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Characteristics of Nitrobenzene Degradation by *Mycobacterium chelonae* Strain NB01

OH, YOUNG-SOOK[1], YOUN-HEE LEE, JUNG-HYUN LEE[2], AND SUNG-CHAN CHOI*

Division of Life Sciences, Hallym University, Chunchon 200-702, Korea
[1]Department of Environmental Engineering & Biotechnology, Myongji University, Yongin 449-728, Korea
[2]Microbiology Lab., Korea Ocean Research & Development Institute, Ansan 425-600, Korea

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Abstract A bacterial strain NB01, isolated from wastewater, was found to utilize nitrobenzene (NB) as the sole source of nitrogen, carbon, and energy. The strain was classified as a member of a high G+C Gram-positive group and identified as *Mycobacterium chelonae* based on an analysis of its 16S rRNA gene sequence. The strain grew on NB with a concomitant release of about 63% of the total available nitrogen as ammonia, suggesting a reductive degradation mechanism. The optimal pH and temperature for degradation were pH 7.0–8.0 and 30°C, respectively. The cell growth was retarded at NB concentrations above 1.8 mM. The degradation of NB followed Michaelis-Menten kinetics within the tolerance range, and the K_m and maximum specific removal rate for NB were 0.33 mM and 11.04 h^{-1}, respectively.

Key words: Biodegradation, kinetics, *Mycobacterium*, nitrobenzene

Nitro-substituted compounds are released into the biosphere almost exclusively from anthropogenic sources. As such, nitroaromatic compounds, including nitrobenzene (NB), are abundantly present in industrial waste streams and surface waters due to their wide use in the manufacturing of rubber chemicals, pesticides, dyes, and pharmaceuticals. NB is relatively toxic and persistent in the environment and listed as an EPA priority pollutant [14].

The bacterial degradation of NB has only been reported in a few species, including *Comamonas* sp. [17, 21] and *Pseudomonas* spp. [5, 6, 9, 10, 20, 24, 27]. This is mainly due to the xenobiotic nature of the nitro group, which strongly withdraws electrons. Therefore, in addition to the stability of the benzene ring, the electron deficiency of the aromatic ring impedes any electrophilic attack by the oxygenases of aerobic bacteria.

Accordingly, in an attempt to isolate a bacterial strain capable of utilizing NB as a sole source of carbon, nitrogen, and energy, wastewater samples were obtained from the Chunchon industrial area in Korea and inoculated into a nitrogen-free broth (NFB) [5]. While incubating at 30°C with shaking, the flask was aerated daily and NB was added directly to the culture medium at 0.1 mM. The enrichment that developed substantial turbidity was serially transferred until stable consortia were obtained. An aliquot of the suspension was spread on mineral salt agar plates and NB was provided in a vapor state. The pure culture exhibiting the fastest growth on NB was designated as strain NB01. The cells were non-motile, rod-shaped, Gram-positive, and catalase-positive. However, they showed negative results with citrate utilization, gelatin hydrolysis, methyl red oxidase, starch hydrolysis, a triple sugar iron agar, urease, and Voges-Proskauer reactions.

The nearly complete nucleotide sequence of 16S rDNA was determined, as described previously [15]. The 16S rDNA sequence was compared to the sequences in the Ribosomal Database Project [18] using the SEQUENCE_MATCH (v. 2.7) option and the GenBank nucleotide database using the Basic Local Alignment Search Tool (BLAST) [1]. Unaligned sequence data from the GenBank database and our present result were aligned manually with pre-aligned sequences downloaded from the RDP. The phylogenetic relationships were estimated using the Phylogenetic Inference Package (PHYLIP version 3.57c) [8]. The Jukes-Cantor evolutionary distances were calculated using the DNADIST program, while dendrograms depicting the phylogenetic relationships were derived using the FITCH program. The sequence of the 16S rDNA of strain NB01 was submitted to the GenBank database under

*Corresponding author
Phone: 82-33-240-1531; Fax: 82-33-242-9300;
E-mail: scchoi@hallym.ac.kr

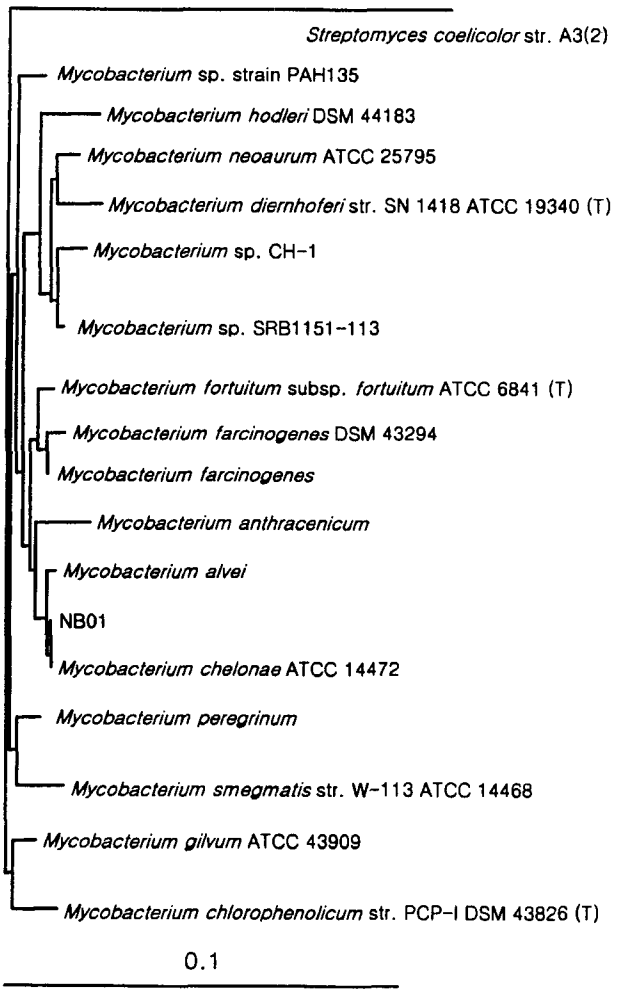

Fig. 1. Phylogenetic tree based on partial 16S rRNA gene sequence data (1,307 bases) showing location of strain NB01.
The scale bar represents 0.1 substitutions per base position.

accession number AY188086. Strain NB01 was classified as a member of a high G+C Gram-positive group and identified as *Mycobacterium chelonae* (X52921) based on the resulting intergeneric phylogenetic tree (Fig. 1). The fact that citrate was not utilized suggested that the strain belonged to *M. chelonae* subsp. *abscessus* [26]. It would appear that this is the first report of NB degradation by a member of the genus *Mycobacterium*.

The NB concentrations in the culture fluids were analyzed by HPLC (Hitachi, Model L-4200H, Japan). The analyses were performed on a 4.6×150 mm COSMOSIL RP-C_{18} column with methanol-water-phosphoric acid (500:500:1 v/v/v) as the mobile phase at 1.5 ml/min. Any compounds including degradation intermediates were monitored with a UV spectrophotometric detector at A_{254}. The concentration of protein was determined by the Bradford method with BSA as the standard [4]. The concentrations of nitrite, nitrate, and ammonia were measured

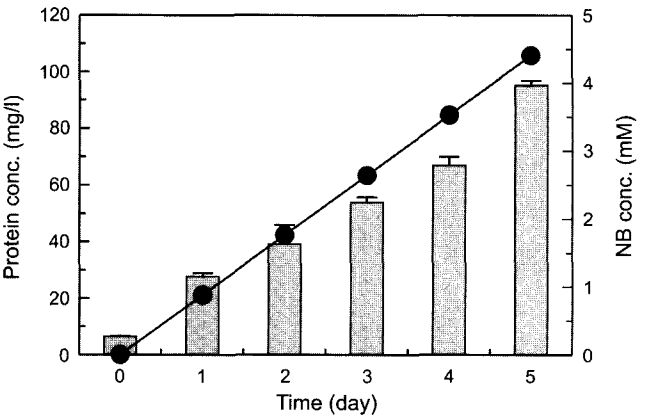

Fig. 2. Growth of *Mycobacterium chelonae* strain NB01.
The strain removed nitrobenzene (●) with a sharp increase in the cell protein (bars). The points plotted are the mean values of duplicates.

spectrophotometrically, as described in Standard Methods [2].

To determine the pH and temperature optima for NB removal, the cells were washed twice with NFB, and tested for NB removal at pH 5-9 and 20-40°C, respectively. All experiments were performed in triplicate, and any abiotic losses of NB were subtracted by sampling an identical yet uninoculated flask. The optimum conditions for degradation were found to be pH 7.0-8.0 and 30°C (data not shown).

To quantitatively determine the growth on NB, a 250-ml Erlenmeyer flask containing 50 ml of NFB was inoculated with 0.1 ml cell suspension (6.5 mg protein/l) and NB at 0.9 mM was added to the flask. When the added NB was completely removed, based on an HPLC analysis, the flask was aerated and additional NB was added to the same concentration (0.9 mM). The cell growth was found to be accompanied by the cumulative removal of NB (Fig. 2), and the yield, Y, on NB was determined as 0.18. The Y value was comparable to that previously obtained with an NB-degrading bacterial consortium composed of 4 distinctive strains, including *Agrobacterium* sp., *Sphingomonas* sp., and *Achrobacterium* sp. [22].

When NB was provided as the sole carbon, nitrogen, and energy source, the strain NB01 rapidly removed NB from the culture fluid after only a slight lag (2.5 h). The initial degradation rate was calculated as 0.137 mM/h (Fig. 3). No nitrite or nitrate was detected in the culture fluid, however, 63% of the nitrogen in the form of NB was converted into ammonia-N. It was highly likely that a considerable amount of the remaining 37% of the NB nitrogen was converted into the nitrogen of the biomass. The degradation of NB by *Comamonas* sp. JS765 produces nitrite exclusively, where the initial reaction is catalyzed by nitrobenzene 1,2-dioxygenase at an initial NB degradation rate of 0.11 mM/h [21]. At a higher initial degradation rate, 0.22 mM/h, the *Pseudomonas*

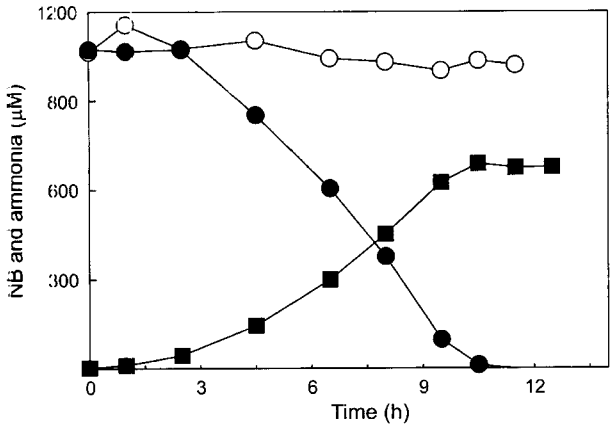

Fig. 3. Disappearance of nitrobenzene and release of ammonia during growth of strain NB01.
The strain removed nitrobenzene (●) with a concomitant release of about 60% of the available nitrogen as ammonia-N (■). An identical yet uninoculated control (○) showed no loss of nitrobenzene during the incubation period. The points plotted are the means of duplicates.

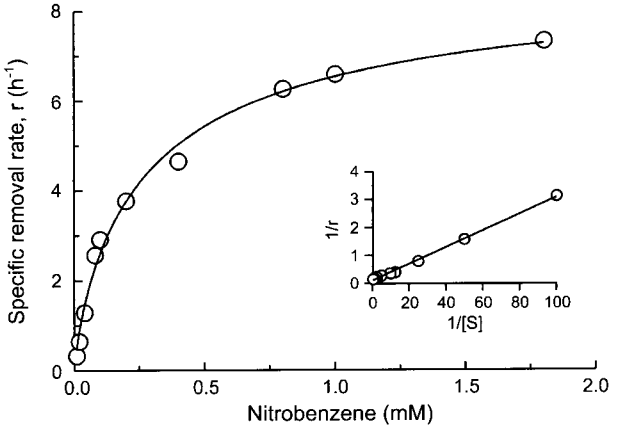

Fig. 4. Degradation kinetic parameters of nitrobenzene-degrading strain NB01.
The nitrobenzene utilization followed Michaelis-Menten kinetics, and a K_m of 0.33 mM and maximum specific removal rate of 11.04 h^{-1} were estimated from a Lineweaver-Burk plot (inset). The reaction was performed at 30°C with an initial biomass of 46 mg protein/l.

pseudoalcaligenes strain converts 1/3 of the available nitrogen into ammonia-N [20]. However, in the latter case, the inoculum density applied was 42 mg protein/l, which was much higher than the value (0.47 mg/l) applied in the current study.

The accumulation of ammonia, but not nitrite, in the media of the NB-grown cultures of the strain NB01 (Fig. 3) suggests that the initial attack on the nitro group was reductive rather than oxidative, as previously reported with *P. pseudoalcaligenes* [20]. The reductive pathway for ring cleavage begins with the partial reduction of NB to hydroxylaminobenzene (HAB) by nitrobenzene nitroreductase [27]. HAB is then rearranged to 2-aminophenol by HAB mutase. Next, 2-aminophenol 1,6-dioxygenase catalyzes an extradiol cleavage of the aromatic ring to produce 2-aminomuconic acid 6-semialdehyde [16], which is subsequently oxidized to 2-aminomuconic acid, and finally ammonia is released from 2-aminomuconic acid by a deaminase [11]. Two other strains of bacteria [25, 28], capable of converting HAB into aminophenols, as in the case of *P. pseudoalcaligenes*, are unable to grow on NB.

The sequence of reactions involved in the reduction of the nitro group into an amine produces highly reactive intermediates. The nitroso and hydroxylamino groups are electrophiles that can interact with biomolecules, causing toxic, carcinogenic, and mutagenic effects [3, 12, 13, 19, 23]. Nishino and Spain [20] reported that ring cleavage, which requires ferrous iron, produces a transient yellow product. Also, in the absence of NAD, the ring fission product is spontaneously converted into picolinic acid, which is not further metabolized. During the growth of the strain NB01 on NB, no accumulation of such metabolites was detected by HPLC, thereby suggesting the ultimate mineralization of NB.

The degradation of NB by the strain NB01 indicated Michaelis-Menten kinetics at NB concentrations below 1.8 mM, however, a certain growth inhibition was observed at over the above concentration. The specific removal rates (r, the amount of NB removed per g protein per h) obtained within the tolerable concentration ranges were plotted versus the corresponding NB concentration values, and the data were analyzed by Lineweaver-Burk equation (Fig. 4). As a result, the best fit for K_m and the maximum specific removal rate (r_{max}) were estimated as 0.33 mM and 11.04 h^{-1}, respectively. Very limited number of NB degradation kinetic studies have been reported, and Somerville *et al.* [27] reported an r_{max} value of 8.86 h^{-1} with a crude lysate of *P. pseudoalcaligenes* JS45.

Dickel *et al.* [7] attempted to treat NB-containing wastewater by converting NB into aniline under anaerobic conditions and then subsequently degrading the aniline under aerobic conditions. However, such a sequential anaerobic-aerobic process would not seem to be cost-effective compared to a single-stage process. Therefore, the higher r_{max} together with no accumulation of metabolites suggests that *M. chelonae* strain NB01 could be applied as a simple and effective treatment for NB-containing wastewater. In addition, the strain NB01 could also be effectively applied for the degradation of NB during a biofiltration process in conjunction with recent bioremediation technologies, including bioventing, biosparging, and biopiles.

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