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Controlled and Functional Expression of the *Pseudomonas oleovorans* Alkane Utilizing System in *Pseudomonas putida* and *Escherichia coli**

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The OCT plasmid encodes enzymes for alkane hydroxylation and alkanol dehydrogenation. Structural components are encoded on the 7.5-kilobase pair *alkBAC* operon, whereas positive regulatory components are encoded by *alkR*. We have constructed plasmids containing fusions of cloned *alkBAC* and *alkR* DNA and used these fusion plasmids to study the functional expression of the *alkBAC* operon and the regulatory locus *alkR* in *Pseudomonas putida* and in *Escherichia coli*. Growth on alkanes requires a functional chromosomally encoded fatty acid degradation system in addition to the plasmid-borne *alk* system. While such a system is active in *P. putida*, it is active in *E. coli* only in *fadR* mutants in which fatty acid degradation enzymes are expressed constitutively. Using such mutants, we found that *E. coli* as well as *P. putida* grew on octane as the sole source of carbon and energy when they were supplied with the cloned complete *alk* system. The *alkR* locus was strictly necessary in *E. coli* as well as in *P. putida* for expression of the *alkBAC* operon. The *alkBAC* operon could, however, be further reduced to a 5-kilobase pair operon without affecting the Alk phenotype in either species to a significant extent. Although with this reduction the plasmid-encoded alkanol dehydrogenase activity was lost, chromosomally encoded alkanol dehydrogenases in *P. putida* and *E. coli* compensated for this loss.

The induction kinetics of the *alk* system was studied in detail in *P. putida* and *E. coli*. We used specific antibodies raised against alkane hydroxylase to follow the appearance of this protein following induction with octane. We found the induction kinetics of alkane hydroxylase to be similar in both species. A steady-state level was reached after about 2 h of induction in which time the alkane hydroxylase accounted for about 1.5% of total newly synthesized protein. Thus, *alkBAC* expression is very efficient and strictly regulated to both *P. putida* and *E. coli*.

The IncP-2 plasmid OCT enables *Pseudomonas oleovorans* to use C_6–C_{12} *n*-alkanes as a sole source of energy and carbon (Baptist *et al.*, 1963). The oxidation of alkanes by *P. oleovorans* is of interest due to the specific characteristics of this organism which grows in the presence of a bulk apolar phase and due to the potential utility of terminal oxidation in the production of long chain terminal alcohols, aldehydes, dicarboxylic acids, and chiral epoxides (De Smet *et al.*, 1983). The strain we use in our studies, *P. oleovorans* TF4-1L, is a cold stable variant of *P. oleovorans* which shows a much higher alkene-epoxidation activity in resting cell suspensions than its parent-strain (Schwartz and McCoy, 1973).

The initial two steps in the oxidation of alkanes are terminal hydroxylation and dehydrogenation of the resulting alkanol. These conversions are catalyzed by inducible enzymes encoded on the OCT plasmid. Studies on the OCT plasmid or its derivatives have shown that the *alk* regulon is encoded by at least two distinct regions (Fennewald *et al.*, 1979), the *alkBAC* operon and the *alkR* locus. The expression of the *alkBAC* operon is positively regulated by the *alkR* gene product(s) in the presence of an inducer *e.g.* *n*-octane or dicyclopropylketone (Fennewald and Shapiro, 1977; Owen *et al.*, 1984). The *alkBAC* operon encodes a membrane-bound alkane hydroxylase (*alkB*), soluble alkane hydroxylase components (*alkA*), and a membrane-bound alkanol dehydrogenase activity (*alkC*) (Benson *et al.*, 1979). In addition, several chromosomal loci have been identified which are involved in alkanol, alkanal, and fatty acid oxidation: *alcA*, *aldA*, and *oic* (Grund *et al.*, 1975; see Fig. 1).

Recently, the cloning (Eggink *et al.*, 1984; Owen *et al.*, 1984) of the *alkBAC* operon and the *alkR* locus was described. The position and structure of the 7.5-kb[1] *alkBAC* operon on a 16.9-kb *Eco*RI fragment was established by R-looping experiments and analysis of translation products in *Escherichia coli* minicells (Eggink *et al.*, 1987). The *alkR* locus was found to be localized on an 18-kb *Eco*RI fragment and analyzed by complementation and marker rescue experiments of *alkR* mutations (Owen, 1986). It is likely that these two *Eco*RI fragments encode all regulatory and inducible biochemical activities that are required for alkane utilization.

The availability of the cloned *alk* genes has allowed the construction of *alkBAC-alkR* fusions on a broad host range vector. In this paper, we describe the introduction of the cloned *alk* genes in *Pseudomonas putida* and in *E. coli*, the kinetics of alkane hydroxylase synthesis in these transformants following induction with octane, and the resulting Alk phenotypes. We report that the *alkBAC* promoter is equally effective in both species and that expression of the *alkBAC* operon is strictly dependent on the presence of *alkR* DNA and inducer. Moreover, synthesis of *alkBAC* peptides results in a fully functional alkane hydroxylation system in both *E. coli* and *Pseudomonas*. Given these results, the *alk* promoter-*alkR* system may be a promising expression system for Gram-negative bacteria.

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[1] The abbreviations used are: kb, kilobase pairs; SDS, sodium dodecyl sulfate.

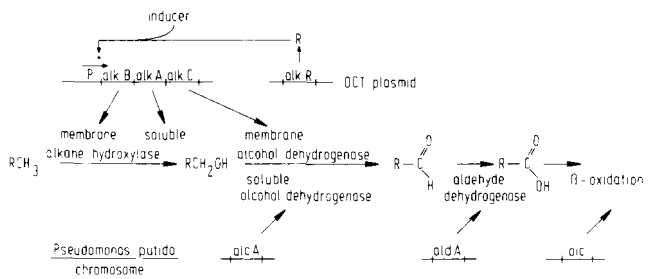

FIG. 1. **Genes and enzymes involved in *n*-alkane oxidation in *P. oleovorans*.** For more detailed information on the structure of the *alkBAC* operon see Fig. 2.

MATERIALS AND METHODS

Bacterial Strains and Media

The bacterial strains and plasmids used in this study are listed in Table I. *E. coli* and *P. putida* cells were grown on L medium or on E-2 medium (Lageveen, 1987) supplied with carbon source (0.2% w/v) and appropriate amino acids (0.01% w/v). Growth on *n*-octane and octanoate was performed at 30 °C on minimal E-2 medium agar plates in closed tins saturated with *n*-octane or octanoate vapor. Tetracycline and ampicillin selection were performed at 15 and 50 μg/ml, respectively.

Recombinant DNA Techniques

Plasmid DNA from *E. coli* and *P. putida* was isolated according to the procedure of Birnboim and Doly (1979). Agarose gel electrophoresis was carried out in Tris-borate EDTA buffer (0.089 M Tris, 0.089 M boric acid, and 2 mM EDTA) (Maniatis *et al.*, 1982). Phage λ *Hind*III fragments were used as molecular weight standards (Bethesda Research Labs GmbH, New Isenburg, West Germany).

Restriction endonucleases and T4 DNA ligase were obtained from Boehringer Mannheim GmbH (Mannheim, West Germany) and Bethesda Research Labs GmbH and used under conditions recommended by the suppliers.

Genetic Procedures

Mobilization of pLAFRI from *E. coli* to *P. putida* was performed according to the triparental mating procedure of Friedman *et al.* (1982). After overnight growth of donor, recipient, and helper strain on an L plate, the exconjugants were selected by replica plating on a minimal medium containing tetracycline, glucose, and the appropriate amino acids.

The preparation of *in vitro* λ phage packaging extracts and the packaging of pLAFRI DNA was done according to the method of Hohn (1979).

We used *E. coli* HB101 for transductions. Strain HB101 was grown overnight in 5 ml of L broth. The cells were centrifuged, resuspended in 5 ml of 10 mM $MgSO_4$, and starved overnight. The *E. coli* cell suspension was mixed with an appropriate amount of *in vitro* packaged cosmids and incubated at room temperature for 15 min. L broth (0.2 ml) was added, and the cell suspension was incubated for 1 h at 37 °C. *E. coli* transductants were plated on L agar containing tetracycline.

Detection of AlkB Gene Product

The induction kinetics of alkane hydroxylase in different strains upon addition of octane was studied by pulse-labeling of cells with [^{35}S]methionine, followed by immunoprecipitation of the *alkB* gene product, and analysis of precipitates by SDS-polyacrylamide gel electrophoresis and fluorography.

Growth Conditions—To exclude possible effects due to previous growth conditions, strains were first grown on E minimal plates during 24 h with a nonrepressing substrate as the carbon source (glycerol in the case of *E. coli* and pyruvate in the case of *Pseudomonas*

TABLE I

Bacterial strains and plasmids

Strain	Relevant genotype or phenotype	Source or reference
E. coli		
HB101	F, *hsdS20, recA13, ara-14 proA2, lacY1, galK2, rpsL20, xyl-5, mtl-1 supE44, leuB6.*	Boyer and Roulland-Dussoix, 1969
DH1	*recA1, endA1, gyrA96, thi-1, hsdR17, supE44*	Maniatis *et al.*, 1982
GEc91	DH1, pGEc29	This study
GEc92	DH1, pGEc47	This study
GEc93	DH1, *fadR*, pGEc47	This study
GEc103	GEc93, cured from pGEc47	This study
GEc137	DH1, *fadR*	This study
GEc139	GEc137, pGEc41	This study
GEc236	GEc137, pGEc29	This study
P. oleovorans TF4-1L		
PPo1	OCT plasmid	Schwartz and McCoy, 1973
GPo12	PPo1 cured from OCT	Kok, unpublished results
GPo15	GPo12, pGEc47	This study
GPo16	GPo12, pGEc41	This study
P. putida		
PpG1	Prototroph (no plasmid)	Chakrabarty *et al.*, 1973
PpS81	*alcA81* (no plasmid)	Grund *et al.*, 1975
PpS124	PpG1 with CAMOCT	Grund *et al.*, 1975
PpS192	*alcA81*(CAMOCT *alkR192*)	Fennewald and Shapiro, 1977
PpS201	*met145 alcA81*(CAMOCT *alkB201*)	Benson *et al.*, 1979
GPp7	PpG1, pGEc47	This study
GPp9	PgG1, pGEc41	This study
GPp10	PpS81, pGEc47	This study
GPp11	PpS81, pGEc41	This study
Plasmid		
pLAFRI	Tc, Tra$^-$, Mob, *cos*, RK2 replicon	Friedman *et al.*, 1982
pRK2013	Km, Tra, ColE1 replicon	Ditta *et al.*, 1980
pGEc29	pLAFRI, *alkBAC* operon	Eggink *et al.*, 1984
pGEc40	pLAFRI, *alkR* locus	Eggink *et al.*, 1984
pGEc41	pLAFRI, *alkBA/alkR*	This study
pGEc47	pLAFRI, *alkBAC/alkR*	This study
pGEc81	pLAFRI, *alkBA/alkR*	This study
pACfadR1	pACYC177, *fadR* locus	DiRusso and Nunn, 1985
pACfadR3	pACYC177, *fadR* locus	DiRusso and Nunn, 1985

species). Cells from a single colony were cultured overnight in test tubes containing 5 ml of E medium and the above mentioned nonrepressing carbon source and transferred to 30 ml of the same medium in 250-ml Erlenmeyer flasks, to give a cell density of about 0.025 mg cell dry weight/ml. Growth was followed at 450 nm in a Zeiss spectrophotometer (Witholt, 1972). At a density of 0.1 mg/ml cell dry weight, 2% (v/v) octane was added in order to induce the formation of alkane hydroxylase.

Pulse-Chase Labeling—Samples were taken from the culture before induction with octane and after induction at 1-h intervals. Samples of 0.5 ml were preincubated during 1 min at 30 °C in Eppendorf cups under magnetic stirring. A mixture of 10–50 μCi of [^{35}S]methionine (specific activity above 1000 Ci/mmol) and cold methionine, calculated to allow continuous uptake during the pulse, was added (Lageveen *et al.*, 1984). Labeling was stopped after 60 s by the addition of 300-fold excess of cold methionine. Samples were withdrawn for the determination of total radioactivity. The remaining cells were centrifuged after 3 min of further incubation, washed with 500 μl of 50 mM Tris-HCl, pH 8.0, resuspended in 100 μl of the same buffer, and stored on ice.

Immunoprecipitation—Labeled cells were converted to spheroplasts by the successive addition of 100 μl of 50 mM Tris-HCl, pH 8.0, containing 0.5 M sucrose at t = 0 min, 5 μl of lysozyme (6 mg/ml) at t = 1 min, and 5 μl of 100 mM EDTA, pH 8.0, at t = 2 min. After incubation on ice for at least 20 min, 200 μl of a 2% SDS buffer was added to the spheroplasts followed by sonication of the suspension for 5 s. Samples for determination of [^{35}S]methionine incorporation were withdrawn, and the proteins were further solubilized by incubation at 70 °C during 10 min.

Radiolabeled samples were prepared for immunoprecipitation as described by Vos *et al.* (1984). 25–100 μl of the resulting radiolabeled sample was incubated with 20 μl of an anti-alkane-hydroxylase serum raised against the purified enzyme (Lageveen, 1987). SDS polyacrylamide gel electrophoresis and fluorography were performed as described by Wensink and Witholt (1981a). Alkane hydroxylase bands were cut out and counted for radioactivity as described by Wensink and Witholt (1981b).

The percentage of alkane hydroxylase was calculated from the amount of radioactivity present in the specific band and the total radioactivity incorporated. This percentage was corrected for background, decay, and relative methionine content in comparison to total cell protein. The percentage of alkane hydroxylase relative to the total newly synthesized protein was calculated. This value can be related directly to the number of copies of this enzyme per cell (Vos *et al.*, 1984).

RESULTS

Cloned alk Sequences of P. oleovorans—From a gene bank of total *P. oleovorans* TF4-1L DNA, established in the broad host range vector pLAFRI, two large *Eco*RI fragments were isolated which contained sequences relevant to alkane oxidation (Eggink *et al.*, 1984). One fragment (pGEc29) of 16.9 kb was able to complement mutations in the *alkB*, *alkA*, and *alkC* loci. Analysis of pGEc29 with R-loop and minicell experiments have revealed the operon structure shown in Fig. 2 (Eggink *et al.*, 1987). Starting from the promoter, the 7.5-kb *alkBAC* operon codes for 41-, 15-, 49-, 58-, 59-, and 20-kDa

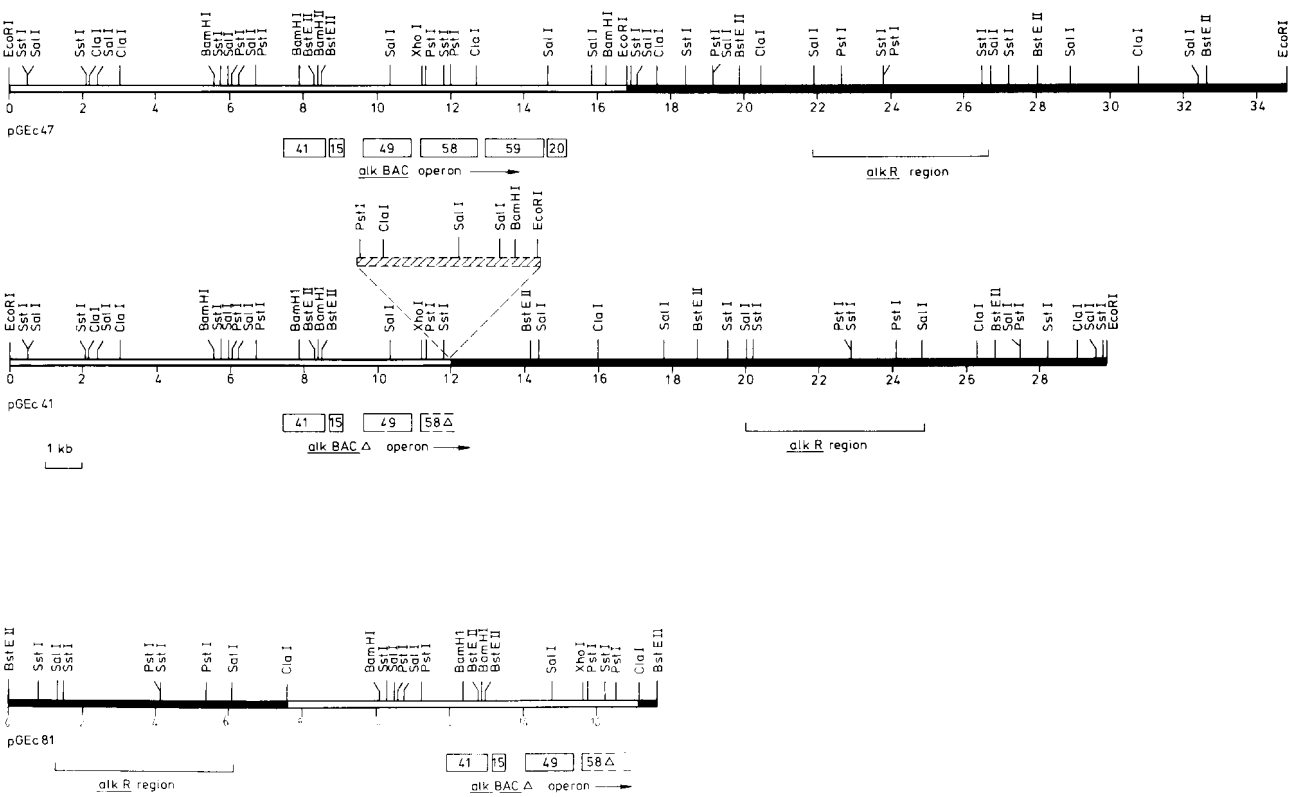

FIG. 2. **DNA inserts in pLAFRI carrying *alkBAC* and *alkR* sequences.** *Open lines* represent DNA sequences derived from the 16.9-kb *Eco*RI fragment containing *alkBAC* sequences. *Solid lines* represent DNA derived from the 18-kb *EcoRI* fragment which contains *alkR*. pGEc47 consists of pLAFRI, the 16.9- and the 18-kb *Eco*RI fragment carrying the *alkBAC* operon and the *alkR* locus, respectively. pGEc41 consists of pLAFRI and a 30-kb *Eco*RI fragment carrying the *alkR* locus and part of the *alkBAC* operon. The *dashed area* down in the *middle* part of the figure refers to the down-stream deletion of the *alkBAC* operon (see "Results"). pGEc81 consists of pLAFRI, an 8.2-kb *Bst*EII fragment carrying the *alkR* locus and 9.7-kb *Cla*I fragment carrying part of *alkBAC*. The position and direction of transcription of the 7.5-kb *alkBAC* operon is given. The *boxes* represent size and position of the proteins encoded by *alkBAC*, and the numbers in the boxes refer to the molecular masses in kiladalton. The region encoding the *alkR* activity is represented by a *thin line*. Note that the *alkR* sequence has the same orientation relative to the *alkBAC* sequence in pGEc41 and pGEc81, whereas its orientation is reversed in pGEc47.

TABLE II

Effect of alkanol dehydrogenase dosage on growth of P. putida and E. coli on octane

Strain	Host	Plasmid	Relevant plasmid genes	Growth on octane vapor[a]
P. putida				
PPo1	*P. oleovorans*	OCT	*alkBAC/R*	++
PpS124	PpG1	CAM-OCT	*alkBAC/R*	++
GPp7	PpG1	pGEc47	*alkBAC/R*	++
GPp9	PpG1	pGEc41	*alkBA/R*	+
GPp10	PpS81 (*alcA*)	pGEc47	*alkBAC/R*	+
GPp11	PpS81 (*alcA*)	pGEc41	*alkBA/R*	±
GPo15	GPo12	pGEc47	*alkBAC/R*	++
GPo16	GPo12	pGEc41	*alkBA/R*	+
E. coli				
DH1				−
GEc137	DH1 (*fadR*)			−
GEc92	DH1	pGEc47	*alkBAC/R*	−
GEc93	DH1 (*fadR*)	pGEc47	*alkBAC/R*	++
GEc139	DH1 (*fadR*)	pGEc41	*alkBA/R*	+
GEc236	DH1 (*fadR*)	pGEc29	*alkBAC*	−

[a] Relative growth was scored by comparing emergence of isolated colonies on minimal plates exposed to octane vapor. ++, normal growth; +, weak growth; ±, leaky growth; −, no growth.

proteins. The 41-kDa protein is alkane hydroxylase, whereas the 15- and 49-kDa proteins are probably soluble components of the alkane hydroxylase. The 58-kDa protein is most likely involved in alkanol dehydrogenase activity. The second *Eco*RI fragment of 18 kb (pGEc40) complements all *alkR* mutations, and genetic analysis has shown that the *alkR* locus is localized on the internal 4.9-kb *Sal*I fragment[2] (1) shown in Fig. 2.

Construction of alkBAC-alkR Combinations—To test expression of the *alkBAC* fragment in various Gram-negative bacteria, it was necessary to coinsert it with the *alkR* fragment, since *alkR* appeared to be absolutely required for *alkBAC* expression. To this end we inserted both *alk* sequences in the same pLAFRI vector to assure coinheritance of both *alk* loci, pGEc29 (containing *alkBAC*) and pGEc40 (containing *alkR*) DNA were *Eco*RI-restricted and ligated in a ratio of 1:1. For the introduction of the recombinant DNA molecules into recipient cells we used *in vitro* packaging. Recombinant plasmids from the resulting tetracycline-resistant *E. coli* transductants were conjugated into PpS201 (*alkB201*), PpS192 (*alkR192*), and PpG1 (no plasmid), and the resulting *P. putida* exconjugants were tested for growth on octane. Four of the recombinant plasmids tested complemented *alkB201* and *alkR192* mutations and enabled PpG1 to grow on octane.

Restriction analysis of these four plasmids showed that three of them (pGEc46, 47, and 52) consisted of pLAFRI and the 16.9 and 18-kb *Eco*RI fragments (Fig. 2). The fourth recombinant, however, had only one *Eco*RI insert of 30 kb (pGEc41). Restriction analysis of pGEc41 revealed a deletion of 5 kb in the fusion between the 16.9 and 18-kb fragments (Fig. 2). As a result of this deletion, the *Eco*RI junction was lost. The deletion stretches into the downstream area of the *alkBAC* operon and ends between the *Sst*I and *Pst*I sites at positions 12.0 and 12.2, respectively. Only a small part, between 0.1 and 0.5 kb, of the 18-kb *Eco*RI *alkR* fragment was lost with the deletion. This means that the *alkR* region located on the internal 4.9-kb *Sal*I fragment is not affected by the deletion.

Phenotype of Strains Equipped with the Recombinant alk Plasmids—*P. putida* strains containing recombinant *alk* plasmids were plated on minimal medium and tested for growth on octane vapor. Table II shows the results obtained for wild-type (Ppo1 and PpS124) and recombinant strains. Examples of such plates exhibiting different growth rates on octane are shown in Fig. 3. Essentially wild-type growth was obtained with the strains GPp7 and Gpo15 containing pGEc47. Therefore, we conclude that the 16.9- and 18-kb *Eco*RI fragments encode all structural and regulatory functions, respectively, for alkane oxidation in *Pseudomonas* and that no other functions encoded by the OCT plasmid are required.

In the partially deleted recombinant plasmid pGEc41, the three distal cistrons, which encode 58-, 59-, and 20-kDa proteins (Fig. 2), are lost. Recombinant strains carrying this plasmid (GPp9 and GPo16) grew on octane, although not as well as compared with GPp7 and GPo15. This shows that the distal half of the *alkBAC* operon is important, but not essential, for growth on alkanes. Previous results suggested that an alkanol dehydrogenase is encoded in this area of the *alkBAC* operon (Fennewald *et al.*, 1979; Owen *et al.*, 1984). To verify this, we introduced pGEc41 and pGEc47 into *P. putida* PpS81, which carries a mutation in the chromosomally encoded alkanol dehydrogenase. Exconjugant strain GPp10 (PpS81, pGEc47) grew at the same rate as GPp9 (PpG1, pGEc41), whereas strain GPp11 (PpS81, pGEc41) showed extremely poor growth on octane (Table II and Fig. 3). This suggests that the distal half of the *alkBAC* operon does indeed encode an alkanol dehydrogenase.

In order to exclude the possibility that the phenotypic characteristics of pGEc41 are caused by unknown effects of the spontaneous deletion, we have made a comparable construct *in vitro*. The internal 8.2-kb *Bst*EII fragment of the 18-kb *Eco*RI fragment was subcloned into the *Bst*EII site of pLAFRI (pGEc74). Then the 9.7-kb *Cla*I fragment carrying *alkBA* sequences was inserted into the *Cla*I site of the *Bst*EII fragment generating pGEc81 (see Fig. 2). The phenotypic characteristics of *P. putida* strains carrying this plasmid are

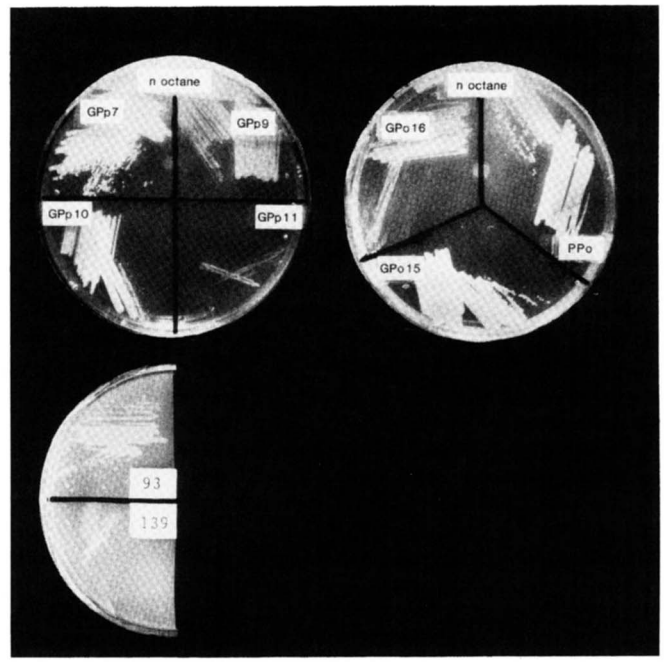

FIG. 3. **Growth of *P. putida* and *E. coli* on octane vapor.** *PPo* is *P. oleovorans* TF4-1L (OCT); *GPo15* is *P. oleovorans* GPo12 (pGEc47); *GPo16* is GPo12 (pGEc41); *GPp7* is PpG1 (pGEc47); *GPp9* is PpG1 (pGEc41); *GPp10* is PPG1 (*alcA81* pGEc47); and *GPp11* is PpG1 (*alcA81* pGEc41). *(GEc)93* is *E. coli* DH1 (*fadR*, pGEc47) and *(GEc)139* is DH1 (*fadR*, pGEc41). The plates with *P. putida* and *E. coli* strains were incubated for 5 and 10 days, respectively, at 30 °C in the presence of octane vapor.

[2] G. Eggink, unpublished results.

the same as those obtained with *P. putida* strains carrying pGEc41.

Growth of E. coli on n-Octane—The expression of the *alkBAC* operon in *E. coli* was studied in *E. coli* DH1 which is *recA* and grows well on minimal medium agar plates with vitamin B1 and an appropriate carbon source. pGEc29 and pGEc47 were introduced into DH1 by means of conjugation, and the resulting strains were designated GEc91 and GEc92, respectively. The tetracycline-resistant exconjugants were plated on minimal medium agar and incubated for 3 weeks at 30 °C in the presence of octane vapor. After 1 week no growth of GEc92 was observed, but after 2 weeks colonies appeared. These colonies were tested for markers and plasmid content. We confirmed these strains, which showed an Alk^+ phenotype (*e.g.* strain GEc93), to be mutated strains of GEc92. Strain GEc91 did not grow on octane even after prolonged incubation on octane. From this we concluded that the *alkBAC* operon can be expressed in *E. coli* only when the *alkR* locus is also present.

To determine the nature of the mutation which enabled *E. coli* to grow on octane, strain GEc93 was cured of pGEc47 by selecting for Alk^- and tetracycline-sensitive phenotype. A cured strain (GEc103) was obtained, into which pGEc47 was introduced again, and a clear Alk^+ phenotype was obtained immediately. From this we concluded that the Alk phenotype of GEc93 was due to expression of the *alk* genes in the presence of a chromosomal mutation. We observed this mutation to be similar to the *fadR* mutants first described by Overath and co-workers (1969), since further tests showed that GEc93 and GEc103 grew well on octanoate, whereas DH1 did not. This suggested that the fatty acid degradation pathway is induced in mutants GEc93 and GEc103 but not in the parent strain DH1. In line with these results, Overath *et al.* (1969) found that expression of the fatty acid degradation (*fad*) genes in *E. coli* is induced only by fatty acids with a chain length longer than C_{12}, but that spontaneous constitutive mutants could be isolated with a frequency of 10^{-5}–10^{-6}, by selection for growth on decanoate. The *fadR* gene product has been shown to be a regulatory protein, which exerts negative control over the fatty acid degradation regulon (DiRusso and Nunn, 1985).

To confirm the relationship between the mutation in GEc93 and the spontaneous *fadR* mutants, we isolated similar spontaneous mutants of DH1 growing on octanoate. When pGEc47 was introduced in such mutants, they immediately showed an Alk^+ phenotype. Thus, *fadR* must be inactivated to allow functional expression of the Alk phenotype in *E. coli*. Further corroboration came from experiments with the *fadR* gene, which was recently cloned by DiRusso and Nunn (1985). After introduction of plasmids carrying the cloned *fadR* gene (pACfadR1 and pACfadR3) in GEc93, the resulting strains lost their ability to grow on octane and octanoate (Table II), although they still carried the *alk* genes.

The *alkBAC* operon encodes two functions: alkane hydroxylation and dehydrogenation of the resulting alkanol. In *P. putida*, the second function is not strictly necessary for growth on octane, since a second alkanol dehydrogenase is encoded on the chromosome. To determine whether the second half of the *alkBAC* operon can also be deleted in *E. coli*, we introduced plasmid pGEc41 into GEc137. Surprisingly, the exconjugant GEc139 grew on *n*-octane, albeit at a lower growth rate (Fig. 3 and Table II). Thus, for growth of *E. coli* on octane, only the alkane hydroxylation system is necessary. As in the case for *Pseudomonas*, *E. coli* seems to be fitted with suitable chromosomally encoded alkanol and aldehyde dehydrogenases. However, we were unable to show growth of *E. coli* on plates with octanol or octanal as the sole source of carbon and energy. It is likely that these substrates are toxic for *E. coli* when added exogenously, since these strains were also unable to grow on glucose in the presence of octanol or octanal vapor.

Induction Kinetics of the Alkane Hydroxylase—The induction kinetics of the *alkBAC* gene products in *Pseudomonas* and *E. coli* were examined by following the appearance of the newly synthesized 41-kDa membrane associated alkane hydroxylase, which is the *alkB* product. To this end, antibodies raised against this protein were used to immunoprecipitate the radiolabeled protein in pulse-labeled cells of cultures induced with octane.

Cells were pulse-labeled with [^{35}S]methionine during growth on pyruvate before and after induction with octane. Following immunoprecipitation, radiolabeled proteins were separated by SDS-polyacrylamide gel electrophoresis and visualized by fluorography. Typical fluorograms of such immunoprecipitates are shown in Fig. 4. Before induction with octane, no 41-kDa band (alkane hydroxylase) could be observed in *Pseudomonas* or *E. coli* immunoprecipitates, not even after prolonged exposure. However, 5 min after addition of octane, alkane hydroxylase could already be detected by immunoprecipitation in both strains. The amount of alkane hydroxylase increased during the induction process as shown in Fig. 4 (*lanes 0–5*).

Quantitation of the 41-kDa band and further calculation of the amount of alkane hydroxylase in comparison to total newly synthesized protein gave the induction kinetics of this specific gene product (Fig. 5). In both *E. coli* and *Pseudomonas* strains, no expression could be detected when *alkR* was absent, which indicates that *alkR* is absolutely required for expression of the *alkBAC* operon. The kinetics of the induction process were nearly identical for both species. There was a rapid increase of the amount of the alkane hydroxylase directly after the addition of the inducer *n*-octane. After about 2 h of growth in the presence of octane, a steady-state level was

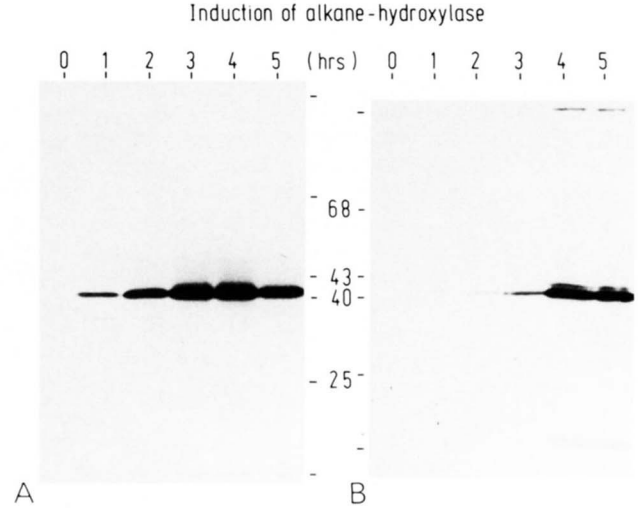

FIG. 4. **Appearance of newly synthesized alkane hydroxylase in *P. oleovorans* (*P. putida*, OCT) and *E. coli* upon induction.** Exponentially growing bacteria were induced with octane. Samples of these cultures were pulse-labeled with [^{35}S]methionine, and alkane hydroxylase was immunoprecipitated as described under "Materials and Methods." The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis followed by fluorography. *A*, *P. oleovorans*. *B*, *E. coli*, GEc93. Time of induction (hours) is indicated above the lanes. Molecular weight standards indicated between the panels are: bovine serum albumin (68,000), ovalbumine (43,000), aldolase (40,000), and chymotrypsinogen A (25,000).

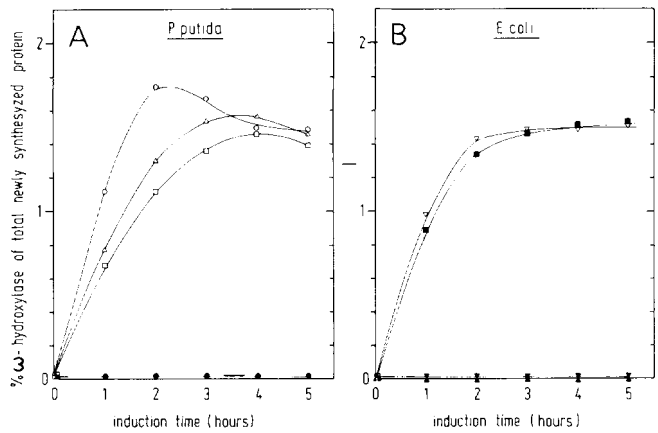

FIG. 5. **Induction kinetics of alkane hydroxylase in *P. putida* and *E. coli*.** Pulse-chase labeling with [^{35}S]methionine, immunoprecipitations, SDS-polyacrylamide gel electrophoresis, and fluorography were done as described under "Materials and Methods." The 41-kDa band was excised from the gel, rehydrated, and counted for radioactivity. The percentage of alkane hydroxylase relative to newly synthesized total protein was calculated for the different time points as described under "Materials and Methods." *A*, *P. oleovorans* noninduced (●); *P. oleovorans* induced (○); PpS 124 (PPG1, CAM-OCT) induced (△); GPp (PpG1, pGEc47) induced (□). *B*, *E. coli* strains: DH1 induced (▼); GEc93 (DH1, *fadR*, pGEc47) noninduced (▲); GEc93 induced (▽); GEc92 (DH1, pGEc47) induced (■).

reached in which the alkane hydroxylase accounted for 25–30% of the total cytoplasmic membrane protein (data not shown) and for about 1.5% of the total newly synthesized cellular protein (Fig. 5).

DISCUSSION

Expression of Cloned alk Genes in Pseudomonas—The availability of the cloned *alkBAC* operon and the *alkR* locus on two *Eco*RI fragments enabled us to reconstitute the *alk* system on a broad host range vector pLAFRI. This recombinant plasmid (pGEc47) endowed *P. putida* with a wild-type Alk phenotype, which implies that no other functions encoded by the OCT plasmid are required for alkane utilization. Moreover, these experiments confirm the genetic indications (Fennewald *et al.*, 1979; Eggink *et al.*, 1984; Owen *et al.*, 1984) that the *alk* genes are clustered in two regions: the *alkBAC* operon contains the structural genes and is under positive regulatory control of the *alkR* region.

The *alkBAC* operon covers about 8 kb of a 16.9-kb *Eco*RI fragment[2] and the *alkR* region was found to reside on a 4.9-kb subclone of an 18-kb *Eco*RI fragment.[3] Thus, it appears that only 13 kb of the very large OCT plasmid are essential for alkane utilization. The size of the OCT plasmid has been estimated to be over 300 kb (Harder and Kunz, 1986) and in our laboratory a size of 400–500 kb was recently found using electron microscopy.[4]

Two recombinant plasmids carrying *alkBAC/R*-derived sequences were introduced in various *P. putida* and *E. coli* backgrounds to study the resulting Alk phenotype. pGEc47 contains the complete *alkBAC* and *alkR* sequences. The second plasmid pGEc41 contains *alkBA* and *alkR* sequences since a spontaneous deletion affected the last three cistrons of the *alkBAC* operon, encoding 58-, 59-, and 20-kDa proteins, respectively. The introduction of these recombinant plasmids into a wild-type *P. putida* strain (PpG1) and a *P. putida* strain lacking the chromosomal alkanol dehydrogenase (PpS81), resulted in some interesting observations (Table II). We observed less than wild-type growth on octane when pGEc47 was introduced in PpS81, which implies that the chromosomally encoded alkanol dehydrogenase is needed to give maximal growth on octane. Introduction of pGEc41 into PpS81 nearly abolished growth on octane, indicating that this plasmid did not encode alkanol dehydrogenase (*alkC*) properly. This result is in agreement with the mapping of *alkC* point mutations (Owen *et al.*, 1984) in the area encoding the 58-kDa protein.

Growth of E. coli on n-Octane—We were surprised to find that introduction of the *alk* system in *E. coli* was sufficient to extend the substrate range of this bacterium towards C_6–C_{12} alkanes. In fact, only the alkane hydroxylase complex (*alkBA*) and the regulatory proteins (*alkR*) were strictly necessary for growth of *E. coli* on octane. *E. coli* itself apparently provides the necessary alkanol and aldehyde dehydrogenation activities. For subsequent fatty acid degradation, however, a mutation in the *fadR* gene is required. This gene encodes a repressor protein of 29 kDa that prevents transcription of the *fad* genes in the absence of long chain fatty acids (DiRusso and Nunn, 1985). Spontaneous mutations in the *fadR* gene may occur at high frequency, resulting in constitutive expression of the *fad* genes (Overath *et al.*, 1969).

P. oleovorans is particularly well suited for growth in media consisting of an aqueous and a bulk apolar phase (De Smet *et al.*, 1983; Lageveen, 1987). When *P. oleovorans* is shifted from glucose or citrate to such a two-phase medium, there are changes in the cell envelope which protect *P. oleovorans* from the bulk apolar phase (De Smet *et al.*, 1983). Given the sensitivity of *E. coli* to apolar solvents such as toluene (De Smet *et al.*, 1978), we had expected that the exposure of *E. coli* to alkanes might well be lethal. The above results indicate that this is not the case: given the appropriate enzymes, *E. coli* is able to oxidize and grow on alkanes. The behavior of *E. coli* in the presence of bulk apolar phases is now being investigated.

Induction Kinetics of the Alkane Hydroxylase—Our results show that *E. coli* is capable of normal regulated expression in *alk* genes of the OCT plasmid. The induction kinetics of the *alkB* gene product alkane hydroxylase were nearly identical in *P. putida* and *E. coli*. After 2 h of induction the 41-kDa alkane hydroxylase reached its maximal level accounting for about 1.5% of the total newly synthesized protein, which corresponds to about 35,000 copies of alkane hydroxylase/cell. Taking into account that the *alk* genes studied are present on low copy number plasmids, such as OCT or pLAFRI, one can conclude that the *alkBAC* operon is efficiently expressed. Comparison of preliminary results on the promoter sequence of the *alkBAC* operon[4] revealed no homology with the *E. coli* consensus promoter but some homology with known *xyl* promoters.

Thus, the *alk* system shows both similarities and differences with other *P. putida*-derived catabolic regulons such as *xyl* and *nah*. The latter are expressed and regulated similarly in *P. putida* and *E. coli*, but the level of expression in *E. coli* is 10–20-fold lower than in *P. putida* (Schell, 1985; Inouye *et al.*, 1983). Although Inouye *et al.* (1985) have found identical transcription initiation sites for the *P. putida xylABC* and *xylDEFG* and *xylR* genes in both *P. putida* and *E. coli*, the induced mRNA levels of *xylABC* and *xylDEFG* are higher in *P. putida* than in *E. coli*, suggesting that inefficient transcription by *E. coli* RNA polymerase accounts for the low expression of *Pseudomonas* genes in *E. coli*. Clearly this is not the case for *alkBAC* genes, which are transcribed efficiently by *E. coli* RNA polymerase.

[3] M. Kok, unpublished results.
[4] G. Eggink and A. Arnberg, unpublished results.

The *alkR* locus is strictly required for the expression of the *alkBAC* operon, since in the absence of *alkR* we could detect neither expression of the *alkB* gene product with antibodies nor growth on octane of *alkBAC* containing *P. putida* strains. Owen (1986) has concluded from complementation and marker rescue experiments that at least three cistrons are involved in transcriptional regulation of the *alkBAC* operon. The complexity of the *alkR* locus makes it even more surprising that the *alk* system is fully functional in *E. coli* as well as in *Pseudomonas*.

In conclusion, the *alkBAC/alkR* system is the first *P. putida* expression system to be described which is controlled identically in *Pseudomonas* and in *E. coli*. It is therefore an interesting candidate for a broad host range expression system, with the added advantage of a well-regulated promoter, which can be induced with inexpensive aliphatic compounds.

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