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Physical Structure and Expression of *alk*BA Encoding Alkane Hydroxylase and Rubredoxin Reductase from *Pseudomonas maltophilia*

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The structural genes of the *Pseudomonas maltophilia alk* system, which are localized on the OCT plasmid were cloned as a 4.2-kilobase pair Hind III fragment. This fragment contains sequences for alkane hydroxylase gene (*alk*B) and rubredoxin reductase gene (*alk*A), respectively. The *alk*B gene encodes a 373-amino acid polypeptide (47.4 kD) that can be expressed at high levels in *Pseudomonas* and *Esherichia coli*. The *alk*BA genes were complemented with alkane hydroxylation in both bacteria. This result shows that *alk*BA gene is essential for alkane hydroxylation since chromosomal loci have been encoded for other enzymes involved in fatty acid oxidation.

Several strains of *Pseudomonas* are able to utilize *n*-alkanes as the sole carbon and energy source by virtue of the OCT plasmid-encoded alkane hydroxylase (1, 2). The gene clusters on the plasmid were found at the *alk*BAC operon that encodes for structural proteins and the *alk*R region that regulates positively their expression in the presence of an inducer, *n*-otane or dicyclopropylketon (3, 4, 5).

The *alk*BAC operon contains the structural genes for membrane alkane hydroxylase (*alk*B), soluble rubredoxin reductase (*alk*A), and membrane alcohol dehydrogenase (*alk*C)(6). However, it is not essential that *alk*BAC operon codes for an alcohol dehydrogenase since this enzyme is also encoded chromosomally. In addition, several chromosomal loci have been encoded for aldehyde dehydrogenase and other enzymes involved in fatty acid oxidation (7).

The cloning of *alk*BAC operon was reported to show the physical structure, genetic content, and expression of the operon (8). Physical mapping of *alk*C::T7 insertion and complementation of *alk*C point mutations with cloned sequences of the *alk*BA fragment were analysed. The operon is transcribed in the order of *alk*BAC under positive regulation by *alk*R regulatory function.

The *alk*BAC operon was cloned as a 16.9-Kb EcoRI fragment and encodes at least six peptides. The *alk*BAC transcript was measured by R-loop electron microscopy (9). Synthesis of *alk*BAC peptides results in a fully functional alkane hydroxylation system in both *Pseudomonas putida* and *Esherichia coli*, and expression of the *alk*BAC operon is strictly dependent on the presence of *alk*R region and inducer (10). The OCT plasmid-encoded alkane hydroxylase gene (*alk*B) and its promoter were identified and sequenced. The transcription initiation site of the *alk*BAC mRNA was determined by nuclease SI mapping (11). The *Pseudomonas oleovorans alk*BAC operon encodes seven proteins, of which at least three are involved in alkane hydroxylase (*alk*BA) and alkanol dehydrogenase (*alk*C) activities. The *alk*A region contains three sequences encoding two related rubredoxins (*alk*F and *alk*G) and aldehyde dehydrogenase (*alk*H) (12).

We have cloned the *alk*BA region from the OCT plasmid of *Pseudomonas maltophilia.* In this paper we present the nucleotide sequence of the *alk*BA region from the OCT plasmid and the resulting model for the structure of the *alk*BA region involved in alkane hydroxylase (*alk*B) and rubredoxin reductase (*alk*A).

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The *alk*BA is expressed fully in *E. coli* since several chromosomal loci have been encoded for alcohol dehydrogenase, aldehyde dehydrogenase, and other sequential enzymes involved in fatty acid oxidation (13). Therefore, only the *alk*BA region is essential for bacterial growth on *n*-alkane since the *alk*BA region was fully expressed in *Pseudomonas maltophilia* and *E. coli.*

MATERIALS AND METHODS

Bacterial strains, plasmids and cultural conditions. Bacterial strains and plasmids are listed in Table 1. The petri-dishes of basal minimal medium (16) were incubated at 30°C in a sealed container under saturated *n*-octane vapor for cell growth. In order to induce alkane hydroxylase, cells were precultured on the basal minimal medium supplemented with 0.2% pyruvate as the carbon source. After shaking cultures at 37°C for 2–3 hours, dicyclopropylketone (DCPK; Janssen Chimica) was added to a concentration of 0.05% (v/v) as inducer (17). Cultures were grown until the end of exponential phase.

Assay of alkane hydroxylase. Cells were harvested by centrifugation at 5,000rpm and resuspended in 20mM Tris-HCl buffer (pH7.4), disrupted using ultrasonic disintegrator (Fisher model 300), and centrifuged for 10 minutes at 7,000rpm. The cell-free supernatant was assayed for alkane hydroxylase activity.

The alkane hydroxylase assay was carried out according to modified methods of Nieboer *et. al.* (18) and Peter & Witholt (19). The reaction mixture consisted of 20mM Tris-HCl and 0.15% CHAPS buffer (pH7.4), 0.1mM NADH, 10μl of octane solution (1% octane in 80% DMSO), and 100 μl crude extract in 1ml volume. To start the reaction, 10 μl of octane solution was added to the reaction mixture. Enzyme activity was measured by decrease in absorbance at 340nm of NADH on spectrophotometer. One unit of alkane hydroxylase activity corresponds the amount of enzyme which oxidizes 1 μmol NADH per minute.

Protein concentrations were determined according to the method of Lowry *et al.*(20) using bovine serum albumin for the standard curve.

DNA manipulation. Plasmid extractions were done according to Birnbolm and Doly(21). DNA was resolved on 0.7% agarose gel with TAE buffer by electrophoresis, and visualized by staining with ethidium bromide. DNA digestion with restriction enzyme, and ligation with T4 ligase were performed according to the supplier's directions (Boehringer Mannhein Biochemicals). DNA transformation was accomplished by the calcium chloride method (22).

DNA sequencing. DNA sequencing was done with the dideoxynucleotide chain termination method according to Sanger *et al.*(23). The sequence strategy is shown in Fig. 1. The Hind III fragment of pPOC122 was used as the source of DNA. The fragments were digested with HindIII, EcoRI, XbaI, BamHI, PstI, and Kpn I and were subsequently inserted into the M13 vectors, M13mp18 and M13mp19. Unlabeled deoxynucleotide triphosphates were purchased from Pharmacia LKB Biotechnology Inc. and dideoxynucleotides as well as deaza-dGTP were obtained from Boehringer Mannheim. Analysis of the nucleotide sequence and comparison of the primary amino acid sequence of alkane hydroxylase and rubredoxin reductase with sequences of other proteins was done with the PC/GENE software package.

RESULTS AND DISCUSSION

Nucleotide sequence of the alkBA cistrons. Bacterial growth on alkanes requires the plasmid-borne *alk* system in addition to a functional chromosomally-encoded fatty acid degradation system(10, 5). From a gene bank of the OCT plasmid DNA of *P. maltophilia* N246, established in the vector pUC19, a 4.2-kb HindIII fragment containing a sequence relevant to alkane oxidation was isolated according to complementation growth on basal minimal medium plus *n*-octane(13). It was designated pPOC122. Fig. 1 shows a restriction map of the 4.2-kb HindIII fragment.

TABLE 1
Bacterial Strains and Plasmids

Strain, plasmid	Relevant genotype or phenotype	Source or reference
P. maltophilia N246	OCT plasmid	Choi *et al.* (14)
E. coli		
NM522	*sup*E *thi*Δ(*lac-pro*AB) *hsd5* F′ [*pro* AB$^+$ *lac* I ^{q}*lac*ZΔM15]	Gough and Murray (15)
SUP2	*alk*BA	Hwang *et al.* (13)
Plasmid		
pPOC122	*alk*BA, Ap^r	This study
M13mp18	Ap^r	
M13mp19	Ap^r	

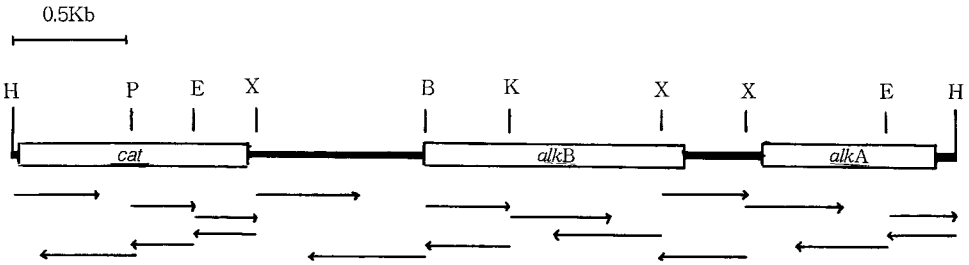

FIG. 1. Restriction map of the cloned *alk*BA fragment and sequencing strategy of *alk* genes in pPOC122. Restriction sites are *Hind*III(H), *Eco*R I(E), *Bam*H I (B), *Pst* I (P), *Kpn* I (K), and *Xba* I (X). The *cat*, *alk*B, and *alk*A represent genes coding for chloramphenicol acetyltransferase, alkane hydroxylase, and rubredoxin reductase, respectively.

The nucleotide sequences of *alk*BA region were determined by Sanger method, submitted to Genbank with accession numbers of U40233 for *alk*B and U40234 for *alk*A, and analyzed. The *alk*B sequence was composed of 1119 nucleotides, which exhibited 62.9% homology with the corresponding sequence for alkane hydroxylase of *Pseudomonas oleovorans*(11). A long open reading frame of *alk*B, starting with an ATG codon at position 67 and ending with a TAA codon at position 1185 was identified. A putative ribosome-binding sequence with 5′-AGGA-3′ was identified at approximately 21 nucleotides upstream from the start to the open reading frame. This open reading frame can encode a polypeptide of molecular weight 47.4 kDa containing 373 amino acid residues, a sequence that exhibits 47.0% homology to that of *P. oleovorans* as shown in Fig. 2 (11).

The *alk*A region located downstream of the *alk*B gene was composed of 791 nucleotides, which showed 32.0% and 56% homologies to corresponding sequences of *alk*F and *alk*G coding for rubredoxin reductase in *P. oleovorans*(12). A open reading frame of *alk*A, starting with an ATG codon at position 1533 and ending with a TAG codon at position 2324, was confirmed. A putative ribosome-binding sequence with 5′-AGGACA-3′ was identified at 23 nucleotides upstream from the starting point of translation in the open reading frame.

The *alk*A open reading frame can encode a polypeptide of molecular weight 35.4 kDa containing 264 amino acid residues in a sequence that exhibited 12% and 21% homologies to those of *P. oleovorans* (12).

The *alk*A region in *P. oleovorans* contains three coding sequences encoding two related rubredoxins(*alk*F and *alk*G) and aldehyde dehydrogenase(*alk*H) (12). However, in our results, we wonder whether the original *alk*A region of *P. maltophilia* was cut or not with restriction enzyme, but only this one open reading frame could be complemented with *alk*FG in *P. oleovorans.* Thus the nucleotide composition of the *alk*A gene in *P. maltophilia* differs considerably from that of the *P. oleovorans* genome suggesting that the *alk* regulon may evolve independently in different organisms.

Expression of alkBA genes in E. coli and Pseudomonas. The expression of *alk*BA genes was studied in *E. coli* SUP2 grown on basal minimal medium agar plates with octane vapor. Recombinant plasmid pPOC122 was introduced into NM522 by means of transformation, and the resulting strain was designated SUP2. The ampicillin-resistant transformants were isolated on minimal medium agar in the presence of octane vapor at 30°C. After 2 days no growth of SUP2 was observed, but after 3-4 days colonies appeared. From this result we concluded that only the *alk*BA region is essential for bacterial growth on *n*-alkane since the *alk*BA region was fully expressed in *E. coli* and *Pseudomonas maltophilia.*

To determine the level of expression of the *alk*B gene, cultures were grown and induced with dicyclopropylketone (DCPK) as described in Materials and Methods. The amount of alkane hydroxylase produced from *P. maltophilia* N246 carrying OCT plasmid and *E. coli* SUP2 was measured by *in vitro* complementation assay(18, 19).

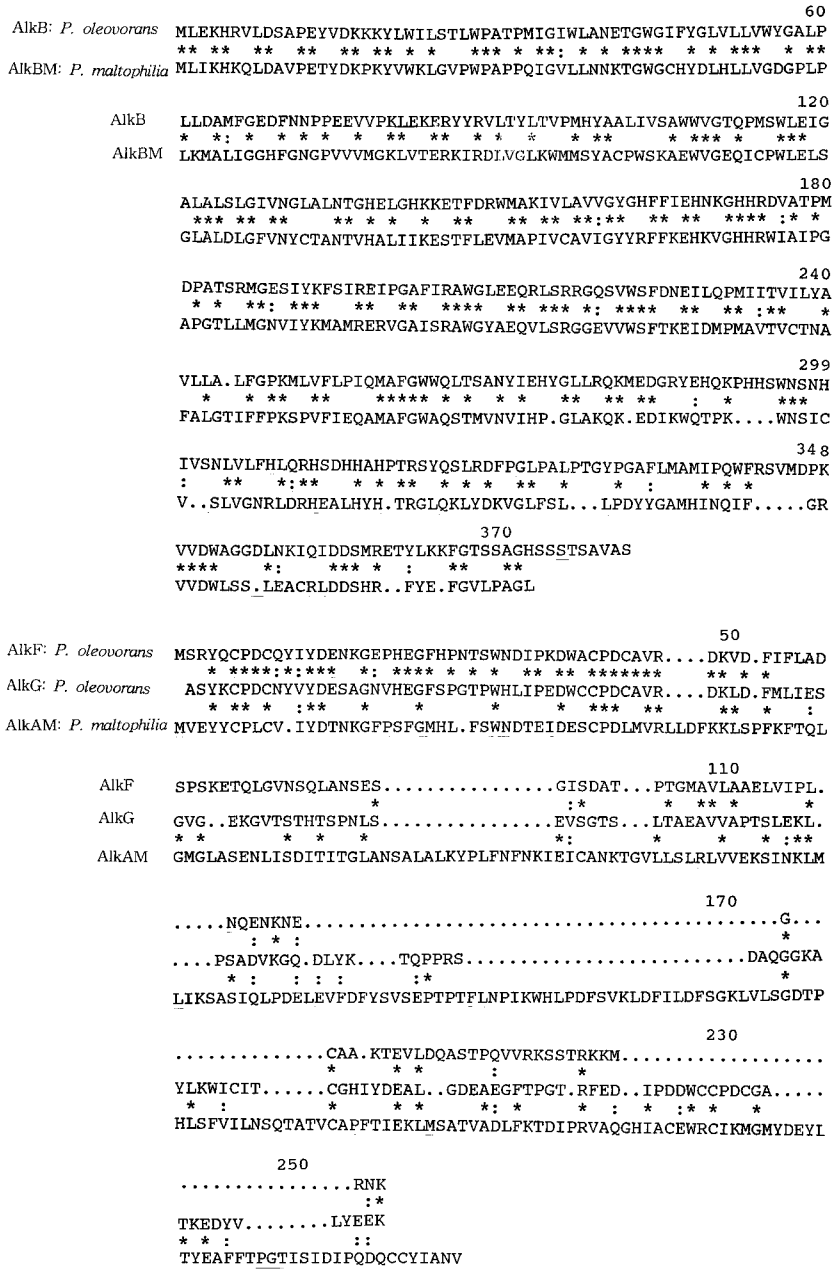

FIG. 2. Comparison of the translation products of alkane hydroxylase and rubredoxin reductase. Amino acids are represented by their one-letter abbreviation. An asterisk * is used to indicate the identical amino acids.

Induction of alkane hydroxylase in *E. coli* by transcription from its own promoter resulted in an alkane hydroxylase accumulation to less than in *Peudomonas maltophilia* N246 as shown in Table 2. However, transcription from the *alk* promoter with *n*-octane resulted in a considerably higher expression level in *E. coli.*

Growth of *E. coli* GEc303 carrying *alk*B gene and its promoter in presence of inducer resulted in the synthesis of the cytoplasmic membrane component of alkane hydroxylase identified as such

TABLE 2
Expression of Alkane Hydroxylase in *Pseudomonas maltophilia* and *E. coli*

Strains	Alkane hydroxylase activity[a] (mU/mg protein)
P. maltophilia N246	11.6
E. coli NM522	0.0
E. coli SUP2	9.8

[a] The specific activity represents unit per mg protein.

in *E. coli*(9, 10) and *Pseudomonas*(4, 10). This result agrees with that in our experiment since the *alk*B gene could be also expressed at high levels in *E. coli* and *Pseudomonas*(11).

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REFERENCES

1. Baptist, J. N., Gholson, R. K., and Coon, N. J. (1963) *Biochem. Biophys. Acta* **69,** 40–47.
2. Chakrabarty, A. M., Chou, G., and Gunsalus, I. C. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 1137–1140.
3. Fennewald, M., and Shapiro, J. A. (1977) *J. Bacteriol.* **132,** 622–627.
4. Benson, S., Oppuci, M., Shapiro, J. A., and Fennewald, M. (1979) *J. Bacteriol.* **140,** 754–762.
5. Fennewald, M., and Shapiro, J. A. (1979) *J. Bacteriol.* **139,** 264–269.
6. Fennewald, M., Benson, S., Oppici, M., and Shapiro, J. (1979) *J. Bacteriol.* **139,** 940–952.
7. Grund, A., Shapiro, J., Fennewald, M., Bacha, P., Leahy, J., Markbeiter, K., Neider, M., and Toepfer, M. (1975) *J. Bacteriol.* **122,** 546–556.
8. Owen, D. J., Eggink, G., Hauer, B., Kok, M., McBeth, D. L., Yan, Y. I., and Shapiro, J. A. (1986) *Mol. Gen. Genet.* **197,** 373–383.
9. Eggink, G., Van Lelyveld, P. H., Arnberg, A., Arfman, N., Witteveen, C., and Witholt, B. (1987a) *J. Biol. Chem.* **262,** 6400–6406.
10. Eggink, G., Lageveen, R. G., Altenburg, B., and Witholt, B. (1987b) *J. Biol. Chem.* **262,** 17712–17718.
11. Kok, M., Oldenhuis, R., Van der Linden, M. P. G., Raatjes, P., Kingma, J., Van Lelyveld, P. H., and Witholt, B. (1989a) *J.Biol Chem.* **264,** 5435–5441.
12. Kok, M., Oldenhuis, R., Van der Linden, M. P. G., Meulenberg, C. H. C., Kingma, J., and Witholt, B. (1989b) *J. Biol. Chem.* **264,** 5442–5451.
13. Hwang, M.-O., Lee, N.-L., and Min, K.-H. (1993) *Kor. Jour. Microbiol.* **31,** 385–390.
14. Choi, S.-Y., Lee, M.-H., Hwang, M.-O., and Min, K.-H. (1991) *Kor. J. Appl. Microbiol. Biotechnol.* **19**(1)**,** 82–87.
15. Gough, J. A., and Murray, N. E. (1993) *J. Mol. Biol.* **166,** 1.
16. Robinson, D. S. (1964) Antonie van Leeuwenhoek *J. Microbiol. Serol.* **30,** 303–316.
17. Benson, S., and Shapiro, J. A. (1975) *J. Bacteriol.* **123,** 759–760.
18. Nieboer, M., Kingma, J., and Witholt, B. (1993) *Mol. Microbiol.* **8**(6)**,** 1039–1051.
19. Peters, J., and Witholt, B. (1994) *Biochim. Biophys. Acta* **1196,** 145–153.
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
21. Bimboim, H. C., and Doly, J. (1979) *Nucleic Acids Res.* **7,** 1513–1523.
22. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *in* Molecular Cloning: A Laboratory Manual, Vol. 1–3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
23. Sanger, F., and Coulson, A. R. (Coulson1975) *J. Mol. Biol.* **94,** 441.