merrero, and Dr. Rafaniello with different lysis enzymes were serially diluted up to 10^7. 5 µl of each sample was run on a 2% agarose gel. The results are shown in Figure 5.3. Using Dr. Rafaniello's method, the 415 bp fragment can be seen on the 10^3 dilution sample

corresponding to cell number of 5 x 10^5; using Dr. Merrero method, the 415 bp fragment can be seen on the 10^5 dilution sample corresponding cells number 5 x 10^3 with either two or three lysis enzymes; using Promega Company method, the 415 bp fragment can be seen on the 10^6 dilution sample corresponding cells number of 5 x 10^2 with two or three lysis enzymes, but the band is faint in the sample with two lysis enzymes. These results indicate that the best method for isolation of *S. leei* is the Promega Company method with three lysis enzymes (lysozyme, lysostaphin, and mutanolysin), and this method will be used to detect the biopsy sample for the incidence of infection of *S. leei*.



Cell	Time (h)
50	72
500	72
5,000	72
50,000	24
500,000	3
5,000,000	1

Figure 5.1. CLOtest of cells of *S. leei.* The different cell amounts of S. leei were added to CLOtest, and then incubated at 37^0C at different time. Then red color indicates the positive result.

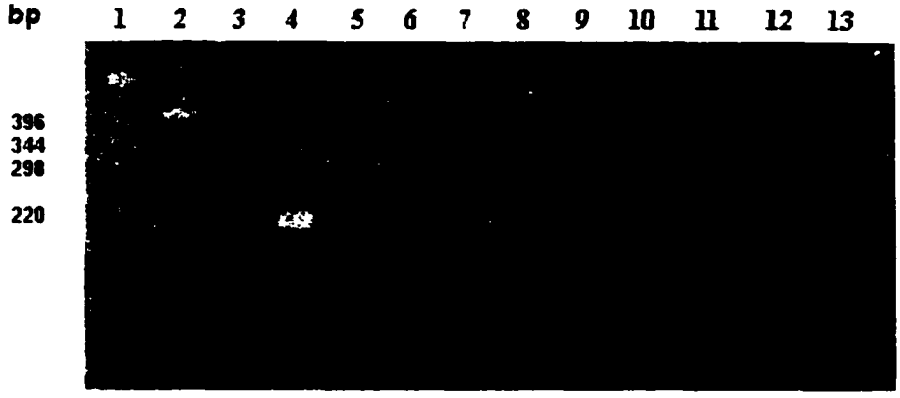
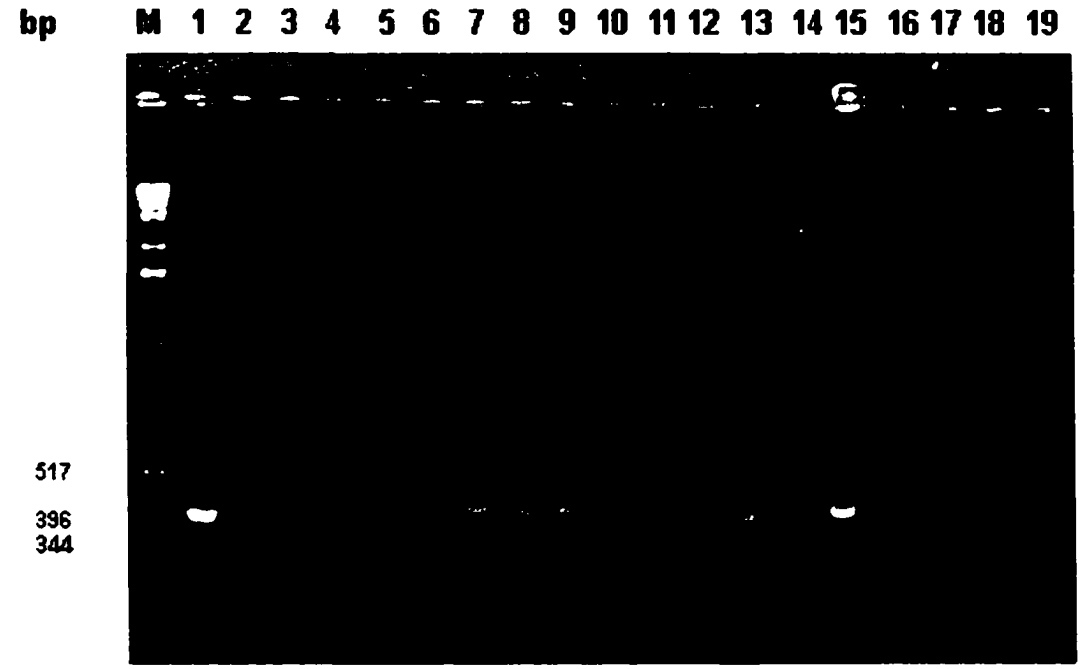


Figure 5.2. PCR products derived from *S. leei* and. *H. pylori* using the primers shown in Table 5.1. The content of primers and genomic DNA in each well used in this experiment are indicated below. Lanes 3, 5, 7, 9, 11, and 13, were controls in which water was added instead of DNA. Sl, *S. leei*; Hp, *H. pylori*. The 415 and 210 bp of PCR products from *S. leei* are present in Lane 2 and 4 respectively. The 294 bp of PCR product from *H. pylori* is present in Lane 12 (faint in this image). The broad and faint bands detected at the bottom of the gel in some lanes are the PCR primers.

Lane	1	2	3	4	5	6	7	8	9	10	11	12	13
DNA	MW	Sl	H_2O	Sl	H_2O	Sl	H_2O	Hp	H_2O	Hp	H_2O	Hp	H_2O
Primer		Sl1a	Sl1b	Sl2a	Sl2b	Hp1a	Hp1b	Sl1a	Sl1b	Sl2a	Sl2b	Hp1a	Hp1b

A



B

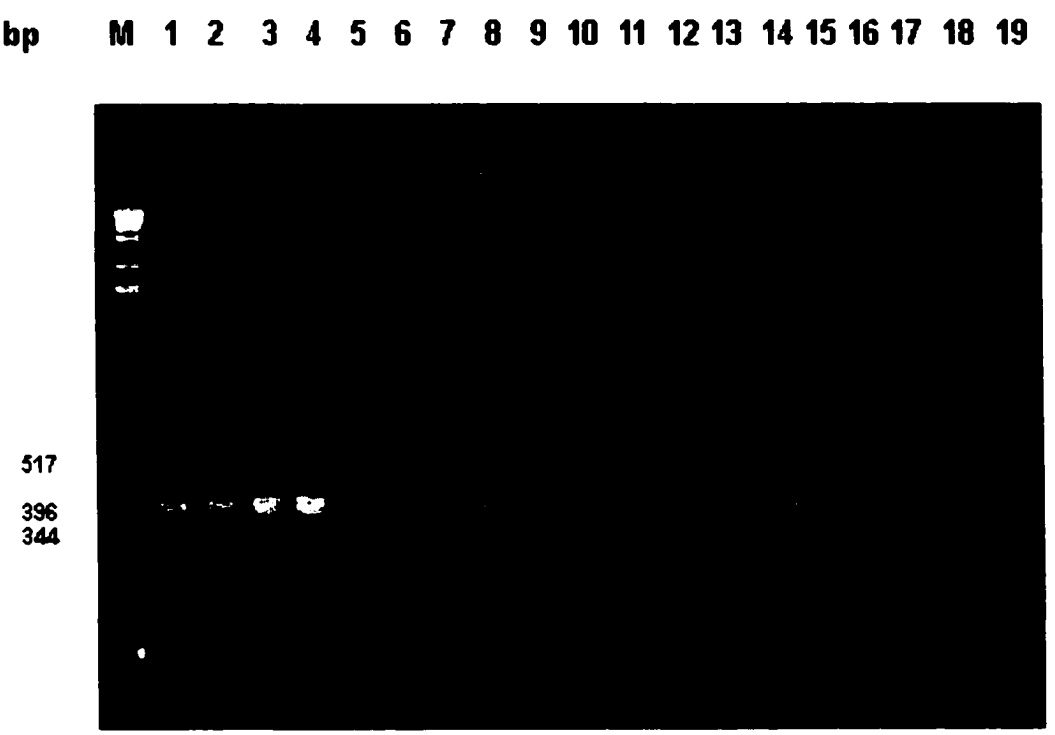


Figure 5.3. PCR results of comparing three isolation method of *S. leei* genomic DNA, the contents of each well are listed in following table. The number 2 or 3 indicates the lysis enzymes, 2, lysozyme and lysostaphin; 3, lysozyme, lysostaphin, and mutanolysin.

Figure	Well	Methods	Dilution (X)
A	1	Dr. Raffaniello	0
A	2	Dr. Raffaniello	100
A	3	Dr. Raffaniello	1,000
A	4	Dr. Raffaniello	10,000
A	5	Dr. Raffaniello	100,000
A	6	Promega-2	0
A	7	Promega-2	100
A	8	Promega-2	1,000
A	9	Promega-2	10,000
A	10	Promega-2	100,000
A	11	Promega-2	1,000,000
A	12	Promega-2	10,000,000
A	13	Promega-3	0
A	14	Promega-3	100
A	15	Promega-3	1,000
A	16	Promega-3	10,000
A	17	Promega-3	100,000
A	18	Promega-3	1,000,000
A	19	Promega-3	10,000,000
B	1	Dr. Marrero-2	0
B	2	Dr. Marrero-2	100
B	3	Dr. Marrero-2	1,000
B	4	Dr. Marrero-2	10,000
B	5	Dr. Marrero-2	100,000
B	6	Dr. Marrero-2	1,000,000
B	7	Dr. Marrero-2	10,000,000
B	8	Dr. Marrero-3	0
B	9	Dr. Marrero-3	100
B	10	Dr. Marrero-3	1,000
B	11	Dr. Marrero-3	10,000
B	12	Dr. Marrero-3	100,000
B	13	Dr. Marrero-3	1,000,000
B	14	Dr. Marrero-3	10,000,000
B	15	water	

Table 5.1. Primers Used for PCR Diagnosis

Primer	Length	Sequence (5' to 3')	Product (bp)
Sl-1a	25	CCATCACCCAATCGTTTAGAGGAGG	
Sl-1b	25	TTTCACTCGTTTTGTCTTGTTCTGC	415
Sl-2a	25	ATGAGCTTTAAAATGACGCAATCCC	
Sl-2b	27	AGCAACTTGCTTGTCATCACGTGTGAC	210
Hp-1a	25	AAGCTTTTAGGGGTGTTAGGGGTTT	
Hp-1b	24	AAGCTTACTTTCTAACACTAACGC	294

Table 5.2. Antibodies Specific Test for Urease of *H. pylori* and *S. leei*

Sera No.	Urease of *H. pylori*			Urease of *S. leei*		
	Pre-Incubation Absorbance	Post-Incubation Absorbance	Decline (%)	Pre-Incubation Absorbance	Post-Incubation Absorbance	Decline (%)
A031	0.98	0.13	86	0.70	0.72	-2.1
A040	1.09	0.13	88	0.64	0.71	-10.3

APPENDIX

Appendix 1. Method for Isolation of Genomic DNA of *S. leei* Using Promega Wizard® Genomic DNA Purification Kit (Technical Manual No. 050)

Materials to Be Supplied by the User
- 1.5ml microcentrifuge tubes
- water bath, 80°C
- water bath, 37°C
- isopropanol, room temperature
- 70% ethanol, room temperature
- water bath, 65°C (optional; for rapid DNA rehydration)
- 50mM EDTA (pH 8.0) (for gram positive bacteria)
- 10mg/ml lysozyme (Sigma Cat. # L7651) (for gram positive bacteria)
- 10mg/ml lysostaphin (Sigma Cat. # L7386) (for gram positive bacteria)

1. Add 1ml of an overnight culture to a 1.5 ml microcentrifuge tube.

2. Centrifuge at 13,000–16,000 × g for 2 minutes to pellet the cells. Remove the supernatant. For Gram Positive Bacteria, proceed to Step 3. For Gram Negative Bacteria go directly to Step 6.

3. Resuspend the cells thoroughly in 480µl of 50mM EDTA.

4. Add the appropriate lytic enzyme(s) to the resuspended cell pellet in a total volume of 120µl. Add 60µl of 10mg/ml lysozyme and gently pipet to mix. The purpose of this pretreatment is to weaken the cell wall so that efficient cell lysis can take place.

5. Incubate the sample at 37°C for 30–60 minutes. Centrifuge for 2 minutes at 13,000–16,000 × g and remove the supernatant.

6. Add 600µl of Nuclei Lysis Solution. Gently pipet until the cells are resuspended.

7. Incubate at 80°C for 5 minutes to lyse the cells; then cool to room temperature.

8. Add 3µl of RNase Solution to the cell lysate. Invert the tube 2–5 times to mix.

9. Incubate at 37°C for 15–60 minutes. Cool the sample to room temperature.

10. Add 200µl of Protein Precipitation Solution to the RNase-treated cell lysate. Vortex vigorously at high speed for 20 seconds to mix the Protein Precipitation Solution with the cell lysate.

11. Incubate the sample on ice for 5 minutes.

12. Centrifuge at 13,000–16,000 × g for 3 minutes.

13. Transfer the supernatant containing the DNA to a clean 1.5ml micro-centrifuge tube containing 600µl of room temperature isopropanol.

14. Gently mix by inversion until the thread-like strands of DNA form a visible mass.

15. Centrifuge at 13,000–16,000 × g for 2 minutes.

16. Carefully pour off the supernatant and drain the tube on clean absorbent paper. Add 600µl of room temperature 70% ethanol and gently invert the tube several times to wash the DNA pellet.

17. Centrifuge at 13,000–16,000 × g for 2 minutes. Carefully aspirate the ethanol.

18. Drain the tube on clean absorbent paper and allow the pellet to air-dry for 10–15 minutes.

19. Add 100µl of DNA Rehydration Solution to the tube and rehydrate the DNA by incubating at 65°C for 1 hour. Periodically mix the solution by gently tapping the tube. Alternatively, rehydrate the DNA by incubating the solution overnight at room temperature or at 4°C.

20. Store the DNA at 2–8°C.

Note:

1. For certain Staphylococcus species a mixture of 60µl of 10mg/ml lysozyme and 60µl of 10mg/ml lysostaphin is required for efficient lysis. However, many Gram Positive Bacterial Strains (e.g. *Bacillus subtilis, Micrococcus luteus, Arthrobacter luteus, Nocardia otitidiscaviarum, Rhodococcus rhodochrous,* and *Brevibacterium albidium*) lyse efficiently using lysozyme alone.

2. At Step 13, some supernatant may remain in the original tube containing the protein pellet. Leave this residual liquid in the tube to avoid contaminating the DNA solution with the precipitated protein.

Appendix 2. Method for Isolation of Genomic DNA of *S. leei* Using Dr. Marrero's Procedure

1. 1 ml of overnight culture is added to a 1.5 ml microcentrifuge tube.
2. Harvest cells by centrifugation at 7,000 rpm for 10 min at 4^0C.
3. Discard supernatant and resuspend cells in 1 ml of cold sterile TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).
4. Centrifuge as in step 2.
5. Resuspend the cells in 0.6 ml cold TE buffer.
6. Add 60 µl of 10 x enzymes (10 mg/ml lysozyme, 1 mg/ml lysostaphin, and 500 U/ml Mutanolysin.
7. Add 10 µl of 2 mg/ml RNAse.
8. Mix and incubate at 37^0C for 30 min.
9. Add 0.6 ml of TE buffer, 20 µl of a 10 mg/ml Proteinase K, and 10 µl of 40% N-lauryl Sarcosine (Na Salt).
10. Seal tube and mix by gentle, repeated inversion.
11. Incubate at 37^0C for 30 min.
12. Split the solution to two microcentrifuge tubes, each is added 0.6 ml of TE-saturated phenol/chloroform/isoamyl alcohol (24:24:1) and mix at room temperature by gentle.
13. Spin at room temperature at 5,000 rpm for 10 min.
14. Carefully remove the aqueous (upper) layer and transfer to a fresh microcentrifuge tube.
15. Repeat the phenol extraction in the previous step.
16. Extract the aqueous phase with 0.6 ml of chloroform/isoamyl alcohol (24:1).
17. Centrifuge as in step 13.
18. Remove aqueous (upper) phase to a fresh microcentrifuge tube.
19. Add 60 µl of 3 M Sodium Acetate, and 2.5 volume of cold Ethanol.

20. Incubate at -70^{0}C for 15 min.

21. Centrifuge at 15,000 rpm for 15 min at 4^{0}C.

22. Carefully discard the supernatant; and drain the tube.

23. Add 0.6 ml of 70% Ethanol to the tube and vortex briefly to wash the pellet.

24. Centrifuge as in step 21.

25. Discard supernatant and drain the tube.

26. Resuspend the pellet in 100 μl of water; to assist in dissolution, the tube may have to be incubated at 65^{0}C for 15 min.

Appendix 3. Method for Isolation of Genomic DNA of *S. leei* Using Dr. Raffaniello Procedure

1 ml of overnight culture is centrifuged at 10,000 g for 5 minutes. The cells are extracted by incubating in 100 µl lysing buffer (20 mM Tris-HCl, pH 8.0) containing 0.5% Tween-20 and proteinase K (0.5 mg/ml) for one hour at 55^0C and 98^0C for 10 minutes to inactivate proteinase K. Following centrifugation (10,000 g for 5 minutes), aliquots (10 µl) of the supernatant are used for analysis by PCR.

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