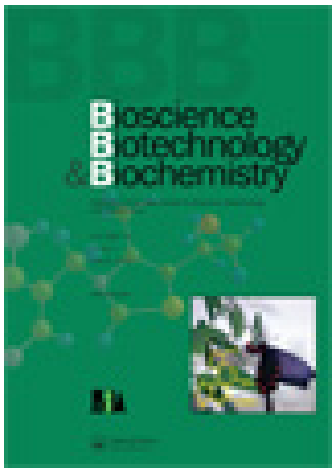

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# Isolation and characterization of an early colonizing *Rhizobium* sp. R8 from a household toilet bowl

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**The bacterial community structure was compared between the third days', one week', and three weeks' biofilm samples from the surface of a household toilet bowl. It was found that the PCR-DGGE band pattern of 16S rRNA gene was dramatically changed after the third day and was not further changed until three weeks. This result suggests that there are early and late colonizing bacterial groups. One of the early colonizers isolated from the third days' sample was *Rhizobium* sp. R8, a closest relative to *Rhizobium giardinii*, which exhibited the highest biofilm formation activity in an artificial urine condition. R8 produced extracellular polysaccharides containing galactose, glucose, and mannose at the molar ratio of 8:1:1, which were probably responsible for the biofilm formation. Its excelled biofilm formation and urease activities together with the lack of nodulation and nitrogen fixing genes in R8 suggest that this strain has been specifically adapted to urine condition in a toilet bowl.**

**Key words:** *Rhizobium*; biofilms; exopolysaccharides; urease; toilet bowls

Biofilms are microbial community structures formed on solid surfaces, which are composed of cells and their extracellular matrices. Forming biofilms is considered an effective strategy for bacteria to proliferate in both favorable and hostile environments.[1,2] The biofilm formation, especially by multispecies microorganisms, is a complex phenomenon in which environmental conditions and microbial interactions govern its temporal and spatial variations. Multispecies interactions can influence the emergence and disappearance of species and therefore play an important role in the shaping of the biofilm communities.[3,4] For example, *Pseudomonas aeruginosa* rapidly spread over the surface via swarming and twitching motility and prevented *Agrobacterium tumefaciens* from invasion and adhesion.[5] *Streptococcus mutans* can serve as the initial colonizer on the tooth surface and together with an *Actinomyces* species promote biofilm growth of *Lactobacillus* in an oral multispecies biofilm.[6] *Streptococcus gordonii* is also a first colonizer on the tooth surface and provides conditions that allow later colonizers including *Porphyromonas gingivalis*, to adhere. Moreover, streptococcal cell wall polysaccharides have been shown to enable other oral bacteria to recognize and coagulate with streptococci, and ultimately form commensal dental plaque.[7,8] These multispecies biofilms are also ubiquitously formed in our living conditions.

Control and prevention of biofilm formation in public and household toilets are important from a hygienic point of view because they can cause biofouling, malodour, and staining and they represent a potential reservoir for pathogens. A great diversity of bacterial community in the biofilms formed on the surface of toilet bowls has been already demonstrated by both culture-dependent and culture-independent methods.[9–12] However, there is only limited information yet available for transient and temporal growth of biofilms on toilet bowls. The aim of this study was to shed light on the transition of biofilm formation on toilet bowls and characterize one of the early colonizers.

## Materials and methods

*Biofilm samples.* Biofilm samples were collected from the inside surface of a household toilet according to the method as previously described.[10] Briefly, after careful cleaning the surface of the toilet bowl, the toilet was normally used by a family for two weeks. The inside surface of toilet bowl was carefully swabbed by a sterile cotton fabric (Japan Textile Evaluation Technology Council) for sampling. Samples were taken on the third day and after one and three weeks. Biofilms were released from the cotton fabric by vigorous vortexing in an aliquot of LP diluent (Nihon Pharmaceutical, Co., Ltd) and spread on R2A agar plates after appropriate dilutions. Bacterial colonies were grown on the plate for a week at 30 °C.

*Culture media and bacterial strains.* R2A medium was used for isolation of bacteria and maintenance

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culture. R2A contains per liter 0.5 g each of Bacto proteose peptone No.3 (Difco), Bacto yeast extract (Difco), casamino acid, glucose, soluble starch, and 0.3 g each of $K_2HPO_4$, sodium pyruvate, and 0.05 g of $MgSO_{4-7}H_2O$ (pH 7.0–7.4). Artificial urine (AU) medium mimicked human urine condition.[13] AU medium contained per liter 1 g of Bacto peptone, 5 g of Bacto yeast extract, 2.1 g of $NaHCO_3$, 10 g of urea, 70 mg of uric acid, 0.8 g of creatinine, 5.2 g of NaCl, 2.3 g of $Na_2SO_4$, 0.95 g of $KH_2PO_4$, 1.2 g of $K_2HPO_4$, 1.3 g of $NH_4Cl$, 0.37 g of $CaCl_2{\cdot}2H_2O$, 0.49 g of $MgSO_4{\cdot}7H_2O$, 1.2 mg of $FeSO_4{\cdot}7H_2O$, 110 µL of lactic acid, 2 mL of 6 N HCl, and pH was adjusted to 6.5. TSB and TY media were used for nutrient rich culture. TSB contained per liter of 17 g pancreatic digest of casein, 3 g of enzymatic digest of soybean meal, 5 g of NaCl, 2.5 g each of $K_2HPO_4$ and dextrose. pH was adjusted to 7.3. TY contained per liter 5 g of Bacto tryptone (Difco), 3 g of Bacto yeast extract, and 0.83 g of $CaCl_{2-2}H_2O$ (pH 6.8–7.0).

*Rhizobium giardinii.* NBRC 107135 [=H152$^T$][14] was used as a reference strain. *Rhizobium* strains H152$^T$ and R8 (waiting for NBRC strain number) were usually cultured in TY medium at 30 °C. When exopolysaccharides were prepared, YM medium was used. YM medium contained per liter 10 g mannitol, 4 g $CaCO_3$, 0.4 g Bacto yeast extract, 0.5 g $KH_2PO_4$, 0.2 g $MgSO_{4-7}H_2O$, 0.1 g NaCl (pH 6.8–7.0). YMA contained per liter 15 g of agar in YM for preparation of solid medium. Other isolated strains were cultured in R2A medium. *Escherichia coli* DH5α was used as a host strain for general DNA cloning. A cloning vector pUC19 was used in *E. coli* DH5α.

*PCR-DGGE analysis.* DNA extraction from the biofilm samples was carried out using ISOIL for Beads Beating (Nippon Gene). Extracted DNA was used as templates for PCR targeting a part of 16S rRNA gene. The nucleotide sequences of the primers were as follows: forward primer with GC-clump (5′-CGCCCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3′) and reverse primer 5′-ATTACCGCGGCTGCTGG-3′. The reaction mixture was composed of 5 µL of 10xPCR buffer, 5 µL of 2 mM dNTP, 3 µL of 25 mM $MgSO_4$, 5 ng of template DNA, 1.5 µL each of forward and reverse primers, 1 µL of KOD Plus-Neo DNA polymerase (Toyobo), and sterile water up to 50 µL in total volume. The template DNA was first denatured for 2 min at 94 °C, followed by 20 cycles of the following steps: denaturation for 10 s at 98 °C, annealing for 30 s from 65 to 55 °C (touchdown by −0.5 °C cycle$^{-1}$), and extension for 12 s at 68 °C (MJ Mini$^{TM}$, Bio-Rad). The 20 cycles were further followed by 10 cycles of above temperature programs with a constant annealing temperature 55 °C. Denaturant gradient gel electrophoresis, DGGE, was performed using D-Code$^{TM}$ universal mutation detection system (Bio-Rad). Denaturant gradient was formed from 30 to 60% where 100% corresponded to 7 M urea and 40% formamide in an 8% polyacrylamide gel (16 cm × 16 cm × 1 mm thickness in 0.5 × TAE). Electrophoresis was carried out at 200 V for 4 h at 58 °C in a tank containing 7 L of 0.5 × TAE buffer. After electrophoresis, the gel was stained for 30 min with 0.01% (v/v) SYBR Gold solution (Molecular Probes), rinsed with water, and photographed on a Fluoroimager 595 (molecular dynamics).

*16S rRNA gene sequence analysis of the bacterial isolates.* Genomic DNA was prepared by Instagene$^{TM}$ MATRIX (Bio-Rad) and used for PCR amplification of 16S rRNA genes. The reaction mixture was composed of 5 µL of 10 × PCR buffer, 5 µL of 2 mM dNTP, 2 µL of 25 mM $MgSO_4$, 2 µL of template DNA, 1 µL [10 pmol/µL] each of 16S rRNA gene general forward 27F and reverse 1492R primers, 1 µL of KOD Plus-Neo DNA polymerase (Toyobo), and sterile water up to 50 µL in total volume. The templates were first denatured for 2 min at 94 °C, followed by 29 cycles of the following steps: denaturation for 10 s at 98 °C, annealing for 30 s at 52 °C, and extension for 45 s at 68 °C. The 29 cycles were followed by a final 3 min extension at 68 °C. Approximately 1,500-bp PCR products were purified with a QIAquick gel extraction kit (Qiagen) and cloned into the *SmaI* gap of pUC19. The nucleotide sequence of the 16S rRNA gene was determined by BigDye terminator cycle sequencing kit ver. 3 on an ABI 3130 DNA sequencer (Applied biosystems). The sequence data were analyzed by GENETYX-WIN version 5.1.1 (Toyobo) and nucleotide BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The 16S rRNA gene sequences of relevant 54 Rhizobia were retrieved from the NCBI database to construct a phylogenetic tree. Phylogenetic trees based on multiple sequence alignments were generated by EBI Clustal W2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and neighbor-joining method using MEGA version 5.5 with 1,000 bootstrap trials.

*Biofilm formation assay.* Pre-culture of each strain was inoculated at 1% into 200 µL of R2A medium in polystyrene 96-well microplates (#167008, Thermo Scientific™ Nunc™) or 3 mL in porcelain crucibles and statically grown to form biofilms at 30 °C for 24 and 48 h. Planktonic cells in the culture liquid were quantified by measuring optical density at 595 nm ($OD_{595}$), and then, the culture was removed by pipet, and the biofilms formed on surface of the vessels were washed with distilled water. The biofilms were stained by 0.1% (w/v) crystal violet for 10 min and washed twice with distilled water. Adherent crystal violet was extracted by an equal volume of 33% acetic acid for 10 min. Biofilm formation activity was determined by measuring absorbance of the solution at 595 nm ($A_{595}$) with a microplate reader (Sunrise, TECAN).

*Urease assay.* Bacterial cells of overnight culture grown in TSB medium were washed twice with equal amounts of 1 × phosphate-buffered saline (150 mM NaCl, 10 mM $Na_2HPO_4$, 20 mM $NaH_2PO_4$, pH 7.0), and an aliquot of the sample (5.0 µL) was spotted on a 1.5% Christensen urea agar plate containing, per liter 1 g of Bacto peptone, 1 g of glucose, 0.5 g of NaCl, 1.2 g of $Na_2HPO_4$, 0.8 g of $NaH_2PO_4$, 12 mg of

phenol red, and 20 g of urea. A plate without 20 g urea was prepared for negative control experiment. After two days' cultivation at 30 °C, urease activity was detected as the formation of a red-colored clear zone around the colony.[13)]

*PCR examination of* nodC *and* nifH *gene.* Existence of *nodC* and *nifH* genes was examined by PCR using NEB One Taq™ 2X master mix. The nucleotide sequences of the primers were as follows: nodCf (5′-AYGTHGTYGAYGACGGTTC-3′) and nodCr (5′- CGYGACAGCCANTCKCTA TTG-3′); nifHf (5′-TACGGNAARGGSGGNATCGGCAA-3′) and nifHr (5′-AGCATGTCYTCSAGYTCNTCCA-3′).[15)] DNA templates were first denatured for 2 min at 94 °C, followed by 30 cycles of the following steps: denaturation for 10 s at 94 °C, annealing for 30 s at 55 °C, and extension for 1 min at 68 °C. The 30 cycles were followed by a final 5 min for extension at 68 °C.

*Nodule formation assay.* Seeds of *Phaseolus vulgaris* "Koi-midori" (Takii & Co., Ltd) were surface-sterilized by soaking for 10 min in 2% (w/v) sodium hypochlorite and 0.02% Tween 20 solution, followed by five times wash with sterile distilled water and germinated on B&D [nitrogen free] medium containing 0.7% agar.[16)] After germination, primary roots of 2-day-old seedlings were inoculated by dripping 200 µL bacterial suspension at about $10^8$ colony forming units/mL. The plants were transferred to 400 mL plant boxes containing expanded vermiculite with an appropriate amount of B&D medium. The plants were cultured for six weeks in a growth chamber at 25 °C with a 12-h photoperiod.[17)] The experiments were performed in triplicates. *R. giardinii* H152$^T$ was used as a positive control for nodulation, and water treatment was used as a negative control.

*Scanning electron microscopy.* Planktonic cell suspension was spotted and fixed on a poly-L-Lysine-coated cover glass. Biofilms were grown in the 35-mm-diameter glass bottom dish (AGC Techno Glass) containing R2A medium at 30 °C for 48 h. Both samples were fixed with 2% glutaraldehyde and dehydrated with ethanol by gradually increasing the concentration, 50, 70, 80, 90, 95, and 100% followed by isoamyl acetate. After $CO_2$ critical point drying with JCPD-5 (JEOL), samples were sputter-coated with gold (JFC-1100, JEOL). Scanning electron micrographs were obtained with S-2400 (Hitachi).

*Physiological characterization of* Rhizobium *strains.* Physiological characterization of R8 was performed according to the standard method[14,18)] with several modifications. Most of the biological tests were done using $\phi$ 18-mm test tubes with 3 mL basal medium containing per liter 1 g of $KH_2PO_4$, 1 g of $K_2HPO_4$, 0.01 g of $FeCl_3{\cdot}6H_2O$, 0.2 g of $MgSO_4{\cdot}7H_2O$, 1 g of $(NH_4)_2SO_4$, 0.1 g of $CaCl_2$, and one each of carbon sources at 0.1% (w/v). When amino acids were tested for nitrogen sources, ammonium sulfate was eliminated and mannitol was used as a carbon source. *Rhizobium* sp. R8 or *R. giardinii* H152$^T$ preculture in TY medium was washed by a newly prepared above test medium and inoculated at initial absorbance at 595 nm, $OD_{595}$ = 0.01. The test tube cultures were shaken at 120 rpm, 28 °C for 4 days. Utilization of carbohydrate and organic acid were determined by cell growth measuring $OD_{595}$. Plate tests for antibiotics resistance and NaCl tolerance, pH dependence, and requirement for calcium were conducted on TY-agar plate.

*Preparation and analysis of membrane proteins.* Biofilm-associated cells were prepared from inner surface of flasks after standing culture in R2A medium at 30 °C for 48 h. Planktonic cells were prepared by shaking culture in TY medium because R8 formed aggregates even in R2A shaking culture. The inoculum size was 1% from fully grown pre-culture. After spin down by centrifuge, cells were re-suspended in 50 mM Tris-HCl (pH 6.8) and disrupted by Multi-beads shocker (Yasui Kikai) using 0.1-mm glass beads. Disruption condition was twelve cycles of 60 s on and 60 s off at 2,500 rpm. After settle down and removal of glass beads by gravity, the disrupted cell suspension was centrifuged at 11,000 × *g* for 5 min to eliminate intact cells. Cytoplasmic and membrane fractions were separated by ultracentrifuge (4 °C, 604,000 × *g*, 120 min). Each fraction was boiled in SDS sample buffer (50 mM Tris-HCl pH 6.8, 6% 2-mercaptoethanol, 10% glycerol, 2% SDS, and 0.01% bromophenol blue) and analyzed by SDS-PAGE using 12% polyacrylamide gel. Electrophoresis was carried out at 100 V for 2.5 h. Protein bands were visualized by Coomassie Brilliant Blue R-250 staining. Proteins from major bands were extracted overnight in 1% SDS/20 mM Tris-Cl (pH 8.0) and applied for N-terminal amino acid sequence analysis by Procise 492 (PerkinElmer) or Procise 491 cLC (Applied Biosystems). The protein sequence was analyzed by protein BLAST homology search program, http://blast.ncbi.nlm.nih.gov/Blast.cgi.

*Preparation and analysis of EPS.* Strain R8 was grown at 28 °C for 96 h, in yeast extracts mannitol agar (YMA) plate,[19)] the colonies were scraped off with disposable cell scraper (#99002, TPP Techno Plastic Products) and suspended in 40 mL distilled water. Bacterial cells were removed by centrifugation and further filtration (0.22 µm pore size Millex GV, Millipore), and clear supernatant containing extracellular polysaccharide (R8-EPS) was pooled. R8-EPS was precipitated by addition of equal volume of ethanol and stored overnight at 4 °C. Resulting precipitates were recovered by centrifuge (7,850× *g*, 4 °C, 20 min) and lyophilized. Purity of polysaccharides was spectrometrically verified by no apparent absorbance at 254 and 280 nm. The recovery yield was about 10 mg/two plates. R8-EPS was hydrolyzed by 2 M trifluoroacetic acid for 4 h at 100 °C under nitrogen atmosphere. Pyridylamination of hydrolyzed R8-EPS was performed according to the method of Hase et al.[20)] with slight modifications.

The pyridylamino (PA)-derivative sugars were separated using a reverse phase column (4.6 × 250 mm CAPCELL PAK MG, Shiseido) with 20 mM ammonium acetate buffer, pH 7.5, containing 2.0% acetonitrile, at a flow rate of 1.0 mL/min, 40 °C. For the detection of PA-derivative sugar, excitation and emission wavelength of 310 nm and 380 nm was used, respectively.

*Sequence data submission.* The 1,467 nucleotides sequence data of R8 16S rRNA gene have been registered to DDBJ/EMBL/GenBank under accession number LC005488.

*Accession numbers of the 16S rRNA sequence data obtained from DDBJ/EMBL/GenBank.* ABB19816, KJ801857, JQ689178, JQ660243, KJ631291, EU730917, JF913979, KF973257, KJ631291, KF358264, EF035074, U71078, U89832, EF061096, AY738130, D14501, AY626395, FJ839677, U28916, U29386, EF141340, DQ835306, AY509899, AM931436, DQ100063, Y10170, AF364068, U89817, AF003375, AF364069, U86343, EU867317, EU256404, EU256434, AM181745, AF041447, D13431, U07934, D14514, X67231, D14509, GU565534, U86344, Y17047, U45329, AY341343, EF440185, EU781656, X73041, AB247615, FJ969841, Z30542, EF125187, DQ454123, EU056823, EF363715, DQ855276, AF025852, EU074168, D11343, GU128881, U69638, X87273.

## Results and discussion

*Change in bacterial community structure of the biofilm samples*

Culture-independent analysis of bacterial community structure in the biofilms formed on toilet bowls was performed by PCR-DGGE using short-length 16S rRNA gene fragments (approximately 200 bp) (Fig. 1). It was shown that the DNA band patterns are quite similar between one and three weeks' biofilm samples (1-w and 3-w), suggesting the stability of microbial community structure during this time period. On the other hand, only a part of them were observed in the third days' sample (3-d). These results suggest that there are a number of later colonizing bacterial groups that prefer conditions formed by early colonizing bacterial groups in 3-d sample. Analysis of these early coming bacterial groups should provide us with knowledge about the initial process of forming biofilms on toilet bowls and also might be a key to control and eliminating stable and recalcitrant biofilm formation. We were thus prompted to isolate early biofilm forming bacteria from the 3-d sample.

*Isolation of early colonizing bacteria*

Eleven candidate strains, R1-R11, were isolated in R2A at 30 °C from the 3-d biofilm sample. The nucleotide BLAST analysis of each partial 16S rRNA gene sequence revealed that these are, R1, *Chryseobacterium* sp. (99.4%: 1371/1378 bp identical to *Chryseobacterium* sp. L7-15, ABB19816); R2, *Microbacterium* sp. (99.2%: 1340/1351 bp identical to *Microbacterium* sp. N54, KJ801857); R3, *Microbacterium* sp. (97.6%: 1335/1367 bp identical to *Microbacterium trichothecenolyticum* A3RC1, JQ689178); R4, *Sphingomonas* sp. (98.5%: 1324/1344 identical to *Sphingomonas* sp. S7-655, JQ660243); R5 *Microbacterium* sp. (98.8% 1348/1365 bp identical to *M. trichothecenolyticum* N3G-5, KJ631291); R6, *Sphingomonas* sp. (99.4% 1333/1341 identical to *Sphingomonas yunnanensis* 215, EU730917); R7, *Sphingomonas* sp. (99.5% 133/1340 bp identical to *S. yunnanensis* 215, EU730917); R8, *Rhizobium* sp. (95.5% 1300/1360 bp identical to *R. giardinii* D30, JF913979); R9, *Microbacterium* sp. (95.1% 770/809 identical to *Microbacterium foliorum* XLST-6, KF973257); R10, *Microbacterium* sp. (97.6% 774/793 bp identical to *M. trichothecenolyticum* N3G-5, KJ631291); and R11, *Microbacterium* sp. (93.9% 1320/1405 bp identical to *Microbacterium oxydans* M2-2, KF358264), respectively. Taking ambiguity of several nucleotide sequences into consideration and similar biofilm formation activities shown below, it is probable that strains R3 and R5, and R6 and R7 are the same strain, respectively.


Fig. 1. 16S rRNA gene PCR-DGGE analyses of R8 and three biofilm samples taken from a household toilet bowl.

Notes: Biofilm samples are: 3-d, on the third day; 1-w, after one week; 3-w, after three weeks. R8, *Rhizobium* sp. R8 isolated from 3-d biofilm sample.

Biofilm forming activity was tested for the eleven strains in three different media, AU, R2A, and TSB (Fig. 2(A)–(C)). It was found that *Rhizobium* sp. R8 exhibited the highest biofilm forming activity in both AU and R2A media, while did not grow on nutrient rich TSB medium. *Microbacterium* sp. R3, R5, R9, and R10 showed the second best activity in AU medium. *Sphingomonas* sp. R4, R6, and R7 had marginal biofilm forming activity in all media. *Chriseobacterium* sp. R1 and *Microbacterium* sp. R2 were poor biofilm

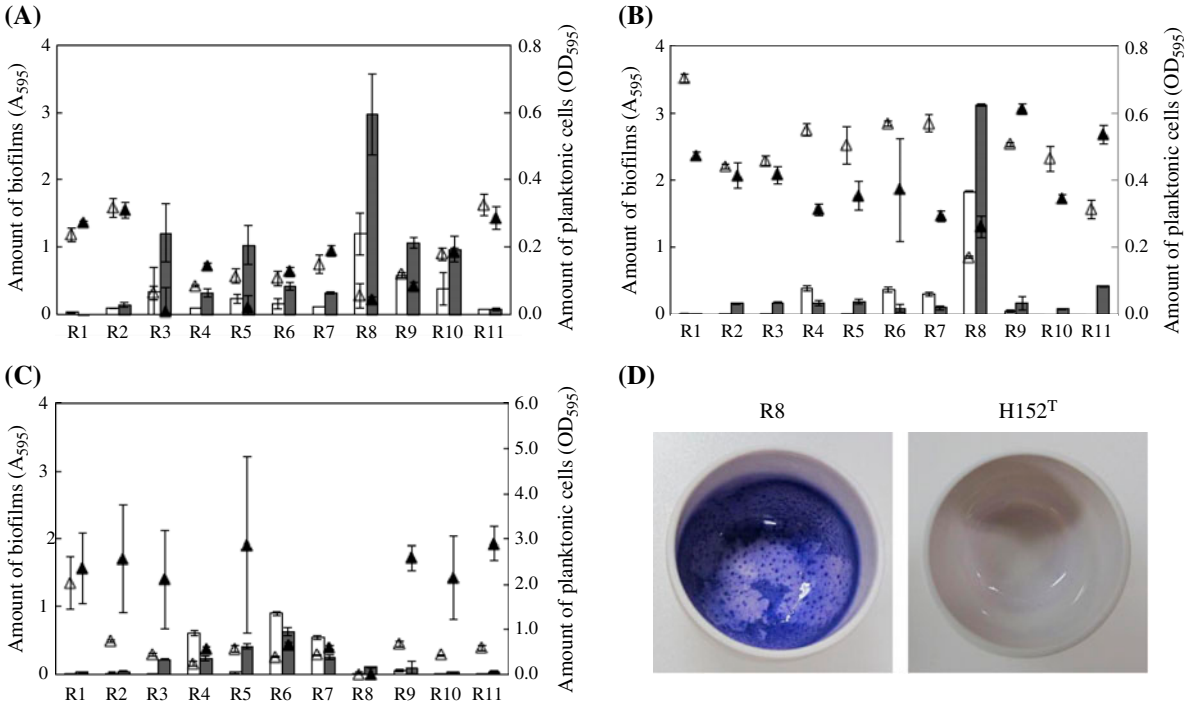


Fig. 2. Biofilm forming activity of isolated bacteria.

Notes: Three medium conditions were examined: (A) AU; (B) R2A; (C) TSB. Bar graphs ($A_{595}$) show the amount of biofilms formed in polystyrene 96-well microplate. Error bar shows SD in triplicate experiments. Triangle shows amount of planktonic cells ($OD_{595}$). Open symbol; 24 h, closed symbol: 48 h. (D) biofilm formation by *Rhizobium* strains on porcelain crucibles. R8 and H152$^T$ are isolated *Rhizobium* strain and the type strain of *R. giardinii,* respectively. Each strain was cultured in 3 mL R2A medium at 30 °C for 24 h. Biofilms formed on the surface were visualized by 0.1% crystal violet staining for 10 min.

former in all the three medium conditions. All the strains showed vigorous growth in TSB medium; however, the biofilm formation was generally poor except for strains R4, R6, and R7. R8 also formed robust biofilms on porcelain crucible whose surface is similar to the toilet bowl (Fig. 2(D)). We thus chose R8 for further characterization and identification of its cellular factors related to the biofilm formation on abiotic solid surfaces. Comparison of the PCR-DGGE band pattern of R8 with 3-d biofilm sample suggested that R8 was not the most dominant bacterium (Fig. 1). We have often faced to the limitation of cultivation method for environmental bacteria, such as the most dominant bacteria could be VBNC, viable but non-culturable.

*Phylogenetic tree analysis and physiological characterization of* Rhizobium *sp. R8*

In order to identify the strain R8, we first determined almost full sequence of the 16S rRNA gene (1,467 bp) and constructed a phylogenetic tree with other related *Rhizobium* type strains (Fig. 3). The 16S rRNA gene sequence of R8 had the highest similarities 99.3% (1,146/1,154 bp) to that of *R. giardinii* H152$^T$, followed by 99.2% (1,143/1,152 bp) to *Rhizobium herbae* CCBAU 83011$^T$. We next compared physiological characterization of R8 with *R. giardinii* H152$^T$. *R. giardinii* H152$^T$ was isolated from a nodule of a leguminous plant *Phaseolus bulgaris* grown in Bretennieres, France.[14] R8 exhibited almost similar assimilation ability of carbohydrates and amino acids to those of H152$^T$ but was slightly different from H152$^T$ in regard to the growth ability on D-dulcitol, requirement of calcium, and drug resistance for kanamycin, gentamycin, and erythromycin (Table 1). Moreover, growth inability on citrate and Luria-Bertani medium and sensitivity to 2% NaCl were different feature from *Rhizobium herbae* CCBAU 83011$^T$.[18] The most significant trait of R8 that differed from *R. giardinii* was urease activity (Fig. 4). Tremendous urease activity should benefit R8 to dominate in the urine conditions on a toilet bowl. It may be also noteworthy that R8 cells formed abundant aggregates when cultured in shaking flask containing R2A medium, while H152$^T$ grew in uniformly dispersed planktonic state.

*Nodule forming activity*

There is increasing number of reports for *Rhizobium* strains that are isolated not from plant roots but from various environments such as activated sludge and bioreactor or contaminated soil environments with aniline, petroleum oil, and triazophos. In such cases, these *Rhizobium* strains often lack *nod* and/or *nif* genes responsible for forming nodules and fixing nitrogen on the roots of leguminous plants.[21–23] R8 was isolated from a household toilet bowl which was different from plant-habiting area. We asked if R8 also did not harbor *nodC* and *nifH* genes. *nodC* encodes *N*-acetylglucosaminyltransferase that is one of the key enzymes to produce Nod factors. *nifH* encodes a dinitrogenase reductase subunit responsible for nitrogen fixation. *nodC* and *nifH* are the most widely used nodulation and nitrogen fixation gene markers, respectively.[15] It was found that PCR for amplifying *nodC* was successful when using template genomic DNA prepared from H152$^T$ but not

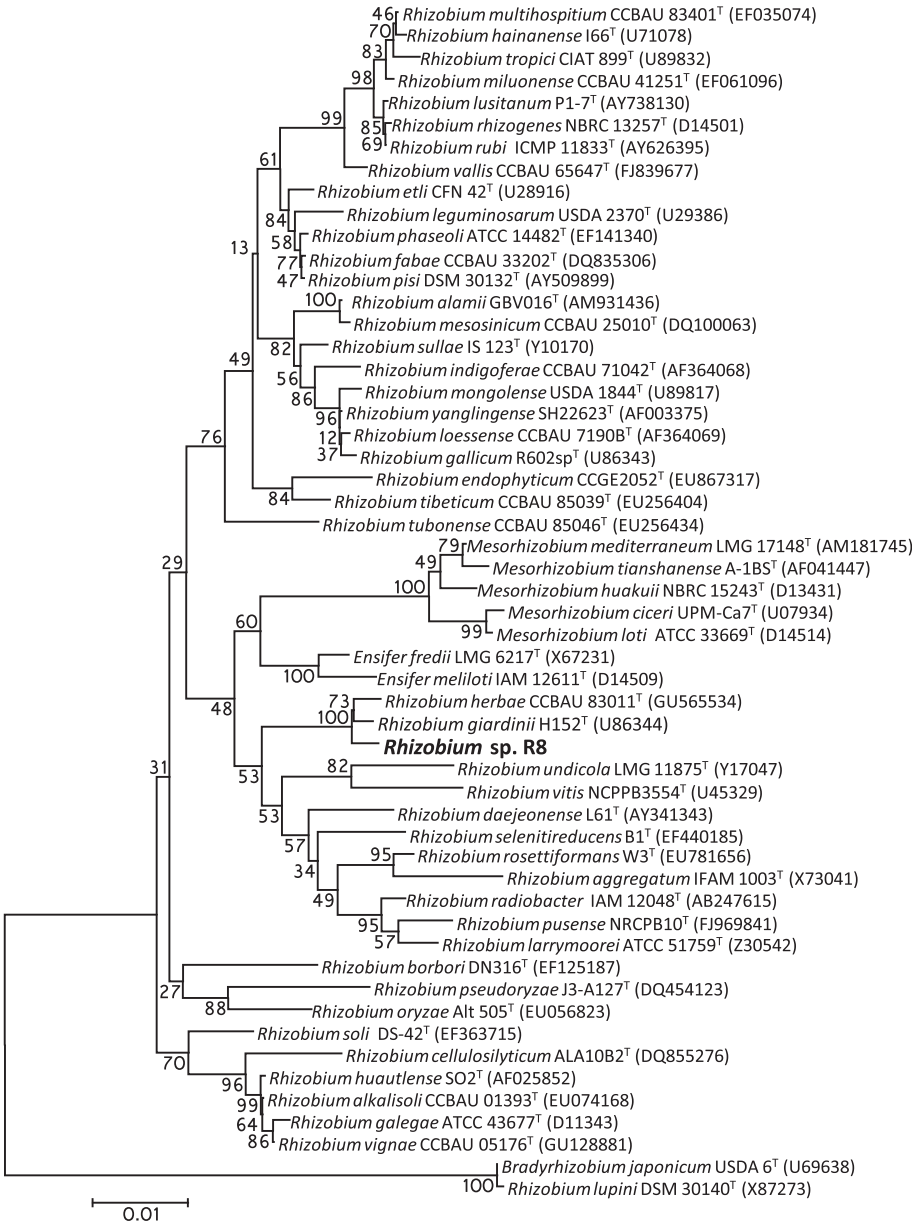


Fig. 3. A phylogenetic tree by neighbor-joining method showing the relationships of genus *Rhizobium* based on 16S rRNA gene sequences.
Note: Bootstrap probabilities (shown as percentages at junctions) were determined from 1,000 samplings.

Table 1. Discriminative features of strain R8 and related strains in the genus *Rhizobium*.

| Characteristics | R8 | H152$^T$ | Characteristics | R8 | H152$^T$ |
|---|---|---|---|---|---|
| D-glucose | + | + | L-glutamate | + | + |
| L-arabinose | + | + | L-glutamine | + | + |
| D-fructose | + | + | L-tyrosine | + | + |
| D-galactose | + | + | L-cysteine | – | – |
| D-*N*-acetylglucosamine | + | + | Glycine | – | – |
| D-gulcuronic acid | – | – | L-tryptophan | + | + |
| D-gulconate | w | + | L-arginine | + | + |
| Lactose | + | + | L-serine | + | + |
| Maltose | + | + | L-histidine | + | + |
| Mannitol | + | + | L-phenylalanine | + | + |
| Glycerol | + | + | 0.5% NaCl | + | + |
| Erythrytol | – | – | 1% NaCl | + | + |
| Dulcitol | + | – | 2% NaCl | – | – |
| DL-lactate | – | – | LB | – | – |
| DL-tartrate | – | – | TY-Ca | – | + |
| D-galacturonic acid | w | w | | | |
| Citrate | – | – | | | |

Notes: LB, Luria-Bertani broth; TY-Ca, TY medium without Ca; +, positive growth; –, no growth; w, weak (slight growth).

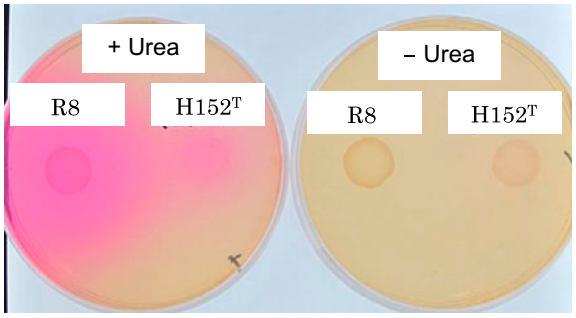


Fig. 4. Urease activity of *Rhizobium* strains.
Notes: Urease activity was indicated by haloformation on Christensen urea agar plate containing 1% urea (left). The plates are after 2 days cultivation at 30 °C.

successful from R8 (figures not shown). As to *nifH*, no positive PCR product was detectable for both H152$^T$ and R8 strains.[15] These results suggest that R8 had lost *nodC* and *nifH* genes. Furthermore, nodule forming activity was examined for *R. giardinii* strains H152$^T$ and R8 by inoculating them to the seedlings of a leguminous plant *P. vulgaris*. It was clearly shown that H152$^T$ but not R8 had ability to form nodules on the roots (Fig. 5).

*Rhizobium* strains can usually establish a symbiotic interaction with leguminous plants under nitrogen-limited soil conditions.[24,25] This host plant-specific interaction results in development of root nodules in which the *Rhizobium* strains differentiate into nitrogen fixing bacteroids.[26] It should be rational decision for *Rhizobium* sp. R8 to have lost abilities of energy consuming nodule formation and nitrogen fixation and acquired high urease activities in urine (soluble nitrogen source) rich conditions. On the other hand, popular nodule forming *Rhizobium* strains including, *R. giardinii* H152$^T$, may maintain urease activity to be ready for the occasion of plant non-associated growth in the environments.

*Membrane protein analysis of R8*

There are examples of comparative proteomic analyses of biofilm-associated cells and planktonic cells. Production of outer membrane proteins, including OmpA-related protein, OmpW family protein, TonB-dependent receptor proteins, and non-fimbrial adhesion YapH, was clearly increased in biofilm-associated cells.[27] Proteins involved in the motility complex, including the flagellins (FlaA, FlaB), the filament cap (FliD), the basal body (FlgG, FlgG2), and the chemotactic protein (CheA), all exhibited higher levels of expression in biofilms than found in stationary-phase planktonic cells of *Campylobacter jejuni*.[28] We hypothesized that the production level of specific proteins including proteinaceous adhesion factors should be increased in membrane or cell surfaces upon formation of biofilms by R8.

Membrane fractions of biofilm-associated cells and planktonic cells were prepared and analyzed their protein production profiles by SDS-PAGE (Fig. 6). There were several membrane proteins produced in different amounts between the samples. It was found that a protein band at approximately 60 kDa, namely MP60, was the most specifically overproduced in the biofilm-associated cells. The N-terminal amino acid sequence of MP60 was determined as 1-KVFGRIELAA, and this sequence was analyzed by protein BLAST program. Although there were proteins whose amino acid sequence was partially similar to this sequence, none of them shared similarity at the N-terminal position. A hypothetical protein (WP_017996705; 143 a.a. = 15.2 kDa) from *Rhizobium leguminosarum* had the sequence 31-KVF*A*RI*DF*AA; however, this protein seemed to be neither a membrane nor a secretion protein based on the analysis by signal peptide prediction program SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). It is probable that MP60 is a unique membrane protein of R8.

*Exopolysaccharides produced by R8*

When we observed the cell structure of R8 by scanning electron microscope, we noticed that R8 cells were incased in abundant extracellular polymers (Fig. 7). It was known that *Rhizobacterium* bacteria effectively produced exopolysaccharides in culture media containing mannitol.[29] When R8 was grown on YMA plate, the colonies became slimy and covered by significant amount of jelly-like exopolysaccharides. The amount of biofilms formed by R8 was also increased by twofolds ($A_{595}$ = 5.3) upon addition of 1% mannitol


Fig. 5. Nodule forming activity.
Notes: Nodules were observed on the root of *P. vulgaris* inoculated by H152$^T$ but not by R8. Arrows indicate nodules. Size bar indicates 2 cm. Experiments were performed in triplicates.

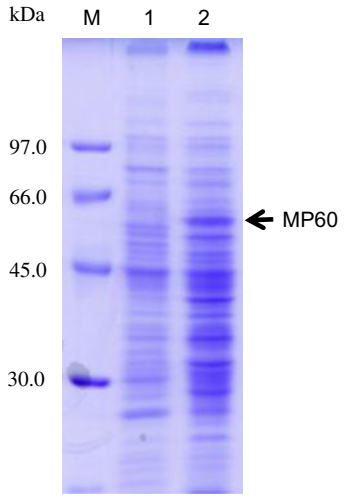


Fig. 6. SDS-PAGE analysis of *Rhizobium* sp. R8 membrane proteins.
Notes: M; Molecular weight marker, 1; planktonic R8 cells, 2; biofilm-associated R8 cells.

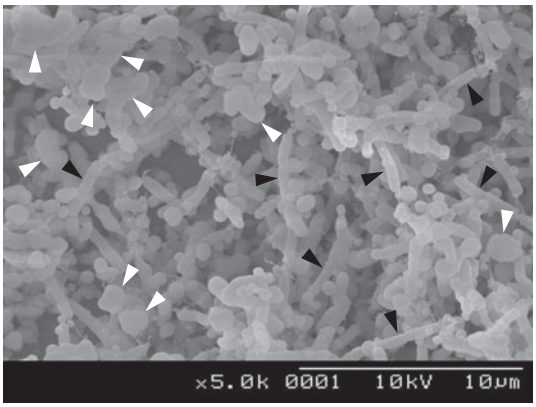


Fig. 7. SEM images of *Rhizobium* sp. R8 biofilm-associated cells.
Notes: White arrows indicate cells that probably produce large amount of EPS, while black arrows indicate cells that produce small amount of EPS.

to R2A medium, suggesting that these mannitol-dependent exopolysaccharides, namely EPS-R8, are also functional biofilm matrices. This dramatic change in the colony morphology and increase in the biofilm formation were not observed for H152$^T$. We were thus prompted to prepare and analyze the structure of EPS-R8.

About 10 mg (dry weight) EPS-R8 was prepared from slime colonies formed on two YMA plates. HPLC analysis of 2-aminopyridyne sugars showed that EPS-R8 is composed of galactose, glucose, and mannose at the ratio of 8:1:1. There is a report that *R. leguminosarum, Rhizobium meliloti, Rhizobium phaseoli,* and *Rhizobium trifolii* commonly produced gel-forming capsular polysaccharides (CPS), which contained galactose, glucose, and mannose at a constant ratio of 4:1:1.[29,30] This ratio was reported unchangeable under different concentration of mannitol. These CPS were reported insoluble in water and prepared from cell surfaces by 1 N NaOH treatment. On the other hand, the EPS-R8 was soluble in water and recovered from cell-free supernatants by simple centrifuge of cell suspension. The CPS structure was shown pseudo-double helix with hexasaccharide repeating unit composed of a main chain →4)-Glc-(1→3)-Man-(1→3)-Gal-(1→, which is doubly branched by single Gal-(1→ side chain at position O-2 and by a Gal-(1→4)-Gal-(1→ side chain at position O-6 of the glucose residue.[31,30] EPS-R8 may contain longer or more galactosyl side chains, which enables to be more soluble in water than CPS.

*R. meliloti* also produced acidic EPS composed of D-glucose, D-galactose, pyruvic acid, and succinic acid in the molar ratios 7:1:1:1 which is called succinoglycan.[32] On the other hand, *R. leguminosarum, R. trifolii,* and *R. phaseoli* produced another group of acidic EPS composed of D-glucose, D-galactose, D-glucuronic acid, and pyruvic acid in the molar ratios 5:1:2:2.[33] Production of these high molecular weight EPS, together with low molecular weight β-l, 2-glucans and acidic oligosaccharides, has been shown to be important for nodule formation by *Rhizobium* bacteria.[34] Our experimental data demonstrated that EPS-R8 did not contain succinate, pyruvate, and uronic acids. This result is not inconsistent with the fact that R8 had lost genes essential for nodulation and nitrogen fixation. It remains to be elucidated whether MP60 together with surface EPS-R8 plays crucial roles staying against water flush and recruiting some friendly neighbors in the multispecies toilet bowl biofilms.

*Proposal of "*Rhizobium *latrinae" sp. nov.*

The 16S rRNA gene of *Rhizobium* strain R8 shares significantly high sequence similarities with *R. giardinii* (99.3%) and *R. herbae* (99.2%), suggesting R8 is a member of species *giardinii* or *herbae*. However, its robust biofilm formation activity, high urease activity, production of novel exopolysaccharide, and loss of nodule forming activity and *nifH* and *nod*C genes are specific traits of R8. Although DNA–DNA hybridization experiment remains to be performed, in order to avoid confusion of R8 with above two species, we propose a non-validated novel species name "Rhizobium latrinae" R8 sp. nov. The word "latrina" means "toilet".

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