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Isolation and Characterization of Nonylphenol-degrading Bacteria

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Abstract

To isolate a nonylphenol (NP)-degrading bacterium, we isolated a single colony from the NP-degrading microbial consortium SW-3, which was previously isolated from an aqueous environment. Ten colonies that exhibited different cell morphologies were isolated and the strains were named SW-3-A, -B, -C, -D, -E, -F1, -F2, -G, -H, and -I. The ability of isolates to degrade NP was evaluated by kinetic analysis by the constant of NP degradation rate (k_1) and the half-life time of NP degradation ($t_{1/2}$). SW-3-F1, -F2, -G, and -I strains were superior at degrading NP. The k_1 and $t_{1/2}$ values of the four strains were sixfold higher and one-sixth lower, respectively, than those of the consortium strain. Additionally, SW-3-F1, -G, and -I strains were tested for their ability to degrade NP during coculture. NP degradation by coculture with a combination of all three strains was inferior to that of culture conducted with single isolates, suggesting that the three strains are antagonistic toward each other during NP degradation.

Key words: Biodegradation, Endocrine disruptor, Microbial degradation, Nonylphenol

Introduction

Nonylphenol (NP) is a ubiquitous pollutant, resulting from the biodegradation of widely used NP polyethoxylate surfactants (Corvini et al., 2004). Polyethoxylate is degraded slowly during aerobic and anaerobic degradation to NP in sewage disposal plants and other aqueous environments (Giger et al., 1984; Ahel et al., 1994; Fries and Puttmann, 2003). NP is an endocrine disruptor due to its weak ability to mimic estrogen, disrupting the natural balance of hormones in affected organisms (Gronen et al., 1999). NP is discharged into streams or coastal waters by industrial wastewater or the sewage disposal process. Its role as an endocrine disruptor has been extensively studied in aquatic organisms (Yadetie and Male, 2002; Karels et al., 2003; Hernandez-Raquet et al., 2007). However, few information is available on the distribution of NP worldwide. In Korea, NP was detected in the range of 113 to 3,890 ng per gram dry weight at Masan Bay, Gyeongnam (Khim et al., 1999), and 6.0 to 119.1 μg per kg from the sediments collected from 11 different rivers (Cho et al., 2004), and 3.6 μg per L in Sihwaho Bay, Gyeongido (Li et al., 2004). These reports revealed that NP is extensively distributed in aquatic environments. Since trace levels of NP in the aquatic environment can act as an endocrine disruptor, technologies that degrade environmental NP are greatly needed.

A biological decomposition method by microorganisms, called bioremediation, has been considered an environmentally favorable method to restore environments contaminated with harmful non-resolvable chemicals (Kim et al., 2004; Lee et al., 2009; Song et al., 2011). Bioremediation decomposes organic toxic substances into water and carbon dioxide (Ripp et al., 2000; Kang and Kim, 2007; Kim et al., 2007; Lee et al., 2009). Several reports have indicated successful bioremediation of NP (Tanghe et al., 1999; Fujii et al., 2000, 2001; Corvi-

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ni et al., 2004; Junghanns et al., 2005; Shi and Bending, 2007). However, most studies have been performed on soil environments. No reports have been published regarding NP bioremediation in aquatic environments. Therefore, this study isolated NP-degrading bacteria from the microbial consortium SW-3, which was previously isolated from an aqueous environment (Song et al., 2011). We also performed a kinetic analysis of NP degradation by individual and groups of bacterial strains.

Materials and Methods

Chemicals

NP (assay >85%) was purchased from Fluka (St. Louis, MO, USA). Other reagents used in the analysis were analytical grade and purchased from a commercial source.

Medium for isolation of NP-degrading bacteria

Yeast nitrogen base (YNB) without amino acids (Difco, Franklin Lakes, NJ, USA) medium was used as a basal medium for the isolation of NP-degrading bacteria as previously described (Fujii et al., 2000, 2001; Corvini et al., 2004). YNB medium containing 100 ppm NP was used for enrichment cultures and YNB agar plates containing 100 ppm NP were used for isolating NP-degrading bacteria.

Isolation and culture of NP-degrading bacteria

To isolate NP-degrading bacteria from the NP-degrading microbial consortium SW-3 (Song et al., 2011), the consortium strain was cultivated in YNB medium containing 100 ppm NP at 25°C under aerobic conditions. 100 uL of the culture was taken at intervals and spread on YNB agar plates containing 100 ppm NP. The agar plate was incubated at 25°C for 7 days. Single colonies grown on the plate were collected for further studies The growth of bacteria in the medium containing 100 ppm NP was measured by a change of optical density at 600 nm.

Identification of NP-degrading bacteria

Bacterial strains isolated from the NP-degrading microbial consortium were identified by their morphological, biochemical, and genetic characteristics. A light microscope (Motic 300; Motic, Richmond, BC, Canada) and a scanning electron microscope (SEM; model S-2400; Hitachi Ltd., Tokyo, Japan) were used for the morphological analysis. VITEK Gram Negative Identification cards (GNI-) or VITEK Gram Positive Identification cards (GNI+) (Biomerieux Inc., St. Louis, MO, USA) were used for physicochemical analysis. Identification of strains was determined through homology analysis of 16S rDNA sequences. Two oligonucleotides (27F: 5′-GTTTGGATCCTGGCTCAG-3′ and 1492R: 5′-AAGGAGGGGATCCAGCC-3′) were used for polymerase chain reaction (PCR) to amplify 16S rDNA (Dunbar et al., 2000). PCR was conducted as follows: 2 μL of 20 pmole each primer, 25 ng DNA template, 0.5 μL Taq polymerase (2.5 U), 5 μL of 10× Taq polymerase buffer, 1 μL of 10 mM dNTP, and 39 μL of dH_2O was denatured for 2 min at 94°C. After denaturation, reactions cycled 25 times at 94°C for 1 min, 52°C for 1 min, and 72°C for 2 min, followed by incubation at 72°C for 5 min. The amplified PCR products were sequenced by SolGent (Daejeon, Korea). Homology searches of sequences were conducted using a ribosomal database (http://www.ncbi.nim.nih.gov/BLAST/).

NP extraction and HPLC analysis

The ability of a bacterial strain to degrade NP was determined by analysis of NP content remaining in medium by high-performance liquid chromatography (HPLC). After inoculation of 1% pre-culture, cells were aerobically cultivated at 25°C. One milliliter of culture was reserved for analysis. Then, 4 mL of deionized water and 15 mL of acetonitrile were added. The solution was mixed for 3 min by a vortex mixer to extract the remaining NP. After extraction, the upper layer was carefully reserved, filtered through a 0.2-μm filter (DISMIC-25AS; Advantec, Tokyo, Japan), and then analyzed by HPLC (Flexar HPLC System; PerkinElmer, Waltham, MA, USA) equipped with a C_{18} reverse-phase column (250 mm × 4.6 mm, I.D. 5 μm; Shiseido Co., Tokyo, Japan). For detection of NP, samples were eluted with 75% acetonitrile in 25% water at a flow rate of 1 mL per minute. Eluates were monitored at 277 nm. Remaining NP was indicated as a percentage value of the total reduced NP peak.

Kinetic analysis of NP degradation

Kinetic analysis of NP degradation under various conditions was estimated according to a first-order model described by the following formula:

$$k_1 = -\{(\ln S/S_0)/t\},$$
$$t_{1/2} = \ln 2/k_1,$$

where S_0 is initial the NP concentration, S is the residual NP concentration at sampling time t, k_1 is the NP degradation rate constant (1/day), and $t_{1/2}$ is the half-life of NP degradation (days). A significant difference was tested by ANOVA (Chang et al., 2007).

Results and Discussion

Isolation of NP-degrading bacteria from the microbial consortium SW-3

To obtain bacterial strains that utilized NP as a carbon source, the NP-degrading microbial consortium SW-3 strain was cultivated and then spread on an agar plate as described in the Materials and Methods. Colonies grown on YNB agar plates containing 100 ppm NP were reserved for further study. Morphology of each colony was confirmed by Gram staining under a light microscope. Ten colonies that exhibited different cell morphologies were obtained by single colony isolation. Strains were named SW-3-A, -B, -C, -D, -E, -F1, -F2, -G, -H, and -I. All strains isolated from the microbial consortium were Gram (-) bacteria except SW-3-F2 (data not shown).

Kinetic analysis of NP degradation by NP-degrading bacteria

Growth of the isolated bacteria was observed in YNB medium containing 100 ppm NP, suggesting that the isolates are capable of using NP as a carbon source (data not shown). As bacterial growth progressed, NP concentrations decreased. For each isolate tested, NP was not detected after 40 days (data not shown). A previous report showed that the *Sphingomonas xenophaga* Bayram strain was capable of degrading over 90% of NP after 2 weeks of incubation (Gabriel et al., 2005) and that the *Sphingomonas* sp. TTNP3 strain degraded over 80% of NP within 2 weeks (Corvini et al., 2004). Additionally, a microbial consortium isolated from an aqueous environment exhibited NP-degrading activity that metabolized 70% of NP after 45 days incubation (Fujii et al., 2000). Due to the complete remediation of NP by the isolated bacterial strains of the present study, we hypothesized that these strains would represent an environmentally favorable technology to restore environments contaminated with NP.

To evaluate NP-degrading activities between the isolates in more detail, a kinetic analysis was performed as described in the Materials and Methods. Based on the analysis of k_1 and $t_{1/2}$ values, all strains except SW-3-H exhibited higher NP-degrading activity as compared to the consortium strain (Table 1). SW-3-F1, -F2 -G, and -I strains exhibited superior NP-degrading ability compared to other isolates and the consortium strain. The k_1 values of SW-3-F1, -F2, -G, and -I strains ranged from 0.340 to 0.456 and were sixfold higher than that of the consortium strain. The $t_{1/2}$ values by the four strains dramatically decreased in the range of 1.5 to 1.7 days. The values were one-sixth that of the consortium strain (Table 1). A previous kinetic analysis of NP degradation in soil reported a k_1 value of 0.054 and a $t_{1/2}$ value of 12.8 at pH 7.0 and 20°C (Chang et al., 2007). These results suggest that isolates SW-3-F1, -F2, -G, and -I will facilitate the production of a starter strain for the biodegradation of NP.

Table 1. Kinetic analysis of nonylphenol degradation by bacteria isolated from nonylphenol-degrading consortium SW-3

Strain	k_1 (1/day)	$t_{1/2}$ (day)	r^2 (correlation coefficient)
Control	0.011	61.8	0.99
SW-3	0.074	9.3	0.99
SW-3-A	0.092	7.5	0.99
SW-3-B	0.102	6.8	0.98
SW-3-C	0.100	6.9	0.99
SW-3-D	0.103	6.7	0.99
SW-3-E	0.159	4.4	0.99
SW-3-F1	0.340	1.7	0.99
SW-3-F2	0.456	1.5	0.99
SW-3-G	0.399	1.7	0.99
SW-3-H	0.054	12.8	0.99
SW-3-I	0.399	1.7	0.99

Nonylphenol (NP) was extracted from yeast nitrogen base medium containing 100 ppm NP as described in Materials and Methods. Control means non-inoculated samples.

Identification of NP-degrading bacteria isolated from the microbial consortium SW-3 strain

Four strains (SW-3-F1, -F2, -G, and -I) that exhibited superior NP-degrading activities were selected for further study. To investigate the cell surface structure of each isolate, morphological features of SW-3-F1, -F2, -G, and -I were identified by SEM analysis. As shown in Fig. 1, SW-3-F1, -G, and -I were bacilli and SW-3-F3 was a coccus. SW-3-F1, -G, and -I were identified as Gram (-) bacilli and SW-3-F2 was a Gram (+) coccus. The biological characteristics of NP-degrading bacteria are listed in Table 2. The SW-3-F1 and -G strains exhibited similar biological characteristics, suggesting that these strains were related (Table 2). The others showed different biological characteristics.

The analysis of biological characteristics suggested that SW-3-F1 and SW-3-G were related. However, these biological characteristics provided limited information regarding the identification of bacteria. Therefore, we performed a genetic analysis using bacterial 16S rDNA. PCR products of 16S rDNA about 1.3 to 1.5 kb were obtained (data not shown). 16S rDNA sequences were matched against 16S rDNA sequences in GenBank using BLAST. SW-3-F1, -F2, -G, and -I exhibited 99% identity with *Ochrobactrum* sp., *Staphylococcus* sp., *Achromobacter* sp., and *Alcaligenes* sp., respectively (Table 3). *Ochrobactrum* sp. is a dominant NP-degrading bacterium in soil (Chang et al., 2007). Community analysis of a NP-degrading bacterial consortium obtained from a textile wastewater pretreatment plant revealed *Achromobacter* sp. (Di Gioia et al., 2008). This is the first study showing that *Staphylococcus* sp. can degrade NP. However, *Sphingomonas* has been reported to bioremediate 4-NP, but was not isolated in this study (Thanghe et al., 1999; Fujii et al., 2001; Corvini et al., 2004; Gabriel et al., 2005).

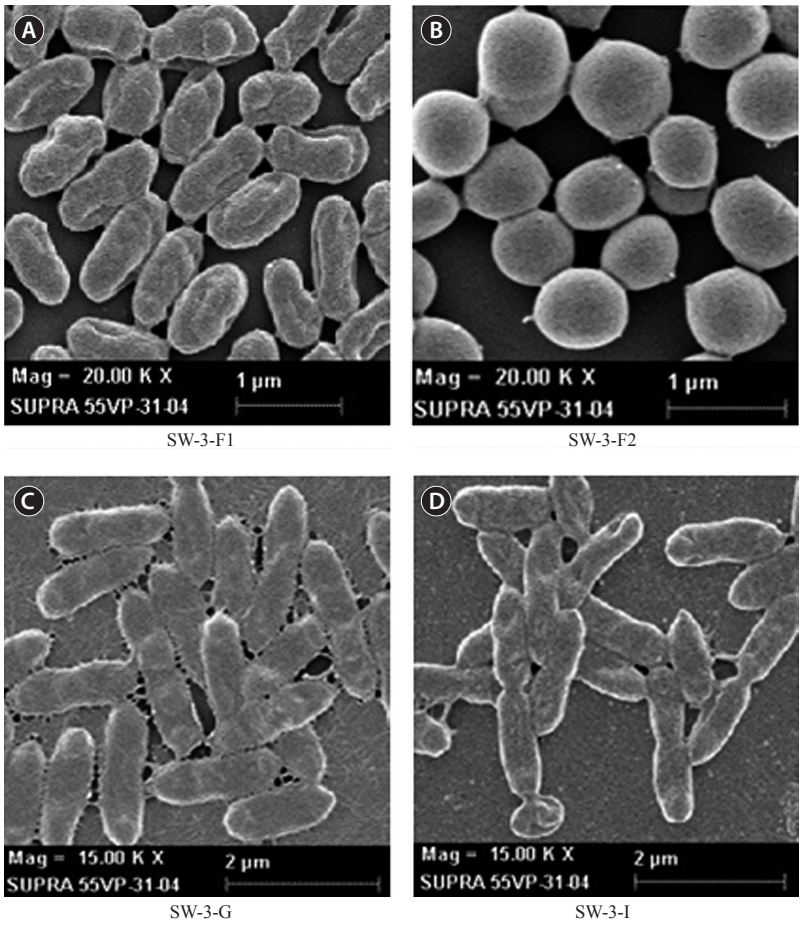

Fig. 1. Scanning electron micrograph of nonylphenol-degrading bacteria isolated from the microbial consortium SW-3. Scale bars represents: A, B = 1 μm; C, D = 2 μm.

NP degradation by coculture of isolates

Kinetic analysis of NP-degrading bacteria revealed that four strains (SW-3-F1, -F2, -G, and -I) were capable of efficiently degrading NP. However, SW-3-F2 exhibited high homology with *Staphylococcus saprophyticus*, a pathogen often implicated in urinary tract infections (Kuroda et al., 2005). Therefore, the SW-3-F1, -G, and -I strains were chosen to investigate the coculture effects on NP degradation. All three strains were mixed in the following variations: SW-3-F1/-G, SW-3-F1/-I, SW-3-G/-I, and SW-3-F1/-G/-I. As bacterial growth progressed, the concentration of NP gradually decreased over 40 days of cultivation. In the case of SW-3-G/-I, SW-3-F1/-G, and SW-3-F1/-G/-I, NP was degraded over 85% within 15 days of incubation (data not shown). By coculture of SW-3-F1/-I, over 85% NP was degraded within 20 days (data not shown). However, NP was still detected in all cocultures after 40 days of incubation. As shown in the above results, the NP-degrading pattern by the coculture of isolates was different according which isolates were mixed together. To evaluate NP-degrading activities between cocultures, a kinetic analysis was conducted as described in the Materials and Methods. The k_1 values by cocultures of isolates (SW-3-G/-I, SW-3-F1/-G, SW-3-F1/-I, and SW-3-F1/-G/-I) ranged from 0.081 to 0.092. This was 1.3-fold higher than the consortium SW-3 strain. The $t_{1/2}$ values steadily decreased to between 7.5 and 8.6 days lower than those of the consortium strain (Table 4). Thus, the efficacy of NP degradation by coculture performed with a combination of three strains was inferior to that of culture with a single isolate. This result suggested that the three strains (SW-3-F1, -G and -I) antagonize NP degradation during coculture (Tables 3 and 4).

NP was not detected in a three-membered (BCaL1, BCaL2, and VA 160 strains) coculture experiment after 25 days of culture (Di Gioia et al., 2004). Indeed, NP degradation was enhanced in BCaL1/BCaL2 cultures by coculturing them with the non-degrading *Bacillus* VA 160 strain. We hypothesize that NP-degrading bacteria isolated in the current study are not suitable to degrade NP under coculture conditions.

Table 2. Biochemical characteristics of SW-3-F1,-F2, -G and I strains isolated from the nonylphenol-degrading microbial consortium SW-3

Mnemonic	Biochemical test	Strain			
		SW-3-F1	SW-3-F2	SW-3-G	SW-3-I
APPA	α-Phe-proarylamidase	-	-	-	-
ADO	Adonitol	+	-	+	+
PyrA	L-Pyrrolydonyl-arylamidase	+	(-)	-	-
IARL	L-Arabitol	+	-	+	+
dCEL	D-Cellobiose	+	-	+	+
BGAL	β-Galactopyranosidase	-	+	-	-
H_2S	H_2Sproduction	-	-	-	-
BNAG	β-N-Acetylglucosaminidase	-	-	-	-
AGLTp	Glutamyl-arylamidasepNA	-	-	-	-
dGLU	D-glucose	+	+	+	+
GGT	γ-Glutamyltransferase	-	-	-	-
OFF	Fermentativeglucose	-	-	-	-
BGLU	β-Glucosidase	-	-	-	-
dMAL	D-Maltose	+	+	+	+
dMAN	D-Mannitol	+	+	+	+
dMNE	D-Mannose	+	-	-	-
BXYL	β-Xylosidase	-	-	-	-
BAlap	β-Alaninearylamidase	-	-	-	-
ProA	L-ProlinearylamidasepNA	+	-	-	-
LIP	Lipase	-	-	-	+
PLE	Palatinose	+	-	+	+
TyrA	Tyrosinearylamidase	-	-	-	-
URE	Urease	-	+	-	-
dSOR	D-Sorbitol	+	-	+	+
SAC	Sucrose	+	+	+	+
dTAG	D-Tagatose	+	-	+	+
dTRE	D-Trehalose	+	+	+	+
CIT	Citrate(sodium)	-	-	-	-
MNT	Malonate	-	-	-	-
5KG	5-Keto-D-gluconate	-	-	-	-
ILATk	L-Lactatealkalinisation	-	+	-	-
AGLU	α-Glucosidase	-	-	-	-
SUCT	Succinatealkalinisation	-	-	-	-
NAGA	β-N-acetyl-galactosaminidase	-	-	-	-
AGAL	α-Galactosidase	-	-	-	-
PHOS	Phosphatase	-	-	-	-
GlyA	Glycine-arylamidase	+	-	-	-
ODC	Omithinedecarboxylase	-	-	-	-
LDC	Lysinedecarboxylase	-	-	-	-
IHISa	L-Histidineassimilation	-	-	-	-
CMT	Courmarate	-	+	-	-
BGUR	β-Glucuronidase	-	-	-	-
O129R	O/129Resistance	-	-	-	-
GGAA	Glu-gly-arg-arylamidase	-	-	-	-
IMLTa	L-Malateassimilation	-	-	-	-
ELLM	Ellman	+	-	-	-
ILATa	L-Lactateassimilation	-	-	-	-

Table 3. Identification of SW-3-F1, SW-3-F1,-F2, -G and -I strains based on the homology search of 16S rDNA sequence

Strain	Reference (accession no.)	Identity (%)
SW-3-F1	*Ochrobactrum anthropi* CCUG 1821(AM114404)	99.0
	Ochrobactrum sp. CA01 (HQ670703)	99.0
	Ochrobactrum sp. lsd01 (GQ180164)	99.0
	Ochrobactrum tritici SCII24 (AM114402)	99.0
SW-3-F2	*Staphylococcus* sp. BQN4T-04 (FJ380997)	99.0
	Staphylococcus sp. 2qH-9 (EU489561)	99.0
	Staphylococcus saprophyticus subsp. saprophyticus BQN1T-01d (FJ380970)	99.0
	Staphylococcus saprophyticus subsp. saprophyticus BAC2101 (HM355690)	99.0
SW-3-G	*Achromobacter* sp. DG (HQ437668)	99.0
	Achromobacter sp. P3 (FJ556879)	99.0
	Achromobacter insolitus CCM7182 (FM999733)	99.0
	Alcaligenaceae bacterium a001-61 (HM468067)	99.0
SW-3-I	*Alcaligenes* sp. MH112 (FJ626643)	99.0
	Achromobacter sp. P3 (FJ556879)	99.0
	Alcaligenes sp. cxh-4 (EF059708)	99.0
	Achromobacter insolitus CCM7182 (FM999733)	99.0

Table 4. Kinetic analysis of nonylphenol degradation by the coculture

Strains	k_1 (1/day)	$t_{1/2}$ (day)	r^2 (correlation coefficient)
Control	0.011	61.8	0.99
SW-3	0.074	9.3	0.99
SW-3-G/-I	0.081	8.6	0.99
SW-3-F1/-I	0.092	7.5	0.99
SW-3-F1/-G	0.086	8.0	0.99
SW-3-F1/-G/-I	0.086	8.0	0.99

Nonylphenol (NP) was extracted from YNB medium containing 100 ppm NP as described in Materials and Methods. Control means non-inoculated samples.

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