

The Role of Urease Activity on Biofilm Formation by *Staphylococcus* sp. T-02 Isolated from the Toilet Bowl

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Urolith, which consists of dirty yellow-colored attachments on the toilet bowl, is associated with a variety of odorous chemicals, including ammonia, and causes disadvantages in daily life. Although largely it is derived from microorganisms, little is known about the microbial processes underlying the formation of urolith. In order to gain insight into the types and the activities of microorganisms present in urolith, culturable bacteria were isolated, identified, and physiologically characterized. One of the isolates exhibited higher ability to produce ammonia when it was grown in artificial urine medium. Phylogenetic and physiological analyses indicated that this strain (T-02) belonged to a new group of *Staphylococcus* species, showing combined phenotypes as between *S. lentus* and *S. xylosus*. T-02 exhibited high urease activity and was capable of growing in the urinary condition by forming robust biofilms. The results of this study indicate that T-02 has successfully adapted itself to the environment of urolith.

Key words: biofilm; *Staphylococcus* sp. T-02; toilet bowl; urease; urolith

Urea is a major nitrogenous waste product of biological actions. For example, urine in the adult human contains over 0.5 M urea.¹⁾ Uric acid, which is released by birds, reptiles, and many terrestrial insects, is also decomposed to urea. In nature, urea is short-lived and rapidly metabolized by microbial activities. It penetrates into bacterial cells by diffusion, where the enzyme urease (urea amidohydrolase; EC 3.5.1.5) catalyzes the hydrolysis of urea, leading to the production of ammonia and carbamate.^{2,3)} The carbamate spontaneously hydrolyzes to another molecule of ammonia and carbonic acid in an aquatic condition. The ammonia is not only used as a nitrogen source, but also contributes to the ecological niche of bacteria. The ammonium becomes protonated to yield ammonium hydroxide ions, and it is believed that these ions increase the pH of the growing habitats or cause direct damage to host tissues during infection by pathogenic bacteria.⁴⁾

One of the serious issues caused by urease-producing bacteria is the formation of infection stones.⁵⁾ In urinary

tract infection, infection stones result from the urease-mediated hydrolysis of urea. The ammonia generated by urease activity causes an elevation of urine pH. As the urine becomes alkaline, inorganic ions present in it, such as magnesium and calcium phosphate crystals, are precipitated. Aggregates of these materials accumulate in the urine and a crystalline bacterial biofilm develops in urinary tract.⁶⁾ Likewise, the crystalline materials, referred as urolith in this study, are deposited on the surface of the toilet bowl. Urolith is a complex of materials composed of porous crystals of inorganic salts and organic compounds, and many bacteria attached to it. Recently it was found that urolith is not a residual of feces but is newly synthesized from splashed urine and microbial activities. Since urolith contains odorous chemicals, including ammonia, organic acids, alcohols, and sulphides, unpleasant smells occur and the appearance of the toilet bowl is spoiled. In addition, the risk of the spread of microbial infection caused by toilet flushing has been suggested.⁷⁾ Hence various cleaning techniques of rigid uroliths have been developed and marketed.

Biofilm formation is a survival strategy of bacteria to colonize a surface.^{8,9)} Unlike planktonic growth under laboratory conditions, bacterial cells in biofilms are encased in gel-like extracellular polymeric substances (EPSs) and are highly tolerant of physicochemical stress. In addition, drastic changes in the cellular process are observed in the environmental fitness of bacteria. By the analogy of urinary tract infection, it is logical that the ureolysis and biofilm formation of bacteria are closely linked with the formation of urolith. However, the microbial communities and activities present in urolith are poorly understood. The objective of this study was therefore to conduct an initial survey of the microorganisms present in urolith, using a cultivation-based method and direct microbial approaches to the mechanisms of urolith formation.

Materials and Methods

Bacterial strains and growth conditions. Artificial urine (AU) medium was used for the cultivation of bacteria.¹⁰⁾ The AU medium contained, per liter, 1 g of Bacto peptone (BD Difco, Franklin Lakes,

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NJ), 5 mg of Bacto yeast extract (BD Difco), 2.1 g of NaHCO₃, 10 g of urea, 70 mg of uric acid, 0.8 g of creatinine, 5.2 g of NaCl, 2.3 g of Na₂SO₄, 0.95 g of KH₂PO₄, 1.2 g of K₂HPO₄, 1.3 g of NH₄Cl, 0.37 g of CaCl₂·2H₂O, 0.49 g of MgSO₄·7H₂O, 1.2 mg of FeSO₄·7H₂O, 110 µl of lactic acid, and 2 ml of 6 N HCl. The pH was adjusted to 6.5. Tryptic soy broth (TSB) medium containing, per liter, 17 g of pancreatic digest of casein, 3 g of enzymatic digest of soybean meal, 5 g of NaCl, 2.5 g of K₂HPO₄, and 2.5 g of dextrose (pH 7.3) was used for isolation and to maintain bacteria.

T-02 and *Staphylococcus epidermidis* type strain ATCC 14990 were grown in AU or TSB medium at 37 °C, kept standing or with shaking of 120 rpm. *Escherichia coli* DH5α was used as host strain for general cloning of the DNA fragments. Cloning vector pGEM-T EASY (Promega, Madison, WI) was used in *E. coli* DH5α.

Phylogenetical analysis of the 16S rRNA gene. The whole-cell lysis PCR amplification method was used to amplify the 16S rRNA gene, using a GenElute Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis, MO). The nearly full-length 16S rRNA gene of T-02 was amplified with KOD Dash DNA polymerase (Toyobo, Osaka, Japan) with forward primer 5'-AGAGTTTCCTGGCTCAG-3' and reverse primer 5'-GGTACCCTTGTTACGACTT-3', which corresponded to nucleotide positions 8 to 27 and 1,510 to 1,492 of the bacterial 16S rRNA genes, respectively. The resulting 1.5-kb PCR products were purified with a QIAquick gel extraction kit (Qiagen, Hilden, Germany) and cloned into pGEM-T EASY. The nucleotide sequence of the 16S rRNA gene was determined by BigDye terminator cycle sequencing on an ABI 3100 DNA sequencer (Perkin-Elmer Applied Biosystems, Wellesley, MA). The sequence data have been deposited in the public database under accession no. AB477242. The 16S rRNA gene sequences of 40 validly described *Staphylococcus* species¹¹ were retrieved from the public database to construct a phylogenetic tree. Distance trees based on multiple sequence alignments were generated with Clustal X 2.0¹² by the neighbor-joining method with 1,000 bootstrap trials.

Gene cloning of T-02 urease. To clone the genes encoding the structure and accessory subunits of T-02 urease, nucleotide primers conserved among the urease genes of *Staphylococcus* species (forward, 5'-AAACCCTGCATTAGACTTCG-3'; reverse, 5'-TCTTCCATAAA-GCCAGCTTC-3') were designed, and a 1.0-kb partial gene fragment spanning the *ureA* and *ureC* locus was amplified using KOD Dash DNA polymerase. After sequencing of the PCR product, neighboring gene regions were obtained by the chromosome-walking PCR technique¹³ with reference to the genome sequences of *Staphylococcus* species available in the public database. The nucleotide sequences of the T-02 urease genes were determined and were deposited in the public database under accession no. AB477241.

Physiological characterization of T-02. T-02 was characterized by the API Staph system (BioMérieux Japan, Tokyo) and the Poa Media oxidase test (Eiken Chemical, Tokyo) following manufacturer's instructions.

Urease assay. For the plate assay, bacterial cells of overnight culture grown in TBS medium were washed twice with equal amounts of 1 × phosphate-buffered saline (150 mM NaCl, 10 mM Na₂HPO₄, 20 mM NaH₂PO₄, pH 7.0), and an aliquot of the sample (5.0 µl) was spotted on a 1.5% Christensen urea agar plate¹⁴ containing, per liter, 1 g of Bacto peptone, 1 g of glucose, 5 g of NaCl, 12 mg of phenol red, and 20 g of urea. After overnight cultivation at 37 °C, urease activity was detected as the formation of a red-colored clear zone around the colony.

For the quantitative assay, bacterial cells were dispersed by vortex and recovered by centrifugation at 7,500 rpm for 10 min at 4 °C. T-02 cells were washed once with 0.1 M K-PO₄ (pH 5.0) and recovered by centrifugation at 7,500 rpm for 10 min at 4 °C. The cells were then washed with cell lysis buffer composed of 20 mM Tris-HCl, 0.5 mM EDTA·2Na, and 0.1 mM NiSO₄·6H₂O (pH 7.8), and suspended in a small volume of the same buffer. After determination of cell densities (OD₆₀₀), the cells were disrupted using a Multi-Beads Shocker (model MB601US) (Yasui Kikai, Osaka, Japan) at 5 cycles of 2,500 rpm for 30 s at an interval of 30 s. The samples were centrifuged at 20,000 × g for 5 min at 4 °C, and the supernatants were used in the enzyme assay.

Quantitative measurements of urease activity were done spectrophotometrically using a coupled assay with glutamate dehydrogenase (GLDH), as previously described.¹⁵ One unit of urease activity was defined as the amount of urease from Jack bean (Toyobo Enzymes, Osaka, Japan) that causes the formation of 2 µmole of ammonia per min at 37 °C. Specific activities were calculated as units of urease per ml per density of dispersed bacterial cells (OD₆₀₀). Each data point was the average of triplicate experiments.

Biofilm formation. Biofilm formation was tested as described previously,¹⁶ with some modifications. An overnight culture was inoculated (1%) into 300 µl of AU or TSB medium in a 1.5-ml microcentrifuge tube (TC131615, Nippon Genetics, Tokyo). After standing cultivation at 37 °C for 24 h, the pellicles and the medium were removed from the tube and their turbidities were determined (OD₆₀₀). The surfaces of the biofilms were rinsed with distilled water and stained with 500 µl of 0.1% w/v crystal violet (CV) solution for 20 min. The CV solution was removed and the tube was washed twice with distilled water. Biofilm formation was observed by light microscopy. The CV attached to the biofilm was dissolved in 400 µl of 33% acetic acid and quantified by measurement of the absorbance at 590 nm. Each data point was the average of triplicate experiments.

Results and Discussion

Isolation and characterization of strain T-02

Culturable bacteria were collected from urolith attached to the surface of the toilet bowl at a private company (Saraya, Osaka, Japan). After several subcultures on a 1.5% TSB agar plate, bacterial colonies were examined for morphological characteristics, including color, shape, size, and surface properties. Isolated strains were subjected to plate assay¹⁴ to check ammonia production due to urease activity roughly. One strain exhibited strong ability to produce ammonia, and phylogenetic analysis showed that this strain belonged to the genera *Staphylococcus* (Fig. 1). We designated this strain T-02 and, used it in this study.

The 16S rRNA gene sequence of T-02 was determined and aligned with those of 40 valid *Staphylococcus* species¹¹ available in the public database (Fig. 1). T-02 and *S. lentus* exhibited sequence similarity of 100% according to the 16S rRNA gene sequences, and branched from the clade comprising *S. vitulinus*, *S. sciuri*, *S. fleurettii*, and *S. pulvereri*, all of which are known to belong to the cytochrome *c* oxidase-positive "sciuri" group of *Staphylococcus* species.¹⁷ Analysis of 16S rRNA gene sequences has generally been used to study the phylogenetical relationships among bacteria, but this method is not adequate to distinguish closely related species.¹⁸ We further characterized T-02 by physiological tests (Table 1).

The physiological features of T-02 and related strains (*Staphylococcus xylosum* and *Staphylococcus lentus*) are summarized in Table 1. T-02 exhibited properties of carbohydrate metabolism nearly identical to those of *S. lentus*, but it differed from *S. lentus* with regard to enzymatic properties, including alkaline phosphatase-positive, urease-positive, and notably cytochrome *c* oxidase-negative, which were rather similar to those of *S. xylosum*. These results indicate that T-02 is a novel *Staphylococcus* species belonging to but distinct from the sciuri group.

Biofilm formation of T-02

The toilet bowl is repeatedly showered by urine and flushed by water. Urine contains a large quantity of

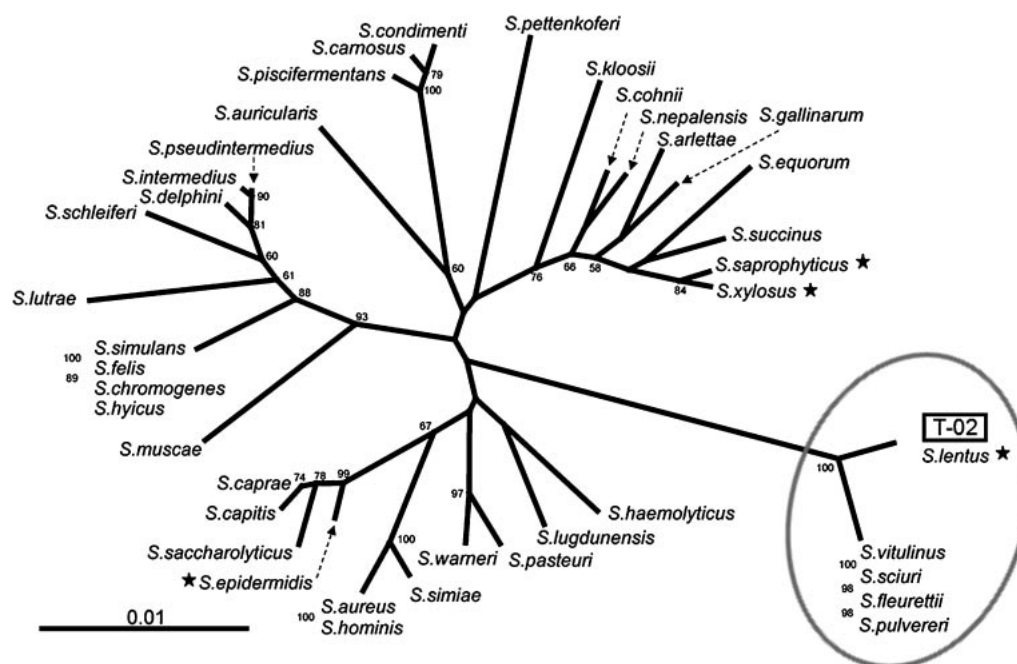


Fig. 1. Phylogenetic Tree Based on the 16S rRNA Gene Sequences of the Genus *Staphylococcus*.

The 16S rRNA gene sequences of the 40 previously identified valid *Staphylococcus* species¹¹⁾ were retrieved from the public database and compared with that of T-02. Bootstrap values greater than 50% are shown at the nodal branches. The cytochrome *c* oxidase-positive sciuri group,¹⁷⁾ including T-02, is circled. The reference strains described in the text are indicated by asterisks (*S. epidermidis*, *S. xylosus*, *S. lentus*, and *S. saprophyticus*). A scale bar of 0.01 nucleotide substitution per site is shown.

Table 1. Physiological Identification of T-02

Positive and negative ratios of T-02 were scored according to the API Staph system (BioMérieux, Tokyo) and the Poa Media oxidase test (Eiken Chemical, Tokyo). The % of reactions positive was denoted for two related strains (*S. xylosus* and *S. lentus*).

Acidification tests	<i>S. sp</i> T-02	<i>S. xylosus</i> (% of reactions positive)	<i>S. lentus</i> (% of reactions positive)
D-Glucose	+	100	100
D-Fructose	+	100	100
D-Mannose	+	92	100
Maltose	+	81	100
Lactose	+	85	100
D-Trehalose	+	95	100
D-Mannitol	+	90	100
Xylitol	±	30	7
D-Melibiose	+	9	99
Raffinose	+	11	100
D-Xylose	+	82	100
Sucrose	+	87	100
α-Methyl-D-glucoside	-	10	28
N-Acetyl-glucosamine	+	80	100
Nitrate reduction	+	82	92
Alkaline phosphatase	+	75	21
Acetyl-methyl-carbinol	+	67	57
Arginine dihydrolase	-	5	0
Urease	+	90	1
Cytochrome <i>c</i> oxidase	-	≤10	≥90

nitrogenous compounds, including urea, and is neutral to slightly acidic. It is evident that alkalization of urine pH directed by the hydrolysis of urea plays a major role in the protection of bacteria from urinary conditions.²⁾ Hence we examined the urease activity of T-02 by plate assay and compared it with that of *S. epidermidis* ATCC 14990, which was utilized as the reference strain of urease-positive and biofilm-forming bacteria (Fig. 2A).

When overnight cultures of T-02 and *S. epidermidis* were spotted on a 1.5% Christensen urea agar plate, red-colored clear zones appeared around the colonies of both strains. The extent of the colored zone was significant in T-02, indicating that T-02 is a hyper ammonia-producing strain.

Attachment to the solid phase is also an important trait of bacteria grown on the surface of the toilet bowl, and presumably it contributes to the formation of urolith. We tested the biofilm formation of T-02 and *S. epidermidis* by the CV staining method (Fig. 2B). When each strain was grown in TSB medium kept standing, T-02 exhibited lesser biofilm-forming abilities, while *S. epidermidis* formed thick biofilms that located mostly at the air/liquid interface. Biofilm assays in AU medium indicated that T-02 cells adhered throughout the surface of the polypropylene tube and formed a substantial biofilm. On the other hand, *S. epidermidis* showed severe growth arrest and resulted in only marginal biofilm formation. These results reveal the high propensity of T-02 to revert to solid phase in AU medium, and might reflect an ecological fitness of T-02 to grow in association with urolith on the toilet bowl.

Gene organization of T-02 urease

Next we examined the gene organization of T-02 that is responsible for the hydrolysis of urea. The majority of bacterial ureases, but not all, are similarly composed of three structural subunits, α , β , and γ , which are encoded by the genes for *ureC*, *ureB*, and *ureA* respectively.³⁾ It is also known that additional accessory proteins encoded by the *ureD/E/F/G* genes are required for the proper assembly of mature enzymes. DNA fragments containing the T-02 urease gene cluster were obtained by chromosome-walking PCR, and the nucleotide sequences were determined (Fig. 3A). The order and the

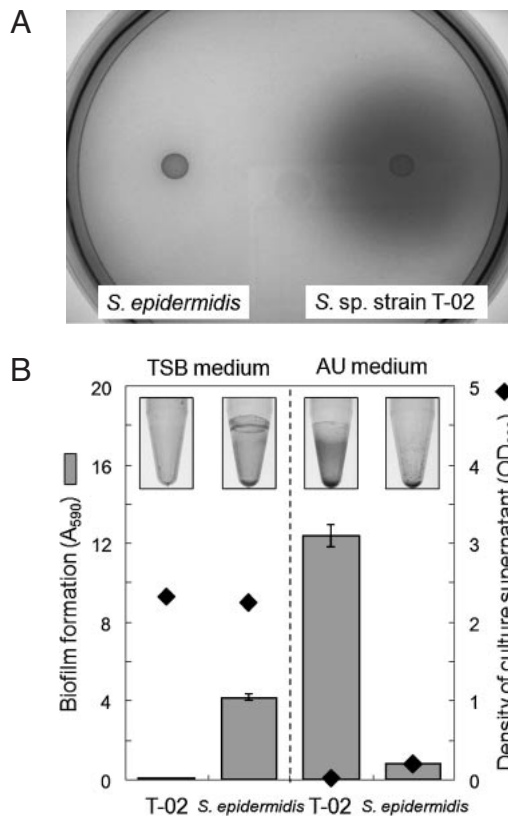


Fig. 2. Urease Activity and Biofilm Formation of T-02.

A, Plate assay of urease activity. The urease activities of *S. epidermidis* (left colony) and T-02 (right colony) were detected on a 1.5% Christensen urea agar plate.¹⁴⁾ The red-colored zone around the colony indicates ammonia production due to urease activity. B, Comparison of biofilm formations between T-02 and *S. epidermidis*. For the assay of biofilm formation, each strain was grown in AU or TSB medium kept standing for 1 d. After staining of a 0.1% crystal violet solution, the amounts of biofilm were observed by light microscopy and calculated (bar graphs). The densities of culture supernatant (lozenges) were also determined. The error bars indicate standard error of the means.

deduced amino acid sequences of the structural and accessory subunits of the T-02 urease encoded by the *ureA/B/C/E/F/G/D* genes displayed significant homologies with those of other bacteria.^{3,19)} For instance, the α subunit of the T-02 urease exhibited up to 88% and 85% identity with the UreC proteins of *Staphylococcus saprophyticus* ATCC 15305²⁰⁾ and *S. epidermidis* ATCC 12228²¹⁾ respectively. We did not find any notable features of T-02 in terms of the genomic organization of the urease operon.

Urease activity of T-02

Previous analysis has revealed that the level of urease activity is constitutive in some organisms but is also induced in response to environmental conditions.²⁾ To determine how the urease activity of T-02 is regulated by growth conditions, exponential and stationary-phase cells of T-02 grown in different media (AU, AU without urea, and TSB) were recovered from shaking and standing conditions and subjected to quantitative assay of urease activity (Fig. 3B). The results showed that the urease activities of T-02 varied among the growth periods, and were slightly induced by standing conditions but not substantially changed. Higher levels of urease activity were observed in T-02 cells grown in AU medium kept standing, in which most of the T-02 cells adhered to the surface of the tube and formed robust biofilms (Fig. 2B). When urea was omitted from the AU medium, the overall activities of urease were roughly reduced to 50% of the levels of the original AU medium in spite of the standing or shaking condition. The detailed mechanisms remain unknown but these observations suggest that biofilm formation as well as high quantities of urea recruit high levels of urease activity in T-02 cells.

Essential factors for the biofilm formation of T-02

To verify further the components responsible for biofilm formation under the urinary condition, T-02 was grown in modified AU medium and tested for biofilm

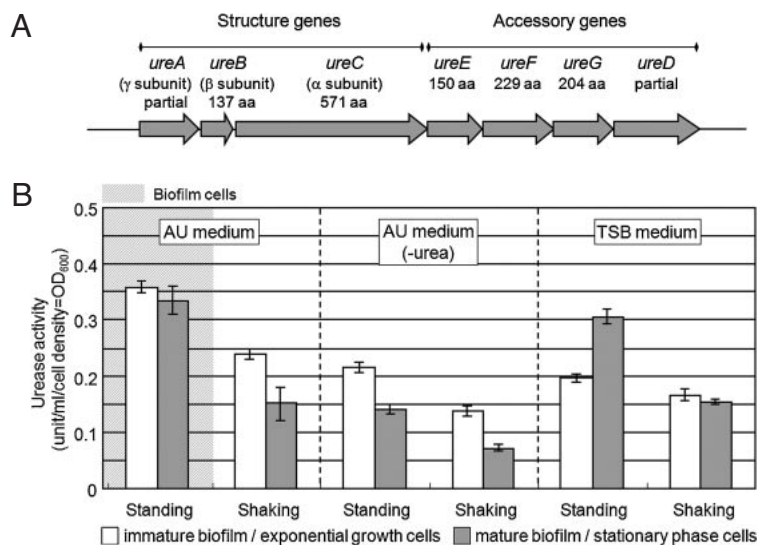


Fig. 3. Characterizations of T-02 Urease.

A, Gene organization of T-02 urease. Arrows indicate the direction of transcription. Protein coding regions for structural and accessory subunits of T-02 urease are shown (*ureA/B/C/E/F/G/D*). B, Urease activities of T-02 grown under various conditions. T-02 was grown in AU medium with and without 10 g/l urea, or in TSB medium under standing or shaking conditions for 1 d. Urease activities were assayed by the urease-GLDH method.¹⁵⁾ The error bars indicate standard error of the means.

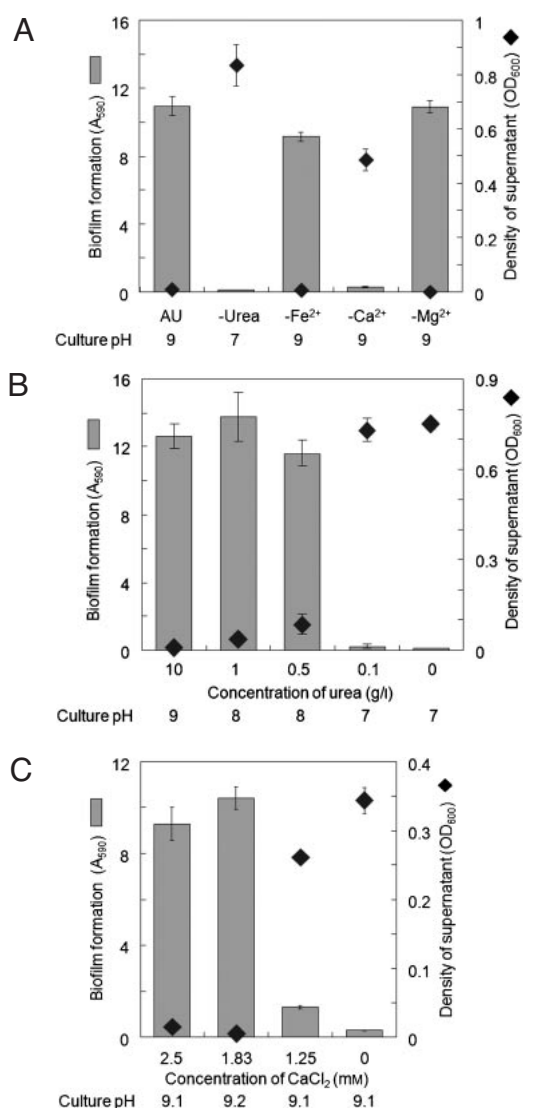


Fig. 4. Effects of the Components in the AU Medium on the Biofilm Formation of T-02.

A, T-02 was grown in original or modified AU medium without urea or divalent cations (Fe^{2+} , Ca^{2+} , and Mg^{2+}) kept standing for 1 d. B, Effect of urea on the biofilm formation of T-02. T-02 was grown in modified AU medium by changing the concentration of urea (the original AU medium contained 10 g/l urea). C, Effect of Ca^{2+} on the biofilm formation of T-02. T-02 was grown in modified AU medium by changing the concentration of $CaCl_2$ (the original AU medium contained 2.5 mM $CaCl_2$). The amounts of biofilm (bar graphs), the densities of the culture supernatant (lozenges), and culture pHs (values under the panel) were calculated. The error bars indicate standard error of the means.

formation (Fig. 4A). In the absence of urea, most of the T-02 cells remained in the culture supernatant and this resulted in lesser biofilm formation. This result indicates that urea is not essential for the planktonic growth of T-02, and it appeared to trigger the attachment of T-02 cells to the solid phase. Previous studies have found that sediments of crystal materials are critical for the formation of crystalline biofilm under the urinary condition, where divalent cations can stabilize the biofilm matrix through electrostatic interactions.⁶⁾ Hence each of the three major divalent cations, Fe^{2+} , Ca^{2+} , and Mg^{2+} , was removed from the AU medium. The results showed that normal biofilm formed when Fe^{2+} or Mg^{2+} was omitted from the AU medium, while depletion of Ca^{2+} abrogated biofilm formation. Both Ca^{2+} and Mg^{2+}

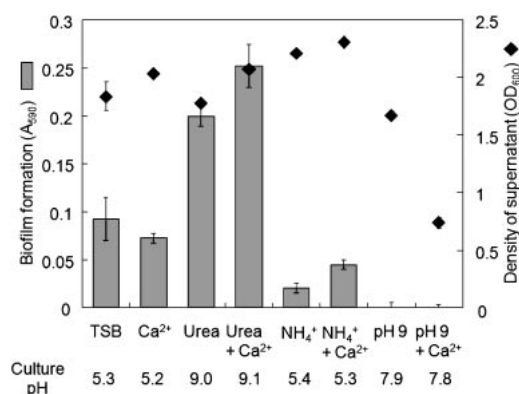


Fig. 5. Effects of Urinary Components on the Biofilm Formation of T-02 in TSB Medium.

T-02 was grown in modified TSB medium that contained 2.5 mM $CaCl_2$, 10 g/l urea, 10 g/l NH_4Cl , or combinations of these, as described under the panel. TSB medium was also buffered with 30 mM Tris-HCl and the pH was adjusted to 9.0. The amounts of biofilm, the densities of the culture supernatant, and the culture pHs were calculated as described in Fig. 4.

have been shown to influence the attachment of bacterial cells and subsequent biofilm formation, by serving as cross linkers between extracellular matrixes,²²⁾ but the effects of the two cations are not identical and vary among tested species and conditions.²³⁾ For example, Lattner *et al.* demonstrated that Ca^{2+} had a much stronger affinity to alginate, a major EPS of *Pseudomonas aeruginosa*, than Mg^{2+} .²⁴⁾ In addition, we used $MgSO_4$ in AU medium. Confound effects of SO_4^{2-} that increase the growth of bacteria and consequently enhance biofilm formation have been proposed.²⁵⁾ Although more detailed studies are needed to determine the effects of divalent cations, high levels of biofilm formation by T-02 under the urinary condition should at least in part be attributed to the presence of urea and Ca^{2+} .

Next we examined the effects of altered concentrations of urea in the AU medium and monitored changes in biofilm formation and culture pH (Fig. 4B). The biofilm formation of T-02 significantly dropped, to below a concentration of 0.1 g/l urea, at which alkalization of the culture pH declined to about 7.0, perhaps due to impaired hydrolysis of urea. This further confirms that the hydrolysis of urea is critical to the alkalization of culture pH and profoundly contributes to biofilm formation. As the levels of Ca^{2+} fell, T-02 displayed marginal biofilms below 1.25 mM $CaCl_2$ (Fig. 4C). It is noteworthy that the culture pHs were almost constant at 9.1 even under reduced concentrations of Ca^{2+} (0 and 1.25 mM $CaCl_2$). These observations indicate that normal hydrolysis of urea occurs in the absence of Ca^{2+} , and that Ca^{2+} might be necessary for the biofilm formation of T-02 at post or distinct processes of urease-mediated alkalization of culture pH.

Although, T-02 cells grown in TSB medium exhibited considerable levels of urease activity (Fig. 3B), biofilm formation was scarcely detected (Fig. 2B). This suggests the presence of inhibitors or a lack of the components responsible for biofilm formation in TSB medium. In order to determine the essential factors leading to the biofilm formation of T-02, we supplemented the TSB medium with urinary components and tested for biofilm formation (Fig. 5). The addition of a high quantity of

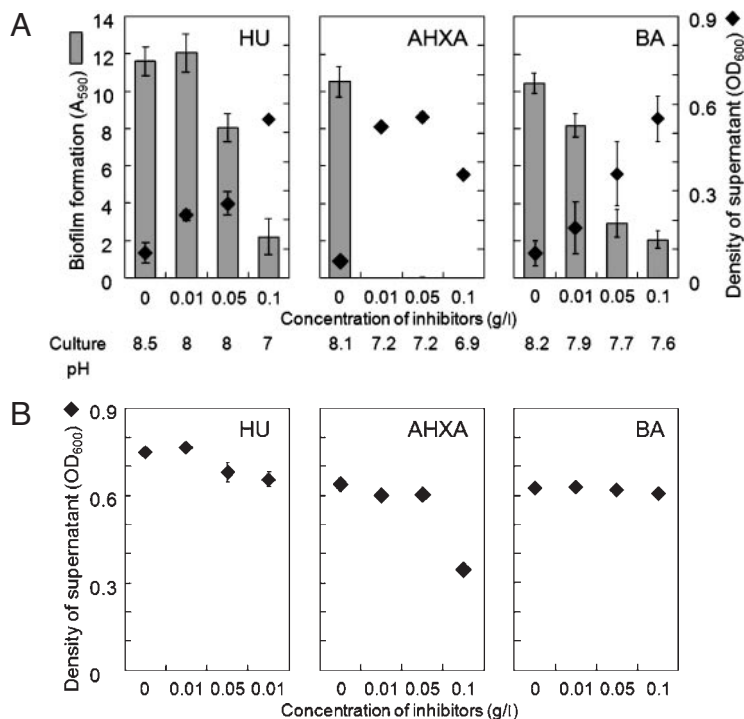


Fig. 6. Effects of Urease Inhibitors on the Biofilm Formation of T-02.

A, T-02 was grown kept standing in modified AU medium containing 0.5 g/l urea and supplemented with various concentrations of urease inhibitors, including hydroxyurea (HU), acetohydroxamic acid (AHXA), and boric acid (BA). B, T-02 was grown standing in AU medium without urea and supplemented with various concentrations of urease inhibitors (HU, AHXA, and BA). The amounts of biofilm, the densities of the culture supernatant, and the culture pHs were calculated as described in Fig. 4. The error bars indicate standard error of the means.

urea (10 g/l) resulted in approximately 2-fold induction of biofilm formation. The addition of 2.5 mM CaCl₂ to this cultivation further prolonged the effect of urea, whereas the addition of 2.5 mM CaCl₂ alone showed no obvious effect. This again supports that the synergy of urea hydrolysis and Ca²⁺ enhances the biofilm formation of T-02. It was found that neither the addition of NH₄⁺ nor artificial alkalization of culture pH resulted in extensive biofilm formation. The process of urea hydrolysis but not the final product of urease enzyme (NH₄⁺) or the result of alkalization of culture pH is necessary for the biofilm formation of T-02.

The levels of biofilm formation by T-02 grown in TSB medium were considerably lower than those in AU medium in spite of the presence of urea and Ca²⁺. Additionally, alkalization of culture pH took place there (Fig. 5). Several explanations are possible, because biofilm formation is a complex communal behavior of bacteria, influenced by many biotic and abiotic factors.²⁶⁾ Previous studies indicate that high levels of citrate (a chelator agent of divalent cations) reduce the rate of crystal formation in urine and render urease-producing bacteria unable to form crystalline biofilms, in which normal alkalization of urine pHs occurs.²⁷⁾ Given this evidence, the involvement of antagonistic effects in the TSB medium acting at after the alkalization of culture pH should be considered.

In vivo inhibition of urease activity

Several compounds have been described as inhibitors of urease.²⁾ Hydroxyurea (HU) is a substrate analog and also an inhibitor of microbial urease. Acetohydroxamic acid (AHXA) is a potent inhibitor of microbial urease, and the mechanism of inhibition is assumed to block the

active site of nickel binding. Boric acid (BA) is a competitive inhibitor of urease and appears to bind the metalcenter. We tested the effects of these urease inhibitors on the biofilm formation of T-02 (Fig. 6A). For the efficacy of the urease inhibitors, T-02 was grown in AU medium containing 0.5 g/l urea. HU and BA inhibited biofilm formation of T-02 in a dose-dependent manner. AHXA significantly suppressed the biofilm formation of T-02 under all the tested concentrations. This agrees with the description of AHXA that it is a stronger urease inhibitor than the others.²⁾ The decline in biofilm formation caused by urease inhibitors showed parallelism with the impaired alkalization of the culture pH. The three compounds effectively inhibited the urease activities in the crude extract of T-02 cells *in vitro* (data not shown), and did not affect the growth of T-02 in AU medium without urea, except for the addition of 0.1 g/l AHXA (Fig. 6B). Thus the data obtained in this experiment can be explained by specific inhibition of urease activity *in vivo*. The inhibitor study indicates that the catalytic activity of urease enzyme contributes to the biofilm formation of T-02 in a urinary environment.

Crystal formation in the biofilm of T-02

We noticed that crystal-like sediments appeared during long-term standing cultivation of T-02 in AU medium (14 d, Fig. 7A). Microscopic analysis of the CV-stained biofilm indicated that large numbers of T-02 cells aggregated with the crystals (Fig. 7B). The crystals were transparent and formed three-dimensional structures. Our preliminary analysis indicates that the main elements of these crystals are calcium, magnesium, and phosphate ions (data not shown); that is, they are very similar to the components of infection stones. Usually,

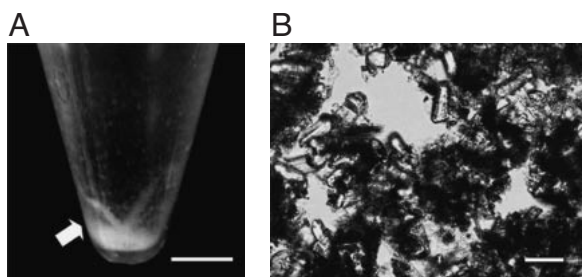


Fig. 7. Crystal Formation in the Biofilm of T-02.

A, Crystal-like sediments developed after 14-d of cultivation of T-02 in AU medium kept standing. The scale bar indicates 0.5 cm. B, T-02 was grown in AU medium kept standing for 24 h at 37°C. The resulting biofilms were stained with 0.1% crystal violet solution, scraped off, and observed under light microscopy. The scale bar indicates 50 μm.

infection stones consist of struvite ($\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$), carbonate apatite ($\text{Ca}_{10}(\text{PO}_4)_6\text{CO}_3$), and monoammonium urate ($\text{NH}_4\text{C}_5\text{H}_3\text{N}_4\text{O}_3$).²⁸ They occasionally occur during urinary tract infection directed by urease-producing bacteria, and facilitate pathogenic or persistent growth under the urinary condition.⁴ If T-02 indeed contributes to the formation of urolith on the toilet bowl and the crystalline materials involve the formation of robust biofilms, the analogy can be applied to bacterial strategies between urinary tract infection and urolith formation on the toilet bowl.

In this study, we characterized a urease-producing strain isolated from urolith on the toilet bowl. The physiological properties of strain T-02 are reminiscent of *S. saprophyticus* type strain ATCC 15305 in view of the high urease activity and the adherent phenotype in the urinary condition.²⁹ *S. saprophyticus* is a uropathogenic bacterium that frequently causes urinary tract infections in young females. The urease activity of *S. saprophyticus* is significantly higher than those of other pathogenic bacteria, such as *S. aureus* and *S. epidermidis*, but comparative genome analyses failed to account for the unique features of the organization of urease genes in *S. saprophyticus*.²⁰ *S. saprophyticus* carries a novel type of adhesive proteins and extensive ion transport systems that may contribute to the expression of high urease activity. *Helicobacter pylori* is a bacterial species capable of growing in very acidic conditions like the human stomach.³⁰ Since urease requires nickel to yield enzyme activity, *H. pylori* uptakes nickel efficiently and maintains urease activity for persistence in its habitat.^{31,32}

In this context, T-02 might have evolved to adapt to the urinary condition by acquiring unique traits that reinforce the basic catalysis of the urease enzyme. Further studies are needed to elucidate mechanisms that involve T-02 and are capable of exhibiting high levels of urease activity in the urinary environment. The data obtained might be applied to prevention or removal procedures for urolith formation on the toilet bowl.

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