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Sporosarcina ureae UREASE: PARTIAL PURIFICATION AND ATTEMPTS TO CLONE THE UREASE GENES

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Sporosarcina ureae UREASE: PARTIAL PURIFICATION AND ATTEMPTS TO CLONE THE UREASE GENES

By

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ABSTRACT

Sporosarcina ureae **UREASE: PARTIAL PURIFICATION AND ATTEMPTS TO CLONE THE UREASE GENES**

By

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Urease was enriched 135-fold and partially characterized from *Sporosarcina ureae*, a gram-positive constitutive urease producer. The K_m was determined to be 62 +/- 20 mM urea with a V_{max} of 770 +/_ 80 umol urea min^{-1} mg^{-1} protein. Gradient SDS-polyacrylamide gel electrophoresis showed the enzyme to possess three subunits, estimated molecular weight 70,000 +/- 3000, 11,000 +/-2000, and 8,000 +/- 2000, indicating this urease of Gram-positive origin more closely resembles ureases purified from Gram-negative microorganisms rather than other gram-positive ureases. However, a urease gene probe from the Gram-negative microbe *Klebsiella aerogenes* failed to hybridize to the *S. ureae* urease genes. Attempts to clone the urease genes, utilizing a variety of vectors and hosts, were unsuccessful. These results suggest the organization of the *S. ureae* urease operon may differ considerably from the urease genes cloned from other microorganisms.

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INTRODUCTION

The experiments discussed in this thesis describe the partial enzyme purification and attempts to clone the urease genes from *Sporosarcina ureae*, a Gram-positive soil microorganism. As an introduction, I describe the significance of soil urease, the sources of urease in soil, the general characteristics of each class of urease, and the molecular biology of ureolysis. As will become apparent, Gram-positive microbial urease and the genes encoding this enzyme have not been adequately characterized despite the importance of urease in soil nitrogen transformations.

Significance of soil urease. The enzyme urease catalyzes the hydrolysis of one molecule of urea to one molecule each of ammonia and carbamate. The molecule of carbamate then spontaneously hydrolyzes to carbonic acid and a second molecule of ammonia (49). Substrate urea is released into the environment through biological actions such as excretion by mammals (74), purine catabolism (75), and degradation of nitrogen-rich amino acids such as arginine. (75). The urea thus released is further degraded in the soil and the resulting ammonia is utilized by both soil microbes and plants. These processes involving nitrogen metabolism require urease activity, thus, the enzyme plays an essential role in nitrogen cycling.

In addition to the biological release of urea into the environment, urea is widely applied as a fertilizer. Agricultural productivity is limited primarily by the availability of fixed nitrogen, thus fertilizers which can be converted to a form of usable nitrogen are widely employed. In the last 20 years, the use of urea as a nitrogen fertilizer has increased dramatically (49, 54), and it is probably the most important

solid fertilizer in world agriculture today (46). In 1987, 14.86 billion pounds of urea were manufactured in the United States, most of which was used in fertilizers (60). Urea has many advantages as a fertilizer: low cost of manufacture, high solubility in water, ease of storage and application (54). However, this increased usage has been accompanied by an increased incidence of phytotoxicity. Applied urea, rapidly hydrolyzed by soil urease, results in an increase in soil pH and the liberation of ammonia. The resulting nitrite and ammonia toxicity, in turn, causes damage to seedlings and young plants (11,18,53,54). These drawbacks cause urea to be an inefficient fertilizer in some soils and can result in reduced crop yields from plant damage. Because of these problems, it has become desirable to find methods to slow this hydrolytic process. One approach is to apply a urease inhibitor in conjunction with the urea fertilizer. A number of compounds have been studied with this objective in mind. Early research indicated that dihydric phenols and quinones decreased soil urease activity (12). Specific urease inhibitors, like hydroxamic acids, have been shown to reduce the rate of loss of ammonia from soil urea (58). In more recent laboratory experiments, several phosphoryl amides and thiophosphoryl amides were shown to inhibit urease, thus retarding urea hydrolysis (10,13,38,46,47,59). Field studies, however, have failed to show an increase in crop productivity with urease inhibitors (71). Regardless, the potential benefits of this approach to increased crop productivity will further research in this area, and an increased understanding of the urease mechanism may lead to development of improved urease inhibitors.

Sources of soil urease. The majority of soil urease is thought to

be of microbial origin, and as many as 17-30% of soil microorganisms can hydrolyze urea (40,54). These organisms include at least one archaebacterium (2), a mycoplasma (63), and many Gram-positive and Gram-negative bacteria (49). In addition, urease is synthesized by many plants and some invertebrates (25,48,57). Urease can be released from disintegrated cells and can be adsorbed onto clay and organic colloids, which demonstrate a high affinity for urease (24,40). Free urease is rapidly degraded by proteases in the soil, but the enzyme appears to be protected when adhered to organic soil constituents (54). This adsorption to soil components increases the longevity of urease in the soil. Other factors which affect urease activity in soil include urea content, moisture content, temperature and pH (47,54).

Urease enzymology. Table 1 summarizes the results of urease studies conducted on enzymes isolated from jack bean, Gram-negative and Gram-positive microorgansims. The first purified urease was that from jack bean in 1926 (67). Of historical note, this was also the first enzyme to be crystallized (67). The first microbial urease to be purified was from *Bacillus pasteurii* in 1954 (37). Purification involved a series of fractionations with ammonium sulfate, calcium phosphate, and acetone. Affinity chromatography, gel filtration and anion exchange resins resulted in less active preparations than obtained with the original method (15,37). Only two other Gram-positive ureases have been purified, those from *Brevibacterium ammoniagenes* (55) and *Arthrobacter oxydans* (61).

The best characterized bacterial urease is that of the Gram-negative organism *Klebsiella aerogenes* (52,68,69,70). In addition, many other Gram-negative microbial ureases have been purified and studied in detail (9,49,51). Purification of enzyme from crude cell extracts of Gram-

Table 1. Selected properties of purified ureases.[1,2]

Organism	K_m (mM)	Specific Activity[3]	Purification (Fold)	Native M_r	Subunit M_r	Subunit Composition	Mechanism[3] (Regulation)	DNA[4] (kb)	References
Eukaryotic									
Jack bean	2.9	3,500		590,000	α-91,000	α_6	NR	NR	5
Prokaryotic									
Gram-positive									
A. oxydans	12.5	219	121	242,000	NR	NR	R	NR	61
B. pasteurii	40-130	1,328	66	230,000	α-43,300	α_4	NR	11	15,34,37
B. ammoniagenes	18-72	3,570	638	280,000	α-47,000	α_6	NR	NR	35
S. ureae	40-80	760	135	260,000	α-70,000 β-11,000 γ-8,000	NR	C	NR	
Gram-negative									
K. aerogenes	2.8	2,200	1,070	224,000	α-72,000 β-11,000 γ-8,000	$\alpha_6\beta_6\gamma_6$	I,R	3.5	48,70
M. morganii	NR	2,431	169	NR	NR	NR	NR	4.2-5.4	48
P. mirabilis	13	2,057	800	212,000- 250,000	α-73,000 β-10,000 γ-8,000	$\alpha_6\beta_6\gamma_6$	I	6.5	9,31,48,62,77
P. stuartii	9.3	3,320	331	230,000	α-73,000 β-10,000 γ-8,000	$\alpha_6\beta_6\gamma_6$	I	4.4-6	30,31
S. rumenantium	2.2	1,019	392	360,000	α-70,000 β-8,000 γ-8,000	NR	NR	NR	48,74

[1] Not all purified preparations represent homogeneous enzymes.
[2] Table is not inclusive of all purified ureases.
[3] C, constitutive; I, inducible; R, repressible.
[4] Length of DNA to which genes have been localized.
[5] umol of urea min^{-1} mg^{-1} of protein.

negative bacteria on diethylaminoethyl (DEAE)-Sepharose, phenyl-Sepharose, and Fast Protein Liquid Chromatography (FPLC) Mono-Q resins has proven to be a generally successful protocol. Urease has a high negative charge at neutral pH and binds tightly to ion exchange resins. Furthermore, it binds more tightly to phenyl-Speharose than many less hydrophobic contaminants, thus making hydrophobic chromatography a useful tool (49).

Most purification schemes, for both Gram-positive and Gram-negative ureases, include low levels (1mM) of EDTA and thiols to prevent inactivation by heavy metal ions and oxidation (6,22,39,42,43,44,55). The enzyme from several microorganisms has proven to be stable for periods longer than one month, if stored in buffers containing EDTA and thiols (49) or glycerol (55) at 0°C. Avoidance of pH and temperature extremes also increases stability of stored urease preparations (49).

Native molecular weights of both Gram-negative and Gram-positive ureases, as measured by gel filtration chromatography, are in the range of 200-250 kDa (49). The native molecular weight of jack bean urease is 590 kDa (1), making it larger than all microbial ureases.

Isoelectric focusing has shown the pI for ureases from *Morganella morganii*, *Providencia stuartii*, *Proteus mirabilis*, *Proteus vulgaris*, and *Providencia rettgeri* to be in the range of 5.1-5.9 (30). Urease from these microorganisms show multiple bands of enzyme activity on native polyacrylamide gel electrophoresis (30,62). There is usually a major band accompanied by 1 to 2 less intense bands. Studies have shown that these bands are not the products of different genes, as mutations in the urease genes result in elimination of all urease activity (31,51). In addition, recombinant urease from cloned *K. aerogenes* (49), *P. mirabilis* (31,77) and *P. stuartii* (50), have given rise to multiple bands on native gel

electrophoresis.

For Gram-positive bacteria, isoelectric focusing indicates a single urease form for both *B. ammoniagenes* and *B. pasteurii* of 4.6 and 4.1, respectively (15,55). In contrast, *A. oxydans* has 4 bands ranging from 4.3-4.7 (61). However, since urease is irreversibly inactivated at low pH, artifactual pI values may have been generated. In addition, multiple activity bands have been reported for crystalline jack bean urease (7). These multiple forms have been shown to represent different aggregation states (1), many of which were dependent on pH, the presence of salt, thiols, or other changes in the buffer. Simple self-aggregation is thought not to be responsible for the multiplicity of bands observed in microbial ureases (49). Attempts to alter the banding pattern by varying buffer components and pH have been unsuccessful.

Jack bean urease consists of one subunit type (α), 91 kDa, with a stoichiometry of α_6 (1,5). In contrast, the ureases purified from *P. stuartii* (51), and *K. aerogenes* (70) show three distinct subunit types: α, 70-75 kDa; ß, 10-12 kDa; and Γ, 8-10 kDa. All Gram-negative ureases characterized thus far have these three subunit types, with the stoichiometry of $[\alpha_1 ß_2 \Gamma_2]_2$ (49). The reported subunit molecular weights and stoichiometry for Gram-positive microorganisms is significantly different from that of Gram-negative microorganisms. Thus far, ureases of Gram-positive organisms have been homopolymeric. *B. pasteurii* is reported to be a tetramer of identical subunits, each with a molecular weight of 65.5 kDa (15). *B. ammoniagenes* is trimeric, each subunit being 67 kDa (55). Subunit composition of urease from *A. oxydans* is as yet unknown (61). It is possible that the differences seen in subunit composition

between Gram-negative and Gram-positive microorganisms are genuine, yet it is also possible the small subunits could have been overlooked in the putative homopolymeric enzymes. Small subunits are poorly resolved from the dye front on gels of less than 10% acrylamide and therefore may easily be missed.

Nickel quantitation has been done with few purified enzymes, but evidence suggests that all ureases may contain nickel (28). The first enzyme shown to contain nickel was jack bean urease, possessing 2 nickel ions per catalytic subunit or twelve nickel per enzyme (17). *K. aerogenes* (70) and *P. stuartii* ureases (51) contain approximately 2 nickel ions per $\alpha_1\beta_2\gamma_2$ structure, hence 4 nickel ions per native molecule. Analysis of *K. aerogenes* urease indicates one active site per $\alpha_1\beta_2\gamma_2$ structure (68), therefore, 2 nickel ions are present per active site, as with jack bean urease.

Studies of nickel content with Gram-positive ureases have indicated that for both *B. pasteurii* (15) and *B. ammoniagenes* (55), there is only a single nickel ion present per subunit. It is known that urease from *A. oxydans* is a nickel-containing enzyme, but its nickel content is unknown (61). Reports of nickel content may not be entirely accurate for Gram-positive microorganisms. No nickel was added to the culture of *B. ammoniagenes* and only 80 nM was added to that of *B. pasteurii* (15,55), therefore, it is possible that insufficient nickel was present and that approximately equal amounts of apoenzyme was purified with the urease.

Urease regulation. In some species, synthesis of urease is repressed by ammonia and nitrogen-rich compounds, like urea, which release ammonia (Table 1). These ureases are derepressed under nitrogen-limiting or

nitrogen-starvation conditions (49). In a second group of microorganisms, urea acts as an inducing agent of urease synthesis. In a third group of microorganisms, urease is constitutively produced and, thus, not affected by levels of ammonia, urea or other nitrogen-rich compounds (49). The latter two groups of ureases, many of which are from Gram-positive organisms, may be the most important for fertilizer decomposition. The nitrogen-repressible ureases, found in many Gram-negative microorganisms, may not make a significant contribution to soil urease content, especially under the high urea conditions found with fertilization.

Molecular biology of ureolysis. The urease genes have been cloned from several Gram-negative organisms and from one Gram-positive organism. The genes were cloned into cosmid, plasmid, or phage vectors, selected for on antibiotic media, and screened for urease activity on indicator plates (21,31,34,50,52,77). Indicator plates contained urea and a pH indicator such as phenol red to detect the increase in pH around the ureolytic colony resulting from the production of ammonia.

Through subcloning of *Sau*3A partial digests of cloned genes (49), deletion mapping (21,31,50,77), Bal31 digests (31), and Tn5 mutagenesis (31,50,51,77), the organization of the Gram-negative urease operon has been intensively studied. Most investigators have found the minimal DNA needed to encode urease is 3.2-6kb (31,50,51,77). Genes are needed to encode not only the structural polypeptides of the native enzyme, but also other proteins which may participate in protein assembly, urea transport, nickel transport and nickel processing (49). Gene regulation depends on adjacent DNA sequences, growth conditions, and host species. In the *P. mirabilis* operon (31), there probably exists a repressor that regulates expression in a manner similar to that of the lac operon. *P. stuartii*

genes, cloned into *Escherichia coli*, were found to be inducible by urea (51). In *K. aerogenes* (52), the genes appear to be regulated via the nitrogen regulation system, as high levels of urease expression are achieved in nitrogen-limited media.

The urease genes of *B. pasteurii* are the only urease genes to be cloned from a Gram-positive microorganism. The DNA was size fractionated and cloned into a positive selection vector and transformed into *E. coli* (34). Screening on R plates (34) yielded a plasmid containing an 11 kb fragment of DNA capable of expressing urease in an heterologous host. Subcloning and restriction mapping of this fragment have not been reported, nor have studies involving regulation, expression or gene organization been performed.

Goals of this research. A purified enzyme is essential for development of new urease inhibitors which are based on knowledge of active site structure and mechanism. The mechanistic studies being pursued today involve urease of Gram-negative origin (68,69). These nitrogen-repressible enzymes may be inappropriate as models in the study of urea fertilizer degradation, thus conclusions drawn from Gram-negative urease studies may not be applicable to the constitutively produced urease of many Gram-positive microorganisms. Knowledge of the Gram-positive urease operon structure and function, also essential for an understanding of urease mechanism and regulation, is severely limited, therefore, studies of agriculturally significant microorganisms need to be pursued.

S. ureae, a Gram-positive microorganism which constitutively produces high levels of urease, would be a suitable representative of an agriculturally significant microorganism. Studies of the *S. ureae* urease operon structure and function may provide important data necessary in the

development of new urease inhibitors.

The goal of this project is to begin to characterize ureolysis in *S. urease*. Initial objectives were to clone the urease genes to determine number, function and regulation of genes, as well to purify of the urease enzyme and analyze of the enzyme subunit composition.

MATERIALS AND METHODS

Bacterial strains. *Sporosarcina ureae* 634 was obtained from Thomas Corner, Michigan State University. *E. coli* VCS257 was obtained from Stratagene. *E. coli* DH1 (27), *E. coli* HB101 (8) and *B. subtilis* BS101 (obtained from Pat Oriel, MSU) served as recipients in transformations. *B. subtilis* BS250, carrying the conjugative transposon Tn916, (obtained from Pat Oriel, MSU) served as the donor in transposon mutagenesis. *K. aerogenes* CG253 was obtained from Boris Magasanik and Alex Ninfa (Massachusetts Institute of Technology), and served as the host for plasmids pKAU11, pKAU12, and pKAU23 (52).

Vectors. Cosmid pWH4 (29), plasmids pBR328 (66), pUC8 (73), and pUB110 (26), were used as cloning vectors. Plasmid pKAU23 (52) served as the probe in DNA labelling experiments. Plasmids pKAU11 and pKAU12 (52) were used in complementation experiments.

Growth conditions. Cultures of *S. ureae* were grown at room temperature in a previously described minimal medium (23) but with the following modifications: K-glutamate at 10 g/L, 50 mM filter-sterilized urea in place of $(NH_4)_2SO_4$, and 10% Brain Heart Infusion broth (BHI) (Difco Labs, Inc., Detroit, Mich.). Alternatively, cultures of *S. ureae* were grown in 100% BHI at room temperature.

The medium used for the transformation of *E. coli* with pBR328 and pUC8 was LB (45) at 37°C. The medium used for the transformation of *B. subtilis* with plasmid pUB110 was DM3 (14) at 37°C. For the infection of *E. coli* with packaged pWH4, NYCZM medium was used, at 37°C (45). For transposon mutagenesis, MMB broth (78) was used at 30°C.

Ampicillin (Ap) and kanamycin (Km), at 50 ug/ml, chloramphenicol (Cm) at 10 ug/ml, and tetracycline (Tc) at 10 ug/ml were used for plasmid

maintenence.

Assays. Expression of urease activity in *E. coli* and *B. subtilis* was detected at 37°C on urease indicator plates consisting of M9 minimal agar (45), pH 6.8, supplemented with 10% (v/v) LB, 100 mM urea, 20 ug/ml phenol red, 1 ml/l trace mineral solution (65) and the appropriate antibiotic. Expression of urease activity in putative transposon mutagenized *S. ureae* was detected at 30°C on the *S. ureae* modified minimal medium, supplemented with 100 mM urea, 20 ug/ml meta cresol purple, pH 8.3, or 20 ug/ml phenol red at pH 6.8, and the appropriate antibiotic.

Urease activity of purified preparations or in sonicated cells was assayed at 625 nm by converting released ammonia to indophenol (79). One unit (U) of urease activity is defined as one umol urea hydrolyzed per minute at 37°C in 200 mM urea, 50 mM HEPES, 10 mM EDTA, pH 7.5 buffer (HE buffer). Protein was assayed as described by Lowry, et al, (41) with bovine serum albumin (BSA) as the standard.

Polyacrylamide gel electrophoresis (PAGE). All PAGE was carried out using the buffers of Laemmli (36), except that SDS was omitted for native gels. Denaturing gels were run by using a 10-15% polyacrylamide gradient resolving gel with a 4.5% stacking gel. Samples were denatured prior to electrophoresis by heating to 100°C in denaturation buffer (36) for 5 minutes. Gels were stained with Coomassie brilliant blue (Sigma) and scanned using a Gilford Response spectrophotometer at 565 nm. Native PAGE was performed by loading 1 U urease activity on a 3% stacking gel and 6% resolving gel, then stained for activity by equilibrating the gel in 1% KH_2PO_4, 0.1% EDTA, 0.02% phenol red (0.5 mM), pH 6.0, followed by incubating the gel in a 1.5% solution of urea. Urease is visualized as red bands on a yellow background.

Large scale growth conditions and urease purification. *S. ureae* was grown in 10 L cultures of modified minimal medium supplemented with 10% BHI at room temperature in either a 14 L Fermentation Design fermenter (Miles Labs) with rapid mixing and aeration or in a 20 L bottle (10 L culture) in a 30°C incubator with rapid shaking. Cells were harvested with a Pellicon concentrator (Millipore Corp., Bedford, Mass), washed once with HED buffer (HE buffer with 1 mM DTT), resuspended in an equal volume of HED buffer and frozen at -20°C. The cells were thawed, disrupted by two passes through a French pressure cell (American Instrument Co., Silver Spring, Md) at 18,000 lb/in^2. Phenylmethylsulfonly flouride (PMSF) was added to a concentration of 1 mM and the disrupted cells were centrifuged at 100,000 x g for 60 min at 4°C. DEAE-Sepharose and phenyl-Sepharose chromatographies were performed on conventional columns at 4°C. Subsequent purification steps were carried out on a Fast Protein Liquid Chromatography (FPLC) system (Pharmacia, Uppsala, Sweden) at room temperature. All resins and columns were purchased from Pharmacia. HED buffer with the stated additions was used in all phases of the purification.

Kinetic parameters of urease. Specific activities of urease were assayed in 200 mM urea in HE, at 37°C, with timepoints at 0, 3, 6, and 9 min. Specific activity was calculated as umol urea min^{-1} mg^{-1}. For K_m determination, the reaction rates for purified urease were measured as the concentration of urea was varied from 1 to 200 mM, and the data were analyzed by the method of Wilkinson (80).

Antisera production. An enriched preparation of urease was run on an SDS gradient polyacrylamide gel as stated, and stained with Coomassie brilliant blue. The clearly resolved bands corresponding to the small

subunits of the enzyme were cut from the gel, destained, fixed in 2% glutaraldehyde, and lyophilized. This preparation was then emulsified with complete Freund's adjuvant (76). A female, New Zealand white rabbit was injected 3 times with 50 ug protein at 3 week intervals and bled 2 weeks after the last injection. Antibody was purified using the method of McKinney and Parkinson (48a), and specificity of the antibody was examined via Western blot (4) and Enzyme Linked Immunosorbant Assay (ELISA) procedures (20).

Antibody immunoprecipitation and inhibition assays. The antibody was serially diluted from 1:50 to 1:12,150 in Tris-buffered saline plus Tween (TBST), pH 7.4, containing per liter: 1.4 g Tris, 8.0 g NaCl. 0.2 g KCl, 0.5 g Tween 20. Urease was diluted to 1:20 in HE buffer. Preimmune serum, diluted 1:50 in TBST, was used as the control. Equal volumes of diluted antibody and urease were combined, incubated overnight at 4°C, and urease activity was assayed as previously described. To assay for immunoprecipitation, the above reaction mixture was centrifuged 20,000 x g for 15 min to pellet the antibody-antigen complexes, and the supernatant assayed for urease activity.

Transposon mutagenesis. *B. subtilis* strain BS250, carrying the conjugative transposon Tn916, was mated at 30°C with *S. ureae* by the method of Sen, et. al. (64). The conjugation mixture was diluted as suggested and spread onto LB plates and Tc^r colonies were selected. Donor *B. subtilis* was distinguished from recipient *S. ureae* by examination with light microscopy or by screening on urease indicator plates.

DNA labelling. The pUC 8-derived plasmid, pKAU 23, containing the cloned 3.5 kb fragment carrying the urease genes of *K. aerogenes* (52) was *Bam*H1-digested and the 3.5 kb fragment was gel isolated. This fragment was

either biotin labelled (Bethesda Research Laboratories) or digoxigenen labelled (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) as per manufacturer's instructions, and used to probe whole cell DNA isolated from *S. ureae*. *S. ureae* DNA was digested with restriction endonucleases, electrophoresed on a 1% agarose gel, blotted onto nitrocellulose and immobilized (45). Nitrocellulose blots were hybridized with the labelled 3.5 kb fragment and visualized using the appropriate detection kit for each probe. *K. aerogenes* whole cell DNA, *Bam*Hl-cleaved, was used as a control.

Molecular biological methods. *S. ureae* chromosomal DNA was purified using standard techniques (45). Transformation of *E. coli* was by the method of Hanahan (27), whereas transformation of *B. subtilis* was by the method of Chang (14). Small-scale plasmid preparations were carried out by the rapid method of Kado and Lui (32) or Birnboim and Doly (3), large-scale preparations by the method of Maniatis (45). Some plasmid DNA was further purified by Superose 6 HR10/30 chromatography on an FPLC system (Pharmacia) in STE buffer (10 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA) with a flow rate of 0.5 ml/min. Restriction fragments were isolated from agarose gels by using DEAE paper (19).

Purified *S. ureae* DNA was partially digested with *Sau*3A to yield approximately 40 kb fragments. The digestion mixture was phenol extracted, ethanol precipitated and ligated into *Bam*Hl-cleaved, phosphatase-treated cosmid pWH4 vector. The resulting DNA was packaged into lambda phage using an in vitro packaging system (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) as per manufacturer's instructions. The phage were used to infect *E. coli* VCS257 and Km^r colonies were selected.

For plasmid library constructions, purified *S. ureae* DNA was

partially digested with *Sau*3A to yield approximately 7-10 kb fragments, ligated into *Bam*H1-cleaved, phosphatase-treated pBR328, pUC8, and pUB110 plasmid vectors. pBR328 and pUC8 constructs were transformed directly into *E. coli* DH1 or HB101 via the Hanahan (27) protocol and Ap^r and Km^r colonies were selected, respectively. The pUB110 constructs were transformed into *B. subtilis* BS101 via the polyethylene glycol protoplast fusion protocol (14) and Km^r colonies were selected.

Complementation experiments. Kanamycin resistant *E. coli* VCS257, containing a mixed population of cosmid pWH4 constructs were made competent by the method of Hanahan (27). Plasmids pKAU11 and pKAU12 were purified from *K. aerogenes* (52) by the methods previously described, used to transform competent *E. coli* VCS257 and plated on antibiotic medium.

RESULTS

Purification of urease. The crude extract (195 ml) from 87.6 g (wet weight) of cells was applied to a DEAE-Sepharose column (2.5 by 15 cm) previously equilibrated with HED buffer. The urease was eluted with a 400-ml linear gradient of 0 to 1.0 M KCl in HED buffer, resulting in a single peak of activity at 0.31 M KCl. Peak fractions (136 ml) were adjusted to 2.0 M KCl and loaded onto a pre-equilibrated phenyl-Sepharose column (1.5 by 14 cm). After a wash with 125 ml of 2.0 KCl in HED buffer, the urease was removed with a single-step elution using 150 ml of HED buffer. Peak fractions were combined (53 ml), concentrated to 5.0 ml by ultrafiltration (Amicon; Amicon Corp., Lexington, Mass.) and applied in 1.0 ml aliquots to a Superose 12 (HR16/50) FPLC column. The activity was eluted as a single peak in HED buffer (Fig. 1). Peak fractions were pooled (32 ml) and applied to a Mono-Q HR 10/10 FPLC column. The activity was eluted as a single peak of activity at 530 mM KCl by using a multi-segment KCl gradient in HED buffer. Peak fractions were pooled (6 ml), diluted 1:2 with HED buffer, and applied to a Mono-Q HR 5/5 FPLC column. Activity was eluted as a single peak at 500 mM KCl by using a multi-segment gradient as before (Fig. 2). Peak fractions were pooled (8 ml) and stored at 0°C. The purification procedure and results are summarized in Table 2.

Analysis of urease by gel electrophoresis. Denatured samples of purified urease were electrophoresed by using a SDS-10 to 15% polyacrylamide gradient gel (Fig. 3). Three polypeptides were observed, with approximate molecular weights 70,000 +/- 3000, 11,000 +/- 2000, and 8,000 +/- 2000. These subunit weights are similar to those found in several other bacterial ureases (Table 1).

Samples of *S. urease* urease were run on non-denaturing gels and

FIG. 1. Superose 12 Fast Protein Liquid Chromatography of *S. ureae* urease. Active fractions from phenyl-Sepharose chromatography were pooled and chromatographed as described in the text. Aliquots of the 1 ml fractions were assayed for urease activity ( ), and A_{280} was monitored (-).

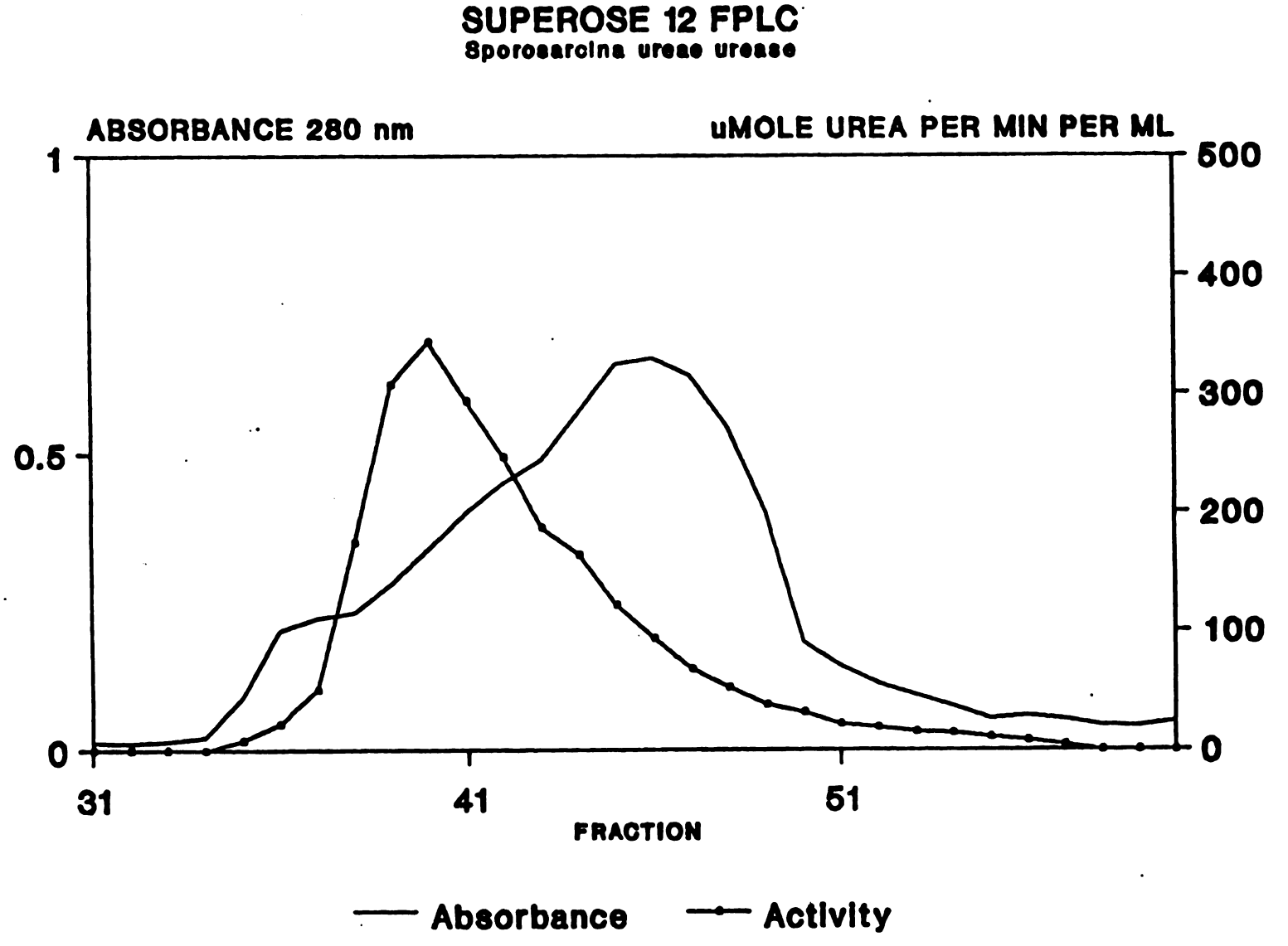

SUPEROSE 12 FPLC
Sporosarcina urea urease
ABSORBANCE 280 nm
uMOLE UREA PER MIN PER ML
1
0.5
0
500
400
300
200
100
0
31
41
51
FRACTION
Absorbance
Activity

Fig. 1

FIG. 2. Mono-Q Fast Protein Liquid Chromatography of *S. ureae* urease. Active fractions from Mono-Q HR 10/10 chromatography were pooled and chromatographed as described in the text using a KCl gradient (---). Aliquots of the 1 ml fractions were assayed for activity ( ), and A_{280} was monitored (-).

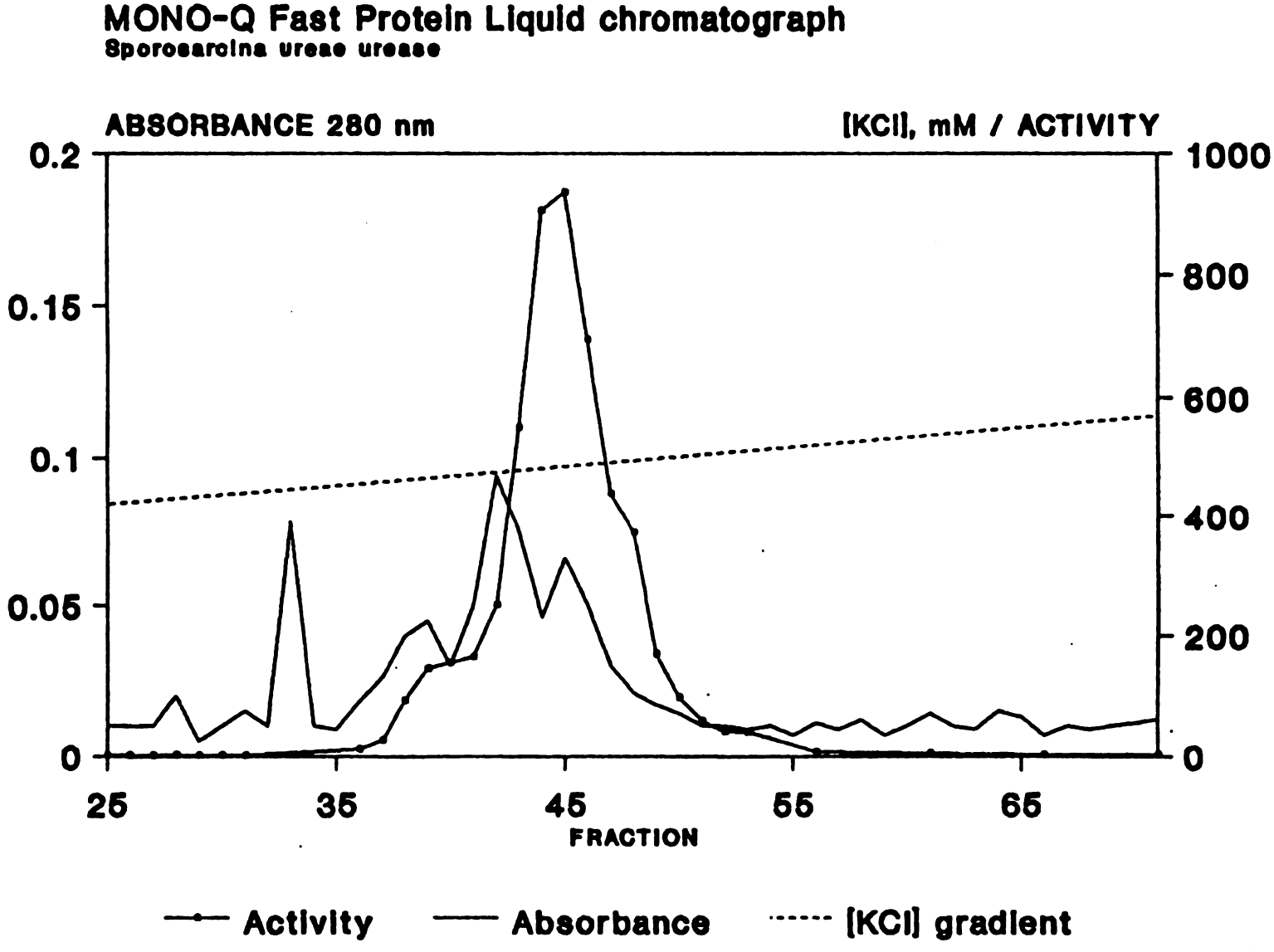

MONO-Q Fast Protein Liquid chromatograph
Sporosarcina urease urease
ABSORBANCE 280 nm
[KCl], mM / ACTIVITY
0.2
0.15
0.1
0.05
0
1000
800
600
400
200
0
25
35
45
55
65
FRACTION
Activity
Absorbance
[KCl] gradient

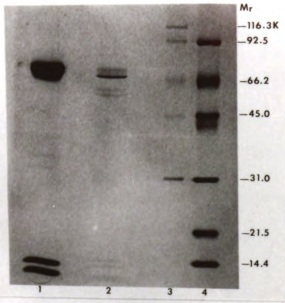
Fig. 2

Table 2. Partial purification of *Sporosarcina* urease urease.

Purification Step	Specific Activity (umol urea min^{-1} per mg)	Purification (fold)	Total Activity (umol/ml)	Total Protein (mg)	Recovery (%)
Crude extract	5.62	1	13,700	2,400	100
DEAE-Sepharose	11.9	2	11,390	950	83
Phenyl-Sepharose	27.8	5	10,314	370	75
Superose 12	164	29	5,400	33	39
Mono-Q (HR 5/5)	760	135	3,300	4.5	25

FIG. 3. Sodium dodecyl sulfate-polyacrylamide gradient gel of purified urease. A sample of purified enzyme was run as described in the text by using 8 ug of protein and then stained with Coomassie brilliant blue. The standards (lanes 3, 4) used were myosin, B-galactosidase, phosphorylase-B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme (Bio-Rad Laboratories, Richmond, Calif.). Lanes: 1, purified *K. aerogenes* urease; 2, purified *S. ureae* urease. Numbers on the right indicate molecular weights.

FIG. 4. Native polyacrylamide gel of purified *S. ureae* urease. Samples of the purified enzyme was run as described in the text by using 1 unit of the enzyme (lane 1), or 0.5 units (lane 2) and then visualized with phenol red and 1.5% urea solution.



3

4

stained for activity (Fig. 4). Three major bands of activity were seen, with several additional faint bands.

Kinetic parameters. A K_m of 62 +/- 20 mM urea and a V_{max} of 770 +/- 80 umol of urea min^{-1} mg^{-1} were obtained for purified *S. ureae* urease.

Antisera production. The titer of the anti-urease antibodies was determined to be less than 450 via an ELISA (Fig. 5). Western blot analysis indicated the antibodies were specific to the small subunits of the purified urease (Fig. 6a). Cross-reactivity with components present in the crude extract, but not in the purified urease preparation, was also observed (Fig. 6b). Additional injections of the more highly purified urease failed to elicit an antigenic response to any of the urease subunits. Specific antibodies to minor contaminants in the more highly purified urease were observed in all subsequent antibody preparations (Fig. 6c).

Immunoprecipitation and inhibition assays. Urease did not precipitate in the presence of antibody nor was it inactivated by antibody at a dilution of 1:50. Urease incubated with pre-immune serum or with TBST alone exhibited no inactivation.

Transposon mutagenesis. The goal of these experiments was to find a urease positive *S. ureae* colony carrying Tn916 which could then be used as the donor in subsequent matings to nonmutagenized *S. ureae*, reasoning that conjugation would be more efficient between two *S. ureae* cells than between *S. ureae* and *B. subtilis*. Once matings between Tc-resistant and Tc-sensitive *S. ureae* were accomplished, urease-negative mutants would be identified. In an attempt to facilitate screening, the conjugation mixture was plated onto indicator plates containing 100 mM urea, 20 ug/ml phenol red, and 10 ug/ml tetracycline, pH 6.8, and incubated at 30°C. Significant

FIG. 5. ELISA titration of anti-urease antibodies. Enzyme activity represented as as function of antiserum dilution in urease coated wells.

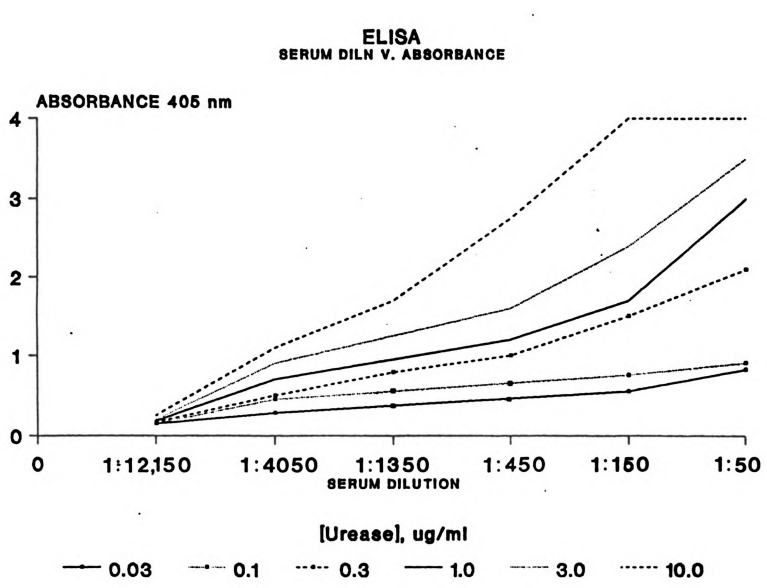
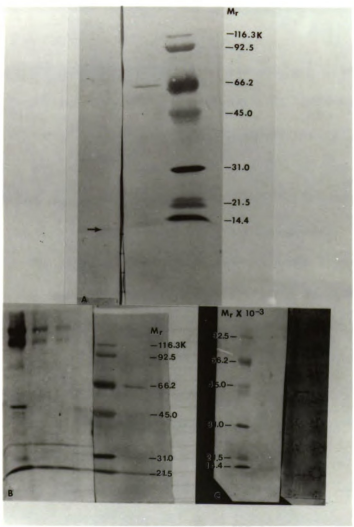

ELISA
SERUM DILN V. ABSORBANCE
ABSORBANCE 405 nm
4
3
2
1
0
0
1:12,150
1:4050
1:1350
1:450
1:150
1:50
SERUM DILUTION
[Urease], ug/ml
0.03
0.1
0.3
1.0
3.0
10.0

Fig. 5

FIG. 6. Western blot analysis of *S. ureae* samples using anti-*S. ureae* urease antibodies. Samples of purified urease (A, C), or crude extract (B) were denatured, electrophoresed on an SDS 10-15% polyacrylamide gel, and blocked onto nitrocellulose. The filters were then probed with anti-*S. ureae* urease antibodies and developed by using alkaline phosphatase conjugates. A standard of purified *S. ureae* urease and molecular weight standards were also run and stained for total protein with Amido black. Molecular weight markers are indicated to the right of each figure.

Figure (A): Lane 1; antibody specificity for the small urease subunits, lane 2; urease standard, lane 3; molecular weight standards. Figure (B): Cross-reactivity with proteins in the crude extract, lanes 1-4; crude extract at 1.0 unit, 0.5 units, 0.25 units, and 0.1 units, lane 5; molecular weight standards, lane 6; urease standard. Figure (C): antibodies produced to a minor contaminant (lane 3). Lane 1; molecular weight standards, lane 2; urease standard.


Mr
-116.3K
-92.5
-66.2
-45.0
-31.0
-21.5
-14.4
A
Mr
-116.3K
-92.5
-66.2
-45.0
-31.0
-21.5
B
Mr X 10-3
2.5-
6.2-
5.0-
1.0-
.5-
.4-

6

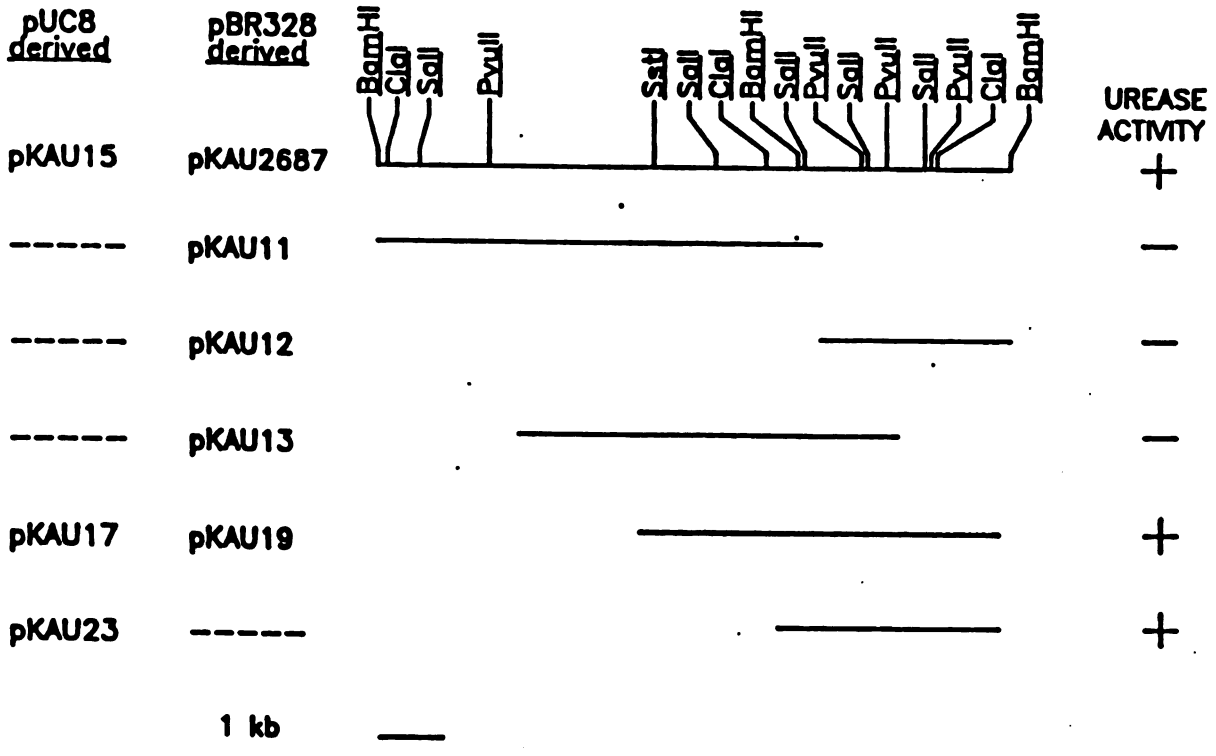
color change on indicator plates was observed, inferring the presence of ureolysis. Since *B. subtilis* does not contain urease, it was assumed the color change was due to the presence of Tc^r *S. ureae*. The color change was not discretely located around individual colonies, but diffused throughout the medium, thus making bright-field microscopic examination necessary. All colonies examined by brightfield microscopy were *B. subtilis*.

To overcome the problem of the rapid and diffuse color change which occurred with phenol red at pH 6.8, a different pH indicator was selected and the buffering capacity and pH of the medium was increased. Meta-cresol purple (MCP) was used as the pH indicator and the pH was raised to 8.3, the preferred pH of *S. ureae*. Again, the color change was diffuse and no *S. ureae* colonies were identified by visual examination.

To test for the possibility that *B. subtilis* was responsible for the color change in the indicator media, despite being urease negative, a pure culture of *B. subtilis* BS250 was diluted and spread onto both phenol red and MCP plates, with and without urea. Upon overnight incubation at 30°C, the color change was again observed in the presence and absence of urea, with both indicators, thus the increase in pH and the accompanying color change occurred by means other than ureolysis.

DNA Labelling. The biotin labelled 3.5 kb fragment from pKAU23 (Fig. 7) was tested for sensitivity by self-hybridization to unlabelled, denatured 3.5 kb insert DNA from plasmid pKAU23. The probe was able to detect 5 pg homologous DNA using the BRL DNA detection system as per manufacturer's instructions. The probe was hybridized to Southern transfers of endonuclease restricted chromosomal *S. ureae* DNA, with *Bam*H1-cleaved *K. aerogenes* whole-cell DNA (1 ug/lane) as the control. A band was

FIG. 7. Restriction map of cloned *K. aerogenes* urease gene fragments. A restriction map is presented of a 10 kb DNA fragment containing the *K. aerogenes* urease genes. Subclones of the DNA fragments were generated and tested for the presence (+) or absence (-) of urease activity based on results from indicator plates. Reprinted with permission (52).


pUC8 derived
pBR328 derived
BamHI
ClaI
SalI
PvuII
SstI
SalI
ClaI
BamHI
SalI
PvuII
SalI
PvuII
SalI
PvuII
ClaI
BamHI
UREASE ACTIVITY
pKAU15
pKAU2687
+
-----
pKAU11
-
-----
pKAU12
-
-----
pKAU13
-
pKAU17
pKAU19
+
pKAU23

+
1 kb

Fig. 7

detected at 3.5 kb in the control lane, but no bands were seen in lanes containing *S. ureae* DNA. The stringency of the hybridization was reduced and the duration of incubation and the concentration of probe were both increased in an attempt to overcome the possible lack of homology between the probe and *S. ureae* DNA. Again, a band was detected at 3.5 kb in the control lane, but no other bands were seen.

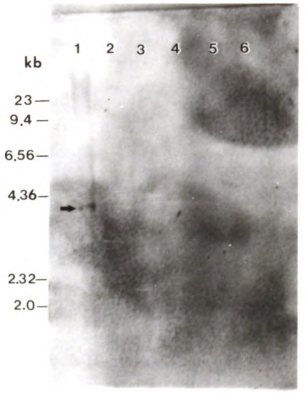
To increase sensitivity, the 3.5 kb fragment was labelled with digoxigenin. Sensitivity testing was conducted as before, the sensitivity of this probe being 0.1 pg of homologous DNA. Hybridization of Southern transfers was conducted at 68°C as recommended by the manufacturer, and at 42°C with 50% formamide to enhance binding of nonhomologous DNA. Only the control lane showed hybridization with the probe (Fig. 8).

Molecular biology. Results of library screening are summarized in Table 3. Incubations were up to 48 hours to allow for low expression of the gene in an heterologous host. Incubations were done at 30°C, the preferred temperature for *S. ureae*, and at 37°C, the preferred temperature for the host strain. Indicator plates contained 100 mM urea for all *E. coli* transformants. 100 mM urea is in the range of *S. ureae* urease K_m. When screening *B. subtilis* transformants, indicator plates containing 200 mM urea were used to compensate for the host's lack of a urea permease, and to enhance diffusion of the urea through the cell wall (34).

Isopropylthiogalactoside (IPTG) was used as an inducer when screening the pUC8 library. Cultures of VCS257 containing cosmid constructs were heated for 20 min at 42°C in an attempt to inactivate the cI repressor protein of pWH4 (45).

Recombinant colonies were screened initially on Christensen's urea agar, then on M9 + 10% LB and 100% LB, both with phenol red, pH 6.8.

FIG. 8. Southern blot analysis of *S. ureae* DNA using *K. aerogenes* urease gene probe. Whole cell DNA was purified from *S. ureae* and restricted with endonucleases. 0.3 ug of restricted DNA was electrophoresed on a 1% agarose gel, blotted onto nitrocellulose, and probed with the digoxigenin-labelled 3.5 kn fragment isolated from pKAU23. The filter was developed by using the digoxigenin detection system (BRL). Whole cell *K. aerogenes* DNA, restricted with *Bam*Hl, was run as the control (lane 1). A molecular weight standard of *Hind*III-restricted DNA was also run and stained with ethidium bromide. Molecular weight markers are indicated to the left of the figure. Lanes 2 - 6 contained *S. ureae* DNA restricted with: 1, *Eco*RI; 2, *Bam*Hl; 3, *Sal*I; 4, *Hind*III; 5, *Sst*I; 6, *Pst*I.



8

Table 3. Results of indicator plate screening of recombinant libraries.

Vector	Vector Size (kb)	Marker	Insert Size (kb)	Host	Transformation Efficiency	Percent Inserts	Number Screened	Grease Positive
pUB110	4.55	Km^r	5	BS101	200 cfu/ug	70	200	0
pUC8	2.7	Ap^r	3-8	DH1/HB101	10^7 cfu/ug	60	2500	0
pBR328	4.9	Ap^r	3-8	DH1/HB101	10^7 cfu/ug	80	2500	0
pUB4	6.4	Km^r	40-45	VCS257	10^6 cfu/ug	75	1000	0

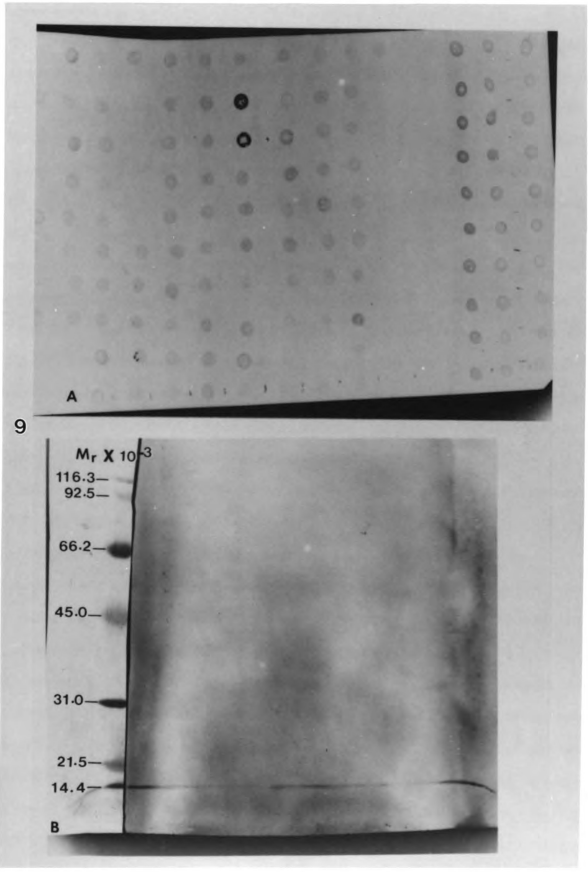
Colonies were rescreened on M9 + 10% LB and 100% LB, both with meta-cresol purple, pH 8.3, to account for the higher pH preferred by *S. ureae*.

Colonies containing pWH4 constructs were also screened with antibodies to *S. ureae* urease, first by *in situ* hybridization (35), followed by Western blot analysis (4) of promising colonies (Fig. 9a, 9b).

Complementation experiments. Kanamycin resistant VCS257 colonies were chosen and small-scale plasmid preps were performed. The plasmid DNA was digested with *Bam*H1, electrophoresed on 1% agarose gels and visualized under ultraviolet light. Only resident cosmid constructs were observed, indicating transformation with pKAU11 and pKAU12 was not successful.

FIG. 9a. *In situ* antibody analysis of pWH4 library. Recombinant VCS257 were grown on nitrocellulose filters on agar. The filters were then incubated in chloroform to lyse the colonies, and bacterial debris was washed away. Filters were blocked in 1% powdered milk in TBST, and then incubated with anti-*S. ureae* urease antibodies and developed by using alkaline phosphatase conjugates (4).

FIG. 9b. Western blot analysis of pWH4 library. Samples of crude extract were denatured, electrophoresed on an SDS 10-15% gradient polyacrylamide gel, and blotted onto nitrocellulose. The Western blot was probed with anti-*S. ureae* urease antibodies and developed by using alkaline phosphatase conjugates. The darkly stained band at bottom of blot is the dye front.


A
9
Mr X 10-3
116.3
92.5
66.2
45.0
31.0
21.5
14.4
B

DISCUSSION

Urease localization. Measurement of spent cell medium, the resolubilized membrane pellet, and cell extracts demonstrated that most of the urease appears to be cytoplasmic and none is secreted by *S. ureae*. This result contrasts with the unsupported statement by Varner (72) that *S. ureae* urease is an extracellular enzyme.

Urease purification. The protocol provides a highly enriched enzyme with a final specific activity of 760 umol of urea min^{-1} mg^{-1} protein. When compared to the ureases of other Gram-positive microbes, this specific activity is higher than that of some, but considerably lower than others (Table 1). The enzyme was enriched 135-fold with an overall recovery of 25%. *S. ureae* urease is estimated to be approximately 75% homogeneous on the basis of polyacrylamide gel electrophoresis. This protocol represents a significant improvement over previous (unpublished) purification schemes from this lab. Earlier purifications utilized HE or other buffers with 1 mM 2-mercaptoethanol (2ME), resulting in a maximum 31-fold enrichment with a total recovery of 23% (data not shown). The substitution of 1 mM DTT for 2ME leads to more effective protection against oxidation (16); these results suggest that the loss of activity seen in previous preparations was a result of oxidation. Activity was restored in oxidized *K. aerogenes* urease by dialyzing the enzyme against DTT (Julie Breitenbach, Hausinger, unpublished). Similar experiments with *S. ureae* urease purified with 2ME were unsuccessful in stimulating enhanced activity. Once activity is lost from *S. ureae* urease it cannot be restored, thus prevention of activity loss during purification is more crucial than regeneration of activity once the enrichment is complete. The incorporation of DTT into the purification scheme proved successful in

this regard. Importantly, because DTT is a urease inhibitor (68), assays were carried out by diluting the enzyme into HE buffer which did not contain this thiol.

SDS-PAGE of *S. ureae* urease shows three distinct polypeptides, which are presumably analogous to the three *K. aerogenes* urease subunits. These three polypeptides account for 75% of the protein staining intensity on SDS-PAGE gels. Subunit molecular weights, estimated from SDS-PAGE, are approximately 70,000 +/- 3000, 11,000 +/- 2000, and 8,000 +/- 2000. Subsequent studies indicate these molecular weights appear to be somewhat smaller, i.e., 66,000 +/- 3000, 10,000 +/- 2000, and 8,000 +/- 2000 (R.P. Hausinger, personal communication). These weights are very similar to subunit molecular weights of several Gram-negative microbial ureases.

The presence of these three polypeptides implies that urease from *S. ureae* may be more analogous in structure to the heteropolymeric ureases seen in Gram-negative organisms than to the homopolymeric ureases described in other Gram-positive microorganisms. These findings lend credence to the hypothesis that the differences seen between Gram-negative and Gram-positive microbial ureases are more artifactual than genuine. As previously discussed, the small subunits are easily missed when the enzyme is electrophoresed on non-gradient SDS-PAGE. A very recent example of the similarity between Gram-negative and Gram-positive ureases, consistent with the findings presented here, is the urease purified from the Gram-positive bacterium *Lactobacillus reuteri* (33). This is an acid urease with three polypeptides, molecular weights 68,000, 16,100, and 8,800, designated α, ß, and Γ, respectively. The native molecular weight of the enzyme was estimated to be approximately 220,000, with a native urease

structure of $(\alpha_1\beta_2\gamma_1)_2$.

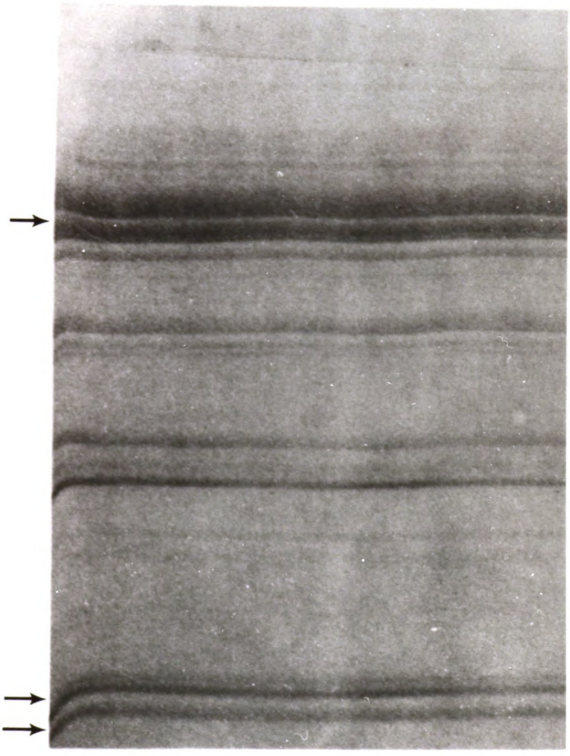
A K_m for *S. ureae* of 61 +/- 20 mM urea is consistent with values obtained by others in this lab utilizing earlier purification protocols of *S. ureae* urease. This K_m is within the range of values determined from crude extracts of other Gram-positive microbial ureases, but well above the values obtained for Gram-negative ureases. The ecological significance of such a high K_m is not clear; it is unlikely such high urea levels would be observed in many environments. However, this enzyme would likely be saturated near feedlots and in soils fertilized with urea.

Native molecular weight studies done previously in this lab (unpublished) place the native molecular weight of *S. ureae* urease at approximately 260 kD + 30 kD. While this is less than the native molecular weight of jack bean urease, it is still within the range of the bacterial ureases (Table 1).

Native PAGE indicates the presence of multiple urease bands when stained for urease activity. The origin of these bands may be similar to the unexplained multiple bands seen with *K. aerogenes* urease and many other ureases (49). As shown in these other cases, the different forms all arise from the same gene product. The multiple bands in *S. ureae* urease could be the result of differential post-translational processing of the same gene product.

Antisera production. The antigen was prepared by isolation from SDS-PAGE in an attempt to obtain the purest protein possible (Fig. 10). At the time of the initial immunization, the purest preparation of urease still contained many contaminanting proteins, thus electrophoresis was chosen as a tool to separate the proteins of interest. While the SDS-PAGE

FIG. 10. Sodium dodecyl sulphate-polyacrylamide preparative gel of small urease subunits.



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band corresponding to the large subunit may have been a better choice as an immunogen, i.e. the large subunit may have been more immunogenic than the small subunits, the close proximity of other protein bands to the large subunit band made isolation of that single band difficult. The bands corresponding to the small subunits were better resolved, making them the better choice as immunogens.

The bands were cut from the gel, cross-linked with glutaraldehyde, and destained thoroughly. No attempt was made to elute the protein from the gel, but rather the gel slice containing the small subunits was lyophilized intact. The polyacrylamide gel increases the immunogenicity of the sample because the polyacrylamide helps to retain the antigen in the animal, thus acting as an adjuvant (76). Once a more purified preparation of the urease was obtained, isolation of the urease bands via electrophoresis was omitted, and the sample from FPLC was used directly to boost the rabbit in an emulsion of Freund's incomplete adjuvant.

The antibody directed against the gel-isolated small subunits was titered at 450. These antibodies, although of low titer, were fairly specific to the small subunits, most likely as a result of the gel isolation procedure. However, in Western blot analysis, some cross-reactivity was seen with high molecular weight polypeptides in the crude extract. Since these high molecular weight polypeptides were not present in the sample used to immunize the rabbit, these results are not indicative of heterologous antibodies being produced in response to a contaminated or heterologous antigen, but rather nonspecific cross-reactivity with the anti-urease antibody.

This cross-reactivity could be the result of the presence of polyacrylamide in the sample used to immunize the animal (76). Since

polyacrylamide is highly immunogenic, nonspecific antibodies may be produced. Another possible explanation is the dose used to immunize the animal may have been too high, which in turn increases the likelihood of cross-reactivity (35). There may also exist in the crude extract a precursor protein that is degraded to form the small subunits. Antibodies recognizing the small subunits would also be likely to recognize this precursor protein. Binding of the antibody to this precursor could account for the results seen with crude extract on Western blots.

Subsequent immunizations with the more highly purified urease failed to increase the titer of the anti-urease antibody, and the titer decreased with time. In addition, a minor contaminant in the more purified urease preparation appeared to be much more immunogenic than any of the urease subunits. On Western blot analysis, crude extracts of *S. urease* probed with these later antibodies indicated the presence of a highly specific antibody to this minor contaminant. There was no cross-reactivity with any other polypeptides in the crude extract, and there appeared to be no antibody directed to any of the urease subunits.

In addition to the immunogenicity of the antigens, another factor to be considered is the responsiveness of the test animal. Some animals may be good responders to certain antigens, while at the same time respond poorly to other antigens (35). Since only one animal was immunized, it is possible the animal was a poor responder, and had immunizations been conducted in more that one animal, a more responsive animal may have been found, thus yielding antibodies of high titer with high avidity.

Immunoprecipitation and inactivation. In other experiments with anti-urease antibodies from the small subunits, it was observed that urease was not inactivated by the antibody. These findings could be the

result of the low specificity of the antibody for urease. However, anti-urease antibody to *K. aerogenes* urease failed to inactivate the *K. aerogenes* urease as well (52). One explanation of this is that urease is a tightly folded molecule with active sites which are not exposed. Antibody to the urease enzyme may not necessarily be directed toward the active sites, but to another, more exposed portion of the enzyme. In addition, the substrate urea is a very small molecule, easily capable of gaining entry to the active site through antibody-antigen complexes. Despite the formation of antibody-antigen complexes, the enzyme retains activity.

If sufficient antigen-antibody complexes are formed, it should be possible to precipitate the urease activity out of solution. Scott Mulrooney, of this lab, succeeded in showing this with *K. aerogenes* anti-urease antibody. With *S. ureae* anti-urease antibodies, no immunoprecipitation was observed. Again, this could be a function of the low specificity or avidity of the antibody for the urease. If few or weak complexes are formed, little precipitation of the urease would occur, leaving the urease activity in the supernatant.

Transposon mutagenesis. Tn916 has been shown to be an effective tool in manipulating the genome of other Gram-positive species, specifically *Bacillus* species (64). Since *S. ureae* is most closely related to *B. pasteurii* on the basis of 16s rRNA (56), it was hoped Tn 916 could be successfully used in generating urease deficient *S. ureae* colonies. All attempts to introduce Tn916 into *S. ureae* were unsuccessful. Possibly, the cell surface receptors which permit conjugation are lacking in *S. ureae*. Nucleases in *S. ureae* may have degraded the foreign Tn916 DNA or the transposon DNA may have been unable to integrate into the host

chromosome.

DNA labelling. Several researchers have found, via DNA hybridization analysis, little genetic similarity between cloned Gram-negative urease genes (49). There are at least four hybridization groups based on DNA hybridization of whole-cell DNA with specific probes from *P. stuartii* (30), *K. aerogenes* (21), and *M. morganii* (Mobley, unpublished). It may be that even less similarity exists between the sequences of Gram-negative and Gram-positive urease genes. This could account for the failure to detect the urease gene or genes of *S. ureae* when whole-cell DNA was probed with the labelled clone from *K. aerogenes*. Control experiments performed to test the sensitivity of both labelling kits indicated that technical errors were not the cause of the problems encountered with this technique.

The biotin-labelled probe was not highly sensitive, detecting only 5 pg of homologous DNA. If only one copy of the *S. ureae* urease gene is present on the Southern blot, it may be difficult to detect with a biotin labelled probe unless there exists a great deal of similarity between the probe and the target sequence. A reduction in stringency of hybridization conditions resulted only in an increase in background on the nitrocellulose filter. To gain increased sensitivity, the digoxigenin-labelled probe was utilized. Again, under conditions of high and low stringency, only the control DNA was detected. These findings suggest there exists a significant amount of dissimilarity between the probe and the target DNA, accounting for the failure of this approach to locate the gene.

Initially, it was thought the 3.5 kb labelled probe contained both structural and regulatory genes for *K. aerogenes* urease (52), i.e. the

recombinant urease possessed all three subunits and was fully functional. However, further studies indicated the 3.5 kb fragment only contains part of the α and none of the ß, Γ or regulatory regions (52). If the probe had contained all the regions originally thought, it may have been possible to locate the *S. ureae* urease genes even if little sequence homology existed or if the *S. ureae* genes were widely distributed over the chromosome. Since the probe contained only part of the α region, this decreased the likelihood of finding the *S. ureae* urease genes via this method. The 5.7 kb fragment, contained in plasmid pKAU 19 (Fig. 7), would have been a better probe as it does contain all the regions the 3.5 kb fragment was assumed to have.

Molecular biology. All attempts to express the urease gene in an heterologous host were unsuccessful. Perhaps, being of Gram-positive origin, the gene was not able to express in the Gram-negative hosts. This seems implausible in view of the fact that a variety of vectors was used in which the size of the insert was varied (4 kb to 40 kb), in which vector promotors were utilized (pUC8), or vector repressors were inactivated (pWH4).

E. coli was the host of choice for a variety of reasons: ease of manipulation, the variety of vectors available, and previously reported success at achieving expression of the Gram-positive *B. pasteurii* cloned urease in an *E. coli* host (34). Since *S. ureae* is most closely related to *B. pasteurii*, it was hypothesized that *S. ureae* urease genes would also be able to express in a Gram-negative host. However, it is possible that *E. coli* lacked the necessary mechanisms to synthesize a functional urease using genes cloned from *S. ureae*. All media contained nickel, known to be required in *K. aerogenes* urease activity (49), and suspected to be

important in *S. ureae* urease activity (61). However, had the nickel processing genes of *S. ureae* not been cloned with the urease structural genes, *E. coli* would not be able to incorporate the nickel into the urease.

Plasmid pBR328 was chosen as an all-purpose cloning vehicle in which expression of the *S. ureae* urease gene would depend on the native promotor being present and functional. Since cloning was performed in an heterologous host, it seemed possible that the *S. ureae* promoter may not be functional, thus plasmid pUC8 was chosen in order to utilize its promotor. Additionally, if only part of the *S. ureae* urease operon was cloned, i.e. the promotor was lacking, the promotor of pUC8 could be used in its stead. The addition of IPTG in the plating medium to derepress the pUC8 represser proved unsuccessful in achieving expression of a cloned *S. ureae* urease gene. The number of recombinant colonies screened, both pBR328 and pUC8, should have been sufficient to account for 99% of the *S. ureae* genome.

Since no expression was obtained with either of the plasmid vectors, it was thought that possibly the *S. ureae* urease genes were more widely distributed in the chromosome, thus the small insert size of these vectors was not sufficient to encompass the entire operon. To overcome this obstacle, a cosmid vector, pWH4, was chosen to allow cloning of fragments up to 40 kb. Again, no expression of the *S. ureae* urease gene was observed. Since pWH4 is a lambda derivative, it possesses the cI repressor, which may have been preventing translation of the insert DNA. To inactivate this repressor, cultures were temporarily heated to 42°C and then plated onto indicator plates. This also failed to produce a urease positive clone.

These findings led to the hypothesis that perhaps some feature of the Gram-negative host was responsible for the lack of expression of the *S. ureae* genes. Plasmid pUB110 was chosen as a vector to allow cloning into a Gram-positive host, *B. subtilis*. Several problems were encountered with the use of this system. The insert size of pUB110 is quite small, only 4.9 kb (26), hence the size of the *S. ureae* genome represented is considerably reduced. This decreased the likelihood of cloning the entire *S. ureae* urease operon intact, especially if the regulatory, structural, and accessory genes were not located in close proximity. It was hoped that the regulatory genes of *B. subtilis* would be homologous enough to *S. ureae* to allow expression if the regulatory genes of *S. ureae* were not present.

Another drawback to this system is the poor efficiency of transformation. Best success was obtained with the polyethylene glycol protoplast fusion protocol (14), but even after many transformations, only a small portion of the total genome had been represented, and all recombinants lacked urease activity. In addition, *B. subtilis* frequently deletes foreign DNA, so that the *S. ureae* urease gene may have been cloned but was deleted or rearranged by the host (Pat Oriel, personal communication).

In addition to the drawbacks of the vectors, some procedural aspects could have caused further problems. When the *S. ureae* DNA was partially digested prior to vector ligation, size fractionation was performed to isolate inserts of the appropriate size for the plasmid vectors, but not for the cosmid vector. Possibly, scrambling of the genome occurred due to the ligation of small pieces to each other or to the larger inserts. There could have been rearrangement of the insert DNA in the cosmid vector due to recombination if the host cell was infected with more than one phage

particle, or if more than one fragment was contained between the cosmid arms. On agarose gels, large inserts of the appropriate size were seen, but small DNA fragments could have been present.

Another possibility for the failure of this method could be a situation similar to that of *U. urealyticum*. The UGA codon is transcribed as a tryptophan in this mycoplasma, whereas these codons are read as stop codons in *E. coli*, thus few sequences are transcribed in full when the DNA is cloned into *E. coli* (5a). It is unlikely that this is the situation with *S. ureae*, but *U. urealyticum*, like other mycoplasmas, did evolve from the Gram-positive bacteria (63), thus one might expect such codon usage in some Gram-positive bacteria. The literature does not exclude this possibility as there are no reports of any previously cloned genes from *S. ureae*.

Perhaps the most likely explanation for the failure to clone and express the urease genes is that the *S. ureae* genes are widely scattered over the chromosome rather than located in a single area. If this is so, this would be the first instance a situation like this has been observed with microbial ureases.

Antibody screening of cosmid library. Recombinant colonies were screened *in situ* initially, and those colonies that appeared promising were then screened via Western blot. None of the colonies analyzed by Western blot showed the presence of any of the three urease subunits. Few recombinants were screened with this method due to the low titer and specificity of the antisera. The antibody was of such low specificity that many of the colonies probed *in situ* appeared to contain elements recognized by the antibody, yet when analyzed via Western blot techniques, none showed the presence of any portion of the urease enzyme.

Complementation experiments. These experiments were attempted in an effort to overcome the possibility that the entire urease operon of *S. urease* was not cloned as one unit. The 7.1 kb fragment in plasmid pKAU11 contained the regulatory regions of the cloned *K. aerogenes* urease operon, while the 2.9 kb fragment in plasmid pKAU 12 was thought to contain the structural genes, as previously discussed. Neither fragment exhibits urease activity, but the entire 10 kb fragment produces a functional urease (Fig. 7). By introducing either of these fragments into a mixed population of hosts with *S. ureae* inserts, it was hoped the structural or regulatory genes of *S. ureae* urease could be located if complementation occurred.

No colonies expressing both Km^r of pWH4 and Tc^R or Ap^R of pKAU 11 or pKAU 12 were observed. Most likely, the host was unable to accomodate both the cosmid and the plasmid vectors, as only the cosmid vector was isolated from colonies after transformation.

CONCLUSIONS

Attempts to obtain a more highly enriched urease from *S. ureae* were successful. The protocol resulted in an 135-fold enrichment of the enzyme with 75% homogeneity. The final specific activity was 760 umol urea min^{-1} mg^{-1}. This is within the range of specific activities of other Gram-positive ureases. Incorporation of DTT into the purification protocol resulted in reduced oxidation of the enzyme, preventing loss of activity.

Based on preliminary studies of the highly enriched enzyme, it appears that *S. ureae* urease more closely resembles ureases purified from Gram-negative microorganisms that those of Gram-positive microbes. *S. ureae* urease appears to be heteropolymeric, with a large subunit and two small ubunits. This raises the possibility that similar small subunits may be present in other Gram-positive ureases but were overlooked on SDS gels. Consistent with this hypothesis is the recently described urease from *L. reuteri*, a Gram-positive urease that contains three distinct subunits.

Attempts to clone the *S. ureae* urease genes were unsuccessful. Failure to achieve expression in a variety of vectors and hosts could indicate that the organization of the *S. ureae* urease genes differ significantly from the urease operons of other organisms studied thus far. Rather than existing in a single discrete operon, it seems possible the *S. ureae* urease genes may be widely scattered throughout the chromosome. It appears that little sequence homology exists between previously cloned urease genes and those of *S. ureae*.

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