Biodegradation of 2,6-Naphthalenedisulfonic Acid by a Floc-Forming Bacterium, *Pigmentiphaga* sp. NDS-1

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Abstract

Two 2,6-naphthalenedisulfonate (2,6NDS) degrading bacteria were isolated from the activated sludge of a wastewater treatment plant and designated NDS-1 and NDS-2. Only strain NDS-1 showed a floc-forming ability in such media as synthetic wastewater. Sequence analysis of the partial 16S ribosomal DNA (rDNA) sequence of strain NDS-1 revealed that it is a strain closely related to *Pigmentiphaga kullae*, which was recently established in the family *Alcaligenaceae*, and that the sequence was in perfect agreement with that of strain NDS-2. Strain NDS-1 could mineralize 2,6NDS and 1,6-naphthalenedisulfonate (1,6NDS) with sulfate ion release, but showed no assimilating or desulfonating activity on 8 other sulfonated naphthalenes, indicating that it has a very narrow substrate specificity. 2,6NDS-induced NDS-1 cells exhibited a high oxygen uptake for 2,6NDS, while non-induced cells showed a very low oxygen uptake, demonstrating that 2,6NDS oxidation is inducible in strain NDS-1.

Key words: *Pigmentiphaga* sp., 2,6-naphthalenedisulfonate, floc-forming, desulfonation

List of abbreviations:

NDS: naphthalenedisulfonate,
NS: naphthalenesulfonate,
LB; Luria-Bertani medium,
DOC: dissolved organic carbon,
HPLC; high performance liquid chromatography,
rDNA; ribosomal DNA.

INTRODUCTION

Sulfonated aromatic compounds are used in large quantities as important intermediates in the synthetic processes of dyes, surfactants, and dispersants. However, they are known to be recalcitrant to biodegradation, because they have C-SO₃⁻ structures that are not ubiquitous in nature^{1,2}. Therefore, discharge of large amounts of sulfonated aromatic compounds may cause serious environmental pollution.

Indeed, many such compounds have been detected in rivers and seepage from waste dumps^{3,4}.

Of sulfonated aromatic compounds, naphthalenesulfonates (NSs) are the major pollutants in wastewaters from azo-dye producing plants and naphthalene refinery processing plants. It has been reported that 7 to 15 % of the organic pollution of the Rhine river is caused by NSs and their metabolites because they are resistant to biodegradation and are incompletely degraded in conventional sewage plants⁵. Consequently, there have been several studies investigating a way of removing NSs from such wastewaters⁶⁻⁹. Ahn *et al.*⁶ reported that, in the practical treatment of naphthalene refinery process wastewater containing mainly 1-naphthalenesulfonate (1NS), natural microbial populations and seeded sludge from

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an adjacent plant treating phenolic compounds showed no growth in aeration tanks. Subsequently, they acclimated bacteria from the bottom soil of an open drainage ditch in the naphthalene refinery process and introduced the bacteria into the treatment system, and achieved a good effluent quality. This indicates that the efficiency of treatments for NSs depends on the ability of the NS degrading bacteria.

In order to obtain bacteria with such particular characteristics, several NS degrading bacteria have been isolated and characterized^{1, 2, 10-13}. However, only a few bacterial strains are able to assimilate naphthalenedisulfonates (NDSs) as the sole carbon source^{12, 13} because NDSs are more resistant to biodegradation than naphthalenemonosulfonates¹⁴. *Moraxella* sp. ASL4¹² and *Pseudomonas* sp. DS-1¹³ could utilize 1,6NDS and 2,6NDS as the sole carbon source and a partial degradation pathway for NDSs has been proposed¹². Desulfonation and partial degradation of NDSs by pure cultures have been also shown^{10, 11}, but the resulting phenolic metabolites tend to polymerize, bind, and adsorb to solid materials such as clay and humic materials⁹, and are consequently more persistent to biodegradation.

In this study, we report the isolation and characterization of 2,6NDS degrading bacteria, one of which has a floc-forming ability that contributes to its survival in biological treatment systems such as activated sludge systems and biofilm systems. In addition, we also show complete degradation (mineralization and desulfonation) of 2,6NDS by this strain.

MATERIALS AND METHODS

Media, culture conditions Activated sludge was taken from the Wakagawa final wastewater treatment facility (Wakayama, Japan) as a bacterial source. The DS medium containing 3.0g of NH_4Cl , 1.0g of K_2HPO_4 , 0.25g of KCl , 0.2g of MgCl_2 , and 0.025g of FeCl_3 , per liter of distilled water was used as a mineral salt medium and Luria-Bertani (LB) medium¹⁵ was employed as a nutrient medium. Solid media were prepared by adding 1.5 % (w/v) agar to liquid media. The

isolated bacteria were incubated at 30 °C on a rotary shaker (120 rpm) unless otherwise stated.

Analytical methods Bacterial growth was monitored by measuring absorbance at 600 nm (O.D._{600}) using a HITACHI spectrophotometer U-2000 (HITACHI, Tokyo). When the isolates formed flocs in the culture, protein concentrations was measured instead of O.D._{600} . This was determined by the method of Lowry-Folin¹⁶ after disrupting the cells on ice for 15 min. by ultrasonication at 20kHz using an ultrasonic disruptor UD-200 (TOMY, Tokyo) and removing the cell debris by centrifugation ($18,000 \times g$, 1 h, 4 °C). Bovine serum albumin was used as the standard. Dissolved organic carbon (DOC) in the culture supernatant was measured by injecting 7 μl of the supernatant into a total organic carbon analyzer TOC-500 (SHIMADZU, Kyoto) and the pH of the culture supernatant was measured using a pH meter model F-22 (HORIBA, Kyoto). The concentration of sulfate and sulfite ions in the culture supernatant was determined using the method described by Bertolacini¹⁷ and the Elman's reagent method¹⁸, respectively. The concentration of 2,6NDS in the culture supernatant was determined using a TOSOH HPLC system (pump, CCPS; controller, SC-8020; detector, UV-8020; column oven, CO-8020; column, Mightysil RP-18 GP Aqua 250-4.6 (Kanto Chemical Co., Inc.) [4.6 mm I.D. $\times$ 250 mm]). The analytical conditions were as follows: column temp., 40 °C; flow rate, 0.8 $\text{ml} \cdot \text{min}^{-1}$; mobile phase, 50 mM sodium sulfate aqueous solution¹⁹; detection wavelength, 254 nm.

Isolation of 2,6NDS degrading bacteria

Batch enrichment culture for isolation of 2,6NDS degrading bacteria was started by adding 1 ml of the activated sludge into 100 ml of DS medium supplemented with yeast extract (200 $\text{mg} \cdot \text{l}^{-1}$) and 2,6NDS (100 $\text{mg} \cdot \text{l}^{-1}$) and shaking the culture. After a week, 10 ml of the culture was centrifuged at $8,000 \times g$ for 10 min. at 4 °C, the resulting pellet was transferred into the same medium and the culture was incubated under the same conditions. This was repeated to give a total of 14 cultivations, each time lowering the concentration of yeast extract and increasing

the concentration of 2,6NDS to give a final 2,6NDS concentration of $200 \text{ mg} \cdot \text{l}^{-1}$, and yeast extract concentration of $0 \text{ mg} \cdot \text{l}^{-1}$. To monitor the degradation of 2,6NDS during the enrichment cultivation, the DOC, pH and concentration of sulfate ions¹⁷⁾ in the culture supernatant were measured as described above. Aliquots of 2,6NDS degrading enrichment culture were spread out onto DS agar plates supplemented with 2,6NDS ($100 \text{ mg} \cdot \text{l}^{-1}$) as the sole carbon and sulfur source, and the plates were incubated at 30°C . Colonies which showed good growth on the plates were independently inoculated into 100 ml of DS medium containing 2,6NDS ($100 \text{ mg} \cdot \text{l}^{-1}$) and the culture was incubated for a week. The 2,6NDS degrading ability of each strain was evaluated as described above (DOC and pH).

Floc-forming ability The IF (index of flocculation) value²⁰⁾ was employed as an index of floc-forming ability. One milliliter of strain NDS-1 pre-cultured in 50 ml of synthetic wastewater, consisting of 200 mg of meat extract, 300 mg of peptone, 15 mg of NaCl, 7.0 mg of KCl, 7.0 mg of CaCl_2 , 5.0 mg of MgSO_4 , 105 mg of NaHCO_3 , 330 mg of 2,6NDS and 1 liter of tap water (pH 7.0), were inoculated into 100 ml of the same culture, and incubated for 10 days. After standing for 30 min. to settle the flocs, the O.D.₆₀₀ of the culture without flocs was measured as O.D._{aus}. Then, the flocs in the culture were disrupted by homogenizing the culture using a homogenizer T25-S2 (IKA-LABORTECHNIK, Germany) at 9,500 rpm for 10 sec., and the O.D.₆₀₀ of the culture was measured again as O.D._{total}. The IF value was calculated as follows:

$$\text{IF}(\%) = 100 \times (\text{O.D.}_{\text{total}} - \text{O.D.}_{\text{aus}}) / \text{O.D.}_{\text{total}}$$

Observation using a scanning electron microscope Floc-forming cells were observed using a scanning electron microscope (SEM). Firstly, floc-forming cells were collected on a cellulose acetate filter ($0.2 \mu\text{m}$), washed twice with sodium phosphate buffer (50 mM, pH 7.2), and then fixed with 2% glutaraldehyde for 2 h. The cells on the filter were washed with the same buffer and then dehydrated stepwise with various concentrations of ethyl alcohol aqueous solutions (50-90 % v/v), with 100 % ethyl

alcohol, and finally with 100 % *t*-butyl alcohol. The cells saturated with *t*-butyl alcohol were frozen in a freezer, and then immediately dried using a freeze-drying device JFD-300 (JEOL, Tokyo). The freeze-dried cells were further sputter-coated with Pt-Pd using an ion sputter model E-1030 (HITACHI), followed by observation using a scanning electron microscope model S-2400 (HITACHI).

Identification of isolated bacteria The isolates were identified according to Bergey's manual of systematic bacteriology, 9th ed.²¹⁾ In addition, to obtain genetic evidence for the identification, the partial 16S ribosomal DNA (rDNA) sequences of the isolates were determined using the method of Rochelle *et al.*²²⁾ Nucleotide sequencing was performed using a Thermo Sequenase Fluorescent Labelled Primer Cycle Sequencing Kit with 7-deaza-dGTP (AMERSHAM PHARMACIA BIOTECH, Tokyo) and a DNA sequencer DSQ-2000L (SHIMADZU, Kyoto) according to the manufacturer's instructions.

2,6NDS degradation test One loop of the isolates was independently inoculated into 50 ml of DS medium containing 2,6NDS (0.6 mM) and each culture was incubated for 3 days. The cells were harvested by centrifugation, washed twice with sodium phosphate buffer (50 mM, pH 7.0) and suspended in 4 ml of the same buffer. A 2,6NDS degradation test was started by inoculating 2 ml of the cell suspension into 250 ml of DS medium containing 2,6NDS (0.6 mM) and shaking the culture on a rotary shaker. A small amount of the culture was taken at specified time intervals and centrifuged to remove the cells. Finally, the DOC, pH, and concentration of 2,6NDS, sulfate ions¹⁷⁾ and sulfite ions¹⁸⁾ in the culture supernatant were determined as described above.

Desulfonation test for sulfonated naphthalenes The isolates were inoculated into LB medium containing 2,6NDS ($200 \text{ mg} \cdot \text{l}^{-1}$) and shaken on a rotary shaker for 3 days. The cells were harvested by centrifugation, washed twice with 50 mM phosphate buffer (pH 7.0), and suspended in an appropriate volume of the same buffer. A small amount (ca. 0.5 ml) of the cell suspension was

inoculated into 5 ml of DS medium containing $100 \text{ mg} \cdot \text{l}^{-1}$ of each sulfonated naphthalene, and the mixture was shaken for 5 days. Then the cells were removed by centrifugation, and the concentration of sulfate ions in the supernatant was determined according to the method described above¹⁷⁾.

Measurement of oxygen uptake rates for substituted naphthalenes The cell suspensions of the isolates were prepared in the same way as in the 2,6NDS degradation test and kept on ice for 2 h. An oxygen uptake test was started by mixing 1 ml of the cell suspension and 29 ml of a phosphate buffer containing each substituted naphthalene ($100 \text{ mg} \cdot \text{l}^{-1}$), pre-heated at 30°C in a 30-ml special glass chamber. Dissolved oxygen in the mixture was monitored continuously using a dissolved oxygen meter YSI Model 58 and a dissolved oxygen electrode Model 5750 (YELLOW SPRING INSTRUMENTS, U.S.A). The rate of oxygen uptake obtained was corrected for the endogenous respiration rate (rate without any substrate). To determine the amount of protein in the corresponding cell suspension, the cell suspension was sonicated to disrupt the cells, the cell debris was removed by centrifugation, and then protein concentration in the supernatant was determined as described above. The rate was expressed as milligram of dissolved oxygen diminution per gram of protein per min.

Nucleotide sequence accession number

The nucleotide sequence of the partial 16S ribosomal DNA of strain NDS-1 was registered in the DDBJ as accession number AB088856.

Chemicals 2,6NDS, naphthalene, 1NS and 2NS were purchased from Nacalai tesque (Kyoto). 1,5NDS, 2,7NDS, 2-hydroxynaphthalene-6-sulfonic acid, 1,3,6-naphthalenetrisulfonic acid and 2-hydroxy-3,6-naphthalenedisulfonic acid were obtained from Kanto chemicals (Tokyo). 1,6NDS was purchased from Tokyo Chemical Industry (Tokyo). 2-naphthylamine-6-sulfonic acid and 2,6-naphthalenedicarboxylic acid were obtained from Wako Pure Chemical Industries (Tokyo). All of the reagents used were with highest purity available.

RESULTS AND DISCUSSION

Isolation of 2,6NDS degrading bacteria

In the enrichment batch cultivation, 2,6NDS degrading bacteria were acclimated by lowering the concentration of yeast extract and increasing the concentration of 2,6NDS stepwise for 3 months. Finally, the culture became turbid rapidly with 2,6NDS as the sole carbon and sulfur source. Figure 1 shows the changes in the concentration of sulfate ions, DOC and pH in the enriched culture. The DOC value decreased to approx. 15 % of the initial amount in 2 days, proving that enriched bacteria utilized 2,6NDS as the sole carbon source. Corresponding to the decrease in DOC, the concentration of sulfate ions increased rapidly and the pH value went down gradually. These results mean that a 2,6NDS degrading enrichment culture was established. Then, aliquots of the culture were spread onto DS agar plates supplemented with 2,6NDS ($100 \text{ mg} \cdot \text{l}^{-1}$) as the sole carbon and sulfur source. About 40 colonies with different shape and color developed on the plates in 5 days. The 2,6NDS degrading ability of each colony was examined in the same DS medium supplemented with 2,6NDS ($100 \text{ mg} \cdot \text{l}^{-1}$). Two strains, designated NDS-1 and NDS-2, showed good growth in the medium and were regarded as 2,6NDS degrading bacteria. When strain NDS-1 was cultured in LB medium or synthetic wastewater²⁰⁾, good flocs were observed in the culture, while strain NDS-2 never formed such flocs under the same culture conditions (see below).

Identification of strain NDS-1 Table 1 summarizes the results of basic taxonomic studies. Strain NDS-1 formed a round, smooth and brownish colony and could grow prototrophically. It was able to utilize a limited number of organic acids, but could not utilize any saccharide tested. From these characteristics, strain NDS-1 was found to have similar morphological and biochemical properties to those of *Alcaligenes* spp. and *Achromobacter* spp. (Table 1). Strain NDS-1 grew within a range of 15°C to 42°C and in a pH range of 6 to 8, and the optimal growth temperature and pH were around 25°C and pH 7, respectively.

Fig. 1 Change in the concentration of sulfate ions, DOC and pH in enrichment culture. DS medium containing $200 \text{ mg} \cdot \text{l}^{-1}$ of 2,6NDS (2,6NDS-DS medium) was used in this experiment. 2,6NDS-DS medium was incubated with bacteria (enrichment culture) or without bacteria (control). Sulfate ions were measured as described in the text. Symbols: ●, DOC (enrichment culture); ○, DOC (control); ▲, sulfate ions (enrichment culture); △, sulfate ions (control); ■, pH (enrichment culture); □, pH (control)

The partial nucleotide sequence of the 16S rDNA of strain NDS-1 (nt 28-1490) revealed that strain NDS-1 belongs to the β -subgroup of *Proteobacteria*. It also showed great similarity to bacterial species in the family *Alcaligenaceae*: *Alcaligenes* sp. STC1 [AB046605], 96.6 %; *Alcaligenes* sp. isolate 151 [AJ002802], 96.5 %; *Achromobacter xylosoxidans* strain AU1011 [AF411020], 96.6 %; *Achromobacter xylosoxidans* subsp. *xylosoxidans* [AF411021], 96.6 %; *Pigmentiphaga kullae* strain K24 [AF282916], 99.4 %. *P. kullae* was recently established as a 1-(4'-carboxyphenylazo)-4-naphthol degrading bacterium, strain K24²³ and other species in the *Pigmentiphaga* genus have not been reported so far. In addition to the highest similarity in the 16S rDNA, several nucleotides at positions that distinguish *P. kullae* from other genera in this family were in perfect agreement between strain NDS-1 and *P. kullae* strain K24 (Table 2)²³. Therefore, we tentatively identified strain NDS-1 as a *Pigmentiphaga* sp., although there were some differences in the utilization of carbon sources (Table 1).

Interestingly, the 16S rDNA sequence of strain NDS-1 was in perfect agreement with that of strain NDS-2, and the morphological and biochemical properties of strain NDS-2 were identical to those of strain NDS-1 (data not shown). Therefore, strain NDS-2 seems

to be identical except for the floc-forming ability of strain NDS-1.

Floc forming ability of strain NDS-1

Figure 2(a) shows the flocs of strain NDS-1 formed in synthetic wastewater in a test tube. Electron microscopic observation demonstrated that strain NDS-1 produced a filamentous material around the cells [Fig.2(b)], and that the material covered the cells like a net when the cell population increased [Fig.2(c)]. The IF value of strain NDS-1, which was proposed as an index of floc forming ability, was 77.3 % in synthetic wastewater containing $200 \text{ mg} \cdot \text{l}^{-1}$ of 2,6NDS and this value was comparable to those of other strong floc-forming bacteria (70-100 %) reported by Fujita *et al.*²⁰ In contrast, the IF value of the non-flocculating strain NDS-2 was less than 2 %.

Practical biological treatments for industrial wastewaters are hampered by the slow growth rate of the bacteria used²⁴, fluctuations in pollutant-feed concentrations²⁵, and the presence of high amounts of inorganic compounds and heavy metals²⁶⁻²⁹. To overcome these problems, immobilized cell techniques and special tanks for culturing the bacterial populations employed have been integrated into practical treatment systems or pilot-plant systems. For instance, in the case of a practical treatment system for 1NS-containing wastewater, a smaller special

Table 1 Differential characteristics of strain NDS-1 and strains of other genetically similar genera

Strains	NDS-1	<i>Pigmentiphaga kullae</i>	<i>Alcaligenes faecalis</i>	<i>Achromobacter xylosoxidans</i> subsp. <i>xylosoxidans</i>
Gram stain	-	-	-	-
motility	+	+	+	+
shape	R	R	R	R
catalase	+	+	+	+
oxidase	+	+	+	+
GC content	68.3%	68.5%	55.9-59.4%	66.0-69.8%
major isoprenoid quinone	ubiquinone Q-8	ubiquinone Q-8	ubiquinone Q-8	ubiquinone Q-8
Hugh and Leifson test	-	NT	-	NT
nitrate reduction to nitrite	-	NT	-	+
nitrite reduction	-	NT	+	+
gelatin liquefaction	-	NT	-	-
arginine hydrolysis	-	NT	-	NT
casein hydrolysis	-	NT	-	NT
starch hydrolysis	-	NT	-	-
urease	-	NT	-	-
Growth at				
4°C	-	-	-	-
35°C	+	+	+	+
40°C	NT	NT	-	+
42°C	+	+	NT	NT
Utilization of :				
L-arabinose	-	-	-	-
D-fructose	-	-	-	±
D-galactose	-	-	-	-
D-glucose	-	-	-	+
D-mannose	-	-	-	±
L-rhamnose	-	-	-	-
sucrose	-	-	-	-
D-xylose	-	-	-	+
adonitol	-	-	-	-
D-mannitol	-	-	-	-
D-raffinose	-	NT	-	-
β-alanine	-	NT	-	±
oxalate	-	NT	-	-
acetate	-	+	+	+
propionate	+	+	+	+
citrate	+	+	+	+
fumarate	+	+	+	+
L-glutamate	+	NT	+	+
α-ketoglutarate	+	NT	±	+
pyruvate	+	+	±	±
salicylate	+	NT	-	-
3-hydroxybenzoate	+	+	-	-
4-hydroxybenzoate	+	+	-	-
iso-valerate	-	NT	±	+
tartarate	-	NT	-	±
glycolate	-	NT	+	-
succinate	+	NT	+	+
DL-malate	+	NT	+	+

Symbols : R, rod shape ; NT, not tested

Table 2 Pattern of selected 16S rRNA signature nucleotides that define genera within the family *Alcaligenaceae*.

Position(s) ^a	NDS-1	<i>Pigmentiphaga kullae</i> K24	<i>Alcaligenes</i>	<i>Achromobacter</i>	<i>Bordetella</i>
43	C	C	G	G	G
123	C	C	U	U	U
238	G	G	A	A	A
399	G	G	C	C	C
412	A	A	U	U	U
1030	A	A	C	C	C
1060	C	C	U	U	U
1197	G	G	A	A	A
1256	C	C	U	U	U
1308	U	U	C	C	C
1329	A	A	G	G	G

a) The indicated positions differentiate *Pigmentiphaga kullae* K24 from the other genera (*Alcaligenes*, *Achromobacter* and *Bordetella*)²⁰. Positions are given relative to the *E. coli* numbering.

Fig. 2 (a) The flocs of strain NDS-1 in synthetic wastewater²⁰
 (b) Strain NDS-1 observed using a scanning electron microscope
 (c) Flocs of strain NDS-1 observed using a scanning electron microscope

aeration tank for the growth of 1NS degrading bacteria was placed prior to the next two larger aeration tanks to obtain good effluent quality⁶. In another case, a two stage airlift-loop bioreactor system was employed for the treatment of model wastewater containing various kinds of NSs, where immobilized *Pseudomonas testosteroni* A3 degraded 1NS and 2NS in the first reactor and immobilized strain RK3 mainly removed the more persistent NDSs in the second reactor¹⁴. The floc-forming ability of strain NDS-1 may contribute to its survival in biological treatment systems for NSs without the need for such special reactors and immobilized cell techniques. Indeed, its floc-forming ability might allow strain NDS-1 to become a stable member in the activated

sludge in the Wakagawa final wastewater treatment facility, in spite of its poor utilization of various carbon sources (Table 1).

2,6NDS degradation by strain NDS-1

Figure 3 shows the changes in concentration of 2,6NDS, DOC, and protein as an index of growth in the 2,6NDS degradation test. The concentration of 2,6NDS (0.6 mM) decreased concomitant with the growth of strain NDS-1, and finally reached 0.14 mM in 120 h. The DOC concentration in the culture also decreased from 68 mg-C·l⁻¹ to 14 mg-C·l⁻¹, indicating that strain NDS-1 utilizes 2,6NDS as the sole carbon source. The carbon content in a decrease of 0.46 mM of 2,6NDS is calculated as ca. 55 mg-C·l⁻¹, which completely corresponds to the decrease in DOC (54 mg-C·l⁻¹). Conversely, the sulfate ion

Fig. 3 Degradation of 2,6NDS by strain NDS-1. A DS medium with 2,6NDS ($200\text{mg}\cdot\text{l}^{-1}$) as the sole carbon and sulfur source was used in this test. Dissolved organic carbon (DOC) was measured using a TOC analyzer. The concentration of 2,6NDS was measured by HPLC and the concentration of sulfate ions was determined as described in the text. Protein concentration was measured as an index of growth according to the Lowry-Folin method.

Symbols: ▲, Growth (protein); ○, DOC; ●, 2,6NDS; □, sulfate ions; △, pH

concentration increased with the consumption of 2,6NDS, while the pH in the culture decreased with the increase in sulfate ion concentration. Thus, the decrease in pH seemed to be caused by sulfate ions liberated into the culture. Sulfite ions could not be detected in the culture supernatant by the method we employed, although Wittich *et al.* detected a very small amount of sulfite ions (up to ca. $35\text{ }\mu\text{M}$) in the degradation of excess amounts of 2,6NDS (10 mM) by *Moraxella* sp. ASL4¹²⁾. In this test, 2,6NDS was not completely degraded. The residual 2,6NDS seem to be due to the breakdown of pH, because resting cells in phosphate buffer (pH 7.2) were able to degrade the same concentration of 2,6NDS completely.

Assimilation and desulfonation range of substituted naphthalenes by strain NDS-1

To determine the assimilation range of substituted naphthalenes by strain NDS-1, an assimilation test of naphthalene and substituted naphthalenes was carried out using DS agarose [$0.8\text{ }\%$ w/v] plates containing $100\text{ mg}\cdot\text{l}^{-1}$ of each substrate as the sole carbon source. Of 12 substrates tested, strain NDS-1 could grow on only 2,6NDS and 1,6-naphthalenedisulfonate (1,6NDS) (Table 3). Similarly, a desulfonation test of sulfonated naphthalenes was also carried out using the cell suspension of strain NDS-1, and showed that free sulfate ions could be detected only when 2,6NDS and 1,6NDS were used as the substrate (Table 3).

These results indicate that this strain has a very narrow substrate specificity for substituted naphthalenes. Other NDS degrading bacteria, strains ASL4 and DS-1, could also utilize 2,6NDS and 1,6NDS, but not any other NDSs^{12, 13)}. In contrast, *Pseudomonas* sp. S-313 was able to desulfonate a wide range of NSs including NDSs, but was unable to utilize any NSs as the sole carbon source¹¹⁾.

Oxygen uptake rates for substituted naphthalenes The oxygen uptake for 2,6 NDS was measured using cells grown on LB medium with or without 2,6NDS (2,6NDS-induced cells or non-induced cells). There was a high oxygen uptake rate for 2,6NDS ($3.33\text{ mg}\cdot\text{O}_2\cdot\text{g of protein}^{-1}\cdot\text{min}^{-1}$) in 2,6NDS-induced cell cultures, but a very low rate ($0.16\text{ mg}\cdot\text{O}_2\cdot\text{g of protein}^{-1}\cdot\text{min}^{-1}$) in the non-induced cell cultures. This suggests that 2,6NDS oxidation in strain NDS-1 is induced by 2,6NDS. Therefore, oxygen uptake for substituted naphthalenes was investigated using 2,6NDS-induced cells. Table 3 shows the oxygen uptake rates for 11 substituted naphthalenes. The oxygen uptake rates for all of other substrates tested except for 1,6NDS and 2,7-naphthalenedisulfonate were lower than that for 2,6NDS. These results also support the narrow substrate specificity of strain NDS-1. However, the rate for 2,6-naphthalenedicarboxylate (2,6 NDC) was relatively higher than those for other substrates due to its structural similarity to 2,6NDS. Wittich *et al.*¹²⁾ also

Table 3 Assimilation, desulfonation and oxidation of substituted naphthalene by strain NDS-1.

Substrate	Assimilation ^{a)}	Desulfonation ^{b)}	Oxygen Uptake ^{c)} (mg-O ₂ ·g of protein ⁻¹ ·min ⁻¹)	Relative Activity ^{d)} (%)
2,6-naphthalenedisulfonic acid	+	+	3.33	100
Naphthalene ^{e)}	-	NT	NT	NT
1-naphthalenesulfonic acid	-	-	0.29	8.8
2-naphthalenesulfonic acid	-	-	0.11	3.3
1,5-naphthalenedisulfonic acid	-	-	0.33	9.9
1,6-naphthalenedisulfonic acid	+	+	4.00	120
2,7-naphthalenedisulfonic acid	-	-	3.19	96
2-hydroxy-6-naphthalenesulfonic acid	-	-	0.19	5.7
2-amino-6-naphthalenesulfonic acid	-	-	0.32	9.7
2-hydroxy-3,6-naphthalenedisulfonic acid	-	-	0.39	12
1,3,6-naphthalenetrisulfonic acid	-	-	0.34	10
2,6-naphthalenedicarboxylic acid	-	NT	0.54	16

a) An assimilation test for naphthalene and substituted naphthalenes was carried out using DS agarose [0.8 % w/v] plates containing 100 mg·l⁻¹ of each substrate as the sole carbon source. Positive (+) shows that strain NDS-1 formed colonies. b) Desulfonation test for sulfonated naphthalenes was carried out using the cell suspension of strain NDS-1. Positive (+) shows that free sulfate ions were detected. c) Oxygen uptake for substituted naphthalenes was investigated using 2,6NDS-induced cells. d) Activity was expressed as the percentage of oxygen uptake rate for 2,6-naphthalenedisulfonic acid. e) Naphthalene was supplied as vapour, putting some crystals on the lid of petri dishes. NT : not tested

reported that the 2,6NDS-grown cells of strain ASL4 co-oxidized its structural analog, 2,6NDC.

The cell extract of strain NDS-1 showed higher oxygen uptake rates for gentisate (0.35 mg-O₂·g of protein⁻¹·min⁻¹) and protocatechuate (0.21 mg-O₂·g of protein⁻¹·min⁻¹), which are key intermediates in the degradation of aromatic compounds. Moreover, strain NDS-1 could grow on gentisate and protocatechuate in addition to 5-sulfosalicylate. Therefore, strain NDS-1 may degrade 2,6NDS through these compounds. An investigation of the degradation pathway of strain NDS-1 is now in progress.

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