*J. Microbiol. Biotechnol.* (2006), **16**(2), 286–290

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY



# Cloning and Characterization of the Urease Gene Cluster of *Streptococcus vestibularis* ATCC49124

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Received: October 18, 2005
Accepted: December 6, 2005

**Abstract** A genomic library of *Streptococcus vestibularis* ATCC49124 was constructed in an *E. coli* plasmid vector, and the urease-positive transformants harboring the urease gene cluster were isolated on Christensen-urea agar plates. The minimal DNA region required for urease activity was located in a 5.6 kb DNA fragment, and a DNA sequence analysis revealed the presence of a partial *ureI* gene and seven complete open reading frames, corresponding to *ureA*, *B*, *C*, *E*, *F*, *G*, and *D*, respectively. The nucleotide sequence over the entire *ure* gene cluster and 3'-end flanking region of *S. vestibularis* was up to 95% identical to that of *S. salivarius*, another closely related oral bacterium, and *S. thermophilus*, isolated from dairy products. The predicted amino acid sequences for the structural peptides were 98–100% identical to the corresponding peptides in *S. salivarius* and *S. thermophilus*, respectively, whereas those for the accessory proteins were 96–100% identical. The recombinant *E. coli* strain containing the *S. vestibularis ure* gene cluster expressed a high level of the functional urease holoenzyme when grown in a medium supplemented with 1 mM nickel chloride. The enzyme was purified over 49-fold by using DEAE-Sepharose FF, Superdex HR 200, and Mono-Q HR 5/5 column chromatography. The specific activity of the purified enzyme was 2,019 U/mg, and the Michaelis constant ($K_m$) of the enzyme was estimated to be 1.4 mM urea. A Superose 6HR gel filtration chromatography study demonstrated that the native molecular weight was about 196 kDa.

**Key words:** *Streptococcus vestibularis*, urease, nickel

*Streptococcus vestibularis*, a Gram-positive, catalase-negative facultative anaerobic bacterium that is a member of the viridans group streptococci, was first isolated from the vestibular mucosa of human oral cavities and described as a new species in 1988 [20]. This microorganism is known to produce urease (urea amidohydrolases [EC 3.5.1.5]), a nickel-containing multisubunit enzyme that catalyzes the hydrolysis of urea to form carbon dioxide and ammonia [14, 15]. This urea hydrolysis by *S. vestibularis* and oral bacteria is thought to play a crucial role in oral microbial ecology and general oral hygiene. For example, the ammonia production from ureolysis and resulting elevated pH could inhibit the initiation and progression of dental caries [18], and ameliorate the glycolytic acidification of dental plaque [4, 9]. The elevated pH values observed in dental plaque as a result of ureolysis may also enhance the deposition of minerals, leading to the formation of calculus [13]. Urea metabolism in gingival crevices and the subgingival environment may also cause mineral deposition on tooth root surfaces and tissue damage, thereby contributing to the development of gingivitis and periodontitis [8]. Nonetheless, *S. vestibularis* was not known to cause any human diseases until a few cases of prosthetic and native valve infectious endocarditis were reported recently [7, 17].

The urease gene clusters of viridans streptococci, including *S. salivarius* [5, 6] and *S. thermophilus* [2, 16], have already been cloned and characterized, and revealed that the overall composition and organization of the urease genes were homologous to those of the majority of other bacterial ureases [15]. Therefore, this study describes the molecular characterization of the urease genes of *S. vestibularis* and the characteristics of the expression of the recombinant enzyme in *E. coli*. In addition a purification scheme for recombinant urease is provided, along with a description of some of the enzyme's biochemical properties.

## MATERIALS AND METHODS

### Bacterial Strains and Growth Conditions

The *Streptococcus vestibularis* ATCC49124 was maintained on a brain heart infusion (BHI) agar (Difco Laboratories,

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Detroit, MI, U.S.A.) supplemented with 7% horse serum. It was also grown aerobically at 37°C in either a BHI broth or trypticase soy (TS) broth, supplemented with 7% horse serum, respectively. The plasmid-bearing *E. coli* DH10B cells were grown in a Luria-Bertani (LB) medium containing 100 μg/ml ampicillin. For the nickel-dependence studies, various concentrations of $NiCl_2$ were added to the LB-ampicillin medium. For the selection of urease-positive recombinant clones, Christensen urea agar (Becton-Dickinson, NJ, U.S.A.) plates were used.

**DNA Manipulations**

The genomic DNA of *Streptococcus vestibularis* ATCC49124 was isolated as follows. Briefly, the cells were grown to the mid-exponential phase in a BHI broth supplemented with 7% horse serum. The cells were then washed once with a 20 mM sodium phosphate buffer (pH 7.0) and incubated with lysozyme (0.2 mg/ml of culture volume) and mutanolysin (Sigma, MO, U.S.A., 0.2 U/ml of culture volume) for 60 min at 37°C. Thereafter, the cells were lysed with 1% sodium dodecyl sulfate (SDS) and proteinase K (50 μg/ml), and the DNA isolated as previously described [1].

To prepare a subgenomic library of *S. vestibularis*, the chromosomal DNA isolated as above was partially digested with Sau3A1 and the resulting DNA fragments were separated on a 0.6% agarose gel. DNA fragments ranging in length from 6 to 9 kbp were isolated using a Gene Clean III kit (Bio101 Inc., CA, U.S.A.). The isolated DNA fragments were then ligated into a BamHI-digested calf intestinal phosphatase-treated plasmid vector pUC19, using T4 DNA ligase. The positive transformants were selected by replica-plating onto Christensen urea agar plates, from which the pink-colored colonies resulting from the production of functional urease were isolated and confirmed using standard urease assays, as described below. A partial restriction map of the cloned insert (~6.8 kbp) in the recombinant plasmid (designated as pSVU101) was constructed using various combinations of the restriction enzymes.

**DNA Sequencing**

The nucleotide sequences were determined using a BigDye™-terminator cycle sequencing kit (Perkin-Elmer, U.S.A.) and ABI Prism®310 Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A.). The clones for the sequence analysis were obtained by generating an exoIII-S1 nested-deletion series using an Erase-A-Base® System (Promega, Madison, WI, U.S.A.). The nucleotide and deduced amino acid sequence data were analyzed using Vector NTI (InforMax, Inc., Frederic, MD, U.S.A.) and BLAST searches at the National Center for Biotechnology Information.

**Expression of *S. vestibularis* Urease in *E. coli***

Recombinant *E. coli* DH10B cells harboring pSVU101 were grown at 37°C in an LB-ampicillin (100 μg/ml) broth supplemented with $NiCl_2$ at concentrations ranging from 0 to 2 mM. The cells were harvested in the late-exponential phase and washed twice with ice-cold 20 mM $KH_2PO_4$-1 mM ethylenediamine tetraacetate (EDTA)-1 mM dithiothreitol (pH 7.0, PED), and then resuspended in the same buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF) at one-tenth of the original culture volume. Thereafter, the cells were disrupted by sonication on ice with 10-sec 200-W pulses for 2 min, and the supernatant was obtained by centrifugation at 20,000 ×*g* for 30 min at 4°C. The urease activity was measured by quantitating the rate of ammonia released from the urea based on the formation of indophenol, which was monitored at 625 nm [19]. The assay buffer consisted of 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 100 mM urea, and 0.5 mM EDTA (pH 7.5). The reactions were initiated by the addition of the enzyme, the concentration of the released ammonia was measured in time aliquots, and the rates were determined by a linear regression analysis. One unit of urease activity was defined as the amount of enzyme required to hydrolyze 1 mmol of urea per min at 37°C. The protein content was measured using either the method of Lowry *et al.* [12] or a MicroBCA™ protein assay kit from Pierce (IL, U.S.A.), with bovine serum albumin as the standard.

**Urease Purification**

The *E. coli* DH10B (pSVU101) cells were grown to the late-exponential phase in an LB broth (2 l) containing 1 mM nickel chloride and 100 μg/ml ampicillin. The cells were then harvested by centrifugation at 8,000 ×*g*, washed twice with an ice-cold 20 mM PED buffer (pH 7.0), and resuspended in 15 ml of PED containing 1 mM PMSF. Thereafter, the cells were disrupted by three passages through a French pressure cell (SLM Instruments, Inc., IL, U.S.A.) at 18,000 lb/in$^2$, and ultracentrifuged at 100,000 ×*g* for 90 min at 4°C to remove the membrane fractions.

Next, the supernatant solution was applied to a column (2.5×15 cm) of diethylaminoethyl-Sepharose Fast-Flow (DEAE-SR FF) equilibrated with a PED buffer at 4°C. The recombinant urease was eluted with a 400-ml linear gradient of 0 to 1 M KCl in a PED buffer, then the pooled sample was concentrated to 2 ml using an Amicon® (MA, U.S.A.) pressure filtration stirred-cell (50 ml) with a YM30 ultrafiltration membrane (MWCO=30,000) and subjected to Superdex HR-200 (1.6×60 cm, Pharmacia, Uppsala, Sweden) gel filtration chromatography in a PED buffer supplemented with 0.15 M KCl. The peak fractions were pooled and dialyzed for 18 h against a PED buffer at 4°C, and then applied to a preequilibrated Mono-Q HR 5/5 column (Pharmacia, Uppsala, Sweden) and eluted with a multistep gradient of increasing KCl (up to 1 M) in the same buffer. SDS-polyacrylamide gel electrophoresis was carried out using the buffers of Laemmli [10] and included a 10–15% polyacrylamide gradient running gel and 4.5% polyacrylamide

stacking gel. The gels were stained with Coomassie brilliant blue R-250.

### Determination of Native Molecular Weight

The native molecular weight of the recombinant *S. vestibularis* urease was estimated using Superose 12 chromatography in a PED buffer (pH 7) containing 0.15 M KCl. The column (1.0×30 cm) was standardized using thyroglobulin, gammaglobulin, ovalbumin, myoglobin, and vitamin $B_{12}$ ($M_r$s=670,000, 158,000, 44,000, 17,000, 1,350; Bio-Rad Labs, U.S.A.).

### Amino Terminal Sequence Analysis

The enzyme subunits from the purified urease were resolved in a 0.75-mm denaturing gel, as described above, and electrophoretically transferred onto an Immobilon®-P membrane (Millipore, CA, U.S.A.) in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS, pH 10)-10% methanol. The bands were visualized by Coomassie blue staining, cut from the membrane, and analyzed using an Applied Biosystem 476A automated sequencer.

### Determination of $K_m$ Value and Nickel Content

The rate of urea hydrolysis was monitored with different urea concentrations, ranging from 2 to 20 mM, and the $K_m$ value determined graphically on a simple Lineweaver-Burk double-reciprocal plot of 1/v vs. 1/[S]. To estimate the nickel content of the enzyme, the purified urease (100 μg) was treated with 20% nitric acid and the sample was vacuum-evaporated several times with the repeated addition of sterile double-distilled water in a rotary vacuum concentrator (MaxiDry-Lyo, Heto-Holten, Denmark). After being resuspended in ultrapure water, the sample was analyzed for its nickel content using an ICP-mass spectrophotometer (VG Elemental, U.K.).

### Nucleotide Sequence Accession Number

The complete nucleotide sequence of the urease gene cluster reported in this article has already been deposited in the GenBank under accession number DQ171937. For brevity, the nucleotide sequence has not been included in this report.

## Results and Discussion

### Cloning and Nucleotide Sequence Analysis of the *S. vestibularis* Urease Gene Cluster

A 6.8 kb DNA fragment including the *S. vestibularis* urease gene cluster was isolated from the subgenomic library as described above, resulting in the recombinant plasmid pSVU101. The complete nucleotide sequences for both strands of the inserted DNA were determined from a series of exoIII-generated nested deletion mutants of the

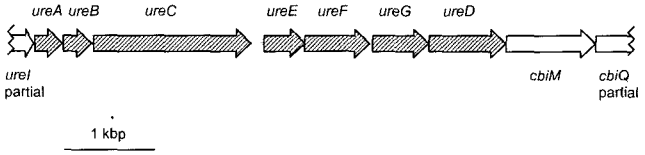


**Fig. 1.** Schematic diagram of cloned *Streptococcus vestibularis* urease gene cluster.

The urease structural genes (*ureA*, *ureB*, and *ureC*) and accessory genes (*ureD*, *ureE*, *ureF*, and *ureG*) are shown as cross-hatched arrows. The partial *ureI* gene was located upstream of *ureA*, yet the presence of the *ureI* product was not required for the expression of active urease in *E. coli*. The co-cloned *cbiM* and partial *cbiQ* genes located downstream are known to be involved in bacterial nickel/cobalt transport [3].

recombinant plasmid. For conciseness, the nucleotide sequence has not been included. Nucleotide sequence analyses and a database (BLAST) search revealed that the cloned insert contained a partial *ureI* gene at the 5'-end, followed by 7 urease genes (*ureA*, *ureB*, *ureC*, *ureE*, *ureF*, *ureG*, and *ureD*) arranged contiguously (Fig. 1), which is consistent with previously characterized urease structural genes and accessory genes [15]. There was also a notable 140-base-pair-gap between the stop codon of *ureC* and the start of *ureE*. One additional complete ORF (*cbiM* or *ureM*) and a partial ORF (*cbiQ* or *ureQ*) were found at the 3'-end region of the cloned insert. These ORFs are known to be part of the five-gene operon of *cbiKLMQO* found in *Actinobacillus pleuropneumoniae* and encode a potential nickel/cobalt ABC-type transporter system [3].

The entire region of the sequenced DNA exhibited a ~95% sequence identity to strains *S. salivarius* (6,583 of 6,866 bp), *S. thermophilus* LMG18311 (6,553 of 6,866 bp), and *S. thermophilus* CNRZ1066 (6,552 of 6,866 bp), respectively. The extent of the sequence identity was even higher (95–100%) when the predicted amino acid sequences were compared with those for the *S. salivarius* and *S. thermophilus* species, as summarized in Table 1. These results were not unexpected, considering these microorganisms belong to the same subgroup of viridans streptococci called the "salivarius" group.

### Expression of *S. vestibularis* Urease in *E. coli*

The urease activities of the crude extracts of *E. coli* cells harboring pSVU101 were examined to characterize the effect of nickel supplementation in an LB medium. It has been previously suggested that the inclusion of components in a rich medium, such as LB, can chelate nickel ions, thereby reducing the nickel ions available for urease activity [11]. The recombinant *E. coli* DH10B (pSVU101) cells were observed to grow well in nickel concentrations up to 2 mM. The urease activity depended strongly on the nickel levels, and peaked at a 1 mM nickel concentration (Fig. 2). Therefore, for the following experiments, the *E. coli* cultures were grown in LB supplemented with 1 mM nickel.

**Table 1.** Percent identity values for predicted amino acid sequences of *S. vestibularis* urease genes in comparison with known protein sequences from *S. salivarius* and *S. thermophilus* species.

| | UreA | UreB | UreC | UreD | UreE | UreF | UreG |
|---|---|---|---|---|---|---|---|
| *S. salivarius* | 99 | 98 | 100 | 96 | 99 | 100 | 100 |
| *S. thermophilus* CNRZ1066 | 100 | 99 | 99 | 95 | 99 | 97 | 100 |
| *S. thermophilus* LMG18311 | 100 | 99 | 99 | 96 | 99 | 97 | 100 |

**Biochemical Characterization of *S. vestibularis* Urease**

The urease enzyme was purified from the cell extracts of *E. coli* DH10B (pSVU101), as summarized in Materials and Methods. This three-column purification protocol yielded a urease preparation with a specific activity of 2,019 U/mg of protein (Table 2). The *S. vestibularis* urease was eluted with a single peak of activity at 0.4 M KCl from both the DEAE- Sepharose and the Mono-Q column, respectively. Therefore, based on the increase in the specific activity of the urease, the enzyme was estimated to have been purified 49-fold. Although the preparation was not completely homogeneous, as shown in the SDS-polyacrylamide gel (Fig. 3, lane 5), there was a major enrichment of the three structural peptides with estimated molecular masses of 64.2, 14.9, and 11.6 kDa, respectively. N-terminal amino acid sequencing analyses confirmed that the smallest (11.6 kDa) peptide was UreA (γ subunit) and the 14.9 kDa peptide was UreB (β subunit), respectively. These values were well within the range of the predicted molecular weights for the structural subunits of the *S. vestibularis* urease, except for the UreB peptide (11,477). The reason for this discrepancy between the apparent and predicted molecular weights for the UreB peptide was unclear, as there were no particular features, such as an unusual content of charged amino acid residues, in the predicted amino acid sequence of the UreB peptide.

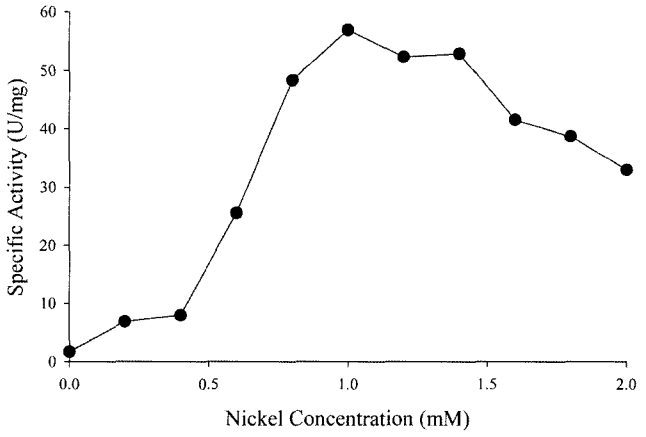


**Fig. 2.** Effect of nickel chloride concentration on urease activity for cells grown in LB.

*E. coli* DH10B cells containing the *S. vestibularis* urease gene cluster in plasmid pSVU101 were cultured for 18 h in an LB medium in the presence of various nickel chloride concentrations. The cells were harvested by centrifugation, disrupted by sonication, and assayed for specific activity.

The native molecular weight for the purified *S. vestibularis* urease was estimated as 196,000±15,000 by Superose 6 gel filtration chromatography, suggesting that the *S. vestibularis* urease exists as either a $(\alpha\beta\gamma)_2$ or $(\alpha\beta\gamma)_3$ structure, based on the calculated molecular mass of 84,587 Da of the enzyme catalytic unit (αβγ).

The $K_m$ for the purified *S. vestibularis* urease was estimated to be 1.44±0.15 mM, which is considerably lower than the reported $K_m$ value (3.7 mM) for *S. salivarius* [5]. The nickel content measured by ICP-mass spectrophotometry was shown to be 1.8±0.2 per catalytic unit, which is similar to other bacterial ureases [15].

Accordingly, this report provided a description of the genes and enzyme characteristics of the urease from *S. vestibularis*, the second human oral streptococci, following *S. salivarius* [5]. High levels of sequence identity and

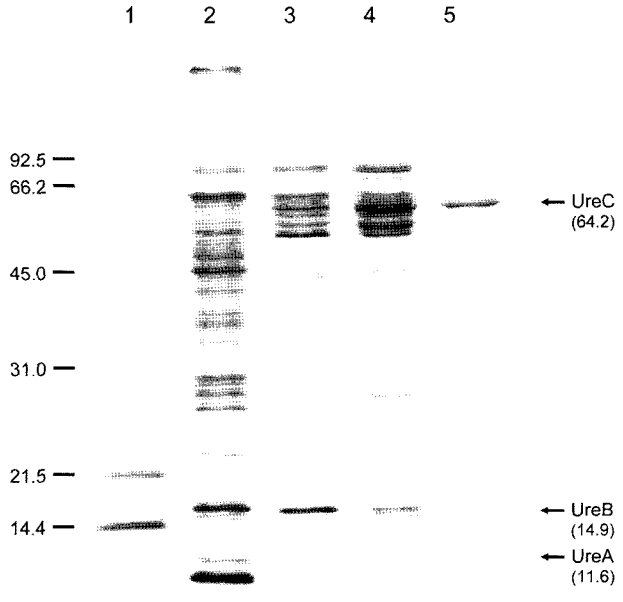


**Fig. 3.** SDS-polyacrylamide gel electrophoresis of recombinant *Streptococcus vestibularis* urease from each purification step.

Cell extracts from *E. coli* DH10B (pSVU101) (lane 2) and aliquots from the DEAE-Sepharose FF pool (lane 3), Superdex HR-200 pool (lane 4), and Mono-Q HR5/5 pool (lane 5) were subjected to SDS-polyacrylamide gel electrophoresis using a 10–15% gradient gel, followed by Coomassie blue staining. The molecular weight markers (lane 1) were phosphorylase b, $M_r$=92,500; bovine serum albumin, $M_r$=66,200; ovalbumin, $M_r$=45,000; carbonic anhydrase, $M_r$=31,000; soybean trypsin inhibitor, $M_r$=21,500; and lysozyme $M_r$=14,400.

**Table 2.** Purification of recombinant *S. vestibularis* urease from *E. coli* DH10B (pSVU101).

| Purification step | Specific activity (U/mg) | Purification (-fold) | Total activity (U) | Enzyme recovery (%) |
|---|---|---|---|---|
| Cell extract | 41 | 1.0 | 46,811 | 100 |
| DEAE-Sepharose | 189 | 4.6 | 38,438 | 82 |
| Superdex HR-200 | 412 | 10.0 | 32,060 | 68 |
| Mono-Q HR 5/5 | 2,019 | 48.8 | 3,429 | 7 |

homology were demonstrated to exist between these two microorganisms, as well as *S. thermophilus* isolated from dairy sources.

A further analysis of the transcriptional regulation of the *S. vestibularis* urease and studies on the functional expression of the urease genes in non-ureolytic oral bacteria are currently under investigation. It will also be interesting to evaluate the effect of ureolysis by these bacterial strains on the general oral microbial ecology and oral health and diseases.

## Acknowledgment

This work was supported by a Research Grant from the Catholic University of Daegu, Korea.

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