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

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Gerrit Eggink, Roland G. Lageveen, Bert Altenburg, and Bernard Witholt

From the Department of Biochemistry, Groningen Biotechnology Center, University of Groningen, Nijenborgh 16, 8747 AG Groningen, The Netherlands

The OCT plasmid encodes enzymes for alkane hydroxylation and alkane 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 alkR gene product was lost, chromosomally encoded alkR 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_2-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 alkane. 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 operon 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 dicyclopentylketone (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 alkane, alkalan, and fatty acid oxidation: alca, alldA, 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 alkBAC operon on a 16.9-kb EcoRI fragment was established by R-looping experiments and analysis of translation products in *Escherichia coli* minicells (Eggink *et al.*, 1984). The alkR locus was found to be localized on an 18-kb EcoRI fragment and analyzed by complementation and marker rescue experiments of alkR mutations (Owen, 1986). It is likely that these two EcoRI 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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HindIII fragments were used as molecular weight standards (Beb-drive and ampicillin selection were performed at 15 and 50 μg/ml, respectively. Growth on n-octane and octanoate was performed at 30 °C on minimal E-2 medium agar plates. The induction kinetics of alkane hydroxylase in different strains upon addition of octane was studied by pulse-labeling of cells with [36S]methionine, followed by immunoprecipitation of the alkB gene product, and analysis of precipitates by SDS-polyacrylamide gel electrophoresis and fluorography.

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 HindIII 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 MgSO4, 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 [36S]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-2 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 oleovoran).

Table I

| Strain | Relevant genotype or phenotype | Source or reference |
|--------|-------------------------------|-------------------|
| E. coli | F, hsdS20, recA13, ara-14 proA2, lacY1, galK2, rpsL20, xyl-5, mit-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, pGEc47 | This study |
| P. putida TF4-1L | OCT plasmid | Schwartz and McCoy, 1973 |
| PP01 | OCT plasmid | Kok, unpublished results |
| PP012 | PP01 cured from OCT | This study |
| PP015 | PP012, pGEc47 | This study |
| PP016 | PP012, pGEc41 | This study |
| P. oleovorans | | Chakrabarty et al., 1973 |
| PP01 | Prototroph (no plasmid) | Grund et al., 1975 |
| PP0124 | PP01 with CAMOCT | Grund et al., 1975 |
| PP0129 | alkB129 (CAMOCT alkR129) | Fenneland and Shapiro, 1977 |
| PP0130 | met145 alkA1 (CAMOCT alkB201) | Benson et al., 1979 |
| GP07 | PpG1, pGEc47 | This study |
| GP09 | PpG1, pGEc41 | This study |
| GP10 | PpS81, pGEc47 | This study |
| GP11 | PpS81, pGEc41 | This study |
| Plasmid | | |
| pLAFRI | Tc, Tra, Mob, cos, RK2 replicon | Friedmann 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 |
| pGEc56 | pLAFRI, alkBA/alkR | This study |
| pACfamR1 | pACYC177, fadR locus | DiRusso and Nunn, 1985 |
| pACfamR3 | pACYC177, fadR locus | DiRusso and Nunn, 1985 |
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specifies). Cells from a single colony were cultured overnight in test tubes containing 5 ml of E medium and the above mentioned non-repressing 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, 1981). 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 [35S]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 [35S]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 EcoRI 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 proteins.

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**FIG. 2.** DNA inserts in pLAFRI carrying alkBAC and alkR sequences. Open lines represent DNA sequences derived from the 16.9-kb EcoRI 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 EcoRI fragment carrying the alkBAC operon and the alkR locus, respectively. pGEc41 consists of pLAFRI and a 36-kb EcoRI 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 BstEII fragment carrying the alkR locus and 9.7-kb ClaI 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 kilodalton. 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.
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 alkyl dehydrogenase activity. The second EcoRI 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 SalI fragment of 18 kb (pGEc41) (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 EcoRI-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 (alkB20I), PpS192 (alkR192), and PpGl (no plasmid), and the resulting P. putida exconjugants were tested for growth on octane. Four of the recombinant plasmids tested complemented alkBZOI (fadR, alca1) which carries a mutation in the chromosomally encoded alkane dehydrogenase. Exconjugant strain GPp10 (PpS81, pGEc47) grew at the same rate as GPp9 (PpG1, pGEc47), 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 alkane 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 BstEII fragment of the 18-kb EcoRI fragment was subcloned into the BstEII site of pLAFRI (pGEc74). Then the 9.7-kb Clal fragment carrying alkBA sequences was inserted into the Clal site of the BstEII 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.](image_url)
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 C12, 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 alkR 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 [³⁵S]methionine during growth on pyruvate 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 [³⁵S]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).](image-url)
lacking the chromosomal alkanol dehydrogenase (PpS81), a spontaneous deletion affected the last three cistrons of the alkBAC operon, encoding 58-, 59-, and 20-kDa proteins, resulting plasmid pGEc41 contains alkBA and alkR sequences since it contains the complete alkBAC and alkR sequences. The sec backgrounds to study the resulting Alk phenotype. pGEc47 that only 13 kb of the very large OCT plasmid are essential respectively. The introduction of these recombinant plasmids for radioactivity. The percentage of alkane hydroxylase relative to the cellular protein (Fig. 5).

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 EcoRI 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., 1973; 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 EcoRI fragment and the alkR region was found to reside on a 4.9-kb subclone of an 18-kb EcoRI fragment. 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.

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 C6-C12 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. putida* 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. putida* is shifted from glucose or citrate to such a two-phase medium, there are changes in the cell envelope which protect *P. putida* from the bulk apolar phase (De Smet et al., 1983; Lageveen, 1987). 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 revealed no homology with the *E. coli* consensus promotor 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.
The \textit{alkR} locus is strictly required for the expression of the \textit{alkBAC} operon, since in the absence of \textit{alkR} we could detect neither expression of the \textit{alkR} gene product with antibodies nor growth on octane of \textit{alkBAC} containing \textit{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 \textit{alkBAC} operon.

The complexity of the \textit{alkR} locus makes it even more surprising that the \textit{alk} system is fully functional in \textit{E. coli} as well as in \textit{Pseudomonas}.

In conclusion, the \textit{alkBAC/alkR} system is the first \textit{P. putida} expression system to be described which is controlled identically in \textit{Pseudomonas} and in \textit{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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