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Insertion Element Analysis and Mapping of the *Pseudomonas* Plasmid *alk* Regulon

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We characterized and mapped new mutations of the *alk* (alkane utilization) genes found on *Pseudomonas* plasmids of the Inc P-2 group. These mutations were isolated after (i) nitrosoguanidine mutagenesis, (ii) transposition of the Tn7 trimethoprim and streptomycin resistance determinant, and (iii) reversion of polarity effects of *alk*::Tn7 insertion mutations. Our results indicate the existence of two *alk* loci not previously described—*alkD*, whose product is required for synthesis of membrane alkane-oxidizing activites, and *alkE*, whose product is required for synthesis of inducible membrane alcohol dehydrogenase activity. Polarity of *alk*::Tn7 insertion mutations indicates the existence of an *alkBAE* operon. Mapping of *alk* loci by transduction in *P. aeruginosa* shows that there are at least three *alk* clusters in the CAM-OCT plasmid—*alkRD*, containing regulatory genes; *alkBAE*, containing genes for specific biochemical activities; and *alkC*, containing one or more genes needed for normal synthesis of membrane alcohol dehydrogenase. The *alkRD* and *alkBAE* clusters are linked but separated by about 42 kilobases. The *alkC* cluster is not linked to either of the other two *alk* regions. Altogether, these results indicate a complex genetic control of the alkane utilization phenotype in *P. putida* and *P. aeruginosa* involving at least six separate genes.

Oxidation of 6- to 10-carbon *n*-alkanes by *Pseudomonas putida* and most *P. aeruginosa* strains depends on the *alk* genes found on OCT, CAM-OCT, and other Inc P-2 plasmids (6, 7, 10, 13). Analysis of plasmid *alk* mutations indicated the existence of at least three genes for specific enzymatic activities—*alkA*, *alkB*, and *alkC* (3)—as well as one or more regulatory genes (8). The *alk* genes appear to constitute a single regulon because noninducible, constitutive, or altered inducer specificity phenotypes in various mutants are the same for all three assayable plasmid-determined activities: soluble alkane hydroxylase component ($AlkA^+$ activity), membrane alkane hydroxylase component ($AlkB^+$ activity), and membrane alcohol dehydrogenase (8).

In this paper we summarize further results on the genetic analysis of the *alk* system. The data suggest additional complexity in the system. The characteristics of new nitrosoguanidine-induced mutations generally confirm earlier conclusions, but the properties of one of these mutants indicate the existence of a new regulatory gene, *alkD*, whose product appears to be essential for synthesis of membrane alkane-oxidizing activities. Analysis of polar effects caused by insertion of the Tn7 trimethoprim (Tp) resistance transposon into *alk* genes indicates the existence of an *alkBAE* operon, where *alkE* is a new locus determining synthesis of membrane alcohol dehydrogenase activity. Linkage studies with Tn7 inserts show that there are at least three distinct *alk* clusters—an *alkRD* regulatory region, the *alkBAE* operon, and the *alkC* alcohol dehydrogenase locus. The *alkRD* and *alkBAE* clusters are linked but separated by about 25 megadaltons of DNA. These results correct the mistaken conclusion that all *alk* loci are tightly clustered (8), and the mapping results support the idea that products of *alk* regulatory genes operate at a distance.

Both our recombinational analysis and polarity studies depended on the isolation of Tn7 insertions into or near the *alk* loci of the CAM-OCT plasmid (9). In particular, the use of Tn7 insertions close to, but not within, the *alk* loci merits emphasis. Without these artificially constructed linked markers, we would not have been able to determine the relative locations of various *alk* genes.

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MATERIALS AND METHODS

Bacterial strains. The basic strains used in these experiments are listed in Table 1. Congenic series of strains for physiological and genetic tests were constructed by crossing CAM-OCT or pBP5::Tn*401* plasmids (7) carrying various *alk* alleles into the same *P. putida* (PpS) or *P. aeruginosa* (PAS) hosts. Growth tests and biochemical assays were carried out in the PpS338 background. Transduction analysis utilized PAS102 derivatives as donors and PAS75 derivatives as recipients.

Media, bacteriophage, and transduction methods. These have been described previously (2, 3, 8, 10, 16). Phage F116C is a clear-plaque mutant of F116L (12). F116C transduces lysogenic recipients with the same efficiency as F116L but is easier to grow on confluent lysis plates.

Genetic analysis and strain construction. (i) "Orgy" method for isolating *alk*-linked Tn*7* insertions. Crosses between PAS278 and PAC5 yield *thr*$^+$ *his* Tp^r exconjugants, most of which contain CAM-OCT::Tn*7* plasmids that arose in transposition events in the donor (9). Many of these plasmids will contain Tn*7* inserted near enough to an *alk* gene to be cotransducible with an appropriate *alk* mutation by F116L. Accordingly, we plated out such crosses on PA–glucose–histidine–trimethoprim (1 mg/ml) agar to yield at least 1,000 colonies per plate. These colonies were then washed off without purification, the resulting orgy cultures were used to prepare F116C orgy lysates by confluent lysis methods, and these lysates were then used to transduce various *alk* lysogens to *alk*$^+$. The *alk*$^+$ Tp^r transductants were identified by replica-plating, and the *alk*-Tn*7* linkage was confirmed in each case by crossing the CAM-OCT::Tn*7* plasmids to *P. putida*, scoring the plasmid-determined phenotypes, retransfer to PAS102, and repeating transductions with pure donor cultures. The two linked Tn*7* insertions used in this paper are called Tn7_{320} and Tn7_{322}. Both were isolated after orgy lysate transduction of a PAS75 derivative carrying the CAM-OCT *alkD208* plasmid to *alk*$^+$. The locations of these insertions are deduced from the data described in the text.

(ii) Two-factor crosses. To measure the cotransduction of the Tn7_{320} and Tn7_{322} insertions with various *alk* mutations, we grew F116C on PAS320 and PAS322, used these lysates to transduce PAS75 derivatives carrying CAM-OCT *alk* plasmids to *alk*$^+$, and then tested the *alk*$^+$ transductants for inheritance of Tn*7* by replica-plating to TYE–trimethoprim (1 mg/ml) agar. The data in Fig. 1 are expressed as the percentage of *alk*$^+$ transductants which are also Tp^r.

(iii) Three-point mapping experiments. To do these experiments, we constructed related series of CAM-OCT *alk* plasmids carrying the *alk*-linked Tn7_{320} or Tn7_{322} insertion by transducing PAS75 derivatives harboring various CAM-OCT *alk* plasmids to Tp^r with F116C grown on PAS320 or PAS322, identifying the Alk$^-$ Tp^r transductants, and crossing the plasmids to PpS338 and then to PAS102 (selecting Cam$^+$ exconjugants) to serve as transductional donors. Our results indicate that the Tn*7* insertions in the CAM-OCT::Tn*7* *alk* plasmids derived this way are the same as those in the parental *alk*$^+$ plasmids. F116C lysates grown on CAM-OCT::Tn7_{320} *alk* or CAM-OCT::Tn7_{322} *alk* strains were used to transduce PAS75 derivatives carrying various CAM-OCT *alk* plasmids, and the *alk*$^+$ transductants were selected on PA–histidine agar in the presence of heptane vapors, stabbed into the same selective medium, and tested for inheritance of Tn*7* by replica-plating to TYE–trimethoprim (1 mg/ml) plates. We only scored crosses where *alk*$^+$ transductants clearly outnumbered revertants, and the cotransduction data were corrected for the number of *alk*$^+$ Tp^s revertants found on control plates. Map orders of the *alk* mutations and the Tn*7* insertion were determined by standard methods.

(iv) Reversion tests. Isolation of spontaneous and UV-induced revertants has been described before (8). Stimulation of reversion by UV light (250 to 500 ergs/mm^2) was accomplished by irradiating selection plates directly. Hence, individual UV-induced revertants arose independently of each other. We have not corrected for UV killing under these conditions (survival not determined) so that the increase in the numbers of revertant colonies is only a minimal estimate of the effectiveness of UV as a mutagen. The selection of revertants on PA agar in the presence of nonanol vapors picks up either clones which can synthesize an active plasmid-determined alcohol dehydrogenase or those in which the chromosomal *alcA* mutation has reverted or been suppressed. Hence, it is necessary to test the plasmids in these clones by replica-mating (9) to a second *alcA* host (e.g., from the PpS338 background to PpS597 or PpS598).

(v) Deletion mapping. In transductional crosses between *thr-102* (CAM-OCT *alk*) donors and *his-5* (F116L) (CAM-OCT Δ*alk*) recipients, we used transduction to *his*$^+$ as a control. A cross was only considered definitely negative when we obtained no *alk*$^+$ and at least 100 *his*$^+$ transductants. Because we used lysogenic recipients, these crosses could be carried out by spotting F116C lysates on at least 10^9 recipient cells spread on either PA–glucose agar (to select *his*$^+$) or PA–histidine agar incubated in the presence of heptane vapors (to select *alk*$^+$).

Enzyme induction, in vitro assays and growth tests. Most of the methods of enzyme induction, in vitro assays, and growth tests have been described previously (4, 5, 8, 10, 16). To score alkane- and alcohol-growth phenotypes semi-quantitatively, we used congenic strains derived from PpS338 carrying either an *alk*$^+$ plasmid, an *alk* plasmid, or no plasmid at all. These strains were streaked on sectors of PA–tryptophan agar and incubated in the presence of octane or nonanol vapors at 32°C, and the emergence of isolated colonies was scored daily. Specific alkane hydroxylase activity is expressed as 100 × nanomoles of [^{14}C]nonane oxidized per minute per milligram of protein. Specific membrane alcohol dehydrogenase activity is expressed as micromoles of dichlorophenol-indophenol reduced per minute per milligram of protein in response to nonanol substrate.

RESULTS

Classification of additional nitrosoguanidine-induced *alk* mutations. We have already described *alkA*, *alkB*, and *alkC* mutations which block synthesis of, respectively, AlkA$^+$, AlkB$^+$, or membrane alcohol dehydrogenase activities (3). Additional strains carrying new mutations in these classes are listed in Table 1. We have also described a group of regulatory mutations which lead to a noninducible phenotype for all three activities (8). We will now designate these mutations *alkR*, until they can be further distinguished by mapping or complementation tests. Some *alkR* mutations revert to various phenotypes which have altered inducer recognition specificity (8). We have recently isolated a similar mutant directly from wild-type strains after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) mutagenesis and call the mutation

TABLE 1. *Bacterial strains*[a]

Strain	Genotype	Source or reference
P. aeruginosa		
PAC5	*his-5*	P. H. Clarke
PAS75	*his-5*(F116L)	PAC5 lysogen
PAS102	*thr-102*	8
PAS278	*thr-102*(CAM-OCT) (RP4::Tn*7*)	9
PAS320	*thr-102*(CAM-OCT::Tn*7*$_{320}$)	This paper
PAS332	*thr-102*(CAM-OCT::Tn*7*$_{322}$)	This paper
P. putida		
PpG1	Prototroph (no plasmid)	I. C. Gunsalus
PpS104	*leu-104*	PpGl·NTG[b]
PpS208	*alcA81 met-145*(CAM-OCT *alkD208*)	10
PpS338	*alcA81 trp-338*	2
PpS353	*alcA81 trp-338*(CAM-OCT::Tn*401*$_{353}$)	2
PpS597	*alcA437 his-597*	S. Benson, Ph.D. thesis, (University of Chicago, Chicago, Ill., 1978)
PpS598	*alcA437 met-598*	S. Benson, Ph.D. thesis, (University of Chicago, Chicago, Ill., 1978)
PpS601	*leu-104*(pMF585)	6, 8
PpS784	*alcA437 his-597*(pMF585 *alkB784*)	PpS601·NTG × PpS597
PpS794	*alcA437 his-597*(pMF585 *alkB794*)	PpS601·NTG × PpS597
PpS1016	*alcA437 met-598*(pBP5::Tn*401*)	W. Prevatt
PpS1025	*alcA81 trp-338*(CAM-OCT::Tn*401*$_{353}$ *alkC1025*)	PpS353·NTG
PpS1027	*alcA81 trp-338*(CAM-OCT::Tn*401*$_{353}$ *alkC1027*)	PpS353·NTG
PpS1029	*alcA81 trp-338*(CAM-OCT::Tn*401*$_{353}$ *alkB1029*)	PpS353·NTG
PpS1031	*alcA81 trp-338*(CAM-OCT::Tn*401*$_{353}$ *alkB1031*)	PpS353·NTG
PpS1033	*alcA81 trp-338*(CAM-OCT::Tn*401*$_{353}$ *alkA1033*)	PpS353·NTG
PpS1037	*alcA81 trp-338*(CAM-OCT::Tn*401*$_{353}$ *alkC1037*)	PpS353·NTG
PpS1039	*alcA81 trp-338*(CAM-OCT::Tn*401*$_{353}$ *alkC1039*)	PpS353·NTG
PpS1045	*alcA81 trp-338*(CAM-OCT::Tn*401*$_{353}$ *alkC1045*)	PpS353·NTG
PpS1047	*alcA81 trp-338*(CAM-OCT::Tn*401*$_{353}$ *alk-1047*)	PpS353·NTG
PpS1165	*alcA81 trp-338*(pBP5::Tn*401 alkR1165*)	PpS1016·NTG × PpS338
PpS1167	*alcA81 trp-338*(pBP5::Tn*401 alkB1167*)	PpS1016·NTG × PpS338
PpS1195	*alcA81 trp-338*(pBP5::Tn*401 alkB1195*)	PpS1016·NTG × PpS338

[a] The Alk$^+$ parental IncP-2 plasmids have been described previously (2, 6, 7, 13). We have omitted a large number of *P. putida* strains carrying CAM-OCT *alk*::Tn*7* plasmids (9), revertant plasmids from these insertion mutants, and revertant plasmids isolated from PpS208. We have also left out the *P. aeruginosa* strains constructed for mapping by introducing various plasmids into PAC5, PAS75, and PAS102. Some mutant plasmids were isolated by crossing pMF585 or pBP5::Tn*401* out of NTG-treated cultures into PpS597 or PpS338 and screening exconjugants for Alk$^-$ clones.

[b] The name of a strain followed by ·NTG indicates a culture of that strain mutagenized by NTG.

alkR1165 (Table 2). The fact that we can isolate a mutant with altered inducer recognition specificity directly from wild type suggests that a change in a single regulatory gene product can confer this novel phenotype. Genetic results given below show that all *alkR* alleles map together in a small region of the CAM-OCT plasmid.

In addition to *alkA*, *alkB*, *alkC*, and *alkR* mutants, we also have two NTG-induced mutants which lack AlkB$^+$ and alcohol dehydrogenase activities but are normally inducible for AlkA$^+$ activity. At first we classified these as strains carrying polar mutations, but further study has shown that this classification is incorrect. One of these mutants (PpS1047) does not revert to wild type and so may have a double mutation. The other mutant reverts to wild type, and we have designated the mutation *alkD208*. The enzymatic profile of a strain carrying *alkD208* is given in Table 3, and the results of reversion analysis are summarized in Table 4. The *alkD208* mutation gives both *alk*$^+$ and *alkB* revertants when selected only for recovery of alcohol dehydrogenase. (Selection only for recovery of AlkB$^+$ activity in an *alcA*$^+$ background gives uniquely *alk*$^+$ revertants.) This observation originally suggested to us that *alkD208* was a polar mutation of the *alkB* gene (3, 8, 19), but mapping of *alkD208* and further characterization of the *alkB* revertants clearly shows that this reversion pattern reflects a genetic peculiarity of the *alkD208* mutation itself rather than a polar effect (see below).

Characterization of *alk*::Tn*7* mutants. A previous paper reports the isolation of a series of CAM-OCT *alk*::Tn7 plasmids in *P. aeruginosa* (9). Because the *P. aeruginosa* strains we used contain chromosomal genes for growth on primary aliphatic alcohols, we had to adopt a special strategy to identify Tn7 insertions that inactivated plasmid alcohol dehydrogenase synthesis without affecting alkane hydroxylase activity. We did this by growing F116C-transducing phage on an unselected (orgy) population of CAM-OCT::Tn7 exconjugants, transducing *alk* recipients to Alk$^+$ (i.e., hydroxylase positive) in *P. aeruginosa*, and then testing the transductants for plasmids which have an alkane-, alcohol-negative growth phenotype after transfer of Cam$^+$ determinants to an *alcA P. putida* host lacking a chromosomal function needed for alcohol growth. In this way, we identified two putative *alkC*::Tn7 insertions, *alk-1170* and *alk-1345*. In addition, we later found that two very leaky *alk*::Tn7 insertion plasmids isolated directly in *P. aeruginosa* (CAM-OCT *alk-1128* and *alk-1132*) also appear to contain *alkC*::Tn7 insertions.

The results of growth tests and enzyme assays on *P. putida alk*::Tn7 mutants and their alcohol-positive revertants are summarized in Tables 5 and 6. All hydroxylase-negative (alkane growth-negative) insertion mutants are also alcohol dehydrogenase (alcohol growth) negative. This suggests that the hydroxylase cistrons are upstream of one or more dehydrogenase cistrons in an operon. This interpretation is consistent with the fact that some of the insertions revert to alcohol-positive, alkane-negative phenotypes. All insertions lacking AlkB$^+$ activity also lack AlkA$^+$, but there are three AlkA$^-$ AlkB$^+$ mutants (*alk-1117*, *alk-1122*, and *alk-1131*). This indicates that *alkB* is upstream of *alkA* in an operon, which is consistent with the isolation of AlkB$^-$ AlkA$^+$ polarity revertants from AlkB$^-$ AlkA$^-$ insertions (*alkB*::Tn7) and of AlkB$^+$ AlkA$^-$ polarity revertants from AlkB$^+$ AlkA$^-$ insertions (*alkA*::Tn7). Because polarity reversion generally occurs by deletion of the inserted element (14), both *alkA*::Tn7 and *alkB*::Tn7 mutants yield AlkA$^-$ AlkB$^-$ revertants which

TABLE 3. *Enzymatic profile of PpS338(CAM-OCT alkD208)*[a]

Inducer	Alkane hydroxylase activity			Alcohol dehydrogenase activity
	Alone	+AlkA$^-$B$^+$ extract	+AlkA$^+$B$^-$ extract	
None	<1	<1	<1	<0.5
Octane	<1	47.0	<1	<0.5

[a] Determination of alkane hydroxylase in the presence of an *alkA7* (AlkA$^-$ AlkB$^+$) extract measures AlkA$^+$ activity and in the presence of an *alkB181* (AlkA$^+$ AlkB$^-$) extract measures AlkB$^+$ activity (3).

TABLE 2. *Characterization of an alkR1165 strain*[a]

Plasmid genotype	Growth on:									Alkane hydroxylase activity on inducer:		
	Alkanes					Alcohols						
	C_6	C_7	C_8	C_9	C_{10}	C_8OH	C_9OH	$C_{10}OH$	$C_{12}OH$	None	Octane	Decane
alk$^+$	+	+	+	+	+	+	+	+	−	<0.5[b]	158[b]	13[b]
alk-1165	−	+	+	−	−	−	−	−	−	<0.5	18	<0.5

[a] Growth and induction were tested on PpS338(CAM-OCT) and PpS1165 as previously described (8).
[b] Data of Fennewald and Shapiro (8).

TABLE 4. *Reversion of alkD208 strains*[a]

Plasmid genotype	Chromosome *alcA* allele	Revertants/10^9 cells plated on:			% Nonanol reversion events on plasmid	Revertant plasmid-determined phenotypes		
		UV	Octane	Nonanol		Octane positive, nonanol positive	Octane negative, nonanol positive	Octane positive, nonanol negative
alkD208	+	+	ND	ND	ND	124	0	0
alkD208	−	−	1	28				
	−	+	45	239	59	126	94	0

[a] Quantitative reversion data were collected as described in Materials and Methods. Selection in an *alcA* background on nonanol plates will also yield *alcA*$^+$ revertants; so it is necessary to characterize revertant clones by replica mating to distinguish plasmid reversion events (*alkD*$^+$) from chromosomal reversion events (*alcA*$^+$). Revertant plasmids were scored after transfer into an *alcA* recipient, and the last three columns indicate the phenotypes of these exconjugants. If any octane-positive revertants isolated in the *alcA*$^+$ background had regained only hydroxylase activity, they would not have transferred nonanol or octane growth-positive characteristics to an *alcA* strain and so would have been scored in the last column of the table. ND, Not determined.

have deletions of both *alkA* and *alkB*. Altogether, these results indicate the existence of an operon transcribed in the order *alkB-alkA*-alcohol dehydrogenase gene. Genetic results described below confirm the close linkage of *alkB* and *alkA* mutations.

The isolation of hydroxylase-positive (AlkA$^+$ AlkB$^+$), dehydrogenase-negative insertion mutants, such as *alkC1170*::Tn*7* and *alkC1345*::Tn*7*, was consistent with such an operon structure. However, two lines of evidence showed that *alkC* mutations are not in the same operon as *alkB* and *alkA*. One consists of the genetic experiments described below which show that *alkC* is not linked to *alkBA*. The second is the phenotypic characterization of various alcohol-negative mutants. Plasmids carrying all hydroxylase-negative *alk*::Tn*7* insertion mutations lead to a tight growth-negative phenotype in *alcA P. putida* hosts on nonanol-PA agar (no visible colonies after 72 h of incubation at 32°C). In contrast, plasmids carrying all *alkC* mutations, both NTG-induced and caused by Tn*7* insertion, give a leaky growth phenotype in *alcA P. putida* hosts on nonanol-PA agar (colonies one-eighth to one-third the size of wild type after 72 h at 32°C). The leaky phenotype is particularly significantly in the *alkC*::Tn*7* mutants because we assume that they have a null phenotype for the *alkC* defect. Although we do not yet have mutations in it, we assign *alkE* as the name of the alcohol dehydrogenase locus downstream of *alkB* and *alkA*. Neither *alkC* nor polar insertion mutants have membrane alcohol dehydrogenase activity in extracts detectable above the background of our assay.

The class of AlkB$^-$ AlkA$^-$ dehydrogenase-negative *alk*::Tn*7* mutants which uniquely revert to *alk*$^+$ (i.e., do not give polarity revertants) can be explained by insertions into *alkR*, into the *alkBAE* promoter, or into an alcohol dehydrogenase gene upstream of *alkB*.

Mapping the *alk* mutations. By the same orgy transduction method used to isolate *alkC*::Tn*7* insertions, we isolated a series of Tn*7* insertions linked to *alkD208* which caused no detectable change in the alkane growth phenotype. Two of these *alk*-linked insertions, $Tn7_{320}$ and $Tn7_{322}$, were crossed with several *alk* point mutations. Because of the presence of multiple copies of Tn*7* in a CAM-OCT::Tn*7* cell (9), we could not do reciprocal crosses easily. So we used *alk*$^+$$Tn7_{320}$ or *alk*$^+$$Tn7_{322}$ donor lysates and lysogenic *alk* recipients. Cotransduction frequencies are summarized in Fig. 1. The mutations tested clearly map in two clusters: *alkR* and *alkD* close to $Tn7_{320}$ (33 to 80% cotransduction) and far from $Tn7_{322}$ (0.8 to 7.1% cotransduction), and *alkB* and *alkA* nearer to $Tn7_{322}$ (12 to 21% cotransduction) than to $Tn7_{320}$ (0.7 to 1.8% cotransduction). These results agree with previous transduction data on the tight linkage of regulatory mutations with *alkD208* (8) and support the idea of an *alkBAE* operon. The distance between the two clusters also supports the conclusion that *alkR* and *alkD* direct the synthesis of diffusible gene products required for expression of both hydroxylase and dehydrogenase activities. These observations rule out polar effects as the explanation for pleiotropy of *alkR* and *alkD* mutations.

The relative order of sites within the two clusters was determined by reciprocal three-factor crosses using the Tp^r determinants of $Tn7_{320}$ and $Tn7_{322}$ as unselected outside markers. The results clearly establish the following orders: $Tn7_{320}$-*alkR252-alkR256-alkR184-alkR192-(alkA7, alkA1033)-(alkB201, alkB205)* and

TABLE 5. *Characterization of alk::Tn7 strains by growth and reversion tests*

alk::Tn7 mutation	*alcA* allele	Growth on:		No. of nonanol-positive revertants			
		Octane	Nonanol	Total	Plasmid reversions	Octane positive	Octane negative
A1117	−	−	−	168	108	25	83
	+	−	+				
R1118	−	−	−	128	6	6	0
	+	−	+				
B1119	−	−	−	332	2	2	0
	+	−	+				
B1120	−	−	−	256	8	6	2
	+	−	+				
B1121	−	−	−	123	5	1	4
	+	−	+				
A1122	−	−	−	56	44	14	30
	+	−	+				
R1123	−	−	−	248	179	179	0
	+	−	+				
R1124	−	−	−	255	237	237	0
	+	−	+				
R1125	−	−	−	232	176	176	0
	+	−	+				
R1126	−	−	−	240	229	229	0
	+	−	+				
R1129	−	−	−	126	3	3	0
	+	−	+				
B1130	−	−	−	239	44	13	31
	+	−	+				
A1131	−	−	−	ND	ND	ND	ND
	+	−	+				
C1128, C1132, C1170, C1345	−	−	−	ND	ND	ND	ND
	+	+	+				

[a] CAM-OCT *alk*::Tn7 plasmids were crossed from *P. aeruginosa* (8) into either an *alcA* or an *alcA*$^+$ *P. putida* host (PpS338, PpG1), and the exconjugants were scored for growth on octane and nonanol. The *alk-1128*, *-1132*, *-1170*, and *-1345* plasmids behave like hydroxylase-positive, dehydrogenase-negative *alkC* mutant plasmids (5), while all others determine a typical hydroxylase-negative, dehydrogenase-negative growth phenotype. Reversion of *alcA* strains carrying most of these plasmids was induced by UV irradiation, nonanol-positive revertants were selected, and the plasmids in these clones were tested by replica mating to a second *alcA* strain. The number of plasmid reversion events (i.e., clones which transferred nonanol-positive determinants) compared to total revertants tested indicates the frequency of *alk* reversion relative to *alcA* reversion (presumably the same in all strains). Polarity revertant plasmids determine the octane-negative, nonanol-positive phenotype after transfer, while *alk*$^+$ revertant plasmids determine the octane-positive, nonanol-positive phenotype. We did not test reversion of *alkC1128*::Tn7, *alkC1132*::Tn7, *alkC1170*::Tn7, or *alkC1345*::Tn7 because of their leaky growth on nonanol (see text). ND, Not determined.

Tn7$_{322}$-*(alkA7, alkA1033)-(alkB201, alkB204)*. The position of *alkD208* in the *alkRD* cluster is not clear from the data, partly because this mutation recombines poorly with *alkR* mutations.

Figure 2 summarizes the results of deletion mapping of markers within the *alkBA* cluster. The Δ*alkB841*, Δ*alkB873*, Δ*alkB874*, Δ*alkB884*, and Δ*alkBA845* deletions were isolated in alkane-negative, alcohol-positive revertants of an *alkD208* strain (cf. Table 4). The other deletions were isolated in polarity revertants of *alkB*::Tn7 and *alkA*::Tn7 mutants. The transduction and deletion mapping results confirm the linkage of *alkB* and *alkA*. Since Δ*alkBA* deletion strains grow on primary alcohols, there is no gene located between *alkB* and *alkA*, whose product is essential for utilization of these substrates.

We tried to demonstrate linkage between *alkC* and other *alk* mutations by the following experiment. F116C phage grown on *alkC* donors were used to transduce *alk P. aeruginosa* (where chromosomal gene products supplant the need for *alkC* expression in growth on alkanes) to alkane positive, and then the transductant plasmid *alkC* genotype was tested by crossing to an *alcA P. putida* strain (with selection for Cam$^+$ determinants). With *alkC*::Tn7 donors, we also tested for cotransduction of the Tp^r determinant.

Neither *alkC1170*::Tn7, *alkC1345*::Tn7, nor five NTG-induced *alkC* mutations yielded

TABLE 6. *Enzymatic characterization and growth of alk::Tn7 mutants, polarity revertant strains, and alkD208 revertants*[a]

alk allele	Parental genotype	Reversion to *alk*$^+$	Alkane hydroxylase activity			Alcohol dehydrogenase activity	Relative growth[b]	
			Alone	+AlkA$^+$B extract	+AlkA B$^+$ extract		Octane	Nonanol
alk$^+$			179	ND[c]	ND	37.0	+	+
alkA1117::Tn*7*	*alk*$^+$	+	6	120	7	<0.5	−	−
alkA1150	*alkA1117*::Tn*7*	ND	3	38	4	23.5	−	+
Δ*alkBA1151*	*alkA1117*::Tn*7*	−	1	4	2	34.6	−	++
alkA1171	*alkA1117*::Tn*7*	+	9	88	3	57.3	−	+
Δ*alkA1181*	*alkA1117*::Tn*7*	−	<1	27	2	42.2	−	+/++
Δ*alkBA1182*	*alkA1117*::Tn*7*	−	6	2	2	45.7	−	++
alkA1183	*alkA1117*::Tn*7*	+	4	65	2	48.6	−	+
alkA1184	*alkA1117*::Tn*7*	+	5	86	3	48.3	−	+
Δ*alkA1185*	*alkA1117*::Tn*7*	−	9	97	2	22.7	−	+
alkA1264	*alkA1117*::Tn*7*	+	16	172	10	23.8	−	+
alkA1265	*alkA1117*::Tn*7*	+	5	128	4	31.0	−	+
alkA1266	*alkA1117*::Tn*7*	+	2	171	16	11.5	−	+
alkA1267	*alkA1117*::Tn*7*	+	8	173	7	21.6	−	+
alkA1268	*alkA1117*::Tn*7*	+	10	182	5	51.3	−	+
alkA1269	*alkA1117*::Tn*7*	+	9	104	8	11.0	−	+
alkR1118::Tn*7*	*alk*$^+$	+	<1	<1	<1	<0.5	−	−
alkB1119::Tn*7*	*alk*$^+$	+	<1	<1	<1	<0.5	−	−
alkB1120::Tn*7*	*alk*$^+$	+	<1	<1	2	<0.5	−	−
alkB1149	*alkB1120*::Tn*7*	+	<1	<1	62	0.8	−	++
Δ*alkBA1179*	*alkB1120*::Tn*7*	−	<1	<1	4	34.8	−	+/++
Δ*alkB1180*	*alkB1120*::Tn*7*	−	<1	<1	77	47.5	−	++
alkB1121::Tn*7*	*alk*$^+$	+	<1	2	1	<0.5	−	−
alkB1147	*alkB1121*::Tn*7*	ND	<1	<1	69	4.3	−	+
alkB1148	*alkB1121*::Tn*7*	+	<1	<1	13	23.0	−	+/++
Δ*alkB1172*	*alkB1121*::Tn*7*	−	<1	<1	138	22.5	−	+
alkB1174	*alkB1121*::Tn*7*	ND	<1	<1	249	44.4	−	+
alkA1122::Tn*7*Δ[d]	*alk*$^+$	+	<1	74	<1	<0.5	−	−
Δ*alkBA1145*	***alkA1122*::Tn*7*Δ**	−	<1	<1	<1	1.6	−	++
Δ*alkBA1146*	*alkA1122*::Tn*7*Δ	−	<1	3	<1	1.9	−	+
Δ*alkBA1249*	*alkA1122*::Tn*7*Δ	−	<1	<1	3	10.4	−	+
Δ*alkBA1250*	*alkA1122*::Tn*7*Δ	−	<1	<1	1.7	42.5	−	+
Δ*alkBA1251*	*alkA1122*::Tn*7*Δ	−	<1	<1	<1	32.5	−	+
Δ*alkBA1252*	*alkA1122*::Tn*7*Δ	−	<1	<1	<1	2.82	−	+
Δ*alkBA1253*	*alkA1122*::Tn*7*Δ	−	<1	<1	2	52.8	−	+
Δ*alkBA1254*	*alkA1122*::Tn*7*Δ	−	<1	<1	2	35.3	−	+
Δ*alkBA1255*	*alkA1122*::Tn*7*Δ	−	<1	<1	2	29.7	−	+
alkR1124::Tn*7*Δ[d]	*alk*$^+$	+	<1	<1	<1	<0.5	−	−
alk$^+$	*alkR1124*::Tn*7*Δ		233	ND	ND	20.2	+	+
alkC1128::Tn*7*	*alk*$^+$	ND	187	ND	ND	<0.5	Very wk	Very wk
alkB1130::Tn*7*	*alk*$^+$	+	<1	<1	<1	<0.5	−	−
alkB1144	*alkB1130*::Tn*7*	ND	<1	<1	60	1.2	−	++
Δ*alkBA1176*	*alkB1130*::Tn*7*	−	<1	2	4	1.8	−	++
alkB1177	*alkB1130*::Tn*7*	+	<1	<1	241	13.8	−	+/++
Δ*alkB1256*	*alkB1130*::Tn*7*	−	2	<1	92	44.6	−	+
Δ*alkB1257*	*alkB1130*::Tn*7*	−	<1	<1	73	15.4	−	+
Δ*alkB1258*	*alkB1130*::Tn*7*	−	<1	<1	115	13.2	−	+
alkB1259	*alkB1130*::Tn*7*	+	<1	<1	94	17.5	−	+

TABLE 6—*Continued*

TABLE 6. *Enzymatic characterization and growth of alk::Tn7 mutants, polarity revertant strains, and alkD208 revertants*[a]

alk allele	Parental genotype	Reversion to *alk*$^+$	Alkane hydroxylase activity			Alcohol dehydrogenase activity	Relative growth[b]	
			Alone	+AlkA$^+$B$^-$ extract	+AlkA$^-$B$^+$ extract		Octane	Nonanol
alkA1131::Tn7	*alk*$^+$	+	<1	82	<1	<0.5	−	−
alkC1132::Tn7	*alk*$^+$	ND	206	ND	ND	<0.5	Very wk	Very wk
alkC1170::Tn7	*alk*$^+$	ND	134	ND	ND	<0.5	Very wk	Very wk
alkC1345::Tn7	*alk*$^+$	ND	157	ND	ND	<0.5	Very wk	Very wk
Δ*alkB841*	*alkD208*	−	<1	<1	79	1.4	−	Wk
Δ*alkBA845*	*alkD208*	−	<1	2	<1	0.6	−	+
alkB866	*alkD208*	+	<1	<1	98	16.6	−	Wk/+
Δ*alkB873*	*alkD208*	−	<1	<1	24	24.3	−	+
Δ*alkB874*	*alkD208*	−	<1	<1	22	28.3	−	+
Δ*alkB884*	*alkD208*	−	<1	<1	31	26.4	−	+

[a] CAM-OCT plasmids carrying the various mutations were introduced into the PpS338 background and induced cultures tested for enzymatic activity were as described in the text.

[b] Relative growth scored by comparing emergence of isolated colonies on plates exposed to octane or nonane vapors. +, Normal growth; −, no growth; ++, faster-than-normal growth (Fig. 4); wk, slower-than-normal growth; very wk, leaky mutant growth.

[c] ND, Not determined.

[d] The *alkA1122* and *alkR1124* alleles are Tp^s mutations isolated after transposition of Tn7 into CAM-OCT (8). These data indicate that *alkA1122* is a polar insertion mutation.

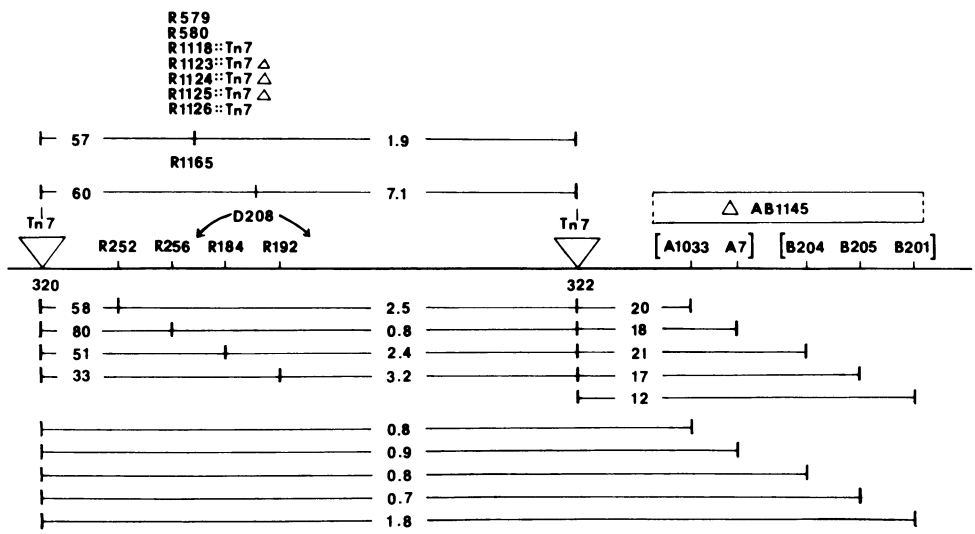

FIG. 1. *Transduction map of the alkRD and alkBA clusters. Each of the numbers in the horizontal bars indicates the percentage of F116 cotransduction of the linked Tn7 insertions and the alk*$^+$ *allele for each of the alk point mutations. The relative order of the mutations in the alkRD cluster and the positions of alkA and alkB were determined by three-factor crosses. The alkR579 and alkR580 alleles determine a constitutive phenotype and are >98% cotransducible with the alkD208 site (8). Transduction of the alkR::Tn7 alleles with F116 grown on alkR579 and alkR580 strains yields more than 89% constitutives among the alkane-positive transductants, indicating that the insertion mutations are located in the alkRD cluster.*

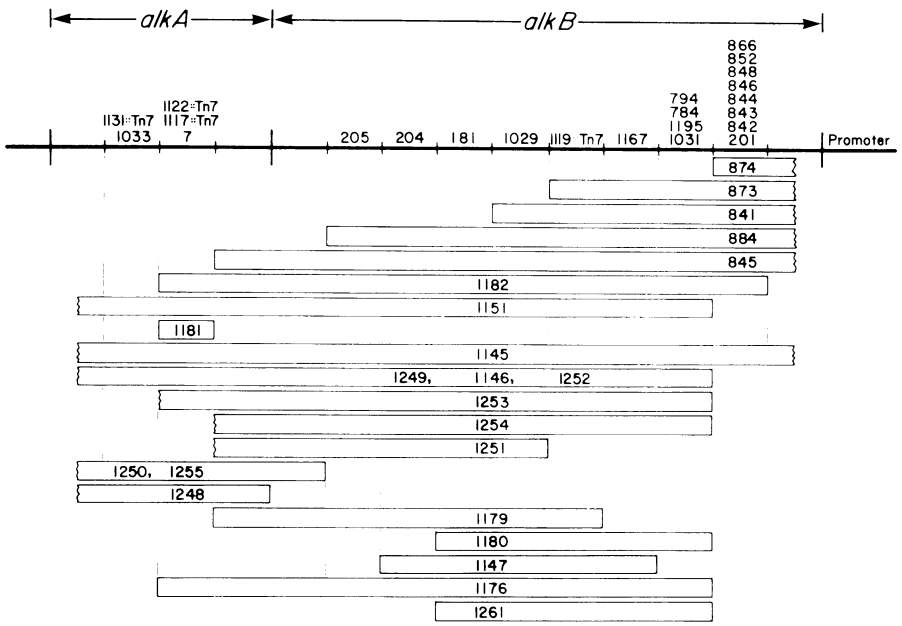

FIG. 2. *Deletion map of the alkBA cluster. Point mutations were mapped against alk deletions by F116 transduction in P. aeruginosa as described in the text. The origins of the various deletions are given in Table 6. The absence of alkA or alkB segments which contain no point mutations is inferred from the AlkA⁻ or AlkB⁻ phenotype of the relevant deletion strain (Table 6). The position of the promoter is based on polarity effects of alkB::Tn7 and alkA::Tn7 mutations as described in the text. Staggered ends to deletion bars indicates uncertainty as to the extent of the deletion.*

hydroxylase-positive, dehydrogenase-negative transductants with *alkA7*, *alkA1033*, *alkB201*, *alkB204*, *alkB205*, *alkD208*, and *alkR252* recipients (at least 90 transductants tested in each cross). So it appears that the *alkC* mutations are located in at least one other *alk* region on the CAM-OCT plasmid.

Crosses of *alk*::Tn7 mutations with Δ*alkBA845* and Δ*alkBA1145* generally confirm the classification of triply negative insertions as *alkB*::Tn7 or *alkR*::Tn7 on the basis of reversion behavior. Those that give hydroxylase-negative polarity revertants do not recombine with either the Δ*alkBA845* or Δ*alkBA1145* deletions (i.e., are *alkB*::Tn7), and those that only revert to wild type recombine with both deletions (i.e., are not *alkB*::Tn7). Only *alkB1119*::Tn7 did not give either polarity revertants or recombinants with deletions and has been mapped in the *alkB* locus (Fig. 2). Because we have only isolated two plasmid-linked revertants of an *alkB1119*::Tn7 strain (Table 5), our failure to find polarity revertants is not significant. Transduction experiments with two constitutive *alkR* alleles (*alkR579* and *alkR580*; 8) confirm the presence of *alk-1118*, *alk-1123*, *alk-1124*, *alk-1125*, and *alk-1126* in the *alkRD* cluster (≧89% cotransduction).

Characterization of *alkD208* partial revertants. An *alkD208* strain gives AlkB⁻ partial revertants (about 40% of all alcohol-positive plasmid reversion events). These could have resulted from suppressor mutations which did not relieve the hydroxylase defect. If this were true, then the partial revertants should contain two mutations, *alkD208* and the suppressor mutation. They would, therefore, be unable to recombine with *alkD208* to yield *alk*⁺ recombinants. However, phage grown on four independent partial revertants will transduce *alkD208* to *alk*⁺; so they have actually lost the original *alkD208* mutation. In other words, ca. 40% of all *alkD208* → *alk*⁺ reversion events appear to be associated with the concurrent appearance of a new mutation in *alkB*. We do not have an explanation for this phenomenon, but the transduction data given in Fig. 1 indicate that *alkD208* is not an inversion. It is perhaps significant that all *alkB* mutations derived from *alkD208* affect one or more sites at the promoter-proximal end of *alkB* (Fig. 2). As reversion tests and mapping experiments show, some of the *alkB* revertants carry deletions of the right end of the *alkBA* cluster (Fig. 2). The majority of *alkB* revertants will subsequently revert to alkane positive, but we do not yet know whether these second-step re-

vertants are fully wild type. Both *alkB*$^+$ and *alkB* alcohol-selected revertants of *alkD208* appear to have an intact *alkBAE* promoter and control region because expression of alcohol dehydrogenase depends on induction. (These strains will only grow on the noninducing substrate dodecanol in the presence of inducer [10].)

Differences in the alcohol phenotypes of deletion mutants in the *alkBA* regions. Careful inspection of the data summarized in Table 6 indicates that the *alkBAE* operon contains additional cistrons, regulatory sites, or both. Plasmids carrying some deletions determine poor growth on nonanol, some determine wild-type growth, and others determine better than wild-type growth (Fig. 3). When induced cultures of strains with these plasmids are assayed for membrane alcohol dehydrogenase activity, at least two classes can be distinguished: those giving approximately wild-type activity (≧10 U) and those giving significantly reduced activity (0.5 to 5 U). Whereas replicate assays show variability (due to factors such as degree of induction and instability of the enzyme in extracts), repeated assays of a given strain are always consistent within these two classes.

Table 7 summarizes the alcohol phenotypes (both growth and assayable activity) for the mutants described in Fig. 2 and Table 6. In addition to the clearly mutant *alkC*::Tn7 and *alkB*::Tn7/*alkA*::Tn7 phenotypes, there are at least six classes we can distinguish. There is no clear correlation between relative nonanol growth and assayable dehydrogenase activity. Both assay classes (low and normal) are found among all three growth classes. There is also no clear correlation between either of these phenotypic variables and the regions of the *alkBA* cluster removed by deletion. However, most of the plasmid deletions determine an alcohol phenotype similar to that of a strain carrying an *alk*$^+$ plasmid. These include several *BA* deletions. Hence, there apparently are no alcohol dehydrogenase genes or regulatory sites between *alkB* and *alkA*.

TABLE 7. *Alcohol phenotypes determined by mutations in the alkBA region*

Nonanol growth[a]	Alcohol dehydrogenase activity	Mutant
−	<0.5	All *alkA*::Tn7, *alkB*::Tn7
Very wk	<0.5	All *alkC*, *alkC*::Tn7
Wk	0.5–5	Δ*alkB841*
Wk/+	>10	*alkB866*
+	0.5–5	*alkB1147*, Δ*alkBA1146*, Δ*alkBA1252*, Δ*alkBA845*
+	>10	*alk*$^+$, *alkA1150*, *alkA1171*, *alkA1183*, *alkA1184*, Δ*alkA1185*, *alkA1264-1269*, Δ*alkB1172*, *alkB1174*, Δ*alkBA1249-1251*, Δ*alkBA1253-1258*, *alkB1259*, Δ*alkB873-874*, Δ*alkB884*
+/++ or ++	0.5–5	Δ*alkB1144*, *alkB1149*, Δ*alkBA1145*, Δ*alkBA1176*
+/++ or ++	>10	Δ*alkA1181*, *alkB1148*, *alkB1177*, Δ*alkBA1151*, Δ*alkBA1179*, Δ*alkB1180*, Δ*alkBA1182*

[a] See footnote *b* of Table 6.

All of the alcohol-positive polarity revertants from *alkB*::Tn7 and *alkA*::Tn7 appear to have inducible alcohol dehydrogenase expression because they will only grow on dodecanol, a noninducing substrate, in the presence of inducer (cf. reference 10).

Nature of polarity revertants from *alk*::Tn7 mutants. The data summarized in Tables 5 and 6 and Fig. 2 show that polarity of *alkB*::Tn7 and *alkA*::Tn7 insertion mutations can be relieved by deletion of the transposon. In addition, *alkA1117*::Tn7, *alkB1120*::Tn7, *alkB1121*::Tn7, and *alkB1130*::Tn7 strains give polarity revertants which can further revert to an alkane-positive phenotype. We do not know yet if these secondary revertants are fully *alk*$^+$. In these cases, it may be that relief of polarity can occur without complete loss of the Tn7 element to yield nonpolar insertions. However, relief of polarity from these four mutants is associated with loss of the Tp^r determinant from CAM-OCT in

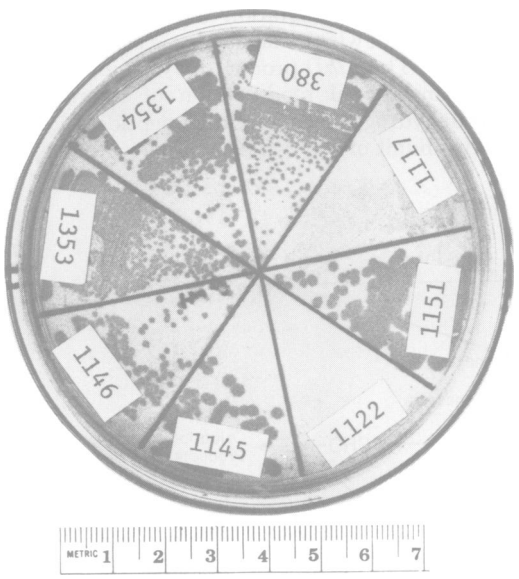

FIG. 3. *Growth of P. putida (CAM-OCT alk) strains on nonanol. Strain 380 carries an alk*$^+$ *plasmid. The other numbers indicate the alk alleles in each strain. These plates were incubated 60 h at 32°C in the presence of nonanol vapors.*

all hydroxylase-negative cases tested: *alkA1117* ::Tn7 (18 Tp^s/18 revertants), *alkB1120*::Tn7 (4 Tp^s/4 revertants), *alkB1121*::Tn7 (6 Tp^s/6 revertants), and *alkB1130*::Tn7 (9 Tp^s/9 revertants).

DISCUSSION

Our current picture of the genetic control of alkane oxidation by *alk*$^+$ *P. putida* strains is summarized in Fig. 4. The results presented above and in earlier publications (3–5, 8) indicate that at least six different *alk* genes are involved in determining that Alk$^+$ phenotype. The functions controlled by these *alk* loci are as follows: *alkA*—synthesis of soluble alkane hydroxylase component, probably rubredoxin (3, 4, 15); *alkB*—synthesis of membrane alkane hydroxylase component, a 40,000-dalton phospholipid-requiring protein (3, 4, 18; Benson, Oppici, Shapiro, and Fennewald, manuscript in preparation); *alkC*—synthesis of membrane alcohol dehydrogenase and growth on alcohols (5); *alkD*—synthesis of membrane alkane hydroxylase component and membrane alcohol dehydrogenase (not required for normal synthesis of soluble alkane hydroxylase component); *alkE*—synthesis of membrane alcohol dehydrogenase and growth on alcohols; *alkR*—inducer recognition and activation of *alkBAE* transcription (8).

It is possible that several of these loci contain more than one cistron and also that future mutant hunts will uncover other *alk* genes. (Our collection still lacks *alkE* point mutations and only contains two mutations in *alkA* and one in *alkD*.) We know from analysis of membrane peptides that the *alkBAE* cluster controls the synthesis of at least four inducible proteins (Benson et al., manuscript in preparation). However, since regulation of *alkBAE* expression is sufficient to explain induction of all three assayable alkane-oxidizing activities, we do not know whether synthesis of the *alkC*, *alkD*, or *alkR* gene products is constitutive or regulated.

Recombination analysis indicates that *alk* loci map at three distinct regions on the CAM-OCT plasmid. One region contains *alkR* and *alkD*, and it is weakly linked to the *alkBAE* region (Fig. 1). We have not measured the cotransduction of the two clusters directly, but the data collected with the $Tn7_{322}$ insertion located between the two clusters suggest a distance of at least 42 kilobases (kb) if we assume that F116C encapsidates random segments of the CAM-OCT plasmid. Tn7 is roughly 12 kb in length (1), and F116L encapsidates ca. 55 kb of DNA (12). So the effective size of host DNA in a transducing particle containing Tn7 is about 43 kb. By the empirical formula of Wu (20), the *alkD208*–$Tn7_{322}$ distance is 25.2 kb, and the $Tn7_{322}$–*alkA1033* distance is 17.8 kb. The *alkC* mutations all appear to be far from both the *alkRD* and the *alkBAE* clusters. We do not yet know whether they all map in a single region of the plasmid DNA.

From our mapping results, it is clear that the pleiotropic effects of *alkR* and *alkD* must be mediated by diffusible protein products. As we have argued previously (8), *alkR* must encode at least one positive regulatory protein. In fact, *alkR* mutations identify two different functions—inducer recognition and *alkBAE* activation—each of which may involve a separate gene product. The *alkD* product may be involved in posttranslational modification of hydroxylase and dehydrogenase membrane peptides, a common subunit of the two enzymes, or required to

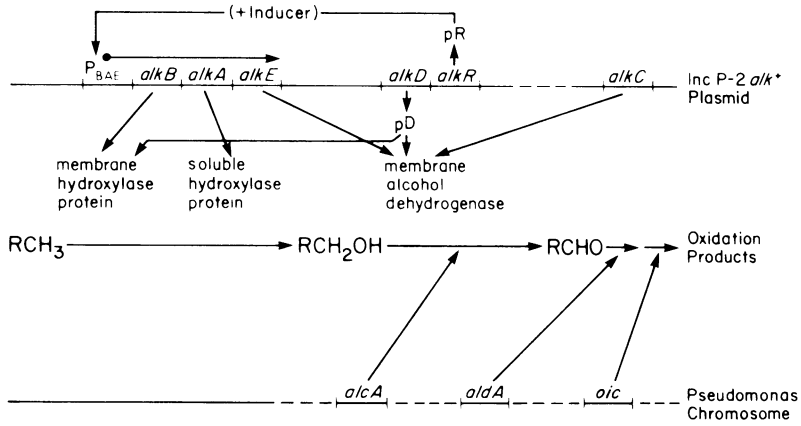

FIG. 4. *Scheme of the genetic control of alkane oxidation in alk*$^+$ *P. putida strains. The roles of the various gene products are discussed in the text and in references 3, 5, 6, and 8. Reference 8 gives the arguments for a positive regulatory function of the alkR gene product(s).*

form an active membrane complex. Evidence for an *alkBAE* transcriptional unit together with normal inducibility of AlkA$^+$ activity in an *alkD208* strain (Table 3) argue strongly against a role for the *alkD* gene product in regulation of transcription. The partial reversion of *alkD208* to AlkB$^-$, dehydrogenase positive, clearly results from a genetic peculiarity of this particular mutation and does not reflect the nature of the *alkD* function because all of these partial revertants have lost the original *alkD208* mutation. The fact that our only *alkD* mutation has unusual reversion properties naturally makes conclusions about *alkD* function very tentative until we can study more alleles of this locus. Notwithstanding this reservation, however, it does not seem possible to explain *alkD208* as either a peculiar allele of *alkR* or a multiple mutation.

We do not yet know why both *alkE* and *alkC* gene products are required for biologically functional alcohol dehydrogenase synthesis or why some deletions of the *alkBA* region reduce assayable enzyme levels but do not affect growth on alcohols. Regulation of *alkE* expression is sufficient to account for inducibility of enzyme activity, and the tight alcohol-negative phenotype of *alkB*::Tn*7* and *alkA*::Tn*7* mutants suggests that *alkE* determines synthesis of an intrinsic enzyme protein. The *alkC* gene product may be either a subunit of the dehydrogenase or some kind of processing enzyme which modifies the *alkE*-determined protein. The leakiness of *alkC*::Tn*7* mutants indicates that the *alkC* gene product is not absolutely essential for alcohol dehydrogenase activity. It is possible that the *alkC* gene product can be replaced to a limited degree by the product of a chromosomal gene. The lack of a reasonable correlation between nonanol growth rates and assayable enzyme activity in extracts of both *alkC* and *alkBA* deletion mutants (Tables 6 and 7; Fig. 3) suggests that several proteins may interact to produce the physiologically active dehydrogenase. (On this hypothesis, low in vitro activity could result from rapid inactivation of an incomplete protein.)

The existence of an *alkBAE* operon is the only reasonable explanation for our observations on the polarity of *alkB*::Tn*7* and *alkA*::Tn*7* insertion mutations. A particularly strong prediction of the operon model is that polarity will be relieved by deletion of the Tn*7* element, and that is exactly what we observe (Fig. 2). There appears to be no alcohol dehydrogenase gene located between the *alkB* and *alkA* genes because deletions such as Δ*alkBA1145* and Δ*alkBA1151* confer an alcohol-positive phenotype. Analysis of inducible membrane peptides controlled by the *alkBAE* cluster shows that not all are incorporated into membranes at the same steady-state rate (Benson et al., manuscript in preparation). So there is further evidence suggesting that the *alkBAE* cluster contains additional sites or cistrons.

Both the preceding discussion and Fig. 4 indicate that genetic control of alkane hydroxylase and alcohol dehydrogenase activities is more complex than we anticipated. Synthesis of alcohol dehydrogenase appears to involve the activity of at least four gene products (*alkC*, *alkD*, *alkE*, and *alkR*). Diploid analysis will probably increase this number. This complexity is similar to that of the *nif* gene complex in *Klebsiella*, and the *alkD* locus may play a role parallel to that of the *nifM* and *nifS* genes (17).

The identification of three *alk* clusters in CAM-OCT was a further surprise. We had earlier concluded that *alk* regulatory and structural genes were closely linked because we mistakenly thought that *alkD208* was a polar mutation of *alkB* (3, 8, 19) and because the *alk*$^+$ loci will recombine between different Inc P-2 plasmids (6, 7). It has been popular to think of plasmid degradative pathway determinants as transposable elements which spread between different plasmids by specific recombination mechanisms. If the full complement of *alk*$^+$ loci lies on a single transposable element, it would have to be very large: the 42 kb between *alkRD* and *alkBAE* plus at least 34 kb to *alkC* on one side or the other (<1% cotransduction with *alkR*, *D*, *A*, or *B*). The presence of such a large transposable element would be tolerated on Inc P-2 plasmids, which all contain greater than 150 megadaltons (7, 11). However, the formation of the CAM-OCT plasmid by a single large *alk* insertion into CAM is not compatible with the similarity in *Eco*RI digestion patterns of CAM and CAM-OCT DNAs (7). Our results on Inc P-2 plasmid recombination (6) and on dispersion of *alk*$^+$ loci in CAM-OCT raise two questions about the evolution of this plasmid group: (i) are there incomplete sets of *alk* loci on different Alk$^-$ Inc P-2 plasmids and (ii) why haven't the determinants of this metabolic pathway evolved in a single cluster of linked genes?

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