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Plasmid-Determined Alcohol Dehydrogenase Activity in Alkane-Utilizing Strains of *Pseudomonas putida*

SPENCER BENSON AND JAMES SHAPIRO*

Department of Microbiology, The University of Chicago, Chicago, Illinois 60637

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We have identified an alcohol dehydrogenase activity in *Pseudomonas putida* strains carrying the CAM-OCT degradative plasmid that were grown on octane. The activity is nicotinamide adenine dinucleotide independent, sediments at 48,000 × *g*, and shows 20-fold greater activity with octanol rather than butanol as substrate. The enzyme is inducible by unoxidized alkane and is present only in strains that have the OCT plasmid genes for alkane degradation with a wild-type *alcO* locus. No analogous chromosomal dehydrogenase could be detected. Wild-type and octanol-negative mutants (*alcA*$^-$) without plasmids both contain a constitutive nicotinamide adenine dinucleotide-linked soluble alcohol dehydrogenase activity. This means that *alcA*$^-$ mutants are cryptic for octanol oxidation and suggests that the particulate plasmid-coded alcohol dehydrogenase activity is active on surface- or membrane-bound substrate.

Our previous physiological studies on alkane oxidation in *Pseudomonas putida* indicated the existence of a specific inducible alcohol dehydrogenase activity involved in hydrocarbon metabolism (7). To obtain a full description of the alkane oxidation pathway and its regulation in this species, identification and assay of this postulated enzyme was essential.

P. putida strains can grow on straight-chain primary aliphatic alcohols from propanol to dodecanol as sole carbon and energy source (7, 8). We and others have described mutants (*alcA*$^-$) that have lost the ability to grow on alcohols of greater than seven carbon atoms (4, 7). Introduction of the OCT or CAM-OCT plasmid into these mutants restores their ability to grow on these alcohols in the presence of inducers of the plasmid-coded alkane hydroxylase (7). These results suggest that the *alcA*$^-$ mutants lack an alcohol dehydrogenase activity that can be replaced by an inducible plasmid-coded enzyme. This hypothesis is strengthened by the results of whole cell oxidation experiments, which demonstrated a constitutive chromosomally determined heptanol-oxidizing activity and an inducible plasmid-determined activity (7). However, neither postulated alcohol dehydrogenase activity had been measured in cell extracts.

In this paper, we report the identification of the inducible alcohol dehydrogenase activity, the presence of which is determined by the CAM-OCT plasmid. The enzyme is found in the particulate fraction of cell-free extracts, is specific for octanol as opposed to butanol, does not use nicotinamide adenine dinucleotide (NAD) as a cofactor, and is lacking in strains with a mutant *alcO*$^-$ plasmid.

MATERIALS AND METHODS

Bacterial strains. The *P. putida* strains used are described in Table 1 and below.

Microbial procedures. Media, culture conditions, growth tests, and mutagenesis procedures are as described by Nieder and Shapiro (8). Camphor was added in the vapor phase by sprinkling a few crystals in the lid of a petri dish and incubating in a closed tin.

Bacterial conjugations. Crosses were performed as follows: an overnight TYE broth culture of the donor strain was diluted (1:50) in the same medium and mixed with an equal volume of an overnight broth culture of the recipient. The mixture was allowed to stand at room temperature for 30 min, after which an aliquot was plated on selective medium. These plates were incubated at 32 C until exconjugant colonies appeared; for growth on camphor this was generally 72 to 96 h after plating. Strains PpS367 and PpS368 were constructed in two steps: first PpS173 was crossed to a *trp*$^-$*str*^{r} strain, and then a resulting camphor-positive (*cam*$^+$) *trp*$^-$*str*^{r} recombinant was crossed to PpG1 and its *alcA*$^-$ mutant PpS81, and *cam*$^+$*trp*$^+$ recombinants were selected.

Chemicals. Most of the chemicals used have been described previously (2, 7, 8). 2,6-Dichlorophenolindophenol (DCPIP) and phenazine methosulfate (PMS) were purchased from Sigma Chemical Co.

Preparation of cell extracts for alcohol dehydrogenase assays. Cells were grown on plates and harvested as previously described (2, 7). Centrifuged cell pellets were resuspended in 0.038 M tris(hydroxymethyl)aminomethane (Tris) (pH 7.4) and stored frozen at −20 C until use. Cells were

then thawed at room temperature and sonically treated in six 20-s bursts at 0 C. For fractionation, the resulting crude extract was centrifuged at 5,000 × *g* for 10 min at 4 C. The supernatant was removed and recentrifuged at 48,000 × *g* for 60 min at 2 C. The resulting supernatant was called the S48 fraction; the pellet was suspended in 0.038 M Tris (pH 7.4) and called the P48 fraction. Fractions were maintained in an ice bath and assayed immediately unless otherwise noted. Reducing agents were not added to the buffer because they interfered with the DCPIP reduction assay. Under these conditions we have found that activity in the P48 fraction is lost rapidly, even during storage at −20 C.

Alkane hydroxylase assay. Alkane hydroxylase assays were performed as described in Benson and Shapiro (2) with the following modifications. (i) Assays were carried out on intact cells. (ii) The [1-^{14}C]nonane substrate was purified by elution from a silicic acid column in tridecane; the tridecane does not interfere with nonane hydroxylation and permits the preparation of labeled substrate at a specific activity of 4 × 10^5 counts/min per μmol. (iii) The amounts of [1-^{14}C]nonane and tridecane in the 1-ml reaction mixture were 0.25 μmol and 0.75 μmol, respectively. Specific alkane hydroxylase activity is expressed as nanomoles of [1-^{14}C]nonane hydroxylated per minute per milligram (dry weight) of cells.

Protein determinations. Protein determination was done by the biuret II method using bovine serum albumin as a standard (6). The determination method was shown to be linear in a concentration range of 0.2 to 2.0 mg/ml.

Alcohol dehydrogenase assays. (i) DCPIP reduction method. The DCPIP reduction method used is basically the one described by Tassin et al. (9). Unless otherwise noted, the assay system contained 3 ml of 0.038 M Tris buffer (pH 7.4), 1.15 μmol of substrate, 0.65 μmol of PMS, 0.27 μmol of KCN, and 0.3 μmol of DCPIP. This solution was allowed to equilibrate for several minutes before the reaction was initiated by the addition of 50 to 100 μl of extract. The activity was assayed by measuring the decrease in absorbance of the DCPIP at 600 nm with a Varian model 635 spectrophotometer equipped with a recorder. Activity is expressed as micromoles of DCPIP reduced per minute per milligram of protein, at room temperature. The calculations are based on an extinction coefficient of 20.6 × 10^3/mol per cm at 600 nm for DCPIP.

(ii) NAD reduction method. The assay system contained 1 ml of 0.038 M Tris buffer (pH 7.4), 1 μmol of substrate, and 0.5 μmol of NAD. The reaction was initiated by the addition of 50 to 100 μl of cell extract. The activity was assayed by measuring the increase in absorbance at 340 nm. The equipment used was the same as described above. Activity is expressed as micromoles of NAD reduced per minute per milligram of protein × 10^2, using an extinction coefficient of 6.22 × 10^3/mol per cm for NADH at 340 nm.

RESULTS

Isolation and characterization of *alcO* mutants. Our previous results showed that alkane-negative, nonanol-negative mutants could be isolated from OCT$^+$ and CAM-OCT$^+$ *P. putida* strains only if they already carried a mutation in the chromosomal *alcA* locus (7). We concluded that these mutants had lesions in a plasmid alcohol dehydrogenase gene called *alcO*. However, the same phenotype could result from the loss of the plasmid or from a pleiotropic mutation that also affected the plasmid alkane hydroxylase loci. To identify those strains specifically mutated for alcohol utilization, we assayed 15 mutants for alkane hydroxylase activity. To our surprise, only two showed activity: PpS116 with the OCT plasmid and PpS173 with the CAM-OCT plasmid. The other 13 mutants still had their plasmids as shown by reversion or retention of the camphor phenotype from the CAM-OCT plasmid; they presumably contain either polar or regulatory mutations. Because the camphor utilization genes on the plasmid in PpS173 facilitated genetic analysis, we concentrated our efforts on this strain. It was necessary to confirm our in vitro enzyme assay by an in vivo growth test. Using camphor as our selected marker, we transferred the mutant plasmid into *alcA*$^+$ and *alcA*$^-$ strains and

TABLE 1. *Growth phenotypes of strains carrying alcA$^-$ and alcO$^-$ mutations*

Strain	Genotype[a]	Growth on:		
		Octane	Nonanol	Nonanal
PpG1	*alcA*$^+$ (no plasmid)	−	+	+
PpS81	$alcA^-_{81}$ (no plasmid)	−	−	+
PpS70	$alcA^-_{1737}$ (OCT *alk*$^+$*alcO*$^+$)	+	+	+
PpS116	$alcA^-_{1737}$ (OCT *alk*$^+$$alcO^-_{116}$)	−	−	+
PpS145	$alcA^-_{81}$ (CAM-OCT *alk*$^+$*alcO*$^+$)	+	+	+
PpS173	$alcA^-_{81}$ (CAM-OCT *alk*$^+$$alcO^-_{173}$)	−	−	+
PpS181	$alcA^-_{81}$ (CAM-OCT alk^-_{181}*alcO*$^+$)	−	+	+
PpS367	*alcA*$^+$ (CAM-OCT *alk*$^+$$alcO^-_{173}$)	+	+	+
PpS368	$alcA^-_{81}$ (CAM-OCT *alk*$^+$$alcO^-_{173}$)	−	−	+
PpS370	*alcA*$^+$ (*OCT alk*$^+$$alcO^-_{116}$)	+	+	+

[a] The plasmids and the genetic designations are as described in Grund et al. (7).

determined their growth phenotypes on aliphatic substrates. The results in Table 1 show that strains with the plasmid from PpS173 are able to grow on alkanes when the chromosome is wild type for growth on alcohols but not when it carries an *alcA*$^-$ mutation. Hence, the mutation on the plasmid does not affect the conversion of alkanes to alcohols and is specific for the alcohol to aldehyde step. In other words, the mutation on the CAM-OCT plasmid from PpS173 defines the *alcO* locus and makes it possible to identify a corresponding enzyme activity.

Plasmid-coded alcohol dehydrogenase activity. Alkane-utilizing *P. aeruginosa* strains contain a number of different dehydrogenases with in vitro activity for aliphatic alcohols, but the specifically inducible enzyme has several properties which make it distinguishable from the other activities. It is NAD(P) independent, it is found in the pellet after centrifugation at 48,000 × *g*, and it reduces DCPIP in the presence of substrate and PMS (9). Since preliminary experiments indicated that the *P. putida* NAD-linked alcohol dehydrogenase(s) are not related to the presence of the OCT or CAM-OCT plasmid, we decided to search in extracts of alkane-grown CAM-OCT$^+$ *P. putida* cells for an activity analogous to the inducible *P. aeruginosa* alcohol dehydrogenase. The results of our search are summarized in Table 2. The crude extract contains an alcohol dehydrogenase activity that is stimulated sixfold by the presence of PMS with octanol as a substrate (experiment i). The majority of this activity is found in the particulate fraction (experiment ii), where it displays a greater than 20-fold specificity for octanol as opposed to butanol (experiment iii). In contrast, the activity that remains in the supernatant shows only about a twofold specificity for octanol over butanol (experiment iii) and has a very high background activity. Since NADH can reduce DCPIP, this supernatant activity is probably due to one or more soluble NAD-linked alcohol dehydrogenases (see below).

Table 3 shows that the particulate activity occurs at high levels only in CAM-OCT *alcO*$^+$ strains that have been grown in the presence of inducers of alkane-oxidizing activity (7). The induction ratio is always greater than 20-fold. (The activity seen for pyruvate-grown PpS145 in this experiment was unusually low.) The fact that the hydroxylase-negative mutant PpS181 is induced by silicic acid-purified octane confirms our previous observation that unoxidized alkane molecules induce both plasmid enzymes (2, 7). In either *alcA*$^+$ or *alcA*$^-$ genetic backgrounds, the *alcO*$^-$ plasmid from strain PpS173 lacks the inducible alcohol dehydrogenase activity. The activity was not missing because of induction problems; alkane hydroxylase activity showed a greater than 200-fold induction for both PpS173 and PpS367 in these experiments. Together with data which indicate that the plasmid *alk* and *alcO* loci are part of a single operon (M. Fennewald, S. Benson, and J. Shapiro, unpublished observations), these results strongly suggest that the particulate enzyme is

TABLE 2. *Nature of the alcO gene product activity*[a]

Expt	Substrate	PMS	Substrate-dependent DCPIP reduction activity[b]		
			Crude extract[c]	P48[d]	S48[d]
i	Octanol	+	12.72		
	Octanol	−	2.09		
ii	Octanol	+		8.66	3.39
iii	Octanol	+		3.84	1.81
	Butanol	+		0.16	0.76

[a] Extracts of strain PpS145 (*alcA*$^-$; CAM-OCT$^+$) grown on octane were prepared, fractionated, and assayed as described in Materials and Methods.

[b] Expressed as micromoles of DCPIP reduced per minute per milligram of protein.

[c] Freshly prepared extract.

[d] Extracts frozen overnight at −20 C before fractionation.

TABLE 3. *Induction of alcO alcohol dehydrogenase activity in mutant strains*

Strain	Growth substrate	Octanol-dependent DCPIP reduction activity in the P48 fraction[a]	Alkane hydroxylase activity
PpG1	Glucose	0.28	NT[b]
alcA$^+$	Octanol	0.23	NT
PpS81	Pyruvate	0.20	NT
alcA$^-$	Pyruvate + octane	0.16	NT
PpS145	Pyruvate	0.02	NT
alcA$^-$ (CAM-OCT, *alk*$^+$*alcO*$^+$)	Octanol	5.83	NT
	Octane	8.12	NT
PpS181	Pyruvate	0.15	NT
alcA$^-$ (CAM-OCT, *alk*$^-$*alcO*$^+$)	Pyruvate + octane	8.45	NT
PpS173	Pyruvate	0.13	0.001
alcA$^-$ (CAM-OCT, *alk*$^+$*alcO*$^-$)	Pyruvate + octane	0.23	0.453
PpS367	Pyruvate	0.16	0.002
alcA$^+$ (CAM-OCT, *alk*$^+$*alcO*$^-$)	Pyruvate + octane	0.29	0.562

[a] Expressed as micromoles of DCPIP reduced per minute per milligram of protein.

[b] NT, Not tested.

the *alcO* gene product. However, we have not rigorously eliminated other possibilities for the *alcO* gene product, such as a positive regulatory function or a role in placing the enzyme in some cellular structure. The induced enzyme always shows a high specificity for octanol over butanol, whereas the low constitutive activity detected in both *alcA*$^+$ and *alcA*$^-$ strains without the plasmid has about the same activity with either substrate.

Attempts to identify the *alcA* gene product. Since CAM-OCT plasmids that code for the presence of alcohol dehydrogenase activity can correct the growth defect of *alcA*$^-$ strains and *alcO* mutant plasmids cannot (Table 1), it was logical to conclude that *alcA*$^-$ mutations lead to a loss of an alcohol dehydrogenase activity. From our whole cell oxidation experiments, we concluded that this activity should be constitutive, and the growth patterns of *alcA*$^-$ mutants indicated that the enzyme would show a chain length specificity similar to that of the plasmid enzyme (7). Searches for octanol-specific reduction of NAD(P) and flavine adenine dinucleotide in both particulate and supernatant fractions of strain PpG1 grown on octanol were negative. The supernatant fraction does contain a constitutive NAD-linked alcohol dehydrogenase activity that is absent in the particulate fraction (Table 4). However, this activity is also found in the *alcA*$^-$ mutant and therefore cannot be the *alcA* gene product. Moreover, the NAD-linked activity shows only about a twofold greater activity with octanol rather than butanol as a substrate. That the *alcO*-determined enzyme does not use NAD as a cofactor is confirmed by the absence of NAD-linked activity in the particulate fraction of strain PpS145 grown on octane. Interestingly, growth of plasmid-carrying strains in the presence of octane appears to depress this NAD-linked activity. The effect is plasmid-specific since it is not observed after exposure of strain PpS81 to octane. We do not know whether this effect is due to a specific regulatory mechanism or is a metabolic consequence of alkane hydroxylation. Analogous constitutive NAD(P)-linked soluble alcohol dehydrogenase activities are found in alkane-utilizing strains of *P. aeruginosa* (1, 9, 11).

TABLE 4. *NAD-linked alcohol dehydrogenase activity*

Strain	Growth substrate	Octanol-dependent NAD reduction[a]	
		S48	P48[a]
PpG1	Octanol	1.85	≤0.01
alcA$^+$	Glucose	1.06	≤0.01
PpS81	Pyruvate	2.35	
alcA$^-$	Pyruvate + octane	3.06	
PpS145	Pyruvate	1.41	≤0.01
alcA$^-$*alcO*$^+$	Octane	0.23	≤0.01
PpS367	Pyruvate	3.33	
alcA$^+$*alcO*$^-$	Octane	0.47	

[a] Expressed as micromoles of NAD reduced per minute per milligram of protein × 10^2. The very low values seen in the P48 fraction are partly the result of particulate NADH oxidase activity.

DISCUSSION

We have described a new NAD-independent aliphatic alcohol dehydrogenase activity in *P. putida*. The corresponding enzyme is specific for longer-chain primary alcohols and appears to be membrane-bound, since it is found in the particulate fraction of sonically disrupted cells (Table 2). The results summarized in Tables 1 and 3 confirm our previous conclusions and show that the presence of this enzyme activity is determined by the alkane-inducible product of the OCT or CAM-OCT plasmid *alcO* locus (7). In its independence of NAD, inducibility, chain length specificity, and cellular location, the *P. putida* plasmid-coded alcohol dehydrogenase is similar to an enzyme found in alkane-utilizing strains of *P. aeruginosa* (9, 11).

Although the plasmid-determined alcohol dehydrogenase can correct the growth defect caused by chromosomal *alcA*$^-$ mutations (Table 1), no corresponding chromosomal activity has been found in uninduced or plasmid-free cultures. These cultures do contain a constitutive activity capable of dehydrogenating octanol (Table 4), but the responsible NAD-linked enzyme(s) is soluble and is found in *alcA*$^-$ mutants. Thus, we still do not know what is the product of the *alcA* locus on the *P. putida* chromosome. However, we predict that it is either (i) an aliphatic alcohol permease that transports octanol across the cytoplasmic membrane to the soluble dehydrogenase(s), or (ii) a particulate alcohol dehydrogenase that can act on surface- or membrane-bound substrate. As yet there is no clear evidence that allows us to distinguish between these possibilities.

The detection of cryptic alcohol dehydrogenase activity in strains that are growth negative on octanol or nonanol (but positive on aldehydes) indicates that aliphatic alcohols do not spontaneously cross the cytoplasmic membrane in physiologicaily significant amounts. We know that one way these substrates can serve as sole carbon and energy sources is through dehydrogenation by the particulate plasmid-determined enzyme. Presumably, this step occurs in the membrane or on the cell surface. The fact that *alcA*$^-$ *(alk*$^+$*alcO*$^-$*)* mutants do not grow on alkanes (Table 1) similarly indicates that alkanes do not enter the cytoplasm before hydroxylation, for if they did the resulting alcohol

product would be a substrate for the soluble dehydrogenase activity. Thus, we propose a model where the initial steps in alkane oxidation occur outside the cytoplasm.

A number of other observations are in agreement with our surface oxidation model. (i) Published data indicate that at least one alkane hydroxylase protein is membrane bound (12), and preliminary studies of the requirements for in vitro complementation between alkane hydroxylase mutants suggest that the enzyme complex is attached to a cellular structure. (ii) Comparison of the products of alkane oxidation in whole cells and crude sonic extracts of *alcA*$^-$ (*alk*$^+$*alcO*$^+$) and *alcA*$^-$ (*alk*$^+$*alcO*$^-$) strains indicate that cellular integrity protects alkane-derived primary alcohol from cytoplasmic dehydrogenase activity. And (iii) there exist chromosomal mutants that have altered surface properties and that are unable to place CAM-OCT plasmid-determined proteins in the proper cellular location for growth on either alkanes, primary alcohols or camphor (M. Fennewald, unpublished data). We are currently attempting to extend these observations. If future experiments confirm the surface oxidation model, then our finding that alkanes are the true inducers of OCT plasmid-coded enzymes (Table 3, references 2 and 7) would suggest that induction involves interactions on the cell surface.

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