Enrichment and Isolation of Surfactin-degrading Bacteria

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Abstract: A total of 100 environmental samples were investigated for their ability to degrade 1 g/L surfactin as a substrate. Among them, two enrichment cultures, which exhibited microbial growth as well as surfactin degradation, were selected and further investigated. After several successive cultivations, nanopore sequencing of full-length 16S rRNA genes with MinION™ was used to analyze the bacterial species in the enrichment cultures. Variovorax spp., Caulobacter spp., Sphingopyxis spp., and Pseudomonas spp. were found to be dominant in these surfactin-degrading mixed cultures. Finally, one strain of Pseudomonas putida was isolated as a surfactin-degrading bacterium. This strain degraded 1 g/L surfactin below a detectable level within 14 days, and C₁₅ surfactin was degraded faster than C₁₆ surfactin.

Key words: biodegradation, biosurfactant, cyclic lipopeptide, Pseudomonas putida, surfactin

1 Introduction

A variety of microorganisms can produce amphiphilic and surface-active compounds, called biosurfactants (BSs), as structurally diverse molecules. BSs are classified as lipoprotein, phospholipid, glycolipid, and fatty acid surfactants based on their chemical structures and microbial origin, and sometimes have a wider range of properties than those of chemically synthesized surfactants [1-3].

Among the BS classes, surfactin, which is the most common cyclic lipopeptide produced by the genus Bacillus [4-5], has received a lot of attention from industries due to its excellent interfacial properties and environmentally friendly characteristics [6-7]. Surfactin is considered to be one of the most powerful BSs thus far, because it can reduce the surface tension of an aqueous solution from 72 to 27 mN/m at concentrations in the order of 10⁻⁵ M [8]. In addition, surfactin is known to act as an ionophore, so alkali metal ions can be trapped in the cyclic peptide of surfactin [9-10]. Specific interaction between surfactin and cesium ion (Cs⁺) has been shown, and highly efficient removal of Cs⁺ from water has been demonstrated [11]. The high cost associated with low productivity has greatly limited the commercial application of surfactin. Therefore, research on enhancing the yield of surfactin, including screening and construction of improved or engineered strains for surfactin biosynthesis, has been performed [12].

However, limited information is available regarding the biodegradation of surfactin, the genus and species of surfactin-degrading microorganisms, and the mechanism of microbial surfactin degradation, although surfactin has been described as an environmentally friendly and biodegradable surfactant. One reason for this is the high cost of surfactin for use as a screening substrate for surfactin-degrading microorganisms. In addition, surfactin is reported to have biological activities, including antiviral [13], antifungal [14], antitumor [15], and hemolytic [16] properties, that may have negative effects on environmental microorganisms.

To our knowledge, one study on the biodegradability of crude surfactin by a pure culture of bacteria has been carried out, which demonstrated that the biodegradation process depends on the initial concentration of surfactin with the highest biodegradability values (>65%) observed at concentrations less than 100 mg/L within 72 h [17]. Biodegradability was investigated for crude surfactin concentrations in the range of 10 to 200 mg/L; however, no information is available on its biodegradation at concentrations higher than 200 mg/L.

The goal of this study was to identify isolates of bacteria that degrade higher concentrations of surfactin. First, we enriched bacterial species able to grow in the presence of 1 g/L surfactin sodium salt from environmental samples. During the enrichment of surfactin-degrading consortia,
bacterial community compositions were monitored using the MinION™ sequencer (Oxford Nanopore Technologies; https://nanoporetech.com), a third generation single-molecule technology sequencer that overcomes short length-associated problems by sequencing the full length of 16S rRNA genes. Finally, we isolated, identified, and characterized a surfactin-degrading bacterium.

2 Experimental

2.1 Materials
Surfactin sodium salt, used as a substrate for screening, was kindly supplied by Kaneka Corporation (Tokyo, Japan), which is a mixture of C13, C14 and C15 homologues. All other commercially available reagents were of the highest purity.

2.2 Apparatus
High performance liquid chromatography (HPLC) analysis of surfactin was performed using an LC-20AD HPLC pump (Shimadzu, Otsu, Japan) equipped with a TSK-GEL ODS-100V column (4.6 × 150 mm, TOSOH, Tokyo, Japan). The mobile phase composed of acetonitrile: 2% (v/v) acetic acid (4:1) was used at a flow rate of 1.0 mL/min, and the column was kept at 30°C during analysis. The samples were detected and quantified at 210 nm using an SPD-20AV UV/VIS detector (Shimadzu).

2.3 Enrichment of surfactin-degrading bacteria
Surfactin-degrading bacteria were enriched from 100 environmental samples (designated No. 1 to 100), primarily soil and plant leaves from areas around Tsukuba, Japan. Approximately 50 mg of each sample was added to 3 ml of surfactin medium in a test tube containing 1 g/L surfactin sodium salt, 2.2 g/L Na2HPO4, 0.8 g/L KH2PO4, 0.2 g/L MgSO4·7H2O, 0.01 g/L CaCl2·2H2O, 0.01 g/L FeSO4·7H2O, and 0.1 g/L yeast extract and incubated aerobically at 30°C on a reciprocal shaker (164 rpm). When the growth of microorganisms was observed, 100 µL of each culture was transferred into new tubes containing freshly prepared surfactin medium (5 mL), and subculture was repeated ten times wherein each culture was incubated for 7–23 days at 30°C. The enrichment process is summarized in Table 1. After the fifth sub-culturing, we selected samples No. 2 and No. 84 whose culture turbidity increased the slowest and fastest, respectively, of the eight samples from the fourth sub-culturing. We then extracted total DNA from these cultures for 16S rRNA gene amplicon sequencing with nanopore technology. Similarly, after the ninth sub-culturing, total DNA was extracted from the cultures to monitor the shifts in bacterial species.

2.4 Degradation of surfactin by enrichment culture
After the fourth and tenth sub-culturing, 500 µL of cultures No. 2 and No. 84 were transferred to flasks containing 30 mL of surfactin medium, or the same medium without surfactin, and incubated for 4 days. Cell growth was evaluated by measuring the optical density (OD) at 600 nm. After removing cells in 1900 µL of culture by centrifugation at 15,000 rpm for 5 min, the supernatant was lyophilized. The resultant powder containing surfactin was dissolved in 190 µL of pure water, and 20 µL of the solution was subjected to HPLC analysis. We determined the decrease in surfactin concentration by comparing the peak area before and after the appropriate cultivation.

2.5 DNA extraction and nanopore sequencing
After the fourth and ninth sub-culturing, total DNA was isolated from 2 mL of both No. 2 and No. 84 enrichment cultures grown on surfactin medium according to standard protocols. To prepare the library, we followed the Oxford nanopore protocol for 16S Rapid Amplicon Barcoding kit (SQK-RAB204), with the use of LongAmp Taq DNA polymerase (New England Biolabs, MA, USA), resulting in the amplification of ~1,500 bp fragments of the full-length 16S rRNA gene.

### Table 1

The process for enrichment of surfactin-degrading bacteria in this study.

| Repeat of subculture | Date            | Incubation period | Sample number remaining |
|----------------------|-----------------|-------------------|-------------------------|
| 1st                  | 30th Nov., 2018 | 7 days            | 100                     |
| 2nd                  | 7th Dec., 2018  | 7 days            | 26                      |
| 3rd                  | 14th Dec., 2018 | 11 days           | 9                       |
| 4th                  | 25th Dec., 2018 | 15 days           | 8                       |
| 5th                  | 9th Jan., 2019  | 20 days           | 8                       |
| 6th                  | 29th Jan., 2019 | 21 days           | 8                       |
| 7th                  | 19th Feb., 2019 | 22 days           | 8                       |
| 8th                  | 13th Mar., 2019 | 23 days           | 8                       |
| 9th                  | 5th Apr., 2019  | 20 days           | 8                       |
| 10th                 | 25th Apr., 2019 | 12 days           | 2                       |
A brand new, sealed R9.4 flowcell was primed using 800 µL of priming mix. The flowcell was equilibrated for at least 5 min before the final DNA library was loaded. The sequencing mix was prepared with 4.5 µL nuclease-free water, 34 µL sequencing buffer, 11 µL DNA library, and 25.5 µL loading beads. A 17-h sequencing was carried out using MinKNOW software.

2.6 Sequencing data analysis
Sequence data analysis and bioinformatics for the nanopore sequencing run were performed as follows. The reads were base-called via the local base-calling algorithm with MinKNOW software. We collected all FASTQ files of passed base-called reads and combined them in one file for further analysis. EPI2ME was used for taxonomic analysis. The datasets generated for this study have been submitted to the DDBJ/EMBL/GenBank databases under accession number DRA011086.

2.7 Identification and characterization of the isolated bacterial strain
After the tenth sub-culture, appropriate dilutions of cultures of No. 2 and No. 84 were spread onto surfactin medium agar plates. The colonies growing on the plates were isolated and checked again for their purity by spreading them on LB medium (Difco) agar plates as well as for their ability to grow on 1 g/L surfactin liquid medium.

Identification of the isolated strain was carried out by TechnoSuruga Laboratory (Shizuoka, Japan), based on 16S rRNA sequence analysis. The 16S rRNA gene sequence of the isolated strain, Pseudomonas putida SF84B, has been submitted to the DDBJ/EMBL/GenBank databases under accession number LC567891.

After pre-cultivation of strain SF84B for 20 days at 30°C using surfactin medium in test tubes, 500 µL of SF84B culture was transferred to flasks containing 30 mL of surfactin medium or the same medium without surfactin, and then incubated for 14 days. As another control, 30 mL of surfactin medium without strain inoculation was also incubated. Two sets of each flask were incubated for reproducibility.

3 Results and Discussion
3.1 Enrichment of surfactin-degrading consortia from environmental samples
In the first screening, 100 environmental samples (No. 1 to 100) of soil and plant leaves were used for enrichment of microorganisms that can grow on surfactin medium. Of the 100 environmental samples, 26 were able to grow on surfactin medium after the first screening. With three further sub-cultures, microbial growth of eight samples was confirmed. Among the eight samples, we focused on No. 2 and No. 84, in which cultures grew the most slowly and most quickly, respectively. To investigate their ability to degrade 1 g/L surfactin for 5 days, we determined the remaining amounts of surfactin by HPLC in the fifth and tenth sub-culturing. As the tendency in decreasing the remaining surfactin is similar, the result obtained in the fifth sub-culturing was shown in Figs. 1 and 2. In Fig. 1, the HPLC profile of the remaining surfactin in the culture at day 0 is shown. Based on our previous report, the three main HPLC peaks correspond to surfactin homologues having C13, C14, and C15 β-hydroxy fatty acids, respectively. As shown in Fig. 2, most of the surfactin was not degraded at day 2 in the sample from culture No. 2, whereas in culture No. 84, 60–90% of surfactin was degraded, depending on its chain length. Finally, surfactin could not be detected at days 4 and 3 in cultures of No. 2 and No. 84, respectively (Figs. 2A and 2B). Considering that an increase in the OD600 values of cultures is concomitant with a reduction of surfactin in the culture medium, it can be considered that surfactin was degraded. The results suggest that surfactin-degrading consortia have the potential for surfactin biodegradation.
3.2 Comparison of the ten most abundant bacteria in two enrichment cultures after four and nine subcultures

A total of 1,030,372 sequences were obtained from four different samples using nanopore sequencing. The species-level phylogenetic analyses of sequence data using EPI2ME software were conducted to clarify the changes in microbial composition after the fourth and ninth sub-cultures of samples No. 2 and No. 84. Of the total sequences, 1,026,394 were classified to known bacteria in databases, and 3,978 were not.

The ten most dominant species in the fourth and ninth sub-cultures were extracted from the sequence library in samples of No. 2, and their relative abundances accounted for 44.55% and 42.52% of the total population, respectively (Table 2). In the fourth and ninth sub-cultures, half of the ten most dominant species were common bacterial species (Variorax paradoxus, Variorax boronicumulans, Caulobacter henricii, Caulobacter mirabilis, and Pseudomonas graminis). After four sub-cultures, V. paradoxus was the predominant species, accounting for 13.76% of the total population, whereas it was the second most predominant in the ninth sub-culture. V. paradoxus has been reported to degrade versatile toxic and/or complex chemical compounds. By contrast, in the ninth sub-culture, C. henricii was the predominant species, accounting for 14.23% of the total population (the third most predominant in the fourth sub-culture).

Similarly, the ten most dominant species in the fourth and ninth sub-cultures were extracted from the sequence library in samples of No. 84, and accounted for 47.62% and 43.50% of the total population, respectively (Table 3). In the fourth and ninth sub-cultures, seven of the ten most dominant species were common species. Also, in both sub-cultures, the most and the second most predominant species were the same, with relative abundances of 12.20%

Table 2 The shift in the ten most abundant bacterial species in the culture of No. 2 sample.

| The 4th sub-culturing | The 9th sub-culturing |
|-----------------------|-----------------------|
| Species               | Relative abundance (%)| Species               | Relative abundance (%)|
| Variovorax paradoxus  | 13.8                   | Caulobacter henricii  | 14.23                 |
| Pseudoxanthomonas mexicana | 9.90                 | Variovorax paradoxus  | 9.75                  |
| Caulobacter henricii  | 5.25                   | Caulobacter mirabilis | 4.75                  |
| Xanthomonas cucurbitae | 3.32                   | Caulobacter vibrioides| 2.98                  |
| Xanthomonas sacchari  | 2.33                   | Pseudomonas plecoglossicida | 2.56          |
| Variovorax boronicumulans | 2.26               | Caulobacter segnis    | 2.28                  |
| Pseudomonas graminis  | 2.03                   | Brevundimonas aurantiaca | 1.96         |
| Brevundimonas aurantiaca | 1.94                | Pseudomonas graminis  | 1.46                  |
| Chryseobacterium pallidum | 1.94                | Variovorax boronicumulans | 1.33       |
| Caulobacter mirabilis | 1.80                   | Acinetobacter calcoaceticus | 1.21         |
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The shift in the ten most abundant bacterial species in the culture of No. 84 sample.

| Species                              | Relative abundance (%) | Species                              | Relative abundance (%) |
|--------------------------------------|------------------------|--------------------------------------|------------------------|
| 1 Sphingopyxis taejonensis           | 12.20                  | 1 Sphingopyxis taejonensis           | 13.90                  |
| 2 Pseudomonas multiresinovorans      | 11.71                  | 2 Pseudomonas multiresinovorans      | 6.64                   |
| 3 Pseudomonas nitroreducens          | 6.20                   | 3 Variovorax paradoxus               | 3.72                   |
| 4 Pseudomonas citronellolis         | 5.90                   | 4 Chryseobacterium defluvii          | 3.34                   |
| 5 Variovorax paradoxus               | 3.53                   | 5 Pseudomonas citronellolis         | 3.25                   |
| 6 Pseudomonas knackmussii            | 2.67                   | 6 Pseudomonas nitroreducens         | 3.20                   |
| 7 Pseudomonas nitritireducens        | 2.41                   | 7 Chryseobacterium hispalense        | 2.72                   |
| 8 Sphingomonas sanxanigenens         | 1.28                   | 8 Bordetella avium                  | 2.70                   |
| 9 Chryseobacterium defluvii          | 0.86                   | 9 Sphingomonas sanxanigenens        | 2.15                   |
| 10 Pseudomonas stutzeri              | 0.85                   | 10 Pseudomonas plecoglossicida      | 1.88                   |

3.3 Isolation, identification, and characterization of a surfactin-degrading strain

After ten successive sub-cultures, a small portion of appropriately diluted resultant cultures (No. 2 and No. 84) was spread onto a surfactin medium agar plate, and different types of colonies were isolated. Three types of colonies from No. 2 and two types of colonies from No. 84 were checked again for their ability to grow in surfactin liquid medium. Among these five types of colonies, only one was confirmed to grow in surfactin liquid medium. The strain was then checked for its purity on LB agar plates and designated SF84B. After 3 days of growth at 30°C on an LB agar plate, morphological analysis revealed that the cells of strain SF84B were 0.8–1.0 µm wide and 1.0–2.0 µm long (Fig. 3).

The 16S rRNA gene sequences of strain SF84B were 99.8–99.9% identical to Pseudomonas putida strains such as KF715 (accession number: AP015029), IHB B 13625 (KP762566), and ATCC12633 (AF094736). Thus, we identified the strain SF84B as Pseudomonas putida. This species is well-known as a degrader of C₆ to C₁₀ n-alkane. Considering that Pseudomonas putida was the 36th most dominant species in No. 84 after the ninth subculturing, the contribution of the strain to surfactin degradation in the mixed culture might seem to be low, but its isolation might be easier than other strains.

3.4 Degradation of surfactin by Pseudomonas putida

Pseudomonas putida SF84B was evaluated for its growth and surfactin consumption in 1 g/L surfactin medium. As shown in Fig. 4, the strain SF84B started to use both C₁₃ and C₁₄ surfactin around day 2, and approximately half and most of these surfactin homologues, respectively, were consumed within 8 to 11 days. By contrast, the strain SF84B started to consume C₁₅ surfactin after day 6, and approximately half and most of the surfactin was used within 10 to 14 days (Fig. 4). To measure growth of strain SF84B, OD₆₀₀ values were determined. The readings showed that its growth was concomitant with surfactin degradation (Fig. 4). In control samples without surfactin or SF84B inoculum, degradation of surfactin and culture turbidity were not observed (data not shown). These results suggest that this strain prefers C₁₅ surfactin as a substrate rather than C₁₃ surfactin, likely because surfactin...
homologues with shorter chain lengths are easier to
degradate, and also that surfactin degradation by a single
strain takes longer than by a mixed culture.

4 Conclusion
Among 100 tested environmental samples, we obtained
two surfactin-degrading enrichment cultures and investiga-
ted bacterial composition using nanopore sequencing. In
the two consortia, bacterial species belonging to Proteo-
bacteria such as Varivorax paradoxus (Betaproteobacte-
ria), Caulobacter henricii (Alphaproteobacteria), Sphi-
gopyxis taejonensis (Alphaproteobacteria), and Pseu-
domonas multiresinovorans (Gammaproteobacteria) were found to be dominant. One strain of Pseu-
domonas putida was successfully isolated from the consortium and examined for its ability to degrade surfactin. The strain degraded 1 g/L surfactin below the detectable level of HPLC analysis within 14 days. This is the first study to
demonstrate surfactin degradation at concentrations
higher than 200 mg/L with a pure culture of a single bacte-
rial strain. Also, the bacterial degradation profiles of re-
spective C13 to C15 surfactin homologues were first exam-
ined, because a previous report assessed surfactin
degradation by measuring the decrease in dissolved
organic carbon with a total organic carbon analyzer.

To reveal the degradation pathway of surfactin, molecular
analysis of the strain is now underway.

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