JOURNAL OF BACTERIOLOGY, Apr. 1985, p. 203–208
0021-9193/85/040203-06$02.00/0

Vol. 162, No. 1

Conjugative Mapping of Pyruvate, 2-Ketoglutarate, and Branched-Chain Keto Acid Dehydrogenase Genes in *Pseudomonas putida* Mutants

PAMELA J. SYKES,[1] JOAN MENARD,[2] VICKI McCULLY,[1] AND JOHN R. SOKATCH[1]*

Departments of Biochemistry and Molecular Biology[1] and Microbiology and Immunology,[2] The University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190

Received 31 August 1984/Accepted 15 January 1985

Branched-chain keto acid dehydrogenase, an enzyme in the common pathway of branched-chain amino acid catabolism of *Pseudomonas putida*, is a multienzyme complex which catalyzes the oxidative decarboxylation of branched-chain keto acids. The objective of the present study was to isolate strains with mutations of this and other keto acid dehydrogenases and to map the location of the mutations on the chromosome of *P. putida*. Several strains with mutations of branched-chain keto acid dehydrogenase, two pyruvate and two 2-ketoglutarate dehydrogenase, were isolated, and the defective subunits were identified by biochemical analysis. By using a recombinant XYL-K plasmid to mediate conjugation, these mutations were mapped in relation to a series of auxotrophic and other catabolic mutations. The last time of entry recorded was at approximately 35 min, and the data were consistent with a single point of entry. Branched-chain keto acid dehydrogenase mutations affecting E1, E1 plus E2, and E3 subunits mapped at approximately 35 min. One other strain affected in the common pathway was deficient in branched-chain amino acid transaminase, and the mutation was mapped at 16 min. The mutations in the two pyruvate dehydrogenase mutants, one deficient in E1 and the other deficient in E1 plus E2, mapped at 22 minutes. The 2-ketoglutarate dehydrogenase mutation affecting the E1 subunit mapped at 12 minutes. A 2-ketoglutarate dehydrogenase mutant deficient in E3 was isolated, but the mutation proved too leaky to map.

Branched-chain keto acid dehydrogenase of *Pseudomonas putida* is a multienzyme complex in the common pathway of branched-chain amino acid metabolism. The complex is composed of three subunits: E1, the dehydrogenase, E2, the transacylase, and E3, lipoamide dehydrogenase. In mammals, only conformational isomers of lipoamide dehydrogenase have been identified (24). However, two structurally and functionally different lipoamide dehydrogenases have been identified in *P. putida* (17). The higher-molecular-weight lipoamide dehydrogenase, LPD-glc, is the specific E3 subunit of 2-ketoglutarate dehydrogenase (17) and is required for glycine oxidation (16). The smaller lipoamide dehydrogenase, LPD-val, is the specific E3 subunit of branched-chain keto acid dehydrogenase. It is of interest to map the genes encoding LPD-val and LPD-glc in relation to genes coding for other enzyme subunits of branched-chain keto acid dehydrogenase to gain insight into the regulation of keto acid metabolism and into the evolutionary relationship of LPD-glc and LPD-val.

Despite its metabolic and genetic diversity, little is known about the arrangement of genes in *P. putida* compared with, for example, *Escherichia coli* and even *Pseudomonas aeruginosa*. One reason for this has been the lack of a suitable range of conjugative vectors in *P. putida*. A number of genes have been mapped in *P. putida* by using the K sex factor (12), and recently, the *P. putida* chromosome was shown to be circular and approximately 103 min in length by using a *P. aeruginosa* plasmid loaded with transposon Tn*501* (2).

In the present study, we isolated a number of mutants involved in keto acid metabolism and identified the enzyme subunits affected. The mutant genes were then mapped on the *P. putida* chromosome by using a recombinant XYL-K sex factor (12) to mediate conjugation. No genes in keto acid metabolism have previously been mapped in *P. putida*, and this is the first report to map genes encoding branched-chain keto acid dehydrogenase in any organism.

MATERIALS AND METHODS

Organisms and growth conditions. Both *P. putida* PpG2 and the donor for the interrupted matings, strain AC143 (12), were obtained from I. C. Gunsalus. *E. coli* NECO 100, used for transposon mutagenesis, was obtained from David Gibson. The mutants and their genotypes are listed in Table 1.

The growth conditions and many of the media were described in earlier publications (9, 18). Valine-isoleucine agar contained 0.3% L-valine and 0.1% L-isoleucine in the basal medium described earlier (9). Pyruvate dehydrogenase mutants were grown in medium with 60 mM acetate, and the 2-ketoglutarate dehydrogenase mutants were grown in GAS medium which contained 10 mM glucose supplemented with 2 mM acetate and 2 mM succinate (18). When antibiotic supplements were added, the final concentrations were (micrograms per milliliter): streptomycin, 100; kanamycin, 90; and chloramphenicol, 50. The final concentration of amino acid supplements was 20 μg/ml. *E. coli* NECO 100 was grown overnight in L broth supplemented with kanamycin. When strain PpG2 was used in transposon mutagenesis experiments, it was grown overnight in L broth with chloramphenicol. Transposon-induced auxotrophs were grown in L broth supplemented with kanamycin and chloramphenicol.

Immunological methods. The preparation of antisera against LPD-val and LPD-glc and double-diffusion experiments have been previously described (18).

* Corresponding author.

TABLE 1. List of *P. putida* strains used in this study

Strain	Genotype[a]	Source or reference
AC143	XYL-K, *met-1*	12
PpG2	Wild type	I. C. Gunsalus
JS112	*bkdAB*	This study
JS113	*bkdA*	This study
JS116	*bct*	This study
JS161	*bkdA*	This study
JS287	*lpdV*	18
JS326	*bkdAB*	This study
JS329	*trp*	This study
JS330	*met*	This study
JS332	*leu*	This study
JS334	*put*	This study
JS335	*hut*	This study
JS336	*arc*	This study
JS342	*pdhAB*	This study
JS343	*pdhA*	This study
JS347	*kgdA*	This study
JS348	*lpdG*	This study
JS401	*trp*::Tn*5*	This study
JS402	*his*::Tn*5*	This study
JS404	*met*::Tn*5*	This study
JS411	*ilv-2*::Tn*5*	This study
JS412	*ilv-3*::Tn*5*	This study
JS419	*leu*::Tn*5*	This study
JS420	*leu*::Tn*5*	This study
JS422	*ilv-19*::Tn*5*	This study

[a] Gene designations of catabolic markers used in this study are: *arc*, arginine catabolism; *bct*, branched-chain amino acid transaminase; *bkdA*, E1 of branched-chain keto acid dehydrogenase; *bkdB*, E2 of branched-chain keto acid dehydrogenase; *hut*, histidine catabolism; *kgdA*, E1 of 2-ketoglutarate dehydrogenase; *lpdG*, LPD-glc, *lpdV*, LPD-val; *pdhA*, E1 of pyruvate dehydrogenase; *pdhB*, E2 of pyruvate dehydrogenase; *put*, proline catabolism. Conventional gene designations were used for auxotrophic markers.

Enzyme assays. The assays for pyruvate, 2-ketoglutarate, branched-chain keto acid, and lipoamide dehydrogenases have been described previously (17, 18). Branched-chain amino acid transaminase was assayed by the method of Duggan and Wechsler (4) with valine and 2-ketoglutarate. The mixture for the E1 assay contained 100 mM potassium phosphate buffer (pH 7.0), 4 mM magnesium chloride, 0.2 mM thiamine pyrophosphate, 5 mM L-valine, 300 μg of phenazine methosulfate per ml, 1.2 mM dichlorophenol-indophenol, enough E1 to yield 0.02 to 0.03 μmol of CO_2 per 15 min, and 1 mM [1−^{14}C]pyruvate, [1−^{14}C]2-ketoglutarate, or [1-^{14}C]2-ketoisovalerate (specific activity, 30,000 cpm/μmol). The reaction was started by the addition of radioactive substrate. The reaction mixture was incubated in 10-ml flasks (Kontes K-882300) fitted with a rubber serum stopper and plastic center wells (Kontes K-882320); the center well contained a strip of filter paper moistened with 40 μl of Protosol (New England Nuclear Corp.). The reaction mixture was incubated for 15 min at 30°C with shaking and then stopped with 0.1 ml of 10 N sulfuric acid injected through the stopper. The flask was shaken for an additional 5 min at which time the filter paper strip was placed in a vial with 10 ml of Econofluor (New England Nuclear Corp.) containing 10% methanol and counted. Specific activity is defined as micromoles of CO_2 produced per 15 min/mg of protein. [1−^{14}C]pyruvate and [1−^{14}C]2-ketoglutarate were incubated for 1 h at 30°C with shaking in a stoppered 10-ml flask as described above to remove $^{14}CO_2$ produced by chemical decarboxylation.

The principle of the E2 assay is that E2 catalyzes transacylation between coenzyme A (CoA) and dihydrolipoamide: Acyl-CoA plus dihydrolipoamide ⇌ acyl-dihydrolipoamide plus CoA. Acetyl and succinyl CoA were prepared from the corresponding anhydrides and CoA as described by Stadtman (20). The concentration of acyl-CoA was determined by the hydroxamic acid assay (20). The reaction mixture for the E2 assay contained (in 1.0 ml) 0.1 M potassium phosphate buffer (pH 7.0), 10 mM dihydrolipoamide prepared as described by Reed et al. (15), 0.03 to 0.13 units of enzyme, and 1 mM acyl-CoA. The reaction was started with acyl-CoA, incubated for 5 min at 30°C at which time the reaction was stopped with 1 μl of redistilled diketene, which converts CoA to acetoacetyl-CoA, mixed, and placed in an ice bath for 3 min. The reaction mixture (0.9 ml) was transferred to a spectrophotometer cell, and 0.3 μmol of NADH in 1% sodium bicarbonate was added. The absorbance was read at 340 nm, and then 4.5 μl of hydroxyacyl-CoA dehydrogenase (Sigma type III from pig heart; 2 mg/ml) was added in a final volume of 1 ml. The absorbance was read again, and the amount of NADH oxidized was calculated from the difference. The specific activity of this assay is defined as micromoles of NADH oxidized per 5 min/mg of protein.

Isolation of mutants. The conditions for nitrosoguanidine mutagenesis and penicillin enrichment were described previously (17). The method of Ornston (13) was used to isolate catabolic mutants. Pyruvate dehydrogenase mutants were obtained by selection after penicillin enrichment in 20 mM lactate broth for growth on 60 mM acetate but not on 20 mM lactate. 2-Ketoglutarate dehydrogenase and LPD-glc–negative mutants were isolated after penicillin enrichment in 10 mM 2-ketoglutarate on the basis of their ability to grow in GAS medium, but not with 10 mM 2-ketoglutarate as the sole carbon source.

Mutations induced by Tn*5* were obtained by the use of pRKTV14, a suicide plasmid in *P. putida* but stable in *E. coli* NECO 100. The plasmid was obtained from David Gibson and was used to isolate auxotrophic mutants of *P. putida* (B. A. Finette and D. T. Gibson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, H127, p. 127). A log-phase culture (1 ml) of *E. coli* NECO 100 in L broth plus kanamycin was mixed with 2 ml of an overnight culture of *P. putida* in L broth. The mixture was filtered onto a 0.45-μm membrane filter, and the filter was left overnight at 32°C on an L agar plate with a soft agar overlay. The cells were dislodged from the filter into 5 ml of L broth, and 0.1 ml of this culture or of a 10^{-1} dilution was plated onto L agar plus kanamycin and chloramphenicol and incubated at 32°C. Chloramphenicol was used to prevent the growth of *E. coli*. The resulting Km^+ Cm^+ recombinant colonies were replica plated onto 10 mM glucose agar containing amino acid pools plus kanamycin and chloramphenicol to characterize the auxotrophic mutants. The percentage of kanamycin resistant colonies which were identified as auxotrophs varied from 0.1 to 0.2% in individual experiments.

Conjugations. The conjugation procedure was that of Mylroie et al. (12). Counterselection against strain AC143 was accomplished by the use of phage pf16, to which the donor was sensitive, and media lacking methionine. The mixture was vortexed vigorously for 30 s to separate mating pairs. The time of entry for individual gene markers was estimated from a plot of number of recombinants per 10^8 donor cells versus time by using data from at least three conjugation experiments for each gene marker. The mutations of auxotrophic strains were mapped by using 10 mM glucose to select for recombinant colonies. The selection system for recombinants of pyruvate genes was 20 mM

lactate. Structural genes for branched-chain keto acid dehydrogenase, branched-chain amino acid transaminase, and 2-ketoglutarate dehydrogenase were mapped with valine-isoleucine agar as the selective medium.

RESULTS

Nutritional phenotypes of keto acid dehydrogenase mutants. Pyruvate dehydrogenase mutants JS342 and JS343, selected for their lack of ability to grow on lactate agar, also were unable to grow on pyruvate agar (Table 2). Pyruvate dehydrogenase mutants grew on GAS medium which is supplemented with 2 mM acetate (18). Presumably this reflects a requirement for acetyl-CoA which can no longer be supplied by pyruvate dehydrogenase (5). It may be significant that neither of the pyruvate dehydrogenase mutants grew on succinate, suggesting that succinate is metabolized via pyruvate. Strain JS347, the mutant lacking 2-ketoglutarate dehydrogenase, failed to grow or grew slowly on a number of compounds thought to be metabolized via the tricarboxylic acid cycle: acetate, pyruvate, lactate, arabinose, and several of the tricarboxylic acid cycle intermediates. In contrast, strain JS348, the *lpdG* mutant, grew well on most compounds tested with the exception of acetate but grew slowly on 2-ketoglutarate.

Keto acid dehydrogenase and branched-chain amino acid transaminase content of mutants. Since mutants defective in branched-chain keto acid dehydrogenase do not grow with valine as the sole carbon source, it was necessary to use GASV medium which allows growth and causes induction of branched-chain keto acid dehydrogenase in these mutants (18). GASV medium contains valine in addition to glucose, acetate, and succinate, and valine is deaminated to 2-ketoisovalerate, the inducer of branched-chain keto acid dehydrogenase (10). The data (Table 3) show that strains JS112, JS113, JS161, and JS326 lacked branched-chain keto acid dehydrogenase even when the assay was supplemented with purified LPD-val. In comparison, strain JS287, a mutant which fails to produce LPD-val, regained full activity when the assay was supplemented with purified LPD-val. These results suggested that the lesions in strains JS112, JS113, JS161, and JS326 were in either E1 or E2 of the complex. Strain JS116, which also fails to grow on valine agar, had only 3% of the branched-chain amino acid transaminase of strain PpG2. All of the mutants listed in Table 3 had normal levels of pyruvate and 2-ketoglutarate dehydrogenases.

TABLE 2. Growth characteristics of pyruvate and 2-ketoglutarate dehydrogenase mutants

Carbon source[b]	Growth characteristics[a] of *P. putida*:				
	PpG2	JS342 *pdhAB*	JS343 *pdhA*	JS347 *kgdA*	JS348 *lpdG*
Acetate	+	+	+	−	−
Pyruvate	+	−	−	−	+/−
Lactate	+	−	−	−	+
Glucose	+	+/−	−	+/−	+/−
GAS	+	+	+	+	+
GA	+	+	+	+	+
GS	+	+/−	−	+	+/−
Arabinose	+	+/−	+/−	−	+
Glutamate	+	+/−	−	−	+
Valine-isoleucine	+	+	−	−	+
Citrate	+	+/−	−	+/−	+
Isocitrate	+	+/−	+/−	+/−	+
2-Ketoglutarate	+	+/−	+/−	−	+/−
Succinate	+	−	−	+/−	+
Fumarate	+	+	−	−	+
Malate	+	−	−	+/−	+
Oxaloacetate	+	−	−	+	+

[a] +, Normal growth; +/−, slow growth; −, no growth. Plates were read at 24 to 36 h.
[b] GA, 10 mM glucose–2 mM acetate; GS, 10 mM glucose–2 mM succinate.

TABLE 3. Subunit activities of branched-chain keto acid dehydrogenase mutants of *P. putida*

Enzyme	Sp act[a] in *P. putida*:					
	PpG2	JS112	JS113	JS161	JS287	JS326
Branched-chain keto acid dehydrogenase	52	0	2	0	6	0
Branched-chain keto acid dehydrogenase + LPD-val	101	4	2	0	246	0
E1	22	0	0	4	45	0
E2	89	3	30	38	146	0
Lipoamide dehydrogenase	565	535	676	260	406	367

[a] Values for branched-chain keto acid dehydrogenase are measured in nanomoles of NADH produced per minute per milligram of protein; values for lipoamide dehydrogenase are measured in micromoles of NADH oxidized per minute per milligram of protein. Other units are described in the text.

Data are shown for assays of E1 and E2 subunits of branched-chain keto acid dehydrogenase as well as total lipoamide dehydrogenase (LPD-glc and LPD-val) in mutants of *P. putida* grown in GASV medium (Table 3). For comparative purposes, there was approximately five times as much branched-chain keto acid dehydrogenase in wild-type PpG2 grown in GASV medium compared with strain PpG2 grown in glucose-mineral salts medium. Strains JS112, JS113, JS161, and JS326 all lacked E1 and contained various amounts of E2. It was not possible to demonstrate a clear-cut deficiency of lipoamide dehydrogenase, even in strain JS287 which is known to lack LPD-val. None of the mutants so far characterized were deficient only in E2.

It was possible that the failure to produce branched-chain keto acid dehydrogenase was due to a failure to metabolize valine to the inducer, 2-ketoisovalerate. To test this idea, we attempted to induce E1 and E2 in these mutants by the following procedure. Strains PpG2, JS112, and JS326 were grown in flasks of GAS medium, harvested, and then transferred to the basal medium with 5 mM 2-ketoisovalerate, the inducer of branched-chain keto acid dehydrogenase (10). The change in the concentration of 2-ketoisovalerate was followed for 5 h, at which time it had disappeared from the flask with strain PpG2 but had changed little in flasks containing the mutants. No E1 was detected in either of the mutants, but the amount of E2 in strain JS326 increased to 44% of that found in strain PpG2. We conclude from these data that there were mutations in E1 and E2 subunits of branched-chain keto acid dehydrogenase.

Pyruvate and 2-ketoglutarate dehydrogenase content of mutants. Enzyme activities of mutants in pyruvate dehydro-

TABLE 4. Subunit activities of pyruvate dehydrogenase mutants of *P. putida*

Enzyme	Sp act[a] in *P. putida*:		
	PpG2	JS342	JS343
Pyruvate dehydrogenase	42	0	11
Pyruvate dehydrogenase + LPD-glc	25	0	1
E1	22	0	1
E2	108	0	72
2-Ketoglutarate dehydrogenase	147	90	141

[a] See Table 3, footnote *a*.

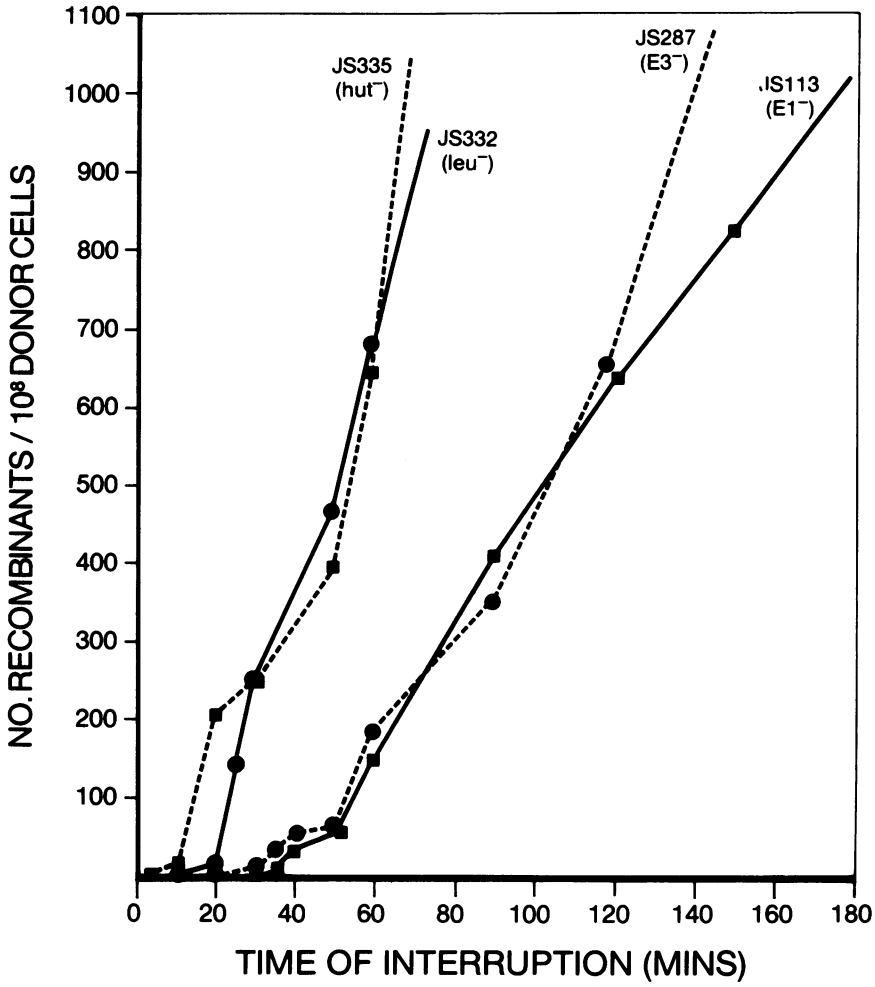

FIG. 1. Time of entry of *hut*, *leu*, *bkdA*, and *lpdV*.

genase are summarized (Table 4). Strain JS342 is an E1-plus-E2 mutant, whereas strain JS343 has a mutation in E1 of pyruvate dehydrogenase. The addition of purified LPD-glc did not restore activity to either of the mutants, indicating that there was no deficiency in lipoamide dehydrogenase of these mutants. Strains JS342 and JS343 had wild-type levels of 2-ketoglutarate dehydrogenase.

Enzyme activities of mutants in 2-ketoglutarate dehydrogenase are shown (Table 5). Strain JS347 has a mutation in E1 of 2-ketoglutarate dehydrogenase with near normal levels of E2. Strain JS348, however, appears to have a mutation in LPD-glc since the addition of purified LPD-glc restored activity of 2-ketoglutarate dehydrogenase to this mutant. Since we had expected that LPD-glc was the E3 subunit of pyruvate and 2-ketoglutarate dehydrogenases, it was surprising that strain JS348 had normal levels of pyruvate dehydrogenase. To confirm that strain JS348 lacked LPD-glc, enzyme extracts were tested for their ability to react with specific antiserum against LPD-glc. An extract of strain JS348 failed to react with anti-LPD-glc, whereas a similarly prepared extract of strain PpG2 formed a precipitate. These results make it clear that strain JS348 has a mutation in LPD-glc. What is not clear is why strain JS348 has a normal amount of pyruvate dehydrogenase, since in *E. coli* one lipoamide dehydrogenase serves as the E3 subunit of both pyruvate and 2-ketoglutarate dehydrogenases (5).

Interrupted matings. The procedure for interrupted matings is described above. Typical plots of colony count per 10^8 donor cells are shown (Fig. 1). The time of entry was extrapolated from the first three time points, giving a significant increase in recombinant colonies. The mutation in strain JS347 was too leaky to map with 2-ketoglutarate as the selective medium; however, it was possible to map the mutation in strain JS347 with valine-isoleucine agar as the selective medium. The logic behind this procedure is that 2-ketoglutarate dehydrogenase is required for valine catabolism since propionyl-CoA is a product of valine catabolism and is oxidized via the tricarboxylic acid cycle (11). Recombinant colonies picked from valine-isoleucine agar and

TABLE 5. Subunit activities of 2-ketoglutarate dehydrogenase mutants of *P. putida*

Enzyme	Sp act[a] in *P. putida*:		
	PpG2	JS347	JS348
2-Ketoglutarate dehydrogenase	72	7	8
2-Ketoglutarate dehydrogenase + LPD-glc	270	2	408
E1	151	0	146
E2	124	87	80
Pyruvate dehydrogenase	69	87	72

[a] See Table 3, footnote *a*.

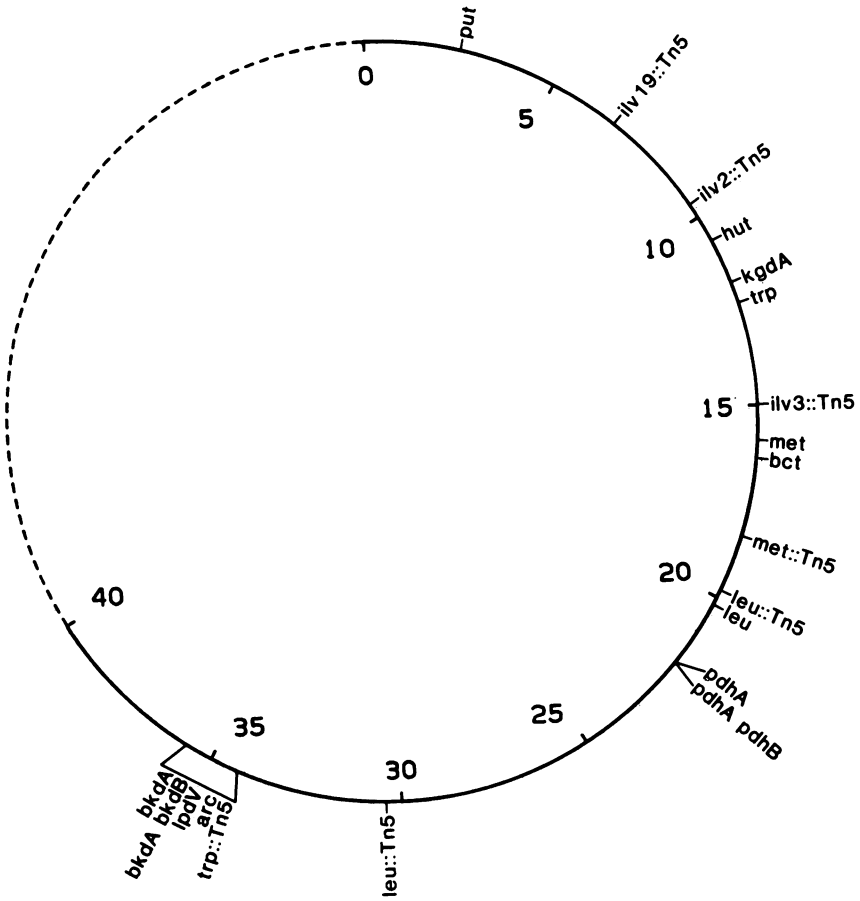

FIG. 2. Map of the chromosome of *P. putida* constructed from times of entry of individual gene markers.

screened for 2-ketoglutarate dehydrogenase activity were found to possess normal levels of that enzyme. Some colonies picked from the donor control plate, which was designed to detect revertants, did not possess 2-ketoglutarate dehydrogenase, but it is not clear what bypass pathway allowed these organisms to grow. It has not been possible to map the mutation in strain JS348, the LPD-glc mutant, since this mutation is too leaky to map with 2-ketoglutarate or valine-isoleucine agar. The map resulting from these studies is shown (Fig. 2). Five gene markers mapped near the 35-min region (Fig. 2). These markers could not be ordered by using the XYL-K conjugation system.

DISCUSSION

The XYL-K recombinant plasmid has been successfully used to map structural genes for the three keto acid dehydrogenases, several auxotrophic genes, and genes for proline (*put*), histidine (*hut*), and arginine (*arc*) catabolism in *P. putida*. The frequency of recombination was ca. $1/10^6$ to 10^7 donors, which is comparable to that obtained by Mylroie et al. (12) with XYL-K and by Dean and Morgan (2) with R91-5::Tn*501* in *P. putida*. Our map of *P. putida* gives an indication of the order of gene markers resolved by using the XYL-K conjugation system between the point of origin and 40 min. From the times of entry of multiple conjugation experiments for each gene marker the time of entry on the map is within approximately ±2 min for the earlier genes up to ±5 min for the later genes. All of our data are compatable with a single point of origin for transfer of the *P. putida* chromosome mobilized by XYL-K. *put* and *hut* are located near the origin of transfer in this study as they were with Mylroie et al. (12). In *P. aeruginosa*, *hut* was mapped at 18 min and *put* was mapped at 22 min from the point of origin of FP2 (6). Not enough common markers were available to compare our map in detail with either that of Mylroie et al., (12) or that of Dean and Morgan (2).

This is the first time that structural genes for branched-chain keto acid dehydrogenase have been mapped in any organism. It also is the first time that pyruvate and 2-ketoglutarate dehydrogenases have been mapped in *P. putida*. It is of interest that the structural gene for LPD-val, the specific E3 subunit of branched-chain keto acid dehydrogenase from *P. putida*, mapped in the same region as did the structural gene for E1. In *E. coli*, there is a single lipoamide dehydrogenase which functions as the E3 subunit for both pyruvate and 2-ketoglutarate dehydrogenases (14). The structural gene for this enzyme, *lpd*, is contiguous with the genes for E1 and E2 subunits of pyruvate dehydrogenase and is located at 2.6 min (5). The genes for E1 and E2 subunits of *E. coli* 2-ketoglutarate dehydrogenase are located at 16.3 min with no *lpd* gene (4). Recently, several papers have appeared from the laboratory of John Guest at Sheffield reporting the primary sequence of all three subunits of pyruvate dehydrogenase complex and of the E1 and E2 subunits of 2-ketoglutarate dehydrogenase (1, 19, 21–23). The sequence data confirm several observations made from genetic data, including the locations of the structural and regulatory genes. However, the most important observation was that there was very little homology between structural genes for the two E1 subunits (*aceE* and *sucA*) but there was

homology between structural genes for the two E2 subunits (*aceF* and *sucB*). These findings suggest that the two E2 subunits had a common ancestor but that the E1 subunits were evolved independently. Recently Lowe et al. (8) presented biochemical and genetic evidence that pyruvate dehydrogenase of *Bacillus subtilis* was actually a dual function complex also responsible for branched-chain keto acid dehydrogenase activity. The findings of Lowe et al. (8) suggest that the evolutionary origin of branched-chain keto acid dehydrogenase might have been pyruvate dehydrogenase. It is unfortunate that so far we have been unable to map the location of LPD-glc since there are a number of interesting questions about the origins of LPD-glc and LPD-val. Peptide maps, antigenic reactivity, genetic data, and amino acid composition all show that LPD-glc and LPD-val are not closely related. In fact, LPD-glc appears to be more closely related to *E. coli* and pig heart lipoamide dehydrogenases than to LPD-val (3). It would have been informative to determine whether *lpdG* mapped with pyruvate or 2-ketoglutarate dehydrogenase. This would have been particularly important since strain JS348, the *lpdG* mutant, had normal pyruvate dehydrogenase (Table 5), whereas *lpd* mutants of *E. coli* lacked both pyruvate and 2-ketoglutarate dehydrogenases.

It is worth commenting on the nutritional phenotypes of pyruvate and 2-ketoglutarate dehydrogenase mutants since these mutants have not been widely studied in *Pseudomonas* spp. Strain JS343, which is a *pdhA* mutant, had a phenotype similar to *P. aeruginosa* PA02851 (7) and failed to grow on lactate, pyruvate, unsupplemented glucose medium, and succinate agar. The latter observation is interesting since it suggests that pyruvate is an intermediate in succinate catabolism in pseudomonads. It is curious that pyruvate dehydrogenase mutants failed to grow on citrate agar, since pyruvate dehydrogenase should not be required for citrate oxidation. Strain JS347, which is a *kgdA* mutant, was unable to grow on unsupplemented media containing carbon sources expected to be metabolized via the tricarboxylic acid cycle, including glucose, arabinose, glutamate, and valine. Strain JS347 did not grow well on glucose agar but did grow on GAS agar which is supplemented with acetate and succinate. The nutritional phenotype of strain JS348, the *lpdG* mutant, was unexpected since it failed to grow only on acetate, although it grew slowly on 2-ketoglutarate and isocitrate. The reason for this nutritional phenotype is not apparent.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AM 21737 and GM 30428 from the National Institutes of Health.

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