# Isolation of naphthalene-degrading bacteria from tropical marine sediments

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**Abstract** Oil pollution is a major environmental concern in many countries, and this has led to a concerted effort in studying the feasibility of using oil-degrading bacteria for bioremediation. Although many oil-degrading bacteria have been isolated from different environments, environmental conditions can impose a selection pressure on the types of bacteria that can reside in a particular environment. This study reports the successful isolation of two indigenous naphthalene-degrading bacteria from oil-contaminated tropical marine sediments by enrichment culture. Strains MN-005 and MN-006 were characterized using an extensive range of biochemical tests. The 16S ribosomal deoxyribonucleic acid (rDNA) sequence analysis was also performed for the two strains. Their naphthalene degradation capabilities were determined using gas chromatography and DAPI counting of bacterial cells. Strains MN-005 and MN-006 are phenotypically and phylogenetically different from each other, and belong to the genera *Staphylococcus* and *Micrococcus*, respectively. Strains MN-005 and MN-006 had maximal specific growth rates ($\mu_{max}$) of 0.082 ± 0.008 and 0.30 ± 0.02 per hour, respectively, and half-saturation constants ($K_s$) of 0.79 ± 0.10 and 2.52 ± 0.32 mg per litre, respectively. These physiological and growth studies are useful in assessing the potential of these indigenous isolates for in situ or ex situ naphthalene pollutant bioremediation in tropical marine environments.
**Keywords** 16S rDNA; naphthalene biodegradation; Monod kinetics; phylogeny; *Staphylococcus*; *Micrococcus*; tropical marine environment

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## Introduction

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds that are known to be cytotoxic, mutagenic, and carcinogenic (Geiselbrecht *et al*., 1996). These hydrocarbons are widely distributed in the environment, and this is due mainly to anthropogenic activities, such as petroleum refining and incomplete combustion of fossil fuels (Berardesco *et al*., 1998). PAH bioremediation has been extensively reviewed as an effective and environmentally benign cleanup technology as it involves the partial or complete bioconversion of these pollutants to microbial biomass, carbon dioxide, and water (Atlas, 1995; Head and Swannell, 1999; Alexander, 1999). A successful bioremediation strategy will have to be based on an in-depth understanding of the factors that influence the biodegradation process and the ecology of pollutant degrading bacteria (Langworthy *et al*., 1998).

Naphthalene, the simplest of PAHs, has long been used as a model compound in PAH bioremediation studies (Hedlund *et al*., 1999). In addition, naphthalene is also considered to be the most toxic of the water-soluble fractions usually present in crude and refined oils (Nagabhushanam *et al*., 1991). Since naphthalene is commonly found in different environments, it is not surprising that these environments have yielded many bacterial isolates capable of degrading naphthalene (Hedlund *et al*., 1999). Common naphthalene-degrading bacteria include *Pseudomonas* spp., *Vibrio* spp., *Mycobacterium* spp., *Marinobacter* spp., and *Sphingomonas* spp. (Berardesco *et al*., 1998; Hedlund *et al*., 1999). Although many naphthalene-degrading bacteria have been isolated, these bacteria may thrive in one environment but may not be able to compete with other microorganisms in another environment as environmental conditions will impose a selection pressure on the types of bacteria that can reside in it. Furthermore, indigenous bacteria have been shown to outcompete other

artificially introduced strains in numerous bioremediation investigations (Iwabuchi *et al*., 1997). Therefore, to implement a successful bioremediation strategy, a detailed evaluation of the roles of the indigenous bacteria should be an integral part of any site investigation (Piehler *et al*., 1999).

This study describes the isolation and characterization of two strains of naphthalene-degrading bacteria from oil-contaminated tropical marine sediments. The strains belong to genera *Staphylococcus* and *Micrococcus* respectively. Their ability to degrade naphthalene *in situ* suggests the possible application of these strains in naphthalene bioremediation schemes.



## Materials and methods

### Isolation procedure

Tropical marine sediments contaminated with marine fuel oil were aseptically collected from a beach in south Singapore and stored at –20°C before use. For enrichment isolation, a 100 ml serum bottle with Teflon coated stopper containing 20 ml ONR7a media (Dyksterhouse *et al*., 1995) and approximately 10 mg/l naphthalene as sole carbon source was aseptically prepared. A two gram (wet weight) portion of sediment was then introduced aseptically. The bottle was stoppered and covered with aluminium foil, and cultured by gentle shaking (100 rpm) (Electric Shaker 40, LABNET®, USA). The cultures were closely monitored and naphthalene concentration was readjusted to 10 ppm periodically. Enrichment cultures were incubated for 2 months at 25°C. After significant growth was observed, the culture media was diluted with ONR7a media ($10^1$- to $10^7$-fold dilutions), and 100 µl of each dilution was spread on an ONR7a agar plate solidified with 1.2% Bacto Agar (Difco Laboratories, USA). Naphthalene was introduced as the sole carbon source during incubation by adding crystals to the plate lids. Plates were inverted and incubated in a 25°C incubator (Sanyo, Japan) and monitored over 6 weeks. Visible colonies were observed after 5 days of incubation. Colonies were transferred to fresh plates, and the incubation process was repeated until pure cultures were obtained. The purity of culture was checked by microscopic examination and pure cultures were preserved in marine broth 2216 (Difco Laboratories, Detroit, Mich.) with 30% sterilized glycerol at –80°C.

### Morphological and phenotypic characterizations

Cells of isolates were observed with both light microscopy (Olympus BX-FLA-3 epifluorescence microscope, Japan) and scanning electron microscopy (Leica stereoscan model 420, UK). Growth at different temperatures and salinities was performed as previously described (Zhuang *et al*., 2001). Gram-stain and the Voges-Proskauer test were performed according to standard methods (Smibert and Krieg, 1994). A non-staining Gram-stain method (Buck, 1982) was also performed to validate the Gram-stain result.

### Monod growth kinetics

The Monod growth kinetics for strains MN-005 and MN-006 were determined separately by counting the total cell numbers with DAPI (4′,6-diamidino-2-phenylindole) stain (Kuwae and Hosokawa, 1999). Cells were grown in ONR7a broth with naphthalene as sole carbon source at 25°C.

### 16S rRNA gene sequencing and phylogenetic analyses

A whole cell direct lysis PCR amplification method was used to amplify 16S rDNA as described previously (Maszenan *et al*., 1997). The 16S rRNA gene was amplified with forward primer Eubac27F and reverse primer Universal 1492R1 (Lane, 1991). The PCR products were purified with BIO101 GENECLEAN® II Kit (BIO101, Canada) according to

the manufacturer's instructions. 16S rRNA sequences were obtained for strains MN-005 and MN-006 using the ABI model 310A DNA sequencer (Applied Biosystems, Perkin-Elmer) and the ABI PRISM® BigDye™ Terminator Cycle Sequencing ready-reaction kit (Applied Biosystems, Perkin-Elmer). Sequence alignments were performed manually with the Genetic Data Environment (GDE) (Smith *et al*., 1994). Distance and maximum likelihood analyses were performed on the final data set with PHYLIP (Felsenstein, 1989), fastDNAml (Olsen *et al*., 1994). A maximum likelihood phylogenetic tree was constructed as described previously (Tay *et al*., 1998). Evolutionary distance bootstrap values were determined using SEQBOOT, DNADIST, FITCH and CONSENSE programs in PHYLIP (Felsenstein, 1993). The GenBank accession numbers of the other sequences used in the phylogenetic analysis are as follows:

*MN-005 – Staphylococcus hominis*, Z26905; *Staphylococcus arlettae*, Z26888; *Staphylococcus auricularis*, Z26889; *Staphylococcus capitis*, Z26940; *Staphylococcus haemolyticus*, Z26896; *Staphylococcus muscae* str. MB4 CCM4175, S83566; *Staphylococcus saccharolyticus*, G576608; *Staphylococcus saprophyticus* subsp. saprophyticus str. NT75, L20250; *Staphylococcus schleiferi*; *Staphylococcus warneri*, G576609; *Staphylococcus xylosus* MAFF911482, G1199956; *Bacillus cereus* IAM12605; G457636.

*MN-006 – Micrococcus luteus* str. Hucker S66, M38242; *Micrococcus* sp. str. S2-07; X86612; *Micrococcus* sp. str. AC-47; X86609; *Micrococcus psychrophilum*; *Micrococcus lylae* str. JL178, X80750; *Arthrobacter ramosus* str. I Gm25, X80742; *Arthrobacter globiformis* str. 168, M23411; *Arthrobacter polychromogenes* str. 2568, X80741; *Arthrobacter nicotinovorans*, X80743; *Arthrobacter ureafaciens* str. NC, X80744; *Arthrobacter sulfureus*; X83409; *Arthrobacter urataxydans*, X83410; *Arthrobacter protophormiae* str. M-570, X80745; *Rothis dentocariasa*, M59055.

## Results and discussion

Two different strains of naphthalene degrading bacteria were successfully isolated from oil-contaminated tropical marine sediments by using the enrichment isolation technique. They were designated as strains MN-005 and MN-006. In 1901, Beijerinck found that a large number of natural and xenobiotic compounds could be degraded by bacteria obtained from the enrichment isolation technique. Bacteria possess this degradative capability either by mutation and natural selection or by induction of the necessary enzymes (Painter, 1974).

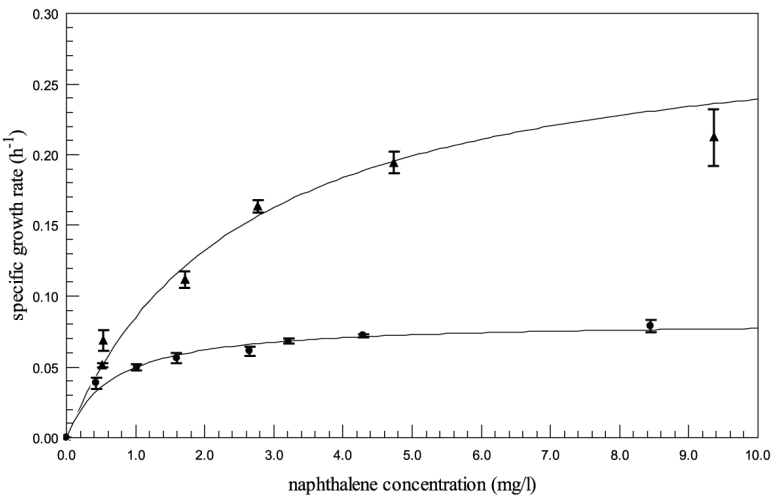


**Figure 1** Monod growth kinetics for strain MN-005 (●) and MN-006 (▲)

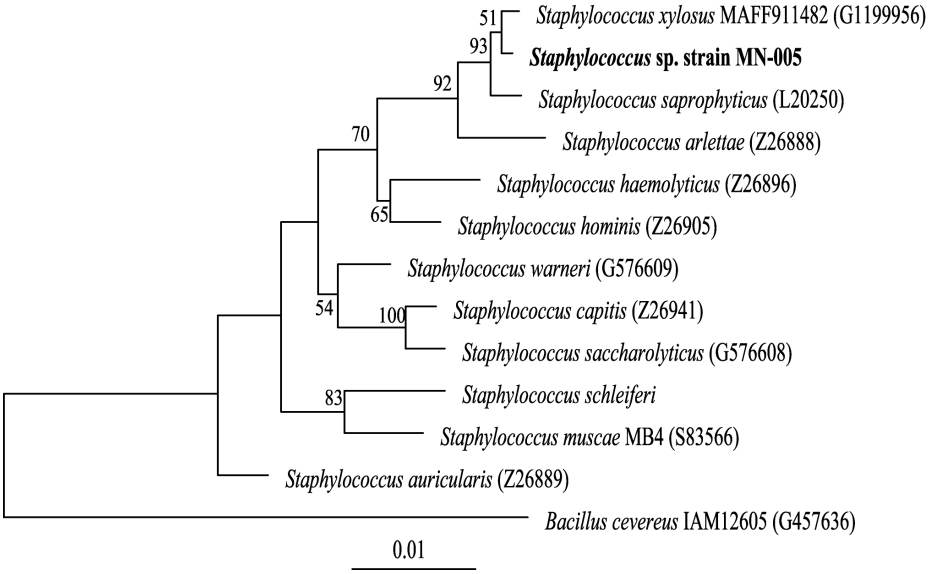


**Figure 2** 16S rDNA phylogenetic tree for strain MN-005. The numbers at the branch nodes are bootstrap values based on 100 resamplings for maximum likelihood. Only bootstrap values greater than 50% are shown. Scale bar = 1 percent nucleotide divergence

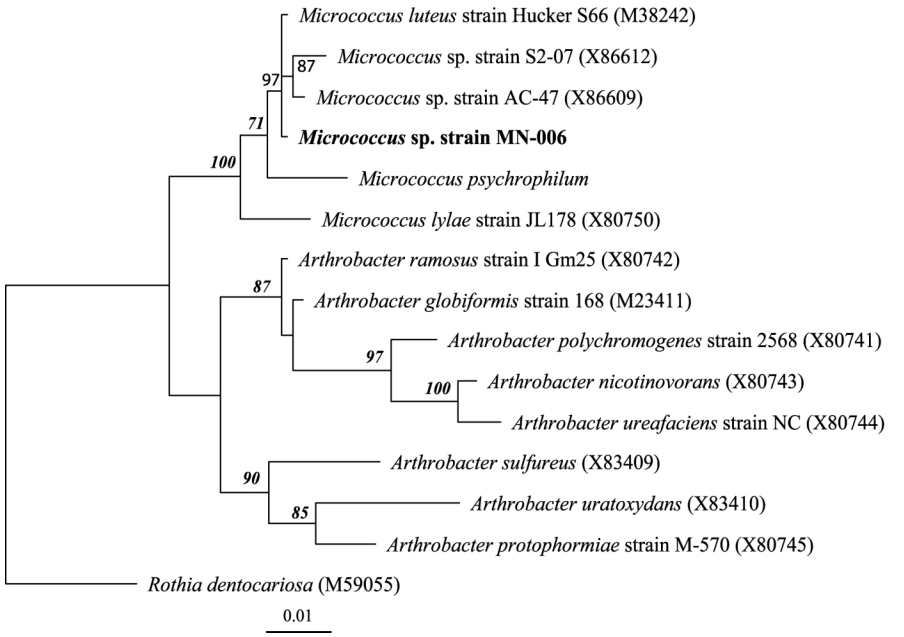


**Figure 3** 16S rDNA phylogenetic tree for strain MN-006. The numbers at the branch nodes are bootstrap values based on 100 resamplings for maximum likelihood. Only bootstrap values greater than 60% are shown. Scale bar = 1 percent nucleotide divergence

Since then, the enrichment isolation strategy has been successfully used to isolate oil and PAH-degrading bacteria from different environments (Bauer and Capone, 1985; Wagner-Döbler *et al*., 1998).

Strain MN-005 is spherical in shape, Gram positive, catalase positive and oxidase negative. Cells ranged between 0.6 to 1.3 μm in diameter when grown in marine broth 2216 media at 25°C. Strain MN-005 grew at salinities ranging from 0.28 to 5.00% and temperatures ranging from 15 to 37°C. Temperatures below 4°C were not tested. Cells were non-motile and flagella were not observed under scanning electron microscopy (data not shown). Strain MN-006 is also spherical in shape, Gram positive, catalase positive and oxidase positive. The cells occur in pairs, in threes and as tetrads. Cell diameters were between 0.4 to 1.0 μm when grown in marine broth 2216 media at 25°C. They grew at salinities ranging from 0.28 to 7.00% and temperatures ranging from 15 to 41°C. Cells were non-motile, and no flagella were detected by scanning electron microscopy (data not shown).

The Monod growth kinetics of strains MN-005 and MN-006 are shown in Figure 2. Strains MN-005 and MN-006 had maximal specific growth rates ($\mu_{max}$) of 0.082 ± 0.008 and 0.30 ± 0.02 $h^{-1}$, respectively, and half-saturation constants ($K_s$) of 0.79 ± 0.10 and 2.52 ± 0.32 mg/l, respectively.

Phylogenetic analysis based on 1264 unambiguous bases revealed that strain MN-005 is a member of the Gram-positive genus *Staphylococcus*. A maximum-likelihood tree for strain MN-005 is shown in Figure 2. The sequence of strain MN-005 was most similar to *Staphylococcus xylosus* and the sequence identity was 99.9%. Phylogenetic analysis based on 1214 unambiguous bases revealed that strain MN-006 is also a member of the Gram-positive genus *Micrococcus*. A maximum-likelihood tree for strain MN-006 is shown in Figure 3. The sequence of strain MN-006 was most similar to that of *Micrococcus luteus* and the sequence identity was 99.8%.

## Conclusions

In this study, two indigenous bacteria were successfully isolated from oil-contaminated tropical marine sediments. Gram positive bacteria have a robust cell wall structure, which can resist salinity fluctuations and extreme environmental conditions. Both strains possess the desired naphthalene degrading capability and robust cell wall property that are important for an effective naphthalene bioremediation strategy in contaminated intertidal marine sediments which are characterized by diurnal variations in temperature, osmotic pressure and nutrient supply.

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