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Gene Structures and Regulation of the Alkane Hydroxylase Complex in *Acinetobacter* sp. Strain M-1

AKIO TANI, TAKERU ISHIGE, YASUYOSHI SAKAI, AND NOBUO KATO*

Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa-Oiwake, Sakyo-ku, Kyoto 606-8502, Japan

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In the long-chain *n*-alkane degrader *Acinetobacter* sp. strain M-1, two alkane hydroxylase complexes are switched by controlling the expression of two *n*-alkane hydroxylase-encoding genes in response to the chain length of *n*-alkanes, while rubredoxin and rubredoxin ruductase are encoded by a single gene and expressed constitutively.

Several strains in the genus *Acinetobacter* are known as *n*-alkane utilizers (4, 10). Among them, our isolate, *Acinetobacter* sp. strain M-1, is characterized by its ability to degrade a variety of *n*-alkanes, including very long chain *n*-alkanes (or paraffin wax) with carbon chain lengths of C_{20} to C_{44} that are in a solid state at ambient temperature (18).

Several pathways have been proposed for the initial reaction of *n*-alkane degradation by *Acinetobacter* strains (1, 2, 4, 5). Previously, we demonstrated three *n*-alkane dioxygenase activities in *Acinetobacter* sp. strain M-1, which had been postulated by Finnerty (5). We assume that these enzymes are involved in the oxidation of *n*-alkanes that are slightly dissolved in the cytosol or oil inclusion of the cell, because the enzymes were found in the soluble fraction of the cell extract of strain M-1. Recently, the genes encoding alkane hydroxylase (*alkM*) (15), rubredoxin (*rubA*), and rubredoxin reductase (*rubB*) (8) in *Acinetobacter calcoaceticus* strain ADP1 were found, and each of the genes was shown to be indispensable for *n*-alkane degradation. These results suggest that a three-component alkane hydroxylase complex participates in *n*-alkane degradation in strain ADP1, which is similar to that in a medium-chain (C_6 to C_{12}) *n*-alkane degrader, *Pseudomonas oleovorans* (24). The difference in the organization of the genes involved in *n*-alkane degradation between *P. oleovorans* and *A. calcoaceticus* strain ADP1 is that these genes are dispersed over the chromosomal DNA in strain ADP1, while they form an operon on a large OCT plasmid in *P. oleovorans*.

We describe here the isolation and characterization of genes in strain M-1 that are homologous to *alkM, rubA*, and *rubB* of strain ADP1. The most characteristic feature of strain M-1 was that two genes encoded alkane hydroxylases and they were differentially induced in response to the chain length of *n*-alkanes.

Cloning of two alkane hydroxylase genes, *alkMa* and *alkMb*, from *Acinetobacter* sp. strain M-1. We intended to clone the alkane hydroxylase-encoding gene from *Acinetobacter* sp. strain M-1 to study the molecular basis of the alkane hydroxylase complex in this organism. We designed the PCR primers mono-N and mono-C (Table 1) based on the highly conserved regions between *alkM* of *A. calcoaceticus* strain ADP1 (15) and *alkB* of *P. oleovorans* (11) and used the chromosomal DNA of strain M-1 as a template. This PCR yielded a 790-bp DNA fragment, and the sequence of the fragment was identical to a part of the *alkMa* gene (see below). Southern blot analysis using the fragment as the probe revealed that the probe hybridized to at least two bands in the genomic DNA of strain M-1 that had been digested with various restriction enzymes (data not shown). The hybridization was performed under low-stringency conditions at 37°C in the buffer from AlkPhosDirect (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, England). From these results, we cloned two alkane hydroxylase genes, *alkMa* and *alkMb*, into pBluescript II SK+ (Stratagene, La Jolla, Calif.) through colony hybridization as described previously (17). The four fragments in pMX4.2, pMC2.2, pMH2.5, and pME3.4 (Table 2) overlapped each other, and the span contained four complete open reading frames (ORFs) and a partial ORF, covering a total of 6,089 bp. The 2.7-kb *Xba*I fragment in pMX2.7 (Table 2) contained two ORFs and a partial ORF over a span of 2,868 bp (Fig. 1). Inverse PCR (13) was performed to amplify the downstream region of *alkMb*. The *Bcl*I-digested chromosomal DNA of strain M-1 was self-ligated and used as a template. The primers used were IPA2up and IPA2dn (Table 1). The resulting PCR-amplified 4-kb fragment was cloned and sequenced. Since the two cloned fragments harboring *alkMa* and *alkMb* could explain all of the hybridizing bands that appeared in the Southern blot analysis, we concluded that strain M-1 has two alkane hydroxylase genes.

The deduced amino acid sequences of *alkMa* and *alkMb* (AlkMa and AlkMb, respectively) showed 52% identity with each other. The eight-histidine motif was conserved in both peptide sequences (20). The hydropathy plots of AlkMa and AlkMb were similar to that of AlkB from *P. oleovorans* (23), suggesting that these proteins are membrane bound (data not shown). A phylogenetic tree of AlkMa and AlkMb with the hydroxylases of other microbes generated by ClustalW is shown in Fig. 2. AlkMa was more similar to AlkM of strain ADP1 (84% identity) than AlkMb was.

In the upstream regions of *alkMa* and *alkMb*, putative transcriptional regulator genes (*alkRa* and *alkRb*, respectively)

* Corresponding author. Mailing address: Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa-Oiwake, Sakyo-ku, Kyoto 606-8502, Japan.

TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristics	Reference or source
pMX4.2	Ap^r; contains 4.2-kb *Xba*I fragment of the gene library including *alkMa* and *alkRa* (Fig. 1)	This study
pME3.4	Ap^r; contains 3.4-kb *Eco*RI fragment of the gene library (Fig. 1)	This study
pMC2.2	Ap^r; contains 2.2-kb *Cla*I fragment of the gene library (Fig. 1)	This study
pMH2.5	Ap^r; contains 2.5-kb *Hin*dIII fragment of the gene library (Fig. 1)	This study
pMX2.7	Ap^r; contains 2.7-kb *Xba*I fragment of the gene library including *alkMb* and *alkRb* (Fig. 1)	This study
pKT231	Km^r, Sm^r	3
pMFY31	Ap^r, Tc^r, Cm^r	6
pMFYalkMa	Ap^r, Tc^r, Cm^r; contains *Pst*I-digested pMX4.2 in the *Pst*I site of pMFY31	This study
pMFYalkMb	Ap^r, Tc^r, Cm^r; contains *Pst*I-digested pMX2.7 in the *Pst*I site of pMFY31	This study

were found. AlkRa showed high similarities with the AraC-XylS type transcription regulators (7), including AlkR of *A. calcoaceticus* ADP1 (15). On the other hand, *alkRb* showed higher similarity with a different type of transcription regulator, OruR of *Pseudomonas aeruginosa*. In the downstream regions of *alkMa* and *alkMb*, the glutathione reductase gene (*gshR*) and alkyl-hydroperoxide reductase gene (*ahpC*, which was found in the inverse PCR-amplified DNA fragment), respectively, were found. These genes encode proteins involved in scavenging reactive oxygen species that may be generated from *n*-alkane oxidation.

Both *alkMa* and *alkMb* function in *A. calcoaceticus* strain ADP1. We attempted to show that *alkMa* and *alkMb* indeed encode functional *n*-alkane hydroxylases. Since several biochemical experiments were not successful, we took advantage of the genetics in *A. calcoaceticus* strain ADP1 (ATCC 33305; synonymous with strain BD413) (9, 21), which has both high transformation frequency and site-specific recombination efficiency.

We constructed the *alkM* disruptant (*alkM*Δ) of *A. calcoaceticus* ADP1 by inserting the kanamycin-resistance (Km^r) cassette. The ca. 3.0-kb DNA fragment containing the alkane hydroxylase gene *alkM* (15) was PCR amplified and cloned from the chromosomal DNA of strain ADP1. The primers used were alkMUp and alkMDn (Table 1). In the *Bcl*I site of *alkM*, the Km^r cassette, which was PCR amplified using pKT231 (3) as a template (Table 2), was inserted, and the resulting plasmid was used to transform strain ADP1. The primers used for amplification were KmNBam and KmCBam (Table 1). Transformation was performed as described by Palmen et al. (14). That the proper gene disruption had occurred was confirmed by Southern blot analysis (data not shown). The *alkM*Δ strain could grow on Luria-Bertani (LB) broth (19) medium containing kanamycin (50 μg/ml), but could not grow on hexadecane.

On the other hand, pMX4.2 and pMX2.7 (Table 2 and Fig. 1) were each digested by *Pst*I (which has a unique site in the multicloning site of the plasmids) and ligated with *Pst*I-digested pMFY31 (6), and the resulting plasmids were pMFYalkMa and pMFYalkMb, respectively. These plasmids had the alkane hydroxylase gene and the corresponding regulator gene from strain M-1, respectively, and each of them was introduced into the *alkM*Δ strain. Transformants were selected in the presence of both ampicillin (50 μg/ml) and kanamycin (50 μg/ml).

When the *alkM*Δ strain was transformed by pMFYalkMa or pMFYalkMb, the ability to grow on solidified M9 medium supplemented with hexadecane vapor in the presence of ampicillin and kanamycin was restored. This was not achieved with the control plasmid pMFY31. All of the transformed plasmids could be recovered from the transformants (data not shown), suggesting that these plasmids were maintained but not incorporated into the chromosomal DNA of strain ADP1. These results show that *alkMa* and *alkMb* could each complement the inability of the *alkM*Δ strain to grow on hexadecane. Therefore, *alkMa* and *alkMb* both encode a functional alkane hydroxylase. However, growth on *n*-alkanes of various lengths did not show a detectable difference between the *alkM*Δ strain carrying pMFYalkMa and pMFYalkMb. Therefore, these in vivo assays using strain ADP1 gave no information on the difference in substrate specificity between the products of the two alkane hydroxylase genes.

Regulation of *alkMa* and *alkMb* expression by *n*-alkanes. The amino acid sequence identity between AlkRa and AlkRb was only 5.0%, while that between AlkRa and AlkR of the heterologous strain ADP1 is 53%. These results raised the possibility that *alkMa* and *alkMb* are regulated in a different manner in strain M-1. Strain M-1 cells were grown on *n*-alkanes of various lengths as a carbon source, and Northern blot analysis was performed using total RNA extracted from these cells and *alkMa*- and *alkMb*-specific probes. Figure 3 clearly demonstrates that (i) neither *alkMa* nor *alkMb* expression was induced when strain M-1 was grown on sodium acetate or on hexadecanol, which induces the *alk* operon in *P. oleovorans* (13) and (ii) *alkMa* and *alkMb* were induced by *n*-alkanes, although in a different manner. *alkMa* expression was induced by solid, very long chain alkanes ($>C_{22}$), and *alkMb* expression was preferentially induced by liquid long-chain alkanes (C_{16} to C_{22}).

Structure of the rubredoxin and rubredoxin reductase genes (*rubA* and *rubB*) in *Acinetobacter* sp. strain M-1. To gain further insight into the molecular structure of the *n*-alkane hy-

TABLE 2. Sequences of primers used in this study

Primer	Sequence[a] (5′→3′)
Mono-N	TTCCGGTGATTGATACGATTATTGG
Mono-C	ACGCGTCGGATAAGCGTGATGATC
IPA2up	AACTATACCTGGTTTGCAGG
IPA2dn	GAAATACAGAGTCGTTTAAAATGC
alkMUp	CGGGGTAAGCATGAATAGCT
alkMDn	CGTACAGCTACTTGGTGGAC
Km-NBam	*GGATCC*GGACCAGTTGGTGATTTT
Km-CBam	*GGATCC*TTAGAAAAACTCATCGAGC
RBDXN	ATGAAAAAGTATCAATGTATCG
RBDXC	TTAAACTTCGATCATTTCAAA
IRBDXN	TCGTAAATCCAACCACAAAC
IRBDXC	TTGCGGCGTTTCAAAAGCTG

[a] The *Bam*HI site is italicized.

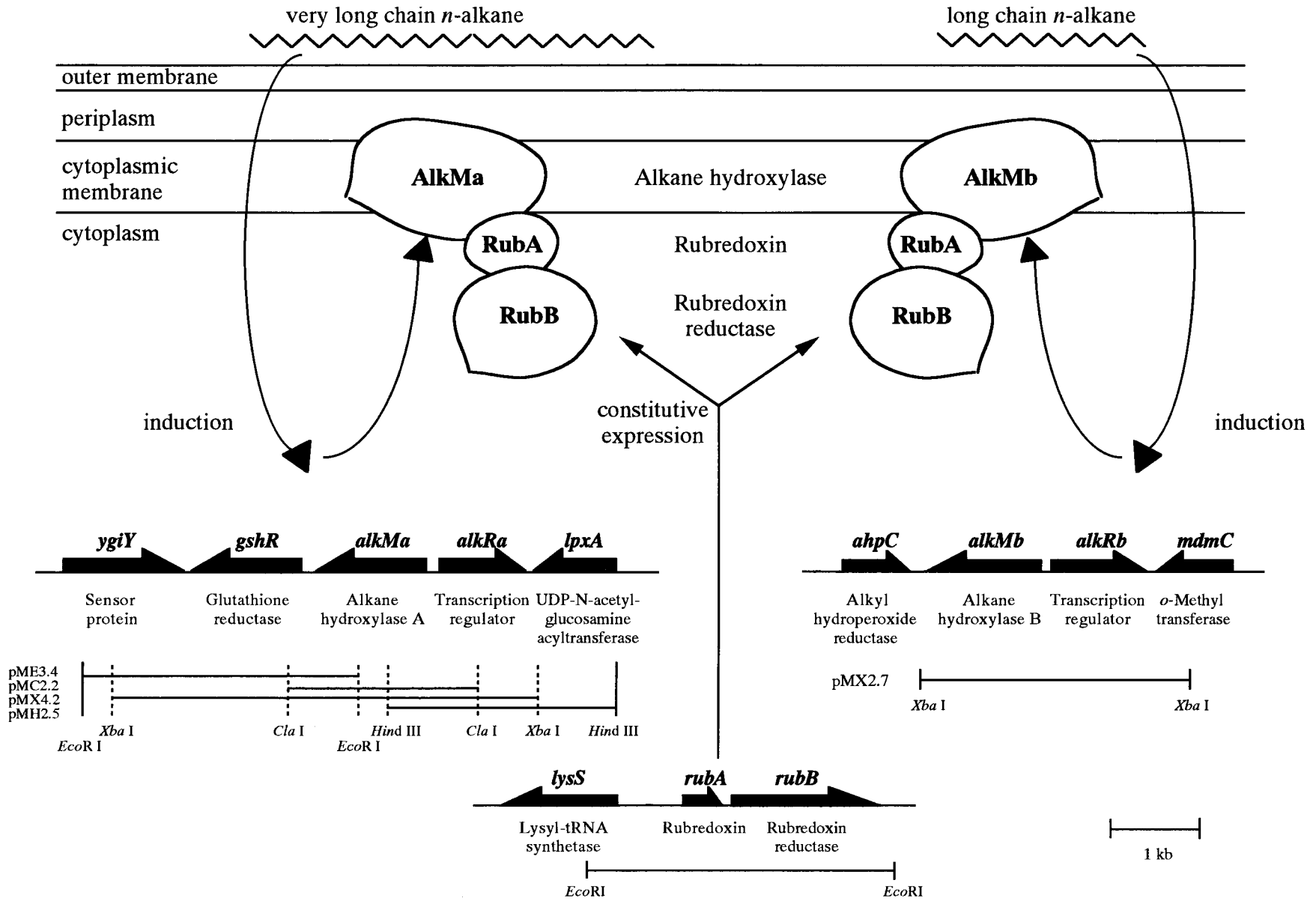

FIG. 1. Gene organization and restriction maps of the cloned regions, including *alkMa*, *alkMb*, and *rubAB* operon, and a proposed model for regulation of the *n*-alkane hydroxylase complex by *n*-alkanes in *Acinetobacter* sp. strain M-1. Thick arrows indicate the orientation of each gene. The downstream region of *alkMb* was sequenced by inverse PCR (see text). The putative products of the genes are indicated below the thick arrows. Very long chain *n*-alkanes and long-chain *n*-alkanes induce *alkMa* and *alkMb*, respectively, and each of the alkane hydroxylase components forms a complex with the constitutively expressed rubredoxin and rubredoxin reductase.

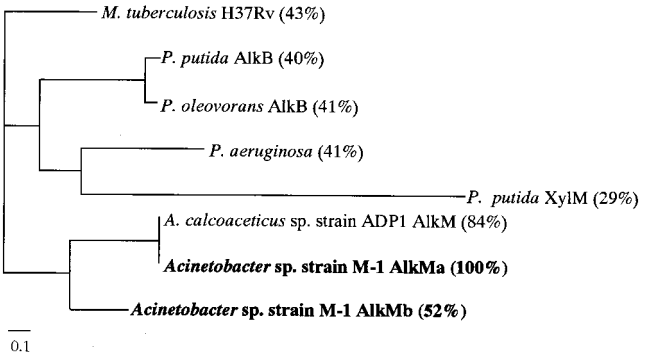

FIG. 2. Phylogenetic tree of alkane hydroxylases. Complete protein sequences of the alkane hydroxylases of several species were selected from the database and aligned using the ClustalW program. The species and proteins are *Mycobacterium tuberculosis* Rv3252c (accession number F70593); *Pseudomonas putida* AlkB (AJ233397); *P. oleovorans* AlkB (X65936); *P. aeruginosa* AE004581; *P. punda* xylene monooxygenase (XylM, A37316); *A. calcoaceticus* strain ADP1 AlkM (AJ002316); and *Acinetobacter* sp. strain M-1 AlkMa and AlkMb (AB049410 and AB049411, respectively). The percent identity values of the deduced amino acid sequences to AlkMa are shown in parentheses.

droxylase complex and its regulation in strain M-1, we cloned the genes *rubA* and *rubB*, encoding rubredoxin and rubredoxin reductase, respectively, using PCR and inverse PCR techniques and chromosomal DNA from strain M-1 as a template. The primers used were RBDXN and RBDXC (Table 1), which had identical sequences to the 5′ and complementary 3′ termini, respectively, of the *rubA* gene of strain ADP1 (8). Inverse PCR was performed to amplify the region surrounding *rubA* using the sequence information. The primers used were IRBDXN and IRBDXC (Table 1). *Eco*RI-digested and self-ligated chromosomal DNA of strain M-1 was used as the template. The cloning experiment yielded one 3.0-kb *Eco*RI fragment harboring two ORFs for *rubA* and *rubB* and one incomplete ORF (Fig. 1). The *rubAB* operon was overexpressed under the *tac* promoter in *Escherichia coli*, and the recombinant rubredoxin and rubredoxin reductase were purified to apparent homogeneity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). The specific activity of the purified recombinant rubredoxin reductase was 1,200 and 1,750 U mg^{-1} toward potassium ferricyanide and rubredoxin, respectively. One unit of activity was defined as the amount of the enzyme that catalyzes the NADH-dependent reduction of 1 μmol of ferricyanide or cytochrome *c* (in the presence of purified rubredoxin) per min.

Genomic Southern analyses of strain M-1 under low-stringency conditions using *rubA* and *rubAB* as probes showed only one hybridizing band in various restriction digests (data not shown). This result suggests that in strain M-1, rubredoxin and rubredoxin reductase are each encoded by a single gene, *rubA* and *rubB*, respectively.

Regulation of the *rubAB* operon. The *rubAB* operon is constitutively expressed in strain ADP1 (12). We performed Northern blot analysis with total RNA extracted from strain M-1 using *rubA* and *rubB* as probes. A hybridizing band was not detectable in strain M-1 that had been grown on any carbon source, including the *n*-alkanes tested, showing that the *rubAB* operon was expressed at a very low level. On the other hand, when the cells were grown on glycerol, hexadecane, or triacontane, the specific activity (toward ferricyanide) of the rubredoxin reductase in the cell extract of parent strain M-1 was 0.43, 0.60, and 0.51 U/mg of protein, respectively. In addition, in the intergenic region of *rubAB*, no transcriptional terminator-like sequence such as an inverted repeat was found. These results suggest that the *rubAB* operon is constitutively expressed in strain M-1.

Two alkane hydroxylase complexes in response to chain length of *n*-alkanes in *Acinetobacter* sp. strain M-1. From these results, we propose a mechanism for the regulation of the *n*-alkane hydroxylase complex by *n*-alkanes in *Acinetobacter* sp. strain M-1 (Fig. 1). According to this model, the organism controls alkane hydroxylase activity in response to the chain length of the substrate by switching the alkane hydroxylase component, AlkMa or AlkMb, without changing other components of the complex, rubredoxin and rubredoxin reductase, which are constitutively expressed. The low sequence similarity between *alkRa* and *alkRb*, which are the putative transcriptional regulators of *alkMa* and *alkMb*, respectively, may also suggest distinct regulatory mechanisms for *alkMa* and *alkMb* expression by *n*-alkanes.

Unfortunately, we have not been able to detect the enzyme activity of the alkane hydroxylase complex in cell extracts of *Acinetobacter* spp. or in the in vitro reconstitution experiment using the recombinant proteins from *E. coli* (data not shown), while the enzyme activity was reported to be detectable in *P. oleovorans* (22). Possible reasons for the failure to detect the activity are (i) poor solubility of the substrate (such as tridecane or longer-chain alkanes) in the reaction mixture in comparison with that used in the assay of *P. oleovorans* alkane hydroxylase (22); (ii) unstable nature of the hydroxylase component (12, 16); and (iii) an unknown factor(s) in the alkane hydroxylase complex of *Acinetobacter* species. An alternative approach to examining the physiological role and substrate specificity of AlkMa and AlkMb may be the use of genetic analyses, such as gene disruption in *Acinetobacter* sp. strain M-1. However, we have not succeeded in deriving disruptants

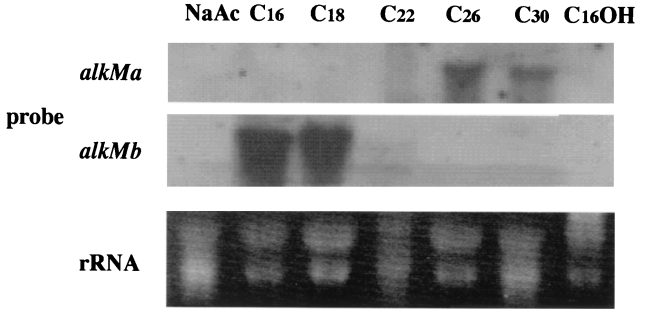

FIG. 3. Northern blot analysis of *alkMa* and *alkMb* in strain M-1 cells grown on various substrates. Total RNA (15 μg) was loaded in each lane, and *alkMa* and *alkMb* transcripts were detected with labeled *alkMa* or *alkMb* fragment, respectively, as a probe. Total RNA was prepared from strain M-1 cells that had been grown on a salt medium containing 1% (wt/vol) sodium acetate (NaAc); 0.5% (vol/vol) *n*-alkane, with the carbon chain length indicated; or 0.5% (wt/vol) hexadecanol (C_{16OH}). rRNA was used as a standard and was visualized by ethidium bromide.

of *alkMa* and *alkMb* due to the very low efficiency of site-specific recombination in this organism.

n-Alkane metabolism in *Acinetobacter* sp. strain M-1 is very complicated due to the diversity and overlapping functions of the enzymes. Genetic and biochemical characterization of *n*-alkane-metabolizing enzymes in *Acinetobacter* spp. will shed light on the poorly understood mechanism of the metabolism of very long chain *n*-alkanes in microorganisms and its regulation.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases under accession numbers AB049411(*alkMa*), AB049412(*alkMb*), and AB049413(*rubAB*).

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