



## Efficacy of forming biofilms by naphthalene degrading *Pseudomonas stutzeri* T102 toward bioremediation technology and its molecular mechanisms

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### ARTICLE INFO

#### Article history:

Received 9 September 2011  
Received in revised form 28 December 2011  
Accepted 29 December 2011  
Available online 28 January 2012

#### Keywords:

Bioremediation  
Naphthalene degradation  
Biofilms  
*Pseudomonas*

### ABSTRACT

In natural environments, bacteria often exist in close association with surfaces and interfaces. There they form “biofilms”, multicellular aggregates held together by an extracellular matrix. The biofilms confer on the constituent cells high resistance to environmental stresses and diverse microenvironments that help generate cellular heterogeneity. Here we report on the ability of *Pseudomonas stutzeri* T102 biofilm-associated cells, as compared with that of planktonic cells, to degrade naphthalene and survive in petroleum-contaminated soils. In liquid culture system, T102 biofilm-associated cells did not degrade naphthalene during initial hours of incubation but then degraded it faster than planktonic cells, which degraded naphthalene at a nearly constant rate. This delayed but high degradation activity of the biofilms could be attributed to super-activated cells that were detached from the biofilms. When the fitness of T102 biofilm-associated cells was tested in natural petroleum-contaminated soils, they were capable of surviving for 10 wk; by then T102 planktonic cells were mostly extinct. Naphthalene degradation activity in the soils that had been inoculated with T102 biofilms was indeed higher than that observed in soils inoculated with T102 planktonic cells. These results suggest that inoculation of contaminated soils with *P. stutzeri* T102 biofilms should enable bioaugmentation to be a more durable and effective bioremediation technology than inoculation with planktonic cells.

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### 1. Introduction

Among the strategies to clean up pollutants it is widely recognized that biological treatments, so called bioremediation technologies, have the advantage over chemical and physical treatments in terms of their compatibility with elemental cycles and thus less impact on the environment (Alexander, 1999). Active on-site bioremediation includes technologies that activate indigenous microbial populations, *biostimulation*, or introduce specific competent foreign bacteria to the contaminated site, *bioaugmentation*. Although bioaugmentation is expected to be a quicker and more effective technology than biostimulation, low fitness and poor colonization of the introduced bacteria to the contaminated sites often make efficacy of bioaugmentation poor (Bouchez et al., 2000; El-Fantroussi and Agathos, 2005). The activity and viability of the introduced bacteria often decreases at the contaminated sites when compared with those observed under laboratory conditions, probably due to diverse environmental stresses (van Veen et al., 1997; Thompson et al., 2005). These include predation by protozoa, competition with other bacteria, unfavorable pH and temperature conditions, and

unavailability of nutrients and oxygen. Catabolite repression by other organic compounds also decreases the expression level of the degradation genes. Bioaugmentation can become more effective if both microbial ecology and population sizes are taken into account (Dejonghe et al., 2001; Silva et al., 2004; El-Fantroussi and Agathos, 2005). One of the options for effective clean-up methods of contaminated sites is the use of carrier materials so as to maintain sufficient activity of inoculants during prolonged periods. There are several reports demonstrating that immobilized or encapsulated cells effectively degrade pollutants at the contaminated sites (Moslemy et al., 2002; Mroziuk and Piotrowska-Seget, 2010). However, the cellular and molecular mechanisms underlying these technologies are largely unknown. The primary purpose of the present study was to demonstrate high performance of naturally self-immobilized cells on surfaces, biofilms, and analyze basic mechanisms as applied in bioaugmentation technology.

Biofilms are structured microbial communities formed on the surface of solid materials or interfaces (Watnick and Kolter, 2000). It has recently been pointed out that environmental microorganisms exist predominantly as biofilms and gain high tolerance to physical, chemical, and biological stresses (Gorbushina and Broughton, 2009). Biofilm-associated cells exhibit specific gene expression, many times controlled by quorum sensing systems, or dormancy, to allow their increases in resistance (Gilbert et al., 1990; Donlan and Costerton, 2002; Rani et al., 2007; Iijima et al.,

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2009; Dickschat, 2010). Thus, forming biofilms is considered a natural strategy of microorganisms to construct and maintain a favorable niche in stressful environments (Thompson et al., 2005; Shemesh et al., 2010). We have speculated that application of biofilms to bioproduction and bioaugmentation process is a simple and rational choice (Morikawa, 2006). Indeed, there are several reports that demonstrate the efficacy of biofilm formation by useful bacteria in the bioremediation and biotransformation technology (Singh et al., 2006; Yamaga et al., 2010). However, to our knowledge there is no report that directly compares degradation activities and persistence in contaminated soils of single species biofilms with that of planktonic cells. When compared with artificially immobilized and encapsulated cells, the advantage of biofilms is that the high density and tolerance of the constituent cell is naturally achieved. Moreover, biofilms can be introduced to contaminated sites free of supports that might cause additional environmental pollution. The secondary purpose of the study was to examine the potential of model biofilms towards developing bioaugmentation technology.

## 2. Materials and methods

### 2.1. Bacterial strains

We examined seven hydrocarbon-degrading bacterial strains in the laboratory for their biofilm forming activities. Characteristics and growth temperature of strains are described in Table 1.

### 2.2. Culture media

Luria broth, containing ( $\text{g L}^{-1}$ ) 10 Bacto tryptone (Difco), 5.0 yeast extract (Difco, Franklin Lakes, NJ) and 10.0 NaCl, was used for general cultivation of bacteria. Y medium was used for biofilm formation and it contained ( $\text{g L}^{-1}$ ) 2.0 Bacto tryptone (Difco), 1.0 yeast extract and 2.0 NaCl, 0.25  $\text{KH}_2\text{PO}_4$ , 0.25  $\text{K}_2\text{HPO}_4$ , 0.1 Ca  $(\text{CH}_3\text{COO})_2 \cdot \text{H}_2\text{O}$ , and 0.1 Mg  $(\text{CH}_3\text{COO})_2 \cdot 4\text{H}_2\text{O}$ . L-agar or Y-agar is a semi-solid medium containing 1.5% agar. BM-medium is a minimal medium used for naphthalene degradation experiments of *Pseudomonas stutzeri* T102, that contained ( $\text{g L}^{-1}$ ) 1  $(\text{NH}_4)_2\text{SO}_4$ , 24.0  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , 9.1  $\text{KH}_2\text{PO}_4$ , 0.2  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , and 0.1% (v/v) vitamin mixture and trace elements before inoculation. Vitamin mixture contained ( $\text{mg L}^{-1}$ ) 0.6 biotin, 0.6 folic acid, 59 inositol, 59 *p*-amino benzoic acid, 59 riboflavin, 120 calcium pantothenate, 120 niacin, 120 pyridoxine-HCl and 120 thiamine-HCl. Trace elements contained ( $\text{mg L}^{-1}$ ) 1  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 3  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 7  $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ , 15  $\text{H}_3\text{BO}_3$ , 17  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 29  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , and 2000 EDTA. pH of the media was adjusted to 7.2 by 2 M NaOH before autoclaving.

### 2.3. Biofilm formation assay

Freshly grown cell cultures were diluted to  $\text{OD}_{600} = 0.3$  in Y medium. Then, 3  $\mu\text{L}$  of the cell suspension were inoculated into

297  $\mu\text{L}$  of Y medium (1%) in 1.5 mL screw capped polypropylene tubes and stood at growth temperatures shown in Table 1. Biofilm formation activity was estimated by the crystal violet staining method with slight modifications (O'Toole et al., 1999). Liquid cultures were carefully removed from the tubes and tubes were rinsed once with distilled water. Then, the biofilms formed inside wall of the tubes were stained with 0.1% crystal violet solution for 20 min at room temperature. After washing twice with distilled water to remove free dye, the dye attached to biofilms was extracted in 300  $\mu\text{L}$  of 95% ethanol and quantified by measuring absorbance at 590 nm.

### 2.4. Scanning electron microscopy

*P. stutzeri* T102 biofilms were formed in a 1.5 mL polypropylene tube at 30 °C for 2 d and fixed stepwise with 5%  $\text{OsO}_4$  and 2% glutaraldehyde in 0.1 M phosphate-buffered saline (pH 7.0). After fixation, the samples were dehydrated by increasing concentration of ethanol and sputter coated with gold. The samples were observed by a Model S-2400 (Hitachi, Tokyo, Japan) scanning electron microscope.

### 2.5. Measurement of colony forming units (CFUs)

Samples from mid-exponential phase shaken cultures in Y medium were serially diluted and spread onto Y-agar plates and incubated at 30 °C for 24 h. Then, the number of colonies was counted to calculate the average CFUs  $\text{OD}_{600}^{-1} \text{ mL}^{-1}$  values for the planktonic cell culture. It is challenging to measure the exact CFUs of biofilms because of being encased in recalcitrant matrices. We adopted a following simple method for this purpose. The biofilms formed in the polypropylene tube or glass bottle (30 °C for 48 h) were removed and dispersed carefully with a disposable cotton pestle and then vigorously vortexed to prepare almost uniform cell suspensions. Microscopic observation warranted quite less amount of cells attached and remained on a cotton pestle. After measuring  $\text{OD}_{600}$  a series of dilution cultures were prepared to determine average CFUs  $\text{OD}_{600}^{-1} \text{ mL}^{-1}$  values of biofilms similarly as described above. These experiments were performed independently at least five times and calculated standard deviation (SD) values.

### 2.6. Naphthalene degradation by biofilms and planktonic cells cultures

Biofilms of T102 were formed in 20 mL glass bottles containing 3 mL Y medium. After cultivation at 30 °C for 24 h without shaking, free planktonic cells were carefully removed from the bottles and the biofilm formed inside walls (ca  $2 \times 10^7$  CFUs) was rinsed once with sterile water. 7 mL of BM medium containing 20 ppm naphthalene were added to each bottle. Bottles were tightly sealed with butyl rubber stoppers and aluminum crimps and incubated at 30 °C without shaking.

Planktonic cells were grown in a 500 mL shaking flask containing 100 mL Y medium. Mid-exponential phase cells were harvested

**Table 1**  
Bacterial strains used in this study and their characteristics.

Strain names	Substrates	Optimal growth temperatures (°C)	References
<i>Pseudomonas stutzeri</i> T102	PAHs including naphthalene	30	Hirano et al., 2004
<i>Xanthobacter polyaromaticivorans</i> 127 W	PAHs including naphthalene	30	Hirano et al., 2004
<i>Oleomonas saganensis</i> HD-1	Alkanes and aromatic hydrocarbons	30	Morikawa and Imanaka, 1993; Kanamori et al., 2002
<i>Gordonia</i> sp. C3	Phthalic acid esters	30	Unpublished
<i>Rhodococcus</i> sp. TMP2	Straight and branched alkanes	20	Kunihiro et al., 2005
<i>Arthrobacter</i> sp. CAB1	Aromatic hydrocarbons	20	Kato et al., 2001
<i>Shewanella</i> sp. SIB1	Aromatic hydrocarbons	20	Kato et al., 2001

PAHs: polycyclic aromatic hydrocarbons.

by centrifugation at 5000g for 10 min at 4 °C and washed and suspended with a small amount of BM medium. 7 mL of BM medium containing the planktonic cells at the same CFUs value of above biofilm samples and 20 ppm naphthalene were prepared in 20 mL glass bottles. The bottles were sealed and kept at 30 °C as described above. All the samples were prepared in triplicate for each sampling time with a negative control containing no cells. Sample bottles were taken every 4 h until 16 h and then every 8 h until 40 h. Remaining amounts of naphthalene in the samples were analyzed by gas chromatography (GC) and total RNA was extracted from the cells for reverse transcriptase PCR analysis as described below.

## 2.7. GC analysis

Whole culture in the bottle was directly extracted with an equal volume of solvent mixture of hexane and acetone (1:1) containing 80 ppm biphenyl as an internal standard. The mixtures were shaken vigorously for 3 min to extract naphthalene, and were centrifuged (3000g, 20 min at room temperature) to separate aqueous and solvent layers. A portion (1 µL, split ratio 50:1) of the solvent layer was analyzed by GC system HP6890 (Agilent, Palo Alto, CA) with a 340 µm × 30 m non-polar capillary HP-1 column and FID detector (GC/FID) or JEOL JMS-DX303 mass spectrometer (JEOL, Tokyo, Japan, GC/MS). Briefly, operating conditions were as follows: oven temperature was increased linearly from 80 to 300 °C at a rate of 10 °C min<sup>-1</sup>. Temperatures for injector and detector were both set at 250 °C. Flow velocity of a carrier He gas was 30 cm min<sup>-1</sup>.

## 2.8. Reverse transcriptase PCR (RT-PCR) analysis

Total RNA was extracted from T102 cells using RNeasy mini kit (Qiagen, Valencia, CA). cDNA was synthesized from 10 µg DNase-treated RNA as previously described (Takei et al., 2008). PCR amplification was performed by a thermal cycler PTC-11 (MJ Research, Waltham, MA) using a primer set for *nahAc* of T102 (forward, 5'-GTGCTAAGCAGGAACGGCTGAA-3'; reverse, 5'-CCA CGA-TAACTGGTCTCGCC-3') or a primer set for 16S rRNA gene (forward, 5'-GACGGGTGAGTAATGCTTGG-3'; reverse, 5'-TGTGCAATATCCCACTGC-3) with KOD-plus DNA polymerase (Toyobo, Osaka, Japan). The PCR condition was 3 min at 94 °C for initial denaturation, followed by 30 cycles of 15 s at 94 °C, 30 s at 58 °C and 30 s at 68 °C, and additional 5 min at 68 °C in the last cycle. The PCR products were separated on 1.5% agarose gels in 1×TBE (45 mM Tris–borate, 1 mM EDTA) and stained by ethidium bromide.

## 2.9. Comparison of fitness between biofilms and planktonic cells in petroleum contaminated soil

Petroleum contaminated soils were taken from Ishikari petroleum field (Hokkaido, Japan) and used for following experiments without sterilization. For Biofilm samples, T102 biofilms were formed in advance at 30 °C for 24 h in 2 mL screw cap polypropylene tubes containing 0.4 mL of Y medium. The liquid culture containing planktonic cells was removed from the tubes leaving biofilms inside wall. 0.5 g of the contaminated soils and the same amount of filtered cell free spent Y medium from planktonic cell culture were added to each tube. Then, two different conditions were set for biofilm samples, one was the “intact biofilms” sample with no treatment, another was “dispersed biofilms” sample that was vortexed for 2 s to detach and disperse the biofilm pieces into the soils. For planktonic cell samples, the 0.5 g of the soils in 2 mL tubes were supplemented with mid-log phase planktonic cell culture containing similar CFUs to above biofilm samples. CFUs in the

samples were adjusted by previously determined CFUs OD<sub>600</sub><sup>-1</sup> values. Soil sample with no inoculation of T102 cells were also prepared as negative control. The soil samples inoculated with intact biofilms, dispersed biofilms, planktonic cells, or no inoculation were incubated at 30 °C according to the naphthalene vapor exposure method (Park and Crowley, 2006). Naphthalene vapor was supplied by placing 0.1 g of naphthalene crystals in the middle of the Petri dishes where each sample tube was loosely capped and placed on a wet paper to avoid desiccation. Solubility of naphthalene in water is approximately 30 mg L<sup>-1</sup> at room temperature. The Petri dishes containing sample tubes and naphthalene crystals were sealed with parafilm, which is permeable to oxygen.

## 2.10. Extraction of bacterial DNA

Total DNA was prepared from liquid or biofilm culture using Insta Gene Matrix kit (Bio-Rad, Hercules, CA). DNA from the soil samples was prepared using ISOIL for beads beating soil DNA extraction kit (NIPPON GENE, Toyama, Japan).

## 2.11. PCR amplification of 16S rRNA genes

PCR amplification was performed by KOD-plus DNA polymerase (Toyobo) with a combination of following primers and template DNA. The primer sequences were 341F with GC-clump (5'-CGCCGGGGCGCGCCCCGGGGCGGGGGCAGGGGGGCTACGGGAGGCGCAG-3'), and 518R (5'-ATTACCGGCTGCTGG-3') in V3 consensus region of 16S rRNA genes (Muyzer et al., 1993). The reaction was carried out according to the following temperature program; initial denature at 94 °C for 2 min, then followed by 20 cycles of denature at 94 °C for 15 s, annealing at temperatures from 65 to 55 °C (touchdown -0.5 °C cycle<sup>-1</sup>) for 30 s and extension at 68 °C for 15 s, and then 8–15 cycles of denature at 94 °C for 15 s, annealing at 55 °C for 30 s and extension at 68 °C for 15 s, and final extension was performed at 68 °C for 2 min. PCR products were separated by normal electrophoresis on 1.5% agarose gels or denaturant gradient gel electrophoresis (DGGE).

## 2.12. DGGE analysis

DGGE was performed using Dcode system (Bio-Rad). PCR amplified 16rRNA gene fragments were separated by 8% polyacrylamide gel with a gradient of denaturant urea and formamide ranging from 20% to 50%. The concentrations of 7 M and 40% (v/v) were defined as 100% of urea and formamide, respectively. Electrophoresis was performed in 0.5 × TAE buffer for 4 h under a condition that the voltage and temperature were maintained at 200 V and 60 °C, respectively. DNA bands were observed and quantified by FluorImager 595 (Molecular Dynamics, Sunnyvale, CA) after SYBR Gold staining (Molecular Probes, Eugene, OR). Density of the DNA band corresponding to *P. stutzeri* T102 16S rRNA gene was estimated by an image software (Image quant 5.0, GE Healthcare, Waukesha, WI) using DNA size marker as a standard (100-bp DNA ladder, Toyobo).

## 2.13. Naphthalene degradation test of soils containing *P. stutzeri* T102

The entire soil sample, 0.5 g, in the 1.5 mL polypropylene tube was transferred into a 50 mL screw capped glass bottle containing 20 mL of BM-medium and 100 ppm naphthalene in methanol solution. Because the petroleum contaminated soils contain significant amounts of various hydrocarbons, addition of 100 ppm naphthalene was necessary to obtain enough signal/noise ratios in GC/FID analysis. Bottles were tightly closed and incubated at 30 °C for 36 h. Extraction of remaining naphthalene and GC/FID analysis were performed similarly as described above.

### 3. Results and discussion

#### 3.1. Biofilm forming activities of bacteria

We first compared the biofilm forming ability of seven hydrocarbon degrading bacterial strains from our laboratory collection. Among the tested bacterial strains, *P. stutzeri* T102, which was isolated from sludge of an oil reservoir tank in Okinawa, formed the most biofilms on the surface of various materials including polypropylene, polystyrene, polyethylene terephthalate, acrylic, and glass bottles. When using a batch culture system for biofilm formation, the amount of T102 biofilms continued increasing even after 120 h (Fig. 1a). On the other hand, six other bacteria formed only a small amount or no biofilms under our conditions. Thus, we chose T102 as a model bacterial strain for further experiments. Gross et al. (2007) tested 69 bacterial strains for biofilm forming capacity (BFC) and showed that most of the strains with BFC greater than 2 belonged to *Pseudomonas*. Our experimental result is consistent with their result.

*P. stutzeri* T102 is capable of degrading PAHs, such as naphthalene and dibenzothiophene (Hirano et al., 2004). *P. stutzeri* are ubiquitous and useful environmental bacteria with high degradation abilities for harmful chemical compounds including biocides, halogenated alcohols, and hydrocarbons (Lalucat et al., 2006). Since T102 is a facultative aerobic strain, it formed ring-shape biofilms near the air–liquid interface on the inner surface of glass bottles (Fig. 1b). However, they formed rather uniform biofilms in small plastic tubes, such as 1.5 mL polypropylene tubes and 96 well polystyrene plates probably due to small depth of the liquid phase with better aeration condition and more or less hydrophobicity and zeta potential of the tube materials (Fig. 1c). Scanning electron microscopy demonstrated that the biofilms of T102 are not flat but highly structured (Fig. 1d and e).

#### 3.2. CFUs of biofilms and planktonic cells cultures

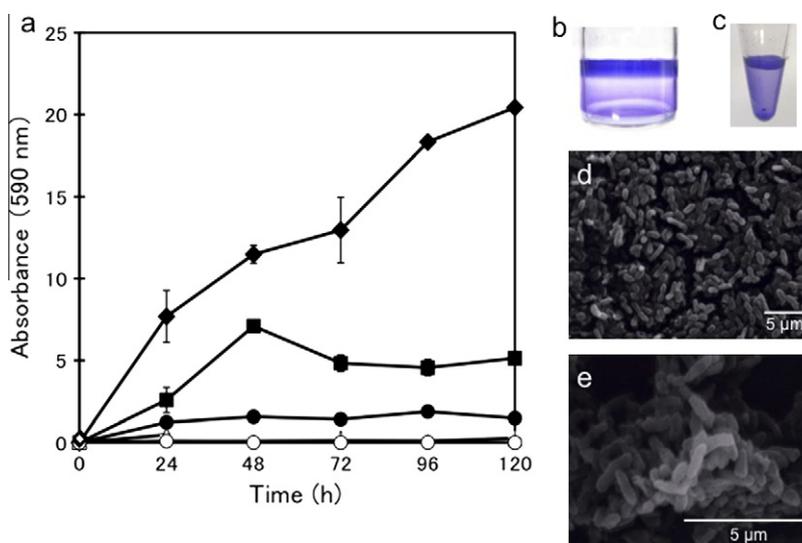
In order to compare metabolic activities of T102 biofilms and planktonic cells, we next asked if the biofilms and planktonic cultures contain different ratios of viable and non-viable cells. We

determined the relationship between turbidity and the viable cell counts of each culture. CFUs were considered as viable cell counts in this experiment. It was found that CFUs  $OD_{600}^{-1} mL^{-1}$  value of T102 biofilms in 20 mL glass bottles was higher,  $9.9 \times 10^7$  ( $SD = 2.7 \times 10^7$ ) than that in 1.5 mL polypropylene tubes,  $3.6 \times 10^7$  ( $SD = 1.2 \times 10^7$ ). This slightly higher viability of biofilms in the glass bottles may be a consequence of robust biofilm formation near the air–liquid interface and/or better nutrient condition than in the small polypropylene tubes. On the other hand, CFUs  $OD_{600}^{-1} mL^{-1}$  value of the mid-exponential phase T102 planktonic cell culture was  $5.8 \times 10^7$  ( $SD = 8.4 \times 10^5$ ). We adjusted the inoculum size based on these values for further experiments.

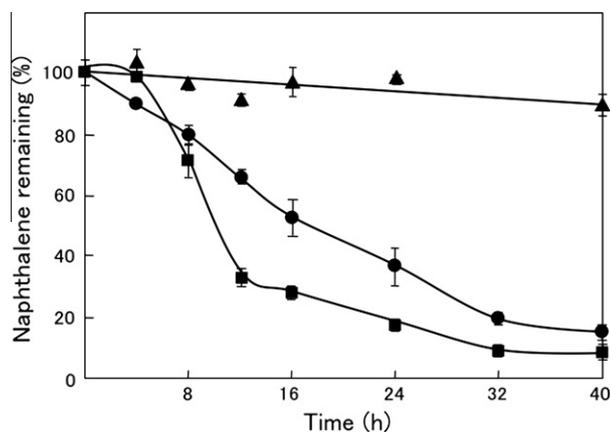
#### 3.3. Naphthalene degradation by T102 biofilms and planktonic cells

There are reports that biofilm-associated cells are more dormant and inactive than planktonic cells (Werner et al., 2004; Lewis, 2007; Rani et al., 2007). This feature of biofilms partly explains their high resistance to environmental stresses (Gilbert et al., 1990). It was thus a concern that T102 biofilms might exhibit less naphthalene degradation activity than planktonic cells. In order to examine this possibility, the activities of T102 biofilm and planktonic cells were compared in a pure liquid culture system (Fig. 2). Experimental results revealed that T102 biofilms did not degrade naphthalene for initial 4 h but after that time they degraded naphthalene faster than planktonic cells. This interesting observation was reproducible in several independent experiments. T102 biofilms degraded 14 ppm, 70% of initial naphthalene (20 ppm), in 12 h and maximum degradation rate was  $1.7 \text{ ppm h}^{-1}$  between 4 and 12 h. On the other hand, T102 planktonic cells started to degrade naphthalene immediately after incubation. The degradation rate was almost constant, about  $0.5 \text{ ppm h}^{-1}$  for 16 h, and it took 28 h for the degradation of 14 ppm of naphthalene. It should be noted that about 1.6 ppm (8%) of the initial naphthalene was absorbed to the butyl rubber septum of the bottle cap and remained un-degradable. This means that naphthalene was almost completely eliminated from the culture of T102 biofilms after 32 h.

We hypothesized that initial delay for degradation by biofilms may be the time that it takes the naphthalene to penetrate through



**Fig. 1.** (a) Biofilm formation by hydrocarbon degrading bacteria. Biofilms were formed at the indicated temperatures in 1.5 mL polypropylene tubes containing 0.5 mL Y medium. Data points are the averages of triplicate assays and error bars represent standard deviations. Symbols are as follows: closed diamond, *P. stutzeri* T102 (30 °C); closed square, *Gordonia* sp. C3 (30 °C); closed circle, *Oleomonas sagaranensis* HD1 (30 °C); open triangle, *Rhodococcus* sp. TMP2 (20 °C); open diamond, *Rhodococcus* sp. T12 (20 °C); cross, *Shewanella* sp. SIB1 (20 °C); open circle, *Arthrobacter* sp. CAB1 (30 °C). The latter four strains did not produce biofilms in the culture systems. (b) Crystal violet staining of *P. stutzeri* T102 biofilms in a 20 mL glass bottle; (c) in a 1.5 mL polypropylene test tube. (d and e) Scanning electron micrographs. All biofilms were formed at 30 °C for 48 h. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 2.** Comparison of naphthalene degradation activity of T102 biofilm and planktonic cell cultures. Data points are the average of triplicate assays; error bars represent standard deviations. Initial CFUs for biofilm and planktonic cell cultures were 21,470,000 and 23,360,000, respectively. Symbols are as follows: triangle, no cells; square, T102 biofilm cell culture; circle, T102 planktonic cell culture.

the extracellular matrix of the biofilm so that the genes responsible for degradation of naphthalene might be induced.

#### 3.4. Expression levels of *nahA* in T102 biofilms and planktonic cells

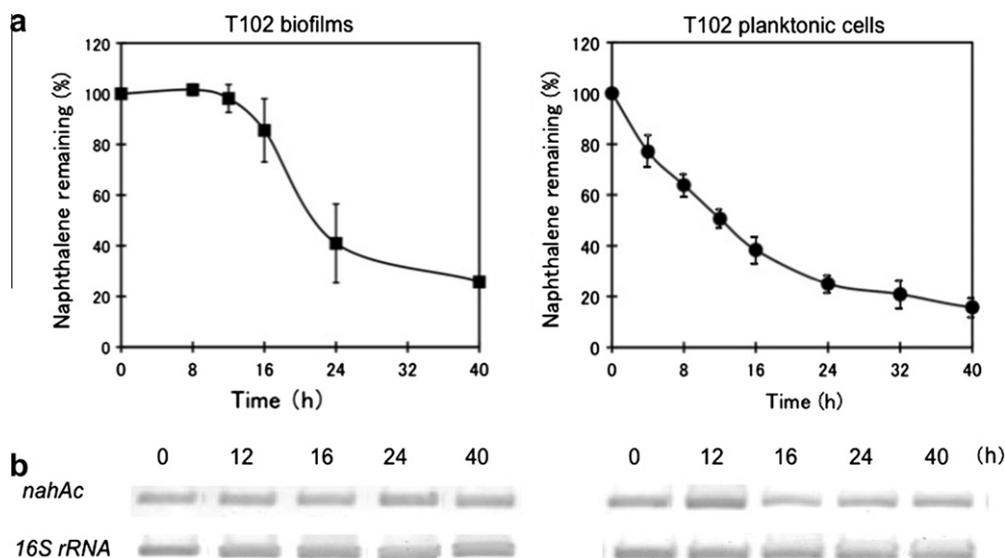
It is known that *nahAa*, *nahAb*, *nahAc*, and *nahAd* form an operon and encode a ferredoxin, ferredoxin oxidoreductase, and naphthalene dioxygenase large and small subunits, respectively (Ensley et al., 1982; Morikawa, 2010). All these genes are essential for aerobic degradation of naphthalene and related compounds. Thus we analyzed the expression level of *nahAc*, which encodes the large subunit of naphthalene dioxygenase, in T102 biofilms and planktonic cells (Fig. 3). Contrary to our hypothesis, *nahAc* in T102 biofilms expressed *nahAc* at constant levels from the start of incubation (time 0) through 40 h. Constant gene expression was also confirmed for *nahAa*, *nahAb*, and *nahAd* (results not shown). These results suggest that the initial lag and subsequent high naphthalene degradation periods exhibited by T102 biofilms are not due to changing gene expression levels of *nahAabcd*. The *nah-*

*Aabcd* operon in *Pseudomonas putida* has been reported to be inducible under regulation of *nahR* (Park et al., 2002). In this experiment expression of *nahA* genes was observed at 0 h, before naphthalene was added to the medium. This result suggests that *nahR* does not exist or does not function in *P. stutzeri* T102, which has been isolated from bottom sludge of a petroleum reservoir tank where naphthalene is always abundant.

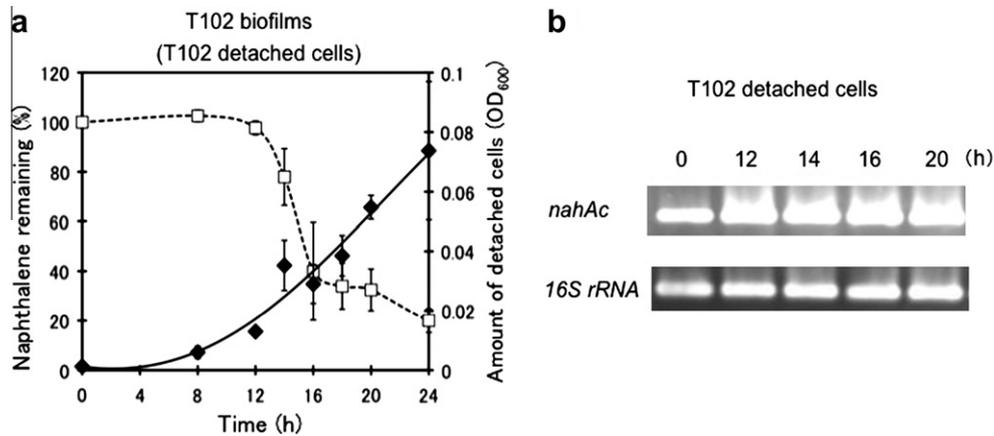
When we carefully observed the culture, we noticed that a part of T102 detached and grew planktonically in the culture bottle containing T102 biofilms. Thus, we carefully separated these detached cells from biofilms, and examined the expression level of *nahAc*. It was found that the expression level of *nahAc* in the detached cells was clearly and significantly higher than that in biofilms from 12 to 20 h (Fig. 4). This result allowed us to conclude that degradation of naphthalene was largely due to the activity of detached cells rather than biofilms. The naphthalene degradation activities of T102 biofilms, detached, and planktonic cells, were determined 0.02, 1.1, and 0.3 pg CFUs<sup>-1</sup> h<sup>-1</sup>, respectively. The degradation activity of the biofilms was much lower than planktonic cells probably due to depletion of oxygen in biofilms where cells were densely packed and poor naphthalene penetration through matrix that encase biofilm-associated cells (Fig. 1c and d).

#### 3.5. Fitness of T102 biofilms and planktonic cells in petroleum contaminated soils

The observation that T102 biofilms were capable of producing highly active detached cells prompted us to test their performance in the natural petroleum contaminated soils, Ishikari, Hokkaido, Japan. In order to estimate the fitness of *P. stutzeri* T102 in the soils, we used comparative DGGE analyses method of the PCR amplified 16S rRNA genes. Although this method is semi-quantitative due to different efficiency of DNA extraction and PCR depending on the bacterial strains, it is useful to analyze population changes of single strains over the time. Moreover, distribution of the DNA bands in DGGE gels provided a rough overview of the bacterial community dynamics upon introduction of T102 biofilm and planktonic cells to the soils. We observed that the “dispersed biofilms” sample maintained a rather dense T102 DNA bands for 10 wk and kept over 40% of its original density level for 8 wk (Fig. 5). The density of T102



**Fig. 3.** (a) Naphthalene degradation activity of T102 biofilm (left) and planktonic (right) cell cultures, respectively. Initial CFUs were 18,850,000 (biofilm culture) and 22,120,000 (planktonic cell culture). Data points are the average of triplicate assays; error bars represent standard deviations. (b) RT-PCR analyses of *nahAc* and 16S rRNA gene expression in the T102 biofilms (left) and planktonic cells (right), respectively.



**Fig. 4.** (a) Naphthalene degradation activity (open square) and the amount of detached cells (closed diamonds) in a T102 biofilms flask. Data points are the average of triplicate assays and error bars represent standard deviations. (b) RT-PCR analyses of *nahAc* and 16S rRNA gene expression in the detached cells. Only the cells sample at 0 h was taken from biofilms.

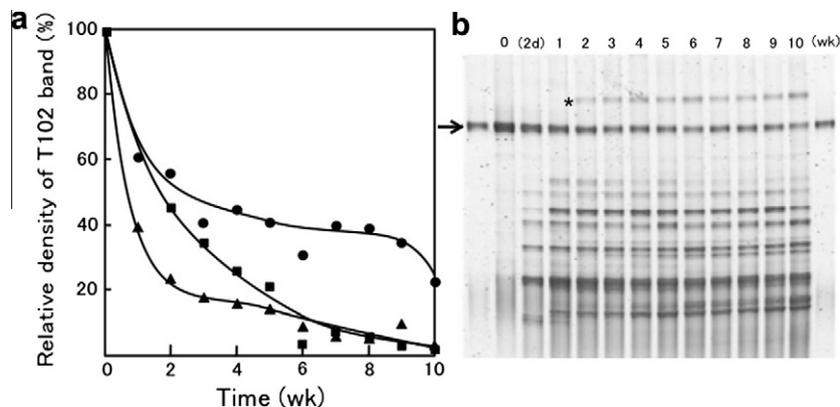
DNA bands of planktonic cells and intact biofilms samples decreased more rapidly than that of dispersed biofilms, and they almost disappeared (ca 2% remained) after 10 wk. The decrease in the amount of T102 DNA bands was more significant within the first 3 wk than later time for all the experimental conditions. The amount of T102 DNA in intact biofilms sample was rather continuously decreased over the period. This may be because the nutrients and oxygen were more rapidly depleted around the sessile intact biofilms than planktonic cells and dispersed biofilms. Duration time of each sample keeping over 20% of the initial density of T102 DNA was 10, 5, and 2 wk for dispersed biofilms, intact biofilms, and planktonic cells inoculates, respectively. These results indicate that dispersed biofilms are more tolerant and stable than intact biofilms and planktonic cells in the petroleum contaminated soils. An increasing density band, indicated by asterisk, in Fig. 5b may be of an indigenous naphthalene degrader since it also appeared in the sample without T102 inoculation (data not shown).

The above experimental results are consistent with previous knowledge that biofilm-associated cells are generally more tolerant to environmental stresses than their planktonic counterparts. The environmental robustness of biofilm-associated cells could be attributed to both specific gene expression and physicochemical toughness given by densely packed cells condition encased in extracellular matrices (Chang et al., 2007; Bossé et al., 2010; Halan et al., 2011). These natural settings of biofilms benefit the member cells by providing favorable niches (Singh et al., 2006). Next, we

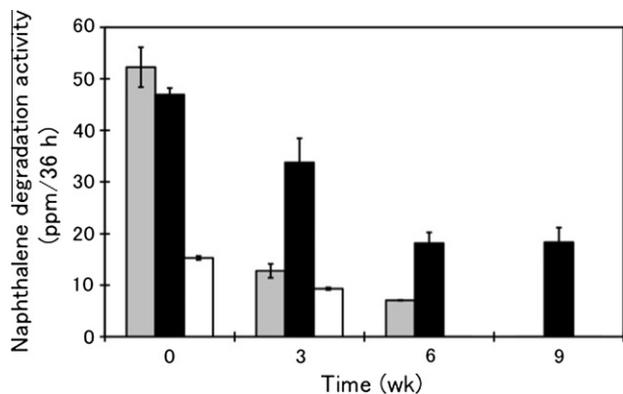
further examined naphthalene degradation activities of the contaminated soils containing either T102 biofilm or planktonic cells.

### 3.6. Naphthalene degradation activity of soils containing T102 biofilms and planktonic cells

As shown above, the liquid culture system inoculated with T102 biofilms exhibited naphthalene degradation activity comparable to or even higher than with T102 planktonic cells (Fig. 2). PCR-DGGE analysis suggested that the population of T102 kept higher levels in the dispersed biofilms than the planktonic cells during incubation for 10 wk in contaminated soils. Thus, we expected that the soil sample containing dispersed biofilms should exhibit higher degradation activity than planktonic cells over the period. But it was difficult to measure naphthalene degradation directly in the contaminated soil samples because the soils contained large amounts of various hydrocarbons and signal/noise ratio in GC analysis was quite low. We decided to measure naphthalene degradation activity of each soil sample in BM-medium containing additional 100 ppm naphthalene (Fig. 6). We found that dispersed biofilms and planktonic cells initially degraded 48 and 52 ppm naphthalene in 36 h, respectively. Their degradation activities gradually decreased as the incubation time increased. When we compared their activities after incubation for 9 wk, dispersed biofilms still degraded 19 ppm naphthalene while planktonic cells and no inoculation samples degraded only <0.1 ppm. Petroleum contaminated soils that were taken from



**Fig. 5.** (a) Comparison of the fitness of T102 cells inoculated differently in contaminated soils. Relative density of 16S rRNA-PCR products band of T102 in each DGGE gel is plotted in the graph. Symbols are as follows: circle, dispersed T102 biofilms; square, intact T102 biofilms; triangle, planktonic cells. (b) DGGE gel data for the dispersed biofilms experiment from 0 to 10 wk. The arrow indicates position for the 16S rRNA-PCR band of T102.



**Fig. 6.** Naphthalene degradation activity of soils with T102 planktonic cells (shaded bar), dispersed T102 biofilms (closed bar), and no T102 cells (open bar). Data points are the average of triplicate assays and error bars represent standard deviations.

Ishikari oil field contained various hydrocarbon compounds such as straight-, branched- and cyclo-alkanes, and PAHs including naphthalene. Thus, it was not surprising that the soils contained significant amount of naphthalene degrading bacteria. Degradation activity of naphthalene with no inoculation could be attributed to these indigenous bacteria. Densitometry of the T102 bands of dispersed biofilms and planktonic cells in DGGE gel showed that 35% and 9% of the initial band intensities remained after 9 wk, respectively. These results suggest that the specific naphthalene degradation activity of dispersed biofilms including detached cells was much higher than planktonic cells in the petroleum contaminated soils after 9 wk.

#### 4. Conclusions

We found that naphthalene degrading *P. stutzeri* T102 formed thick biofilms under laboratory conditions. Naphthalene degradation rate of T102 biofilms was initially low but later became higher than that of T102 planktonic cells. This unique feature of biofilm cultures has never been reported in the literature. Rapid degradation activity of biofilm cultures could be attributed to producing detached cells. We suggest that biofilms are a bacterial cell carrier breeding and releasing super-activated detached cells. Moreover, T102 cells were shown to be more durable and active in the petroleum contaminated soils when they are introduced as biofilms rather than as planktonic cell suspensions.

#### Acknowledgments

The authors appreciate Prof. Roberto Kolter at Harvard Medical School for his helpful comments to the manuscript. This work was supported by the New Energy and Industrial Technology Development Organization (NEDO), the Institute for Fermentation, Osaka (IFO), and KAKENHI (19380189) to M.M.

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