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Characterization of Naphthalene-Degrading *Pseudomonas* Species Isolated from Pollutant-Contaminated Sites: Oxidative Stress During their Growth on Naphthalene

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Abstract Four naphthalene-degrading bacteria (*Pseudomonas* sp. strains O1, W1, As1, and G1) were isolated from pollutant-contaminated sites. Examination of their substrate utilization and analyses of key naphthalene-catabolic regulatory genes revealed that the pathway and regulation of naphthalene-degradation in all four strains resemble those of NAH7 from *P. putida* G7. Superoxide anion production, superoxide dismutase activity, and catalase activity during their growth on naphthalene-amended medium increased significantly, compared with those with glucose-amended medium. Addition of ascorbate, an antioxidant, or ferrous iron (Fe^{2+}) increased the growth rates of all tested microorganisms on naphthalene. Northern blot and HPLC analyses showed that both *nahA* gene expression and naphthalene degradation increased under those conditions. Our data suggest that naphthalene degradation can impose severe oxidative stress, and defenses against oxidative stress would play an important role in the metabolism of naphthalene.

Key words: Ascorbate, biodegradation, oxidative stress, salicylate, transcriptional regulation

The bacterial degradation of naphthalene has been extensively studied [17, 36, 38]. Many naphthalene-degrading bacteria possess an NAH-type plasmid that metabolizes naphthalene via salicylate to acetyl coenzyme A and pyruvate, although new isolates such as *Ralstonia* sp. strain U2 converts naphthalene to central metabolites via gentisate [9, 16, 25, 37]. All NAH catabolic plasmids studied to date encode a single upper operon (genes *nahA-nahF*) and a single lower operon (*nahG-nahM*). The upper operon is responsible for converting naphthalene to salicylate, and the lower operon encodes a pathway for salicylate metabolism, where salicylate is oxidized to catechol, followed by ring cleavage and conversion to TCA cycle intermediates [6, 22, 36].

Induction of both upper and lower operons is controlled by NahR, a LysR-type transcriptional factor. NahR, together with the inducer, salicylate, binds the promoter regions of both operons and activates transcription of *nah* operons [32]. The structure and function of *nahR* appear to be highly conserved among naphthalene-degrading bacteria isolated from a coal tar waste contaminated site [27]. Although the exact transcriptional mechanisms remain to be investigated, the NahR makes direct contact with RNA polymerase to initiate transcription [28]. Inefficient expression of naphthalene catabolic operons could contribute to the accumulation of toxic metabolites, which might decrease the survival rates of naphthalene-degrading bacteria in the laboratory and field conditions [29].

From enrichment culture with naphthalene crystals (0.5%), four naphthalene-degrading bacteria (*Pseudomonas* sp. strains O1, W1, As1, and G1) were isolated from different types of pollutant-contaminated sites (strain O1 from a gasoline-contaminated site in Seoul, South Korea, W1 from a wastewater reservoir in Seoul, As1 from an arsenic-contaminated site in Gwangju, Kyunggi-Do, and G1 from a gas station in Sungnam, Kyunggi-Do). Isolation of naphthalene-degrading bacteria has been described previously [15], except that we used 28°C and 2 days for the enrichment process. Cycloheximide (100 μg/ml) was added to the agar plate to suppress native soil fungi. They could grow on naphthalene and salicylate, but not on gentisate. These data suggest that all strains possess classical naphthalene pathway enzymes, which can degrade naphthalene via catechol into acetyl coenzyme A and pyruvate, in contrast to the recently discovered gentisate pathway [16]. We investigated their naphthalene catabolic genes using the restriction

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fragment length polymorphism (RFLP) analysis. The conserved regions of the known *nahG* genes encoding salicylate hydroxylase from *P. putida* NCIB 9816-4 (pDTG1), *P. fluorescens* PC20, and *Pseudomonas* sp. ND6 (GenBank accession numbers NC004999, AY887963, NC002947, and NC005244, respectively) were used to design the degenerate primer pair nahG-F/nahG-R (nahG-F, 5'-CCARCARGCGGGCSAGSAWGKAG -3'; nahG-R, 5'-GMTSCG YRRNCATGTRCTGSAAGG-3'), which were used for amplifying the *nahG*-like genes. Another primer pair, Ac114F/Ac596R (primer pairs as described previously) [35], was used for amplifying the *nahAc* gene, which encodes a naphthalene dioxygenase. The *nahG* promoter region and *nahR* from four naphthalene degraders were amplified using a degenerate primer pair (pro-R/onahR-R) previously described [28]. In restriction fragment length polymorphism (RFLP) analysis, a PCR fragment using the primer pair (pro-R/onahR-R) was digested with HaeIII and HhaI (Takara, Japan) and was separated on a 3% agarose gel. Consistent with this observation, RFLP analysis and sequencing have shown that partial naphthalene dioxygenase (*nahA*) and salicylate hydroxylase (*nahG*) genes, and the regulatory gene (*nahR*) and their promoters, are 100% identical to those of *P. putida* NCIB 9816-4 (data not shown). To determine the level of naphthalene catabolic transcripts, we performed Northern blot analysis as described previously [24]. The amount of *nahA* mRNA was determined by hybridizing the membrane with an *nahA*-specific [^{32}P]-labeled probe (Invitrogen) prepared by PCR amplification with a primer pair, nahA-F/nahA-R. The probe for *nahG* mRNA was prepared by PCR amplification with the primer pair, *nahG*-F/*nahG*-R. We found that the expression of naphthalene catabolic genes were higher during exponential growth than in stationary strain O1 cells with continuous naphthalene availability (Fig. 1). Glucose-grown strain O1 did not produce the *nahA* and *nahG* transcripts in both the exponential and stationary phases. The data presented here are consistent with RT-PCR data indicating expression of *nahAc* decreased in late-stationary phase cells. The *lux* reporter assay showed that *nahAc* expression decreased in the late-stationary phase, and salicylate degradation also decreased in the stationary phase [8]. Other strains showed the same results as strain O1 (data not shown). The data indicated that the pathway and regulation of naphthalene degradation in all four strains resemble NAH7 from *P. putida* G7.

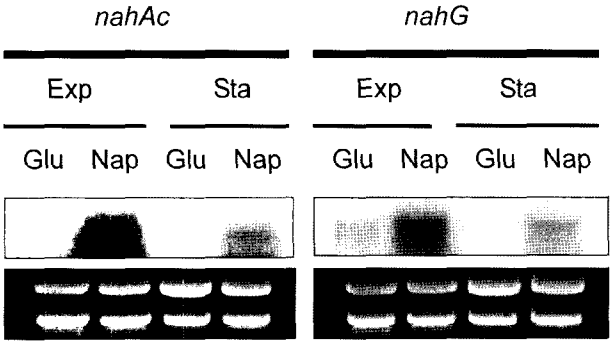

Fig. 1. Northern blot analysis of *nahAc* and *nahG* levels under various conditions.

A. Total RNA of strain O1 culture with either naphthalene or glucose (10 mM) was extracted at the early-exponential phase (OD_{600}~0.3) and stationary phase (16 h): Glu, glucose; Nap, naphthalene; Exp, exponential; Sta, stationary.

To determine their sequences of 16S rRNA genes, PCR amplification of 16S rRNA genes of the naphthalene-degrading bacteria were conducted using universal eubacterial primers (27f and 1492r) [35], except that annealing was performed at 55°C. The PCR fragment was ligated into the pGEM-T Easy vector (Promega, Madison, WI, U.S.A.) following the manufacturer's instructions. Analysis of their partial 16S rRNA sequences has shown that strain O1 is closely related (99% DNA identity) to phenol- and *p*-cresol-degrading *P. putida* PC16 (NCBI accession no. AY918067) isolated from phenol-contaminated areas, and strains W1 and G1 have 100% DNA identity with the 16S rRNA sequence of an ethylbenzene-degrading *P. veronii* CA-4 (NCBI access no. AY081814). The 16S rRNA sequence of strain As1 has 99% DNA identity with that of a PCB-degrading *P. frederiksbergenesis* JAJ28 (NCBI accession no. PFR249382) [2]. The nucleotide sequences of the 16S rRNA from four naphthalene degraders have been deposited in GenBank (accession numbers: DQ767827, O1; DQ767828, W1; DQ767829, As1; DQ767830, G1).

Growth of each strain was monitored by measuring the OD_{600} of the cultures with either naphthalene (crystals, 0.5%) or salicylate (2 mM). Glucose-cultured cells were collected in the stationary phase and then washed three times with autoclaved phosphate-buffered saline (PBS; pH 7.5). Approximately ~10^9 CFU/ml were inoculated into MSB [33] (50 ml) with appropriate carbon sources. The growth rates (h^{-1}) of strains O1, W1, As1, and G1 with naphthalene were 0.29±0.01, 0.28±0.03, 0.31±0.01, and 0.25±0.03, respectively. Although the growth rates of all other tested microorganisms were very similar, strain O1 had a shorter lag phase (~1 h) than the other strains (~5 h). The rate of naphthalene biodegradation was monitored using HPLC analysis, as previously described [28]. Their specific naphthalene degradation rates were very similar to each other (data not shown). The growth rate (h^{-1}) of strains O1, W1, As1, and G1 with salicylate (2 mM) were 0.42±0.04, 0.49±0.05, 0.49±0.01, and 0.42±0.03, respectively. Although strain O1 had a much shorter lag phase (~2 h) than the others (~7 h) in the presence of salicylate, the growth rates of all tested microorganisms were very similar (Fig. 2A). These are probably because their different genetic background, adaptation, and defense mechanisms against naphthalene and salicylate toxicities.

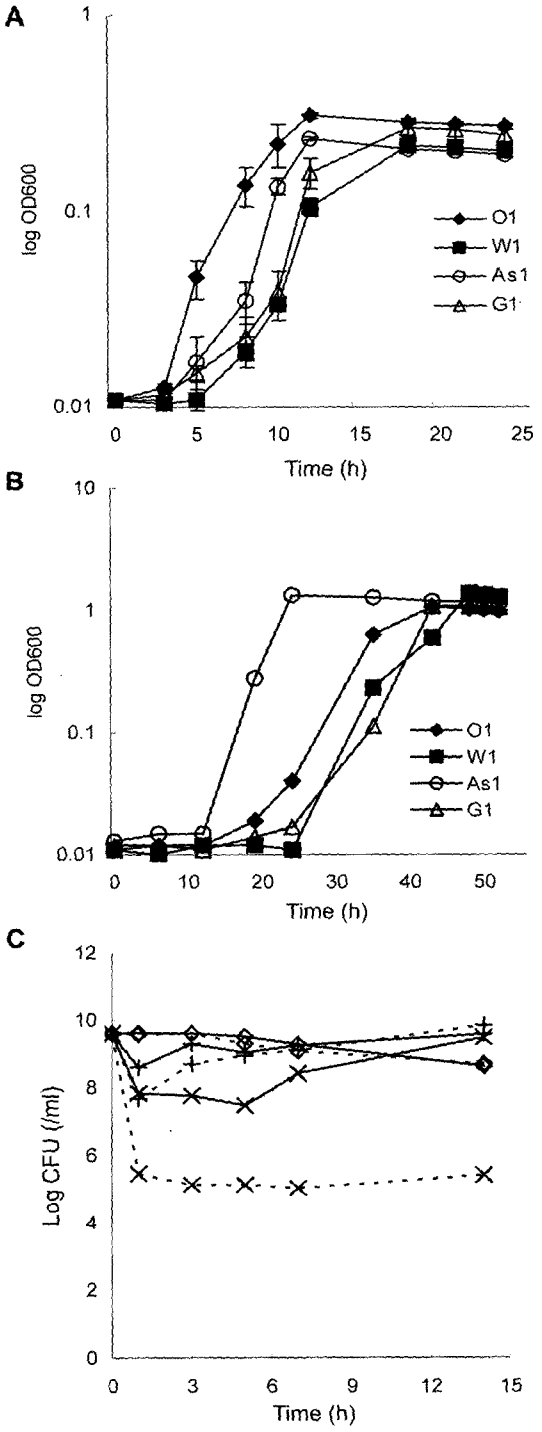

Fig. 2. A. Growth of naphthalene-degrading bacteria on MSB media including salicylate (2 mM). **B.** Growth characterization of naphthalene-degrading bacteria in high concentration of salicylate. Growth curve of each strain was monitored after inoculating 10^9 cells/ml to fresh MSB media amended with salicylate (10 mM) as a sole carbon source. **C.** Survival of strains O1 (---, open symbol) and As1 (—, closed symbol) with various concentrations of salicylate (0 mM, ◇; 2 mM, +; 10 mM, ×) at different time points.
One ml of cells from a 20-ml culture was harvested, serially diluted, and counted after plating on LB medium.

Interestingly, the growth rate of strain O1 was similar to that of strain As1 in the presence of salicylate (2 mM, Fig. 2A), but the growth rate of strain As1 ($0.55\pm0.04\ h^{-1}$) was much greater than that of strain O1 ($0.28\pm0.01\ h^{-1}$) in the presence of a high concentration of salicylate (10 mM; Fig. 2B). Strain O1 also had a longer lag phase than strain As1, suggesting that strain O1 was killed upon exposure to toxic levels of salicylate during its long lag phase. To determine viability after salicylate treatment, we measured the colony forming units by the following method: Glucose-cultured cells were collected in the stationary phase and then washed three times with autoclaved phosphate-buffered saline (PBS; pH 7.5). Approximately, 10^9 CFU/ml was inoculated into MSB (50 ml) containing different concentrations of salicylate and then incubated at 28°C with agitation (220 rpm). Cells at random time-points were harvested and washed with PBS. The harvested cells were diluted and serially plated on a Luria-Bertani (LB) agar plate. Agar plates were incubated, lid down, at 30°C for 15–20 h before colonies were counted. The result indicated that strain O1 had a significant reduction (4-log decrease) in viability upon exposure to a high concentration of salicylate (10 mM) compared with strain As1 (2-log decrease in viability) (Fig. 2C). High concentrations of salicylate cause the death of these *Pseudomonas* strains. The physiology and genetic background affects resistance to both substrates and metabolites. Higher tolerance to toxic substrates is an important issue in using microorganisms for pollutant biodegradation. Strain As1 will be a better candidate for degradation of toxic salicylate-like compounds.

In eukaryotic cells, naphthalene toxicity is associated with oxidative stress [12, 34]. Naphthalene and its metabolites also have a variety of effects on microbial cells [30, 34]. The toxicity of naphthalene and its metabolites could result in loss of viable cells both in liquid and soil conditions, probably because of their intrinsic toxicities and the reactive oxygen species (ROS) generated during its metabolism [29, 30]. Inefficient biodegradation of naphthalene occurred because of decreases in viable cell number [29]. Naphthalene could be toxic to naphthalene degraders under oxygen and nutrient deprivation [1, 11]. The relationship between ROS generation and naphthalene degradation has been poorly explored. We investigated oxidative stress during naphthalene degradation by measuring the amounts of superoxide and catalase activity. Many oxidative stress-generating agents increased expression of antioxidant genes including catalase [23, 26]. Bacterial cultures to oxidative stress exhibited an increase of catalase [14]. Superoxide anion production during their growth on naphthalene was evaluated using the nitroblue tetrazolium (NBT) test described previously [24]. Bacteria (1 ml) suspension (OD_{600}~0.2) was incubated with 0.5 ml of 1 mg/ml NBT for 30 min at 30°C. Then, 0.1 ml of 0.1 M HCl was added and the tubes were centrifuged at 1,500 ×*g* for 10 min. The separated pellets were treated

with 0.4 ml dimethyl sulfoxide (DMSO) to extract the reduced NBT; finally, 0.8 ml PBS (pH 7.5) was added and optical density was determined at 575 nm. Total protein concentration was measured using the Bradford assay [7]. A unit was defined as OD_{575}/mg protein and fold difference was calculated as (unit with naphthalene-cultured cells/unit with glucose-cultured cells). Superoxide anion production increased 7-fold during their growth on naphthalene-amended medium, compared with that with glucose-amended medium (Fig. 3A). Catalase activity was measured by monitoring the decrease at A_{240} resulting from the elimination of H_2O_2, using a UV visible spectrophotometer (Optizen 2120, Mecasys, Korea). The extinction coefficient (ε) for H_2O_2 at 240 nm was 43.6 $M^{-1}cm^{-1}$. The standard reaction mixture for the assay contained 50 mM potassium phosphate buffer (pH 7.2), 20 mM H_2O_2, and 20 µl of crude extracts for a total volume of 3.0 ml. The reaction was performed at 25°C. The amount of enzyme activity that decomposed 1 µmole of H_2O_2 per min was defined as 1 unit (U) of activity [3]. Total catalase activity of naphthalene-grown cells during their exponential phase (OD_{600}~0.2) was increased about 6-fold, whereas glucose-grown cells exhibited lower catalase activity (Fig. 3B) although their growth rates were slightly greater in the presence of glucose than in the presence of naphthalene [growth rate (h^{-1}) with glucose: O1, 0.4±0.03; W1, 0.32±0.02; AS1, 0.35±0.03; G1, 0.33± 0.04]. Treatment of bacterial cultures with salicylate (10 mM) also resulted in a higher level of catalase activity, compared with that with glucose-amended cultures (data not shown).

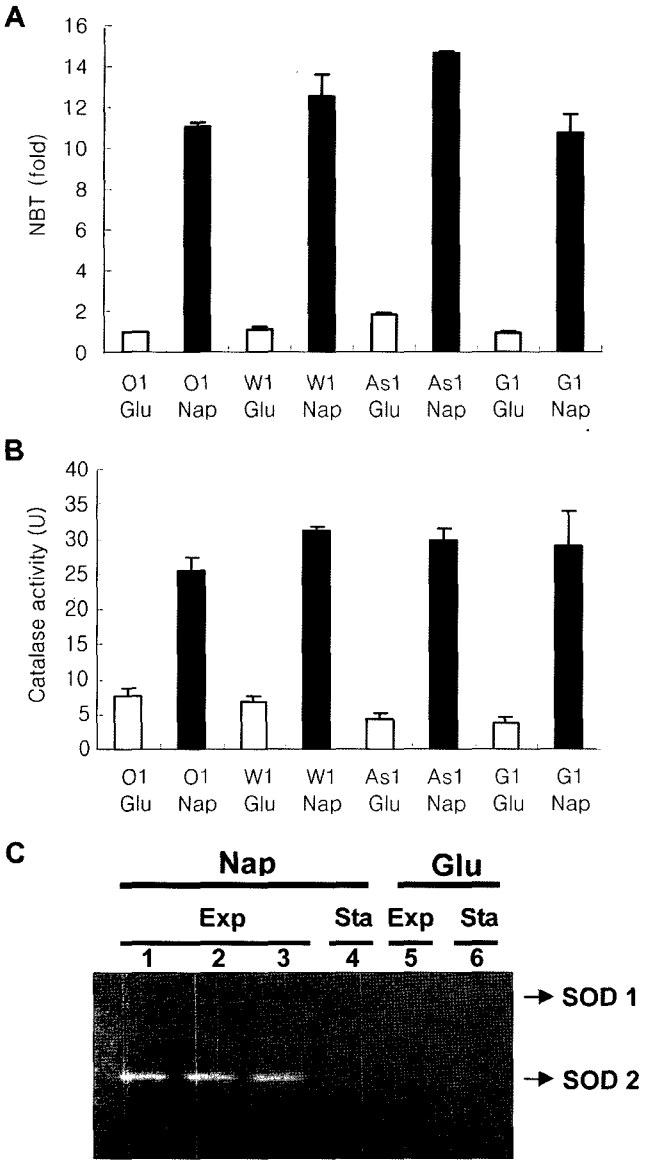

Fig. 3. Generation of superoxide anion during growth on either naphthalene or glucose (10 mM).

A. NBT assay of *Pseudomonas* sp. strains O1, W1, As1, and G1 grown in MSB media containing glucose (10 mM, white bar) or naphthalene (0.5%, black bar). **B.** Catalase activity of the four strains grown in MSB media containing glucose (10 mM, white bar) or naphthalene (0.5%, black bar). **C.** Superoxide dismutase activity in cell extracts from strain O1. Cell extracts (10 µg protein/lane) of strain O1 grown in MSB medium with either naphthalene or glucose as a carbon source were electrophoresed in 10% nondenaturing polyacrylamide gel and then negatively stained for the SOD activity. Either 0.2 mM ascorbate or 0.1 mM $FeSO_4$ was also added to the medium at the exponential phase when necessary. Lane 1, naphthalene (exponential); 2, naphthalene plus 0.2 mM ascorbate; 3, naphthalene plus 0.1 mM $FeSO_4$; 4, naphthalene (stationary); 5, glucose (exponential); 6, glucose (stationary): Glu, glucose; Nap, naphthalene; Exp, exponential; Sta, stationary.

The superoxide dismutase (SOD) activity assay was performed as described by Beauchamp and Fridovich [4]. Supernatants of cell extracts were loaded onto a 10% native polyacrylamide gel in a running buffer made of 25 mM Tris and 192 mM glycine. Twenty µg proteins were resolved at 20 mA for 2 h. Subsequently, the gels were processed for the SOD activity. The gels were first soaked in 2.5 mM NBT for 10 min in darkness under gentle shaking. They were then incubated in a 50 mM potassium phosphate buffer (pH 7.2) containing 28 mM TEMED and 28 µM riboflavin for 15 min, in the dark and under constant agitation. The SOD activity appeared as a white band on blue background gel. Crude extracts of strain O1 showed two types of the SOD, a higher molecular weight SOD (SOD 1) and a lower SOD (SOD 2) under naphthalene-amended media, but not under glucose-amended media (Fig. 3C). The effects of iron addition on the level of SOD expression were previously reported by Kim *et al.* [19]. They provided the evidence that the expression of FeSOD (SOD containing iron) was not influenced by iron availability. However, they established that the *sodB* gene, encoding iron-superoxide dismutase, of *Pseudomonas putida* was highly induced throughout exponentially grown cells in a medium supplemented with ferrous iron, but was downregulated in iron-deficient conditions, such as in stationary phase or 2,2-dipyridyl (iron chelator) treatment. Their observations indicated that iron plays an important role in the transcriptional regulation of the *sodB* gene. Consistent with their observation,

the activity of SOD in the present study was not influenced by iron addition although it is not straightforward why SOD activity remains the same in iron-amended condition.

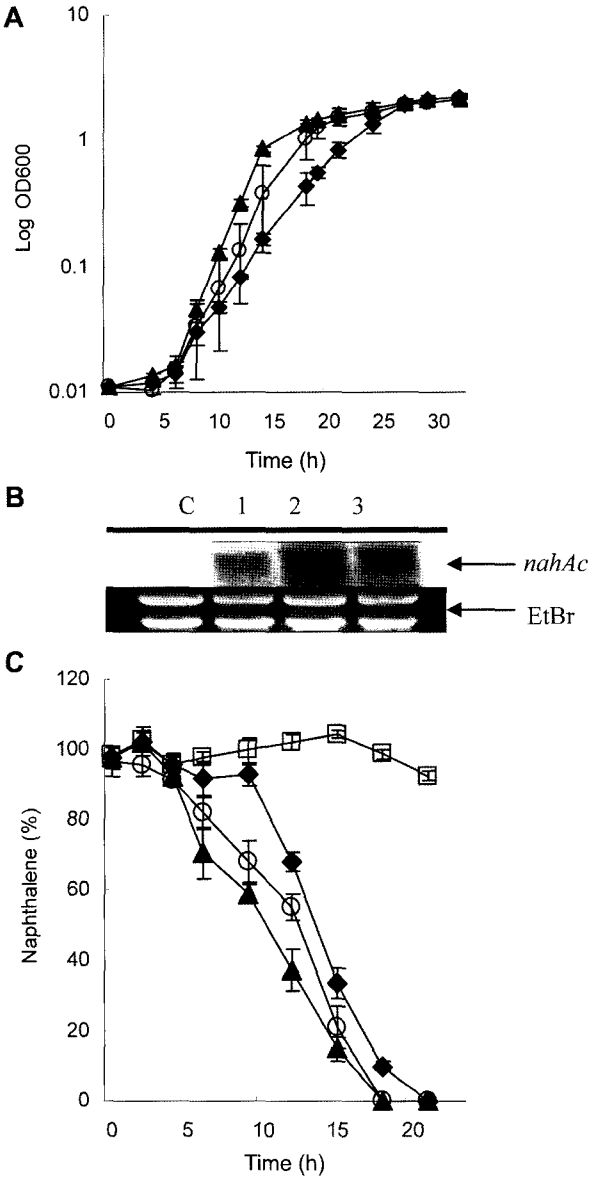

Fig. 4. Effect of ascorbate and ferrous iron on the growth, and expression of naphthalene catabolic genes of strain O1.

A. Growth of strain O1 was monitored in MSB plus naphthalene media amended without (closed diamond) or with either ascorbate (200 μM, open circle) or ferrous iron (100 μM, closed triangle). **B.** Each reagent was added at the incubation time of 8 h. Northern blot analysis was performed to measure the level of *nahAc* expression in various conditions. Total RNA was extracted in exponentially grown cells (OD_{600}~0.2) during the incubation condition described above (lane C, glucose; 1, naphthalene; 2, naphthalene plus ascorbate; 3, naphthalene plus ferrous iron). **C.** HPLC-based measurement of aqueous naphthalene during growth of strain O1 (closed diamond) in MSB media. Degradation of naphthalene during growth of strain O1 in the presence of ascorbate (200 μM, open circle) or ferrous iron (100 μM, closed triangle) was measured. Ascorbate or ferrous ion was added at the incubation time of 4 h; Control (□), no inoculum.

However, we have shown here that glucose-amended cells did not show any SOD activity (Fig. 3C). Interestingly, the stationary phase naphthalene-grown cells did not show any SOD activity (Fig. 3C). The data clearly suggested that naphthalene-amended cells experience more oxidative stress than glucose-amended cells. We tested the effects of the antioxidants, such as ascorbate or ferrous iron, on biodegradation of naphthalene in all four strains. Addition of ascorbate or ferrous iron (Fe^{2+}) to minimal media with naphthalene increased the growth rate of strain O1 dramatically (Fig. 4A; strain O1, 0.30±0.05 h^{-1}; strain O1 plus ascorbate, 0.50±0.02 h^{-1}; strain O1 plus ferrous iron, 0.55±0.04 h^{-1}). The growth rate of all strains on either naphthalene or salicylate was significantly enhanced by ascorbate (200 μM) or ferrous iron (Fe^{2+}; as 100 μM $FeSO_4$) (data not shown). Under oxidative stress conditions, iron in heme and iron-sulfur proteins can be oxidized, leading to protein inactivation [13]. Many iron-sulfur proteins are involved in naphthalene biodegradation [18]. Iron starvation leads to oxidative stress [10, 21]. Iron supplementation of growth media for the *sodAsodB* double mutant of *E. coli* increased the aerobic growth rate and diminished sensitivity toward Paraquat (PQ) [5]. Interestingly, iron taken into the cells from the medium is not available for Fenton chemistry, but is available for reconstitution of iron-sulfur clusters. Therefore, we conclude that ascorbate or ferrous iron helps cells diminish the effect of ROS generated during naphthalene metabolism.

Addition of either ascorbate or ferrous iron increased *nahAc* expression levels dramatically during growth of strain O1 on naphthalene (Fig. 4B). All RNA samples were collected at the exponential phase (OD_{600} ~0.2–0.3). The data indicated that the expression of *nahAc* is dependent not only on bacterial growth phase (Fig. 1) but also on their growth rate (Fig 4B). Along with the above results, HPLC analysis indicated that both ascorbate and iron addition accelerated the rate of naphthalene biodegradation (Fig. 4C). The data demonstrated that oxidative stress during naphthalene metabolism could be alleviated by addition of these antioxidants. We speculated that addition of antioxidants such as ascorbate or iron could provide protective effects on metabolic enzymes and alleviate energy burden for defense against oxidative stress during their growth on naphthalene. Thus, both the naphthalene biodegradation and growth rate are enhanced by addition of antioxidants. The higher level of the *nahAc* expression under these conditions could be explained by two ways: (1) a higher naphthalene-biodegradation might yield higher concentration of salicylate, which is known to be an inducer for naphthalene-catabolic genes [29]; (2) because it has been known that the *nahAc* expression is regulated by housekeeping σ^{70}, the relative amount of σ^{70} is much higher than that of other alternative sigma factors under these favorable conditions for bacterial growth [28]. This is the

first report to show that the growth rate and the expression of the pollutant-catabolic gene are greatly enhanced by antioxidants. We proposed, based on the above observation, that many organic pollutant compounds and their metabolites can impose oxidative stress on either indigenous microorganisms or introduced microorganisms used in bioremediation. This oxidative damage may decrease the activity and/or viability of cells in a polluted environment. Recent proteomics studies have shown that *P. putida* KT2440 increases production of oxidative stress defense proteins such as AhpC, SodB, and Dsb in response to environmental pollutants [20, 31]. Additional studies of the roles of oxidative stress genes in pollutant-degrading microorganisms could improve the survival rate of microorganisms in bioremediation processes.

Acknowledgments

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References

1. Ahn, I. S., W. C. Ghiorse, L. W. Lion, and M. L. Shuler. 1998. Growth kinetics of *Pseudomonas putida* G7 on naphthalene and occurrence of naphthalene toxicity during nutrient deprivation. *Biotechnol. Bioeng.* **59:** 587–594.
2. Andersen, S. M., K. Johnsen, J. Sorensen, P. Nielsen, and C. S. Jacobsen. 2000. *Pseudomonas frederiksbergensis* sp. nov., isolated from soil at a coal gasification site. *Int. J. Syst. Evol. Microbiol.* **50:** 1957–1964.
3. Beers, Jr. R. F. and I. W. Sizer. 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J. Biol. Chem.* **195:** 133–140.
4. Beauchamp, C. and I. Fridovich. 1971. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* **44:** 276–287.
5. Benov, L. and I. Fridovich. 1998. Growth in iron-enriched medium partially compensates *Escherichia coli* for the lack of manganese and iron superoxide dismutase. *J. Biol. Chem.* **273:** 10313–10316.
6. Bosch, R., E. Garcia-Valdes, and E. R. Moore. 2000. Complete nucleotide sequence and evolutionary implications of a chromosomally encoded naphthalene-degradation lower pathway from *Pseudomonas stutzeri* AN10. *Gene* **236:** 65–74.
7. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
8. Burlage, R. S., G. S. Sayler, and F. Larimer. 1990. Monitoring naphthalene catabolism by bioluminescence with nah-lux transcriptional fusions. *J. Bacteriol.* **172:** 4749–4757.
9. Eaton, R. W. 1994. Organization and evolution of naphthalene catabolic pathways: Sequence of the DNA encoding 2-hydroxy-chromene-2-carboxylate isomerase and trans-*o*-hydroxybenzylidenepyruvate hydratase-aldolase from the NAH7 plasmid. *J. Bacteriol.* **176:** 7757–7762.
10. Fereira, F. and N. A. Straus. 1994. Iron deprivation. *J. Appl. Phycol.* **6:** 199–210.
11. Garcia, E. M., I. G. Siegert, and P. Suarez. 1998. Toxicity assay and naphthalene utilization by natural bacteria selected in marine environments. *Bull. Environ. Contam. Toxicol.* **61:** 370–377.
12. Greene, J. F., J. Zheng, D. F. Grant, and B. D. Hammock. 2000. Cytotoxicity of 1,2-epoxynapthalene is correlated with protein binding and *in situ* glutathione depletion in cytochrome P4501A1 expressing Sf-21 cells. *Toxicol. Sci.* **53:** 352–360.
13. Imlay, J. A. 2003. Pathways of oxidative damage. *Annu. Rev. Microbiol.* **57:** 395–418.
14. Imaly, J. A. and S. Linn. 1987. Mutagenesis and stress responses induced in *Escherichia coli* by hydrogen peroxide. *J. Bacteriol.* **169:** 2967–2976.
15. Jeon, C. O., W. Park, W. C. Ghiorese, and E. L. Masden. 2004. *Polaromonas naphthalenivorans* sp. nov., a naphthalene-degrading bacterium from naphthalene-contaminated sediment. *Int. J. Sys. Evol. Microbiol.* **54:** 93–97.
16. Jones, R. M., B. Britt-Compton, and P. A. Williams. 2003. The naphthalene catabolic (nag) genes of *Ralstonia* sp. strain U2 are an operon that is regulated by NagR, a LysR-type transcriptional regulator. *J. Bacteriol.* **185:** 5847–5853.
17. Kahng, H. Y. and K. P. Nam. 2002. Molecular characteristics of *Pseudomonas rhodesiae* strain KK1 in response to phenanthrene. *J. Microbiol. Biotechnol.* **12:** 729–734.
18. Karlsson, A., J. V. Parales, R. E. Parales, D. T. Gibson, H. Eklund, and S. Ramaswamy. 2003. Crystal structure of naphthalene dioxygenase: Side-on binding of dioxygen to iron. *Science* **299:** 1039–1042.
19. Kim, Y. C., C. D. Miller, and A. J. Anderson. 1999. Transcriptional regulation by iron of genes encoding iron- and manganese-superoxide dismutases from *Pseudomonas putida*. *Gene* **239:** 129–135.
20. Krayl, M., D. Benndorf, N. Loffhagen, and W. Babel. 2003. Use of proteomics and physiological characteristics to elucidate ecotoxic effects of methyl tert-butyl ether in *Pseudomonas putida* KT2440. *Proteomics* **3:** 1544–1552.
21. Latifi, A., R. Jeanjean, S. Lemeille, M. Havaux, and C.-C. Zhang. 2005. Iron starvation leads to oxidative stress in *Anabaena* sp. strain PCC 7120. *J. Bacteriol.* **187:** 6596–6598.
22. Lee, D. S., M. W. Lee, S. H. Woo, and J. M. Park. 2005. Effects of salicylate and glucose on biodegradation of phenanthrene by *Burkholderia cepacia* PM07. *J. Microbiol. Biotechnol.* **15:** 859–865.
23. Lee, Y.-D., B.-Y. Moon, J.-P. Choi, H.-G. Chang, B.-S. Noh, and J.-H. Park. 2005. Isolation, identification, and characterization of aero-adaptive *Campylobacter jejuni*. *J. Microbiol. Biotechnol.* **15:** 992–1000.

24. Lee, Y., S. Pena-Llopis, Y. S. Kang, H. D. Shin, B. Demple, E. L. Madsen, C. O. Jeon, and W. Park. 2006. Expression analysis of the *fpr* (Ferredoxin-NADP+ reductase) gene in *Pseudomonas putida* KT2440. *Biochem. Biophys. Res. Commun.* **339:** 1246–1254.
25. Manohar, S., C. K. Kim, and T. B. Karegouder. 1999. Production of salicylic acid from naphthalene by immobilized *Pseudomonas* sp. strain NGK1. *J. Microbiol. Biotechnol.* **9:** 482–487.
26. McConmick, M. L., G. R. Buettner, and B. E. Britigan. 1998. Endogenous superoxide dismutase levels regulate iron-dependent hydroxyl radical formation in *Escherichia coli* exposed to hydrogen peroxide. *J. Bacteriol.* **180:** 622–625.
27. Park, W., P. Padmanabhan, S. Padmanabhan, G. J. Zylstra, and E. L. Madsen. 2002. *nahR*, encoding a LysR-type transcriptional regulator, is highly conserved among naphthalene-degrading bacteria isolated from a coal tar waste-contaminated site and in extracted community DNA. *Microbiology* **148:** 2319–2329.
28. Park, W., C. O. Jeon, and E. L. Madsen. 2002. Interaction of NahR, a LysR-type transcriptional regulator, with the α-subunit of RNA polymerase in the naphthalene degrading bacterium, *Pseudomonas putida* NCIB 9816-4. *FEMS Microbiol. Lett.* **213:** 59–165.
29. Park, W., C. O. Jeon, H. Cadillo, C. DeRito, and E. L. Madsen. 2004. Survival of naphthalene-degrading *Pseudomonas putida* NCIB 9816-4 in naphthalene-amended soils: Toxicity of naphthalene and its metabolites. *Appl. Microbiol. Biotechnol.* **64:** 429–435.
30. Price, C. T. D., I. R. Lee, and J. E. Gustafson. 2000. The effects of salicylate on bacteria. *Int. J. Biochem. Cell Biol.* **32:** 1029–1043.
31. Santos, P. M., D. Benndorf, and I. Sa-Correia. 2004. Insights into *Pseudomonas putida* KT2440 response to phenol-induced stress by quantitative proteomics. *Proteomics* **4:** 2640–2652.
32. Schell, M. A., P. H. Brown, and S. Raju. 1990. Use of saturation mutagenesis to localize probable functional domain in the NahR protein, a LysR-type transcriptional activator. *J. Biol. Chem.* **265:** 3844–3850.
33. Stanier, R. Y., N. J. Palleroni, and M. Dudorhoff. 1966. The aerobic pseudomonads: A taxonomic study. *J. Gen. Microbiol.* **43:** 159–271.
34. Stohs, S. J., S. Ohia, and D. Bagchi. 2002. Naphthalene toxicity and antioxidant nutrients. *Toxicology* **180:** 97–105.
35. Wilson, M. S., C. Bakermans, and E. L. Madsen. 1999. *In situ*, real-time catabolic gene expression: Extraction and characterization of naphthalene dioxygenase mRNA transcripts from groundwater. *Appl. Environ. Microbiol.* **65:** 80–87.
36. Yen, K. M. and C. M. Serdar. 1988. Genetics of napthalene catabolism in pseudomonads. *CRC Crit. Rev. Microbiol.* **15:** 247–268.
37. Zhou, N.-Y., S. L. Fuenmayor, and P. A. Williams. 2001. *nag* Genes of *Ralstonia* (formerly *Pseudomonas*) sp. strain U2 encoding enzymes for gentisate catabolism. *J. Bacteriol.* **183:** 700–708.
38. Zylstra, G. J. and J. J. Dennis. 2004. Complete sequence and genetic organization of pDTG1, the 83 kilobase naphthalene degradation plasmid from *Pseudomonas putida* strain NCIB 9816-4. *J. Mol. Biol.* **341:** 753–768.