Journal of Microbiology and Biotechnology
Vol. 3, No. 4, 252-255, 1993

n-Alkane Utilizing Capability and Location of the Genes for Alkane Hydroxylases in *Pseudomonas maltophilia* N246

CHOI, SOON-YOUNG, MYUNG-HYE LEE, MOON-OK HWANG AND KYUNG-HEE MIN*

Department of Biology, Sookmyung Women's University, Seoul 140-742, and Research Center for Molecular Microbiology, Seoul National University, Seoul 151-742, Korea

***Pseudomonas maltophilia* N246 carrying on OCT plasmid grew on n-alkanes of 6 to 14 carbon atoms, but not on n-alkanes of more carbon atoms. *P. maltophilia* strains with and without OCT plasmid could utilize primary alcohols, aldehydes and fatty acids derived from n-alkane. The N246 strain could also utilize monocarboxylic and dicarboxylic acids, and terminal branched dimethyloctane. Unlike the genes of alcohol dehydrogenase and aldehyde dehydrogenase which were located on both the chromosome and the OCT plasmid, genes for the alkane hydroxylase components were located only on the OCT plasmid in *P. maltophilia* N246.**

Some strains of *Pseudomonas* can utilize n-alkanes as a sole carbon source by means of an initial oxidation of a terminal methyl group followed by oxidation of fatty acids (14,7). *Pseudomonas putida* strains which carry transmissible OCT plasmid can grow on n-alkanes of 6 to 10 carbon atoms (7). This substrate range is strain specific according to the capabilities of the OCT plasmid.

Alkanes, primary alcohols, and other compounds including dicyclopropyl ketone (DCPK) induce whole-cell alkane-oxidizing activity in a *P. putida* strain carrying plasmids (4). Whole cell alkane-oxidizing activity is inducible in *P. aeruginosa* (14) as well as in other alkane-utilizing bacteria (10). In *P. aeruginosa*, the effective inducers include alkane growth substrates, aliphatic diols, and dicyclopropyl compounds (15). Grund *et al.* (4) indicated that the OCT plasmid in *P. putida* codes for inducible alkane-hydroxylating and primary alcohol dehydrogenase activities and that the chromosome codes for constitutive oxidizing activities for primary alcohols, aliphatic aldehydes, and fatty acids.

Recently, the cloning of *alk*BAC operon (3,8) was described. The *alk*BAC operon contains the structural genes of membrane alkane hydroxylase component (*alk*B), soluble alkane hydroxylase component (*alk*A), and membrane alcohol dehydrogenase (*alk*C).

We have previously reported that *P. maltophilia* N246 carried on OCT plasmid which utilizes n-alkane, and that the OCT plasmid was about 118kb long (2).

This report describes the n-alkane metabolism by *Pseudomonas maltophilia* N246 and the location of the genes of alkane hydroxlase components.

MATERIALS AND METHODS

Bacterial Strain

Pseudomonas maltophilia N246 carrying OCT plasmid was isolated from sea water by n-alkane enrichment culture (2).

Media and Culture

Growth experiments were conducted using a minimal medium (Na_2HPO_4 6 g, KH_2PO_4 3 g, NaCl 0.5 g, NH_4Cl 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, and $CaCl_2 \cdot 2H_2O$ 0.015 g per liter; pH 7.4). Maintenance cultures were grown on a complete medium (tryptone 10 g, yeast extract 5 g, and NaCl 5 g per liter). Inocula were prepared by transferring a loopful of cells from the agar slant culture (complete medium) to a flask containing minimal medium with various concentrations of hydrocarbons. Incubation was carried out overnight at 30℃ with agitation (180 rpm).

Chemicals

Alkanes, aliphatic alcohols, aldehydes, and fatty acids with a purity of over 98%, except for 1,6-hexanediol

*Corresponding author
Key words: alkane hydroxylase, OCT plasmid, *Pseudomonas maltophilia*

and undecanoic acid which were 97% pure, were purchased from Sigma Chemical Co. and Fluka Chemical Co.

Growth Tests

All growth tests were carried out on basal minimal plates, and the soluble substrates were added at the concentrations as indicated in the tables. Growth with hydrocarbons was determined after replica-plating the dense patches of bacterial growth from the complete medium. The hydrocarbon substrate was added by placing a few drops on a piece of filter paper in the lid of petri dish. The plates were incubated at 30℃ for 48 hr in sealed tins. Due to the toxicity of aliphatic alcohols and aldehydes, the amount of substrate allowed per plate was limited to 0.02~0.05 ml.

DNA Isolation and Curing Experiment

DNA isolation and plasmid curing with mitomycin C were carried out by the method of Choi et al (1991). All of the DNAs from the strain carrying the OCT plasmid (N246) or the cured strain (N246-0) were isolated by the alkaline disruption method described elsewhere (12). The purified DNAs were separated on a 0.8% agarose gel by electrophoresis.

Fractionation of Supernatant and Cell Membrane

Cell culture was carried out in basal minimal medium containing 0.05% yeast extract plus 0.5% octane. Cells were harvested by centrifugation for 15 min at 10,000 rpm in a microcentrifuge at 0℃. The supernatant (S) was used for extracellular soluble fractionation. The cell pellet was washed twice with 0.02 M Tris-HCl (pH 7.8), and resuspended in the same buffer supplemented with 0.067 M dithioerythritol. Cell-free extracts were prepared by a sonification of the above suspension with a sonicator Model 300 (Fisher) for 30 min at 0 to 4℃. The disrupted cell suspensions were centrifuged at 15,000 g for 30 min to produce supernatant S1 and pellet R. All centrifugations were performed at 0℃ (9).

Determination of Enzyme Activities

Alcohol dehydrogenase and aldehyde dehydrogenase activities were measured by the method of Parekh *et al* (9). Enzymes were assayed spectrophotometically by measuring changes in the absorbance of pyridine nucleotides at 340 nm with a Perkin-Elmer UV-VIS model 552 spectrophotometer. One unit of enzyme was defined as the quantity of enzyme required to cause a change in an optical density of 0.01 per min.

RESULTS AND DISCUSSION

Growth of *P. maltophilia* N246 on Alkanes and Their Oxidative Products

P. maltophilia N246 can grow on n-alkanes of 6 to 14 carbon atoms, but not on n-alkanes of more carbon atoms (Table 1). It was found that the N246 strain carried OCT plasmid (2). As shown in Figure 1, however, we made a strain N246-0 that does not carry the OCT plasmid by curing (2). As we expected, n-alkanes did not support the growth of *P. maltophilia* N246-0 (Table 1). Therefore,the use of n-alkane by strain N246 depends on whether the strain has an OCT plasmid or not. This n-alkane growth pattern is different from that of *P. putida PpG6* (7), indicating that the OCT plasmid of N246 has different characteristics from the plasmid present in PpG6 strain.

P. maltophilia N246 and N246-0 grew on primary alcohols, aldehydes and fatty acids derived from n-alkanes. But it did not grow on 1,6-hexanediol, adipic acid, heptanal, pimelic acid and 2-octanone (Table 2), and

Table 1. Growth ability of *P. maltophilia* strains N246 and N246-0 on n-alkane components as sole carbon source

Strain	No. of carbon atoms in the alkane chain										
	6	7	8	9	10	11	12	13	14	15	16
N246⁺	+	+	+	+	+	+	+	+	+	−	−
N246-0⁺⁺	−	−	−	−	−	−	−	−	−	−	−

⁺Strain N246 carries OCT plasmid. ⁺⁺Strain N246-0 is a derivative strain of N246 which had been cured of OCT plasmid. Growth test was determined from three replicate experiments.

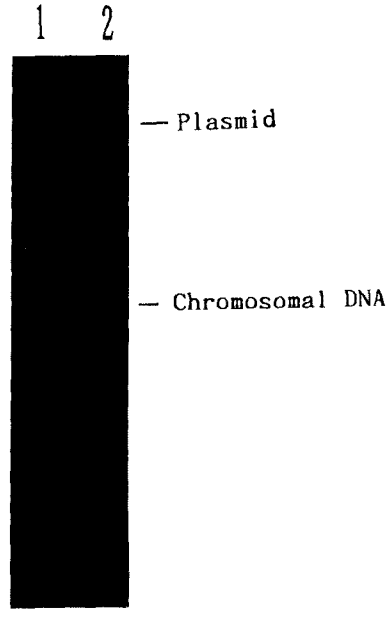

Figure 1. Plasmid pattern from *P. maltophila* N246 (lane 2) and the cured strain (N246-0; lane 1).

Table 2. Growth of *P. maltophilia* N246 on alkane oxidation products

Carbon sources	Growth
Hexane (C_6)	+
Hexanol	+*
1-Hexanal	+*
Hexanoic acid (20 mM)	+*
1,6-Hexanediol (20 mM)	−
Adipic acid (20 mM)	−
2-Hexanone	+
Heptane (C_7)	+
1-Heptanol	+*
Heptanal	−
Heptanoic acid (20 mM)	+
Pimelic acid (20 mM)	−
2-Heptanone	+
Octane (C_8)	+
1-Octanol	+*
Octanal	+*
Octanoic acid (20 mM)	+
2-Octanone	−
Nonane (C_9)	+
1-Nonanol	+*
Nonanal	+
Nonanoic acid (20 mM)	+

+: All substrates were added in the vapor phase except 1,6-hexanediol, adipic acid, pimelic acid, and other fatty acids which were incorporated directly in minimal agar. *: Toxic substrates were added at very low concentrations.
Growth test was determined from three replicate experiments. Symbols: +, growth; −, no growth.

this may be because *P. maltophilia* cannot take up these substrates. On the other hand, *P. putida* PpG6 also grew on primary alcohols, aldehydes, and monocarboxylic acids (7). Taken together, these data suggest that the OCT plasmid of *P. maltophilia* N246 codes for the alkane hydroxylase components.

Utilization of Monocarboxylic and Dicarboxylic Acids.

The observation that the alkane hydroxylase complex of *Pseudomonas* strains can hydroxylate fatty acids (5, 6, 11) suggested that the strains with alkane hydroxylase activity may utilize n-alkane during fatty acid oxidation as an initial step in the formation of monocarboxylic or dicarboxylic acids.

In this study *P. maltophilia* N246 utilized both monocarboxylic and dicarboxylic acids (Table 3). This result agrees with the fact that *P. putida* PpG6 (7) and *P. aeruginosa* (1) could utilize monocarboxylic and dicarboxylic acids. Schaeffer *et al.* (13) devised screening procedures to evaluate alkane-utilizing bacteria which oxidizes 2,7-dimethyloctane (2,7-DMO) and 2,6-dimethyloctane (2,6-DMO) as the sole carbon source. And their isolated bacteria could oxidize branched-chain alkanes. In our study, *P. maltophilia* N246 could also utilize 2,6-DMO as terminal branched dimethyloctane derivatives (Table 3).

Table 3. Growth of *P. maltophilia* N246 on monocarboxylic or dicarboxylic acids, and terminal branched dimethyloctane

Carbon Sources (0.3%)	*P. maltophilia* N246
Monocarboxylic acid	
Palmitic acid	+
Butyric acid	+
Acetic acid	+
Dicarboxylic acid	
Glutaric acid	+
Succinic acid	+
Malonic acid	+
Glyoxylate	+
2,6-Dimethyloctane	+

Symbols; +, growth, Growth test was determined from replicate experiments.

Table 4. Compartment of alcohol dehydrogenase and aldehyde dehydrogenase in cell-free preparation from *P. maltophilia* N246 and N246-0

Fraction		Specific enzyme activities ($\times 10^2$ u/min/mg protein)			
		Alcohol dehydrogenase		Aldehyde dehydrogenase	
		N246	N246-0	N246	N246-0
Soluble	(S)	0.9± 0.2	0.8± 0.3	1.0± 0.2	0.9± 0.2
Particulate	(S1)	2.1± 0.1	1.0± 0.0	1.6± 0.3	1.4± 0.1
	(R)	3.5± 0.3	1.0± 0.6	2.0± 0.1	1.6± 0.4

Culture condition was carried out in basal minimal medium containing 0.05% of yeast extract plus 0.5% octane as Materials & Methods. Mean values from three replicate determinations are shown ± SEM.
(S); Supernatant of extra cellular fraction, (S1); Supernatant after centrifugation of disrupted cell-free extract, (R); Pellet after centrifugation of disrupted cell-free extract.

Location of Genes of Alkane Hydroxylase Components

To determine whether genes of the enzymes are located on the OCT plasmid or on the chromosome, the activities of the alcohol dehydrogenase and aldehyde dehydrogenase in soluble and membrane fraction were measured. As shown in Table 4, the activities of both enzymes were present even in the absence of the OCT

plasmid, indicating that genes of these enzymes may be located on both the plasmid and the chromosome. Interestingly, cells carrying the OCT plasmid had a higher alcohol dehydrogenase activity compared to those without the plasmid. This could mean that the N246 strain contains two genes of each enzyme.

Unlike the genes of alcohol dehydrogenase and aldehyde dehydrogenase, genes of the alkane hydroxylase components were located only on the OCT plasmid. This is because, as shown in Table 1, only the cells carrying the OCT plasmid in N246 strain could grow on a medium containing n-alkane components as the sole carbon source. Thus, it was found that the OCT plasmid carries genes of the alkane hydroxylase components. Recently, we have cloned and determined the sequence of the genes encoding the alkane hydroxylase components from OCT plasmid (unplished results).

Acknowledgement

This work was supported by the KOSEF research grant for SRC (Research Center for Molecular Microbiology, Seoul National University).

REFERENCES

1. **Ali Khan, M.Y., A.N. Hall and D.S. Robinson.** 1964. Products of the oxidation of selected alkanes by a gram-negative bacterium. Antonie van Leeuwenhoek. *J. Microbiol. Serol.* **30**: 417-427.
2. **Choi, S.Y., C.S. Kim, M.H. Lee, M.O. Hwang and K.H. Min.** 1991. Octane biodegradability by crude oil-utilizing bacteria carrying OCT plasmid. *Kor. J. Appl. Microbiol. Biotechnol.* **19**: 82-87.
3. **Eggink, G., R.G. Lageveen, B. Alterburg and B. Witholt.** 1987. Controlled and functional expression of the *Pseudomonas oleovorans* alkane utilizing system in *Pseudomonas putida and Escherichia coli. J. Biol. Chem.* **262**: 17712-17718.
4. **Grund, A., J.A. Shapiro, M. Fennewald, P. Bacho, J. Leahy, K. Markbreiter, M. Nieder and M. Toepier.** 1975. Regulation of alkane oxidation in *Pseudomonas putida. J. Bacteriol.* **123**: 546-556.
5. **Kusunose, M., E. Kusunose and M.J. Coon.** 1964. Enzymatic *w*-oxidation of fatty acids: II. Substrate specificity and other properties of the enzyme system. *J. Biol. Chem.* **239**: 2135-2139.
6. **Mckenna, E.J. and M.J. Coon.** 1970. Enzymatic *w*-oxidation. *J. Biol. Chem.* **245**: 3882-3889.
7. **Nieder, M. and J.A. Shapiro.** 1975. Physiological function of the *Pseudomonas putida* PpG6 alkane hydroxylase: Monoterminal oxidation of alkanes and fatty acids. *J. Bacteriol.* **122**: 93-98.
8. **Owen, D.J., G. Eggink, B. Hauer, M. Kok, D.L. McBeth, Y.I. Yang and J.A. Shapiro.** 1984. Physical structure, genetic contents, and expression of the *alk*BAC operon. *Mol. Gen. Genet.* **197**: 373-383.
9. **Parekh, V.R., R.W. Traxler and J.M. Sobek.** 1977. n-alkane oxidation enzymes of a Pseudomonad. *Appl. Envir. Microbiol.* **33**: 881-884.
10. **Perry, J.J. and H.W. Scheld.** 1968. Oxidation of hydrocarbons by microorganisms isolated from soil. *Can. J. Microbiol.* **14**: 403-407.
11. **Peterson, J.A., M. Kusunose, E. Kusunose and M.J. Coon.** 1967. Enzymatic *w*-hydroxylation. *J. Biol. Chem.* **242**: 4334-4340.
12. **Sambrook, J., E.F. Fritsh and T. Maniatis.** 1989. Molecular cloning: A Laboratory Manual. Cold Spring Harbor.
13. **Schaeffer, T.L., S.G. Cantwell, J.L. Brown, D.S. Watt and R.R. Fall.** 1979. Microbial growth on hydrocarbons: Terminal branching inhibits biodegradation. *Appl. Envir. Microbiol.* **38**: 742-746.
14. **Thijsse, G.J. E. and A.C. van der Linden.** 1958. n-Alkane oxidation by a *Pseudomonas*: Studies on the intermediate metabolism. Antonie van Leeuwenhock. *J. Microbiol. Serol.* **24**: 298-308.
15. **Van Eyk, J. and T.J. Bartels.** 1968. Paraffin oxidation in *Pseudomonas aeruginosa*. I. Induction and paraffin oxidation. *J. Bacteriol.* **96**: 706-712.

(Received October 21, 1993)