

Differential Expression of the Components of the Two Alkane Hydroxylases from *Pseudomonas aeruginosa*

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Oxidation of *n*-alkanes in bacteria is normally initiated by an enzyme system formed by a membrane-bound alkane hydroxylase and two soluble proteins, rubredoxin and rubredoxin reductase. *Pseudomonas aeruginosa* strains PAO1 and RR1 contain genes encoding two alkane hydroxylases (*alkB1* and *alkB2*), two rubredoxins (*alkG1* and *alkG2*), and a rubredoxin reductase (*alkT*). We have localized the promoters for these genes and analyzed their expression under different conditions. The *alkB1* and *alkB2* genes were preferentially expressed at different moments of the growth phase; expression of *alkB2* was highest during the early exponential phase, while *alkB1* was induced at the late exponential phase, when the growth rate decreased. Both genes were induced by C₁₀ to C₂₂/C₂₄ alkanes but not by their oxidation derivatives. However, the *alkG1*, *alkG2*, and *alkT* genes were expressed at constant levels in both the absence and presence of alkanes.

Pseudomonas aeruginosa is a ubiquitous bacterium that can be found associated with soil and aquatic environments and can behave as an opportunistic pathogen for animals and plants (17, 25). This ecological diversity is probably related to its ability to use a wide range of substrates as carbon and energy sources (25). Most *P. aeruginosa* strains isolated from both clinical and nonclinical environments can degrade *n*-alkanes (1, 31, 36). Alkanes are the most abundant family of hydrocarbons in crude oil and are generated by many plants and algae (20). Bacterial metabolism of *n*-alkanes normally proceeds via sequential oxidation of a terminal methyl group to render alcohols, aldehydes, and, finally, fatty acids (20). The best-characterized alkane degradation pathway is that of *Pseudomonas putida* GPo1 (32). In this case, the initial oxidation step is performed by an alkane hydroxylase system composed of a membrane-bound nonheme iron monooxygenase (commonly named alkane hydroxylase) and two soluble proteins, rubredoxin and rubredoxin reductase, which act as electron carriers between NADH and the hydroxylase. Genes encoding related enzymes have been found in several alkane-degrading bacteria (12, 13, 18, 23, 24, 31, 34).

P. putida GPo1 and *Acinetobacter* sp. strain ADP1 contain only one alkane hydroxylase (18, 32). However, other bacterial strains contain several alkane hydroxylases. In this case, the different alkane hydroxylases may have distinct properties or may be induced under different conditions. *Acinetobacter* sp. strain M1 contains two alkane hydroxylases, one of which oxidizes C₁₂ to C₁₆ *n*-alkanes, the other one showing a higher activity with C₂₀ or larger alkanes (12). The expression of each hydroxylase is controlled by a specific transcription factor that responds to the alkanes recognized by the corresponding hydroxylase (29).

P. aeruginosa PAO1, whose genome has been sequenced

(28), contains genes encoding two alkane hydroxylases, two rubredoxins, and a rubredoxin reductase homologous to those of *P. putida* GPo1 (Fig. 1A). All these genes encode enzymes that participate in the oxidation of *n*-alkanes (22, 23, 30), although the regulation of their expression has not been reported. The AlkB1 alkane hydroxylase oxidizes C₁₆ to C₂₄ *n*-alkanes, while AlkB2 is active on C₁₂ to C₂₀ *n*-alkanes (22). Therefore, the substrate specificity of the two enzymes overlaps substantially. This suggests that they may not be induced simultaneously, each one being present under different situations.

To investigate this issue, we analyzed the expression of the components of the two *P. aeruginosa* alkane hydroxylases. Assays were performed on *P. aeruginosa* RR1, a strain isolated from an oil-contaminated site (36), and extended to *P. aeruginosa* PAO1, isolated from an infected wound (16).

Characterization of promoters for *alkB1*, *alkB2*, *alkG1*, *alkG2*, and *alkT* genes. PCR analyses showed that *P. aeruginosa* RR1 contains genes homologous to those of *P. aeruginosa* PAO1 encoding the AlkB1 and AlkB2 alkane hydroxylases, the AlkG1 and AlkG2 rubredoxins, and the AlkT rubredoxin reductase. DNA segments encoding the 5' regions of the strain RR1 *alkB1* and *alkB2* genes were PCR amplified and cloned. In the case of *alkB1*, a primer pair that allowed us to amplify a 425-bp DNA fragment spanning positions –336 to +89 relative to the *alkB1* transcription start site (as determined in this work; see below) was used. This DNA fragment was cloned into pGEM-T Easy (Promega), generating plasmid pPB1R1. With primers containing targets for *EcoRI*, a 566-bp fragment spanning positions –185 to +381 relative to the *alkB2* transcription start site (see below) was PCR amplified and cloned at the *EcoRI* site of pUC18 (21), yielding plasmid pBRR1. A similar strategy allowed us to obtain a 1.9-kbp DNA fragment including *alkG1*, *alkG2*, and the 5' region of *alkT* (nucleotides –206, relative to the *alkG1* transcription start site, to +1034, relative to *alkT* translation start site), which was cloned at the *BamHI* site of plasmid pUC19 (21), generating pRUB1.

Sequencing of these DNA regions showed very few differences between strains RR1 and PAO1. One base pair deletion

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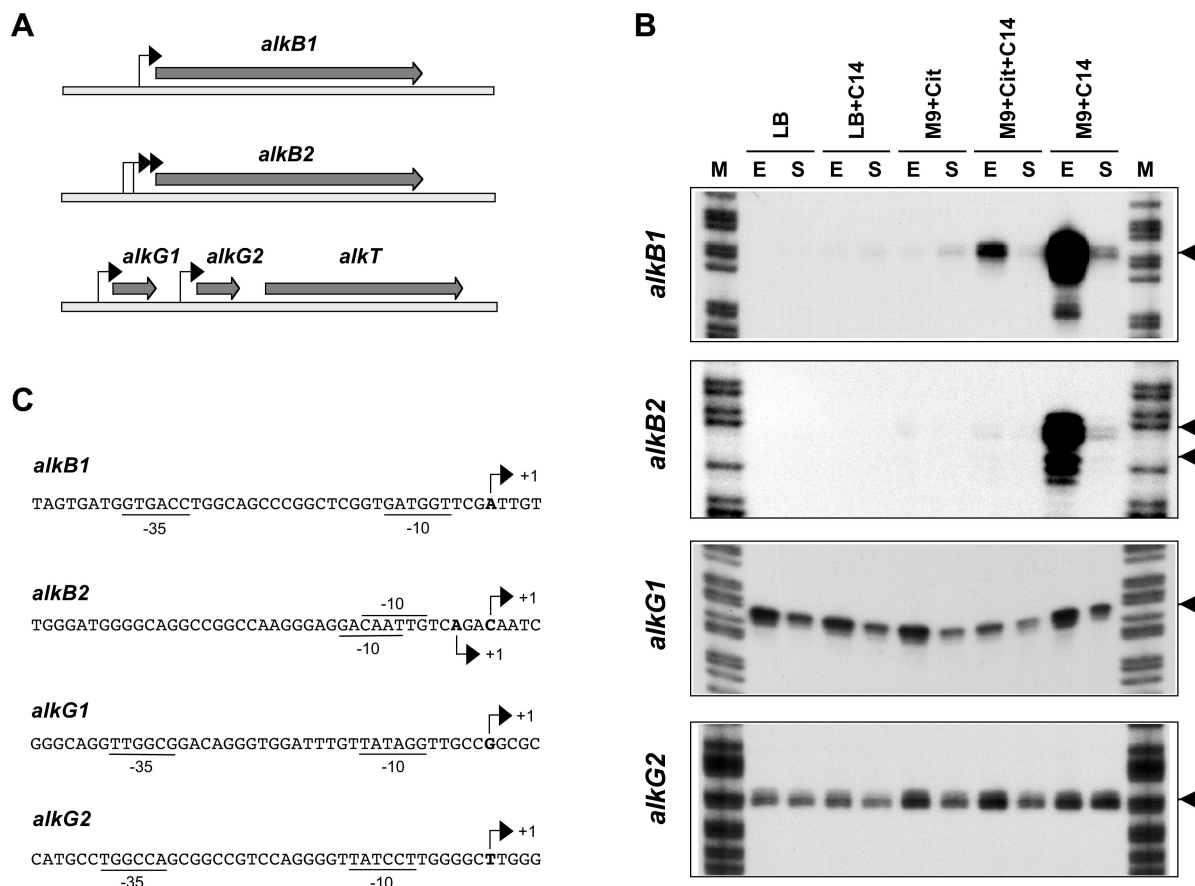


FIG. 1. Promoters for *alkB1*, *alkB2*, *alkG1*, and *alkG2* genes. (A) The genes encoding the two membrane-bound alkane hydroxylases, *alkB1* and *alkB2*, are located on different sites of the chromosome. The genes encoding the two rubredoxins (*alkG1* and *alkG2*) and the rubredoxin reductase (*alkT*) are clustered and map at a different site. Arrows indicate the promoters identified in this work. (B) Identification and expression of the promoters for the *alkB1*, *alkB2*, *alkG1*, and *alkG2* genes in cells grown either in rich LB medium or in M9 minimal salts medium in the absence or presence of tetradecane (C_{14}) or citrate (Cit). Cells were collected at either the exponential (A_{550} of 1.5) or stationary (A_{550} of 5) phase of growth (indicated as E and S, respectively). Transcripts originating upstream of the genes under study were analyzed by S1 nuclease protection assays; the 5' ends of the probes used hybridized at positions +89, +56, +87, and +152 relative to the transcription start sites observed for promoters *PalkB1*, *PalkB2*, *PalkG1*, and *PalkG2*, respectively. Lane M, DNA size ladder. Arrows indicate the transcription start sites observed. No other bands were detected in the gels in addition to those shown. (C) Sequences of the promoters identified in *P. aeruginosa* strain RR1. The transcription start sites observed in panel B are indicated with arrows. Sequences at the -10 and -35 regions showing similarity (at least three matches) to those recognized by the vegetative RNA polymerase are underlined. The ATG translation start sites of *alkB1*, *alkB2*, *alkG1*, and *alkG2* are located at positions +36, +35, +36, and +101, respectively.

was found at position -55 of the strain RR1 *alkB1* gene. No differences were found at the promoter region of *alkB2*. The sequences of *alkG1*, *alkG2*, and the 5' region of *alkT* were also identical to those of strain PAO1 except at the *alkG1* promoter, where positions -2 and -15 were guanosine residues in RR1 and adenosines in PAO1.

To determine the transcription start site of each of these genes, *P. aeruginosa* RR1 was grown at 30°C either in rich Luria-Bertani (LB) medium or in M9 minimal salts medium (21), the latter supplemented with trace elements (2) and a carbon source (either 30 mM citrate, 1% [wt/vol] tetradecane, or a mixture of both). Total RNA was obtained from cells collected at either the exponential (A_{550} of 1.5) or the stationary (A_{550} of 5) phase of growth. The origin and amount of the transcripts corresponding to the genes under study were analyzed by S1 nuclease protection assays as described earlier (35, 37), with equal amounts of RNA in each sample. The single-

stranded DNA probe used, which was added in a large excess to titrate the mRNA, was generated by linear PCR as described before (35), with plasmids pPB1R1 linearized with *EcoRI* (contains the promoter for *alkB1*), pPBRR1 cut with *NdeI* (contains the promoter for *alkB2*), or pRUB1 cut with *BamHI* (contains *alkG1*, *alkG2*, and the 5' region of *alkT*) as the substrate.

As shown in Fig. 1B, strong signals were detected for the *alkB1* and *alkB2* genes in cells growing in minimal salts medium containing tetradecane as the carbon source and collected at the late exponential phase (A_{550} of 1.5). It should be noted that growth of *P. aeruginosa* RR1 on alkanes showed an initial exponential phase until the turbidity reached about 1.2, followed by a period of slower growth that ended when the turbidity reached 5 to 6, when the stationary phase became evident (an example can be seen in Fig. 2). For *alkB1*, a single band was detected corresponding to a promoter in which con-

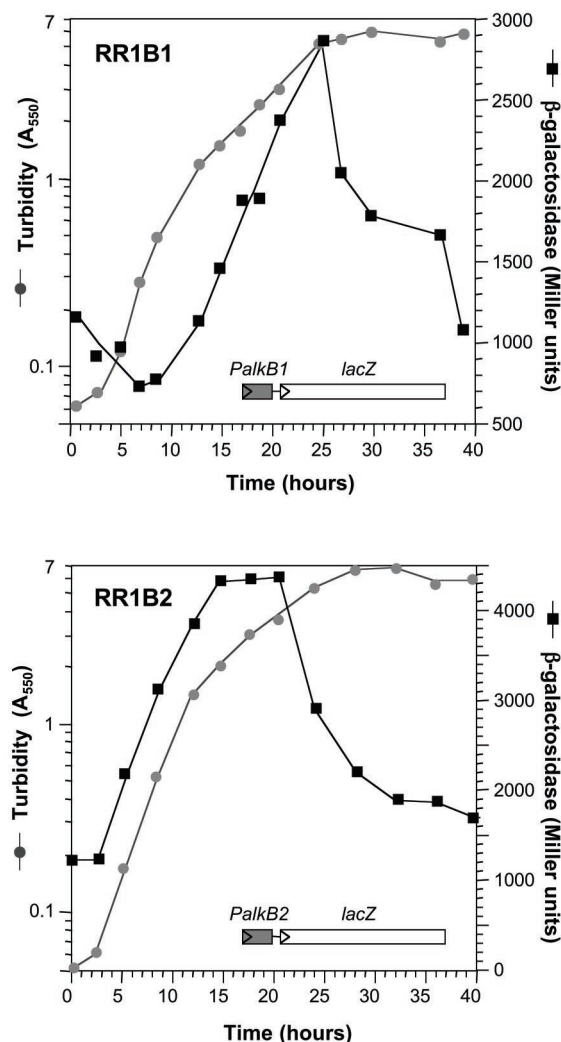


FIG. 2. Expression of *alkB1* and *alkB2* throughout the growth phase. Strains RR1B1 (strain RR1 containing a *PalkB1::lacZ* transcriptional fusion) and RR1B2 (strain RR1 containing a *PalkB2::lacZ* transcriptional fusion) were grown in minimal salts M9 medium containing tetradecane as the carbon source. Samples were taken at different times, and the amount of β -galactosidase present was measured (solid squares). Cells were washed and resuspended in M9 medium without alkanes prior to turbidity measurements (gray circles) to avoid errors induced by nondissolved alkanes.

sensus boxes for the vegetative σ^{70} RNA polymerase could be recognized, although conservation was low (see Fig. 1C). For *alkB2*, two adjacent signals were observed, each composed of two or three bands (Fig. 1B). In both cases, the start site was preceded by -10 regions showing some similarity to the consensus for σ^{70} RNA polymerase, although no -35 consensus boxes were evident (Fig. 1C).

The presence of two moderately conserved -10 boxes suggests that the two signals correspond to overlapping promoters, although it cannot be ruled out that the smaller transcript derives from the larger one. However, since both behaved similarly in all our assays, they are referred to hereafter as *PalkB2*, and coordinates are given relative to the start site of the largest transcript, which was also the most abundant. The

activity of promoters *PalkB1* and *PalkB2* decreased significantly when cells reached the stationary phase of growth (A_{550} of 5 to 6; Fig. 1B). No expression of the *alkB1* and *alkB2* genes was observed when cells were grown in rich LB medium or in minimal salts medium containing citrate as the carbon source, which indicates that their induction requires the presence of alkanes (Fig. 1B). However, addition of tetradecane to these culture media led to a low induction of the two promoters (with citrate) or even to no induction (with LB; Fig. 1B). This is indicative of a catabolic repression control induced by the components of the rich medium and by citrate.

A similar analysis of expression of the *alkG1* and *alkG2* genes, which encode two homologous rubredoxins, indicated that each is expressed from an independent promoter (Fig. 1B). In both cases, the -35 and -10 regions were rather similar to the consensus recognized by σ^{70} RNA polymerase (Fig. 1C). Expression was somewhat weaker at high turbidities but was independent of the absence or presence of tetradecane and was not repressed by the presence of citrate or by growth of cells in rich LB medium. No promoter could be found immediately upstream from *alkT*, which encodes the rubredoxin reductase. The probes used, which comprised the 5' end of *alkT* and the complete *alkG2* gene, were protected in their entire length from S1 nuclease attack (not shown), suggesting that *alkT* is expressed from the *alkG2* promoter.

A putative rho-independent transcriptional terminator was present downstream of *alkG1*, but no such sequences were found between *alkG2* and *alkT*. A reverse transcription-PCR analysis confirmed that *alkT* was expressed in cells growing in all the culture media analyzed, as it did for *alkG2* (not shown). The use of an upstream primer hybridizing to *alkG2* and a downstream primer hybridizing to *alkT* showed that transcripts running through *alkG2* continued into *alkT* (not shown), further supporting that *alkT* is expressed from the promoter for *alkG2*. The constitutive expression of *alkG1*, *alkG2*, and *alkT* contrasts with the controlled expression of *alkB1* and *alkB2* and suggests that the three components of the alkane hydroxylase system are not present in stoichiometric amounts in induced cells. The ratio of AlkB, AlkG, and AlkT has been analyzed only for *P. putida* GPo1, where it was found to be 7:2.5:1 in spite of the fact that the three proteins are coordinately induced (27). This unbalanced stoichiometry suggests that the molecules of the membrane-bound hydroxylase share a limiting number of rubredoxin and rubredoxin reductase molecules, which are soluble proteins that can probably associate and dissociate from the membrane-bound hydroxylase.

Differential expression of *alkB1* and *alkB2* genes. To gain further insight into the expression of *alkB1* and *alkB2*, transcriptional fusions of promoters *PalkB1* and *PalkB2* to the *lacZ* reporter gene were constructed. In the case of *PalkB1*, a DNA fragment was PCR amplified from *P. aeruginosa* RR1 spanning positions -336 to $+10$ relative to the transcription start site (the translation start site is at position $+36$). The primers used introduced artificial sites for *EcoRI* and *BamHI* endonucleases, which served to clone the DNA fragment between the same sites of plasmid pUJ8 (6), yielding plasmid pPAH1 β 1. The *PalkB1::lacZ* fusion was excised from pPAH1 β 1 with *NotI* and cloned at the *NotI* site of pUT-mini-Tn5Sm (6), generating plasmid pPAH1 β 3.

A similar strategy was followed for promoter *PalkB2*. The

DNA fragment cloned into pUJ8 spanned positions -185 to $+23$ relative to the *alkB2* transcription start site (the translation start site is at position $+35$). The plasmid obtained was named pPAH2 β 1. This plasmid was cut with *NotI*, and the *PalkB2::lacZ* fusion was cloned at the *NotI* site of pUT-mini-Tn5Sm, generating pPAH2 β 3. The suicide plasmids containing the *PalkB1::lacZ* and *PalkB2::lacZ* fusions were introduced into *P. aeruginosa* RR1 by transformation (8) to deliver the fusions into the chromosome (these plasmids do not replicate in *P. aeruginosa*). Two transformants were selected for each fusion. Since in each case they generated similar levels of β -galactosidase when grown in the presence of tetradecane, only one of them was selected for further analyses.

The transformant containing the *PalkB1::lacZ* fusion was named RR1B1, while that containing the *PalkB2::lacZ* fusion was named RR1B2. To analyze the expression of the *PalkB1* and *PalkB2* promoters, strains RR1B1 and RR1B2 were grown in minimal salts medium containing tetradecane as the carbon source, and β -galactosidase activity was measured at several points of the growth phase as described earlier (15, 35). Promoter *PalkB1* showed low activity during the early exponential phase of growth, when cells grew at a faster rate (Fig. 2). The activity of β -galactosidase did not increase until the cells approached the late exponential phase, at turbidities of about 1, when the growth rate decreased. At this time, β -galactosidase levels increased steadily and peaked at turbidities of about 5, when culture growth had ceased and the stationary phase was evident. At later times, β -galactosidase levels dropped in spite of the presence of nonmetabolized alkanes in the medium.

Interestingly, promoter *PalkB2* showed a different behavior. It was activated as soon as the cells started to grow, and β -galactosidase levels increased steadily during the early exponential phase, reaching a plateau at turbidities of about 2, when the growth rate had decreased and cells had entered the late exponential phase of growth. The β -galactosidase levels remained high for some time but then declined before cell growth ceased. Cell growth continued until the turbidity reached values of 6 to 7, although β -galactosidase activity declined steadily. This expression pattern agrees with the results of the mRNA analyses presented in Fig. 1B. In both cases, the S1 nuclease protection assays were performed with RNA obtained from cells collected at either the late exponential phase (A_{550} of about 1.5) or the stationary phase (A_{550} of about 5). Therefore, the results indicate that the two *P. aeruginosa* alkane hydroxylases are preferentially induced at different points of the growth phase. *PalkB2* is strongly induced at the start of the exponential phase of growth, while promoter *PalkB1* is not, although both promoters are active during the late exponential phase and are switched off when the cells enter the stationary phase.

It is not self-evident what would be the advantage, if any, of the differential expression of *alkB1* and *alkB2*. Both genes allowed growth at the expense of C_{12} to C_{16} alkanes when individually introduced into a *Pseudomonas fluorescens* derivative in which the equivalent native alkane hydroxylase had been inactivated (22). On the other hand, the membrane component of the alkane hydroxylase is detrimental to cell growth when expressed at high levels (4). The fact that both genes are silenced when growth ceases at stationary phase even if alkanes are still present in the medium agrees with this observation. It may therefore be advantageous to have two alkane hydroxy-

lases, one of which is induced more efficiently when cells grow fast (*AlkB2*), and the other being induced to lower levels when cells grow slowly (*AlkB1*). Alternatively, *AlkB1* may have a higher affinity for oxygen than *AlkB2*, which could explain why *alkB1* is preferentially expressed at high cell densities, when the oxygen supply is limiting.

Induction of promoter *PalkB1* can occur in the absence of RpoS, LasR, RhIR, and RelA. Many *P. aeruginosa* genes that are activated at high cell densities or during the transition from the exponential to the stationary phase of growth are under control of the stationary-phase sigma factor RpoS (9), of the LasR/RhIR quorum-sensing response (5), or of the RelA-mediated stringent response (33). We investigated whether promoter *PalkB1* could be controlled by any of these global regulatory mechanisms. All of them have been studied mainly in *P. aeruginosa* strain PAO1, for which several mutant derivatives are available.

Transcription of the *alkB1* gene in *P. aeruginosa* PAO1 started at the same position as in strain RR1 and followed a similar expression pattern (not shown). Inactivation of the *rpoS* gene (strain PAOS [9]), the *lasR* gene (strain PAOR [11]), the *rhIR* gene (strain PT462 [10]), or the *relA* gene (strain PA Ω R3 [33]) did not impair expression of promoter *PalkB1* (not shown). Therefore, these global regulators are not needed for the expression of promoter *PalkB1* during the late exponential phase. It is unclear what regulates the activity of this promoter. The *P. aeruginosa* genome may contain up to 24 sigma factors, many of which correspond to the subfamily of the extracytoplasmic function sigma factors (14, 28). The role and regulation of most of them are not known. However, known extracytoplasmic function sigma factors coordinate transcription in response to extracytoplasmic stimuli such as environmental signals. Expression of *alkB1* could perhaps depend directly or indirectly on one of these uncharacterized sigma factors. It should be noted that the transcriptional regulators of the *alkB1* and *alkB2* genes have not been identified yet, and their expression could also be regulated.

Promoters *PalkB1* and *PalkB2* are subject to strong catabolic repression. As shown in Fig. 1B, induction of promoters *PalkB1* and *PalkB2* by alkanes was severely inhibited when citrate was present in the growth medium and when cells were grown in a rich medium, suggesting that their expression is controlled by catabolic repression. The effect of other carbon sources was analyzed with strains containing the transcriptional fusions to the *PalkB1* and *PalkB2* promoters. β -Galactosidase levels were determined in late-exponential-phase cultures (A_{550} of 1.5 to 2), when both promoters were active. Glucose, citrate, lactate, succinate, glycerol, and myristic acid repressed *PalkB1* activity 15- to 20-fold. Repression was higher for *PalkB2* (20- to 40-fold). A similar effect occurred in LB medium containing tetradecane. Catabolic repression is a common phenomenon in alkane degradation pathways (3, 13, 26, 35), which indicates that they are not preferred growth substrates for bacteria.

Range of alkanes inducing promoters *PalkB1* and *PalkB2*. Reporter strains RR1B1 and RR1B2 were used to analyze the range of inducers of *PalkB1* and *PalkB2*. To obtain comparable growth rates without interference from the different compounds tested as inducers, cells were grown in a minimal salts medium containing 0.3% (vol/vol) Tween 80 as the carbon

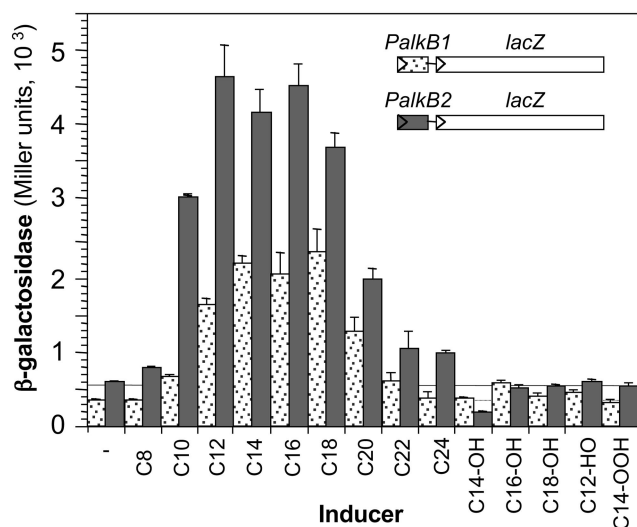


FIG. 3. Inducers of *alkB1* and *alkB2* genes. Strains RR1B1 and RR1B2 were grown in minimal salts M9 medium containing Tween 80 (0.3%, vol/vol) as the carbon source in the absence or presence of different alkanes (C₈ to C₂₄), alcohols (C₁₄-OH, C₁₆-OH, and C₁₈-OH), aldehydes (dodecanal, C₁₂-HO), and fatty acids (myristic acid, C₁₄-OOH). When cultures reached a turbidity of 2.0 (late exponential phase), the levels of β-galactosidase were measured. Liquid hydrocarbons were added at 1% (vol/vol), and solid hydrocarbons were added at 1% (wt/vol). Dotted bars correspond to the values obtained for strain RR1B1 (*PalkB1::lacZ* transcriptional fusion), and gray bars correspond to those for RR1B2 (*PalkB2::lacZ* transcriptional fusion).

source in addition to the inducer. At this concentration, Tween 80 does not generate catabolic repression but allows good growth. Promoters *PalkB1* and *PalkB2* showed similar behavior. In both cases, the highest induction was observed for C₁₂ to C₁₈ alkanes (Fig. 3). Promoter activity decreased almost twofold when C₂₀ was used as the inducer and further diminished for alkanes of longer chain length. Although Tween 80 is a nonionic surfactant that probably facilitates alkane dispersion in the aqueous phase, the low induction achieved by the longer-chain alkanes could be related to their low water solubility. When alkanes were supplied dissolved in dioctylphthalate, induction increased by about twofold for C₂₀, C₂₂, and C₂₄ but not for C₁₈ or smaller alkanes. Octane was a very poor inducer for both promoters. Decane efficiently induced *PalkB2* but was less effective for *PalkB1*. Alcanols, which are the products of alkane oxidation by alkane hydroxylase, did not serve as inducers. Aldehydes such as dodecanal and fatty acids such as myristic acid did not serve as inducers either. It is likely, therefore, that alkanes are the direct inducers of the *alkB* genes. The ability of alkane oxidation products to induce expression of alkane hydroxylases varies in different bacterial species. In *P. putida* GPO1, alkanes and alcanols are good inducers, while alcanols induce poorly and fatty acids induce not at all (7). In *Burkholderia cepacia* RR10, the alkane hydroxylase is induced both by alkanes and by their oxidation products (13), while in *Acinetobacter* sp. strain ADP1, only the nonoxidized alkane can act as an inducer (19).

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