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Cloning and Sequence Analysis of the LPD-glc Structural Gene of *Pseudomonas putida*

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***Pseudomonas putida* is able to produce three lipoamide dehydrogenases: (i) LPD-glc, which is the E3 component of the pyruvate and 2-ketoglutarate dehydrogenase complexes and the L-factor for the glycine oxidation system; (ii) LPD-val, which is the specific E3 component of the branched-chain keto acid dehydrogenase complex and is induced by growth on leucine, isoleucine, or valine; and (iii) LPD-3, which was discovered in a *lpdG* mutant and whose role is unknown. Southern hybridization with an oligonucleotide probe encoding the highly conserved redox-active site produced three bands corresponding to the genes encoding these three lipoamide dehydrogenases. The complete structural gene for LPD-glc, *lpdG*, was isolated, and its nucleotide sequence was determined. The latter consists of 476 codons plus a stop codon, TAA. The structural gene for LPD-glc is preceded by a partial open reading frame with strong similarity to the E2 component of 2-ketoglutarate dehydrogenase of *Escherichia coli*. This suggests that *lpdG* is part of the 2-ketoglutarate dehydrogenase operon. LPD-glc was expressed in *Pseudomonas putida* JS348 from pHP4 which contains a partial open reading frame corresponding to the E2 component, 94 bases of noncoding DNA, and the structural gene for *lpdG*. This result indicates that *lpdG* can be expressed separately from the other genes of the operon.**

Lipoamide dehydrogenases (8, 14, 45) are redox-active disulfide flavoproteins as are glutathione reductases (18), mercuric reductases (4, 22), trypanothione reductase (29), asparagusate dehydrogenase (46), bis-γ-glutamylcysteine reductase (38), and pantethine 4′,4″-diphosphate reductase (39). Redox-active disulfide flavoproteins use NADH or NADPH to reduce a disulfide bond and are homodimers containing 1 mol of flavin adenine dinucleotide per subunit.

The reaction catalyzed by lipoamide dehydrogenases is $E2\text{-}Lip(SH)_2 + NAD^+ \leftrightarrow E2\text{-}LipS_2 + NADH + H^+$, where $E2\text{-}Lip(SH)_2$ is the E2 component of a keto acid dehydrogenase multienzyme complex which contains covalently bound lipoic acid. The E3 component, lipoamide dehydrogenase, catalyzes the NAD^+-linked oxidation of the lipoyl prosthetic group, allowing E2 to recycle (44). In the oxidation of glycine by *Pseudomonas putida*, LPD-glc is required for oxidation of H, a polypeptide which contains lipoic acid (30).

P. putida contains structural genes for three lipoamide dehydrogenases, LPD-glc, LPD-val, and LPD-3. LPD-glc is thought to be constitutively produced, is the L-factor of the glycine oxidation system (30), and is the E3 component of the pyruvate and 2-ketoglutarate dehydrogenase multienzyme complexes (32). LPD-val is the specific E3 component of branched-chain keto acid dehydrogenase and is induced when *P. putida* (31) and *Pseudomonas aeruginosa* (21) are grown in medium containing branched-chain amino acids (21, 31). LPD-3 is expressed in *lpdG* mutants of *P. putida* (14), which have pyruvate dehydrogenase activity but no 2-ketoglutarate dehydrogenase activity. So far, the function of LPD-3 has not been discovered. *Escherichia coli* was thought to have only one lipoamide dehydrogenase, but a second one has recently been purified from a *lpd* mutant (25).

In *E. coli*, the structural gene for lipoamide dehydrogenase, *lpd*, is linked to the pyruvate dehydrogenase operon (37). It is not known whether the structural gene for the second lipoamide dehydrogenase of *E. coli* (25) is linked to an operon. In contrast, the structural genes for the lipoamide dehydrogenases of *Azotobacter vinelandii* (42) and *Pseudomonas fluorescens* (2) are part of the 2-ketoglutarate dehydrogenase operon.

The main objective of the present research was to clone and determine the nucleotide sequence of *lpdG*, the structural gene encoding LPD-glc, and to compare the amino acid sequence of LPD-glc with those of other lipoamide dehydrogenases, particularly those of *P. putida*. The second objective was to determine the operon to which *lpdG* is attached and to study its expression.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. pHP1 contains a 5-kb *Sph*I fragment of *P. putida* DNA which includes the complete *lpdG* gene. pHP3 contains a 2-kb *Pst*I insert from pHP1 with the *lpdG* gene in the orientation opposite to that of the *lac* promoter in pUC series plasmids. pHP4 contains the *P. putida* DNA fragment from pHP3 inserted into the *Hin*dIII and *Sac*I sites of the broad-host-range vector pKT240 (1) which contains a promoterless streptomycin resistance gene. *P. putida* JS348 is Km^s Str^r, and *P. putida* JS348(pKT240) is Km^r Str^s. pHP4 was introduced into *P. putida* PpG2 by triparental mating (13).

Media and growth conditions. *E. coli* and *P. putida* were grown in L broth (19) at 37 and 30°C, respectively, with aeration. Antibiotics were added to the medium, depending on which plasmids the cells contained, as follows: cells containing the pUC series plasmids, 200 μg of ampicillin; cells containing pRK2013, 90 μg of kanamycin per ml; and cells containing pKT240-based plasmids, 50 μg of carbenicillin per ml or 2 mg of streptomycin per ml. The basal medium was described earlier (16, 33), and basal medium

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
P. putida		
PpG2	Wild type	I. C. Gunsalus
JS348	*lpdG*[a]	32
E. coli		
TB1	*ara lacZ* ΔM15 Δ(*lac proAB*) φ80 *hsdR17* (r_K^- m_K^+) *rpsL*	BRL[b]
D5Hα	F^- φ80d *lacZ* ΔM15 Δ(*lacZYA-argF*) U169 *endA1 hsdR17* (r_K^- m_K^+) *recA1 supE44* λ^- *thi-1 gyrA relA1*	BRL
HB101	F^- *hsdS20* (*hsdR hsdM*) *supE44 recA13 thi arg14 leu proA2 rpsL20 lacY1 xyl-5 mtl-5 galK2* λ^-	3
Plasmids		
pUC19	Ap^r *lacZ* ΔM15	47
pUC18	Ap^r *lacZ* ΔM15	47
pKT240	IncQ *mob*$^+$ Ap^r Km^r	1
pRK2013	ColE1 *mob*$^+$ *tra*$^+$ (RK2) Km^r	13
pHP1	*lpdG*$^+$; 5.3-kb insert, in pUC19, with orientation opposite to that of the *lac* promoter	This study
pHP3	*lpdG*$^+$; 2.1-kb insert, in pUC18, with orientation identical to that of *lac* promoter	This study
pHP4	*lpdG*$^+$ Str^r pHP3 insert in pKT240	This study

[a] Gene designation of *lpdG* refers to LPD-glc of *P. putida* PpG2.
[b] Bethesda Research Laboratories.

free of ammonium ion was obtained by omitting ammonium sulfate from the basal G solution. Synthetic media contained basal G solution and salts S plus carbon sources at the following concentrations: 10 mM glucose, 10 mM DL-lysine, 40 mM serine, 30 mM threonine, and 60 mM glycine. N-free basal G solution and Salts S was used in medium containing glucose and glycine. *Pseudomonas* Isolation Agar was obtained from Difco Laboratories.

Enzymes and chemicals. Restriction enzymes, kinases, and phosphatases were purchased from Promega Corporation, Madison, Wis. Radioactive materials used in these studies were purchased from New England Nuclear, Dupont Corp., Boston, Mass. [α-^{32}P]dCTP (3,000 Ci/mmol) was used for nick translation and nucleotide sequencing, [γ-^{32}P]ATP (3,000 Ci/mmol) was used for end labeling of oligonucleotide probes, and [α-^{35}S]dCTP (1,221 Ci/mmol) was used in nucleotide sequencing. Nick translation of DNA was performed according to the manufacturer's recommendation using a kit from Bethesda Research Laboratories. Antibiotics were purchased from Sigma Chemical Co.

DNA preparation. *P. putida* chromosomal DNA was isolated from a single cesium chloride-ethidium bromide centrifugation of a cleared lysate by the method of Clewell and Helinski (9). pUC18 and pUC19 plasmids were isolated by the same method as that described for chromosomal preparations, with the addition of a sodium chloride precipitation step. Alkaline lysis (20) was used for small-scale plasmid preparations.

Probe #1, LPD-val active site

```
Thr Cys Leu Asn Ile Gly Cys Ile Pro
5'  TGC CTG AAC ATC GGC TGC ATT CC  3'
3'  ACG GAC TTG TAG CCG ACG TAA GG
```

Probe #2, LPD-glc N-terminus

```
Met Thr Gln Lys Phe Asp Val Val Val Ile
5'          AAG TTC GAC GTG GTG GTG ATC  3'
```

FIG. 1. Construction of probes used to isolate *lpdG*.

Probe preparation. Two synthetic single-stranded oligonucleotides were prepared by the Molecular Biology Resource Facility of St. Francis Hospital, using an Applied Biosystems DNA Synthesizer (model 380B). Probe 1 encoded the highly conserved redox-active site derived directly from the sequence of *lpdV* (Fig. 1) and was made complementary to the sense strand. When probe 1 was used as a primer, the sequence extended toward the N-terminal coding region. Probe 2 was constructed to match the sense strand encoding the N-terminal amino acids of LPD-glc (7). The N-terminal amino acid sequence of LPD-glc was translated into nucleotide sequence, using the codon preference data derived from *lpdV* (24). When probe 2 was used as a primer, the nucleotide sequence extended toward the active site coding region.

The probes were end labeled (20) with 20 U of T4 polynucleotide kinase, using 50 pmol of oligonucleotide and [γ-^{32}P]ATP (150 μCi; specific activity, 3,000 Ci/mmol), and the mixture was incubated at 37°C for 30 min. The labeled oligonucleotide was separated from [γ-^{32}P]ATP, using a spun Sephadex G25/50 column.

Library construction. A limited restriction enzyme digest of *P. putida* chromosomal DNA by *Sph*I produced a majority of fragments in the 4- to 9-kb range. The DNA was mixed with a complete *Sph*I digest of pUC19 which had been treated with alkaline phosphatase with a chromosome-to-vector ratio of 3:1 and ligated with T4 ligase. The amount of total DNA in the ligations and transformations ranged from 0.02 to 0.5 μg. Ligation was done in a total volume of 69 to 79 μl containing 2 U of T4 ligase, and the mixture was left overnight at 14°C. *E. coli* TB1 cells were made competent by the $CaCl_2$ method (20). Selection for recombinants was achieved by growth on ampicillin.

Colony hybridizations. A total of 3,000 white colonies of *E. coli* TB1 cells containing the *P. putida* PpG2 *Sph*I-digested DNA fragments were plated per screening. The colonies were transferred and lysed by using GeneScreen Plus (New England Nuclear) according to the manufacturer's instructions in a solution of 0.5 N NaOH and neutralized in 1.0 M Tris-HCl, pH 7.5. The nylon membranes were prehybridized with 5× Denhardt solution, 0.7 μg of salmon sperm DNA, 0.5% sodium dodecyl sulfate (SDS), and 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 1 h at 37°C. End-labeled probe 1 or 2 (10^6 cpm) was placed in each hybridization bag at 37°C with slight agitation overnight. The GeneScreen Plus filters were washed by the manufacturer's instructions and placed on Kodak X-OMAT RP film overnight.

All colonies which hybridized to probe 1 during the initial screening of the *Sph*I library were transferred with sterile toothpicks to duplicate grid L-agar plates containing 200 μg of ampicillin per ml plus 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Colonies which hybridized to

both probes were grown in 2 ml of L broth with ampicillin overnight and rescreened with both probes using dot blots. One of the clones isolated, pHP4, contained *lpdG* on a 5.3-kb insert.

Southern blots. *P. putida* PpG2 chromosomal DNA (2 μg) was digested with 5 U of *Sph*I for 2 h at 37°C. The digested samples were electrophoresed in a 0.8% agarose gel in two separate lanes and transferred to GeneScreen Plus membrane as described above. The nylon membrane was cut in half; one half was hybridized to labeled probe 1 and the second half was hybridized to probe 2 at 37°C overnight with slight agitation. The membranes were washed and placed on film as described above, and the film was allowed to expose for 2 days with an intensifier screen.

Determination of nucleotide sequence. The nucleotide sequence was determined by the chain termination method of Sanger et al. (27). The starting materials for subcloning were pHP1 and pHP3. pHP3 was constructed by taking a 2-kb *Pst*I fragment of pHP1, containing the complete gene for *lpdG*, and ligating it in the opposite orientation into pUC18. The set of ordered deletions for sequencing (15) was obtained by digesting the polylinker region of pHP1 and pHP3 with *Bam*HI and *Sac*I. The former produced a site sensitive to *Exo*III, and the latter produced a site resistant to *Exo*III. The digests were then treated with S1 nuclease. The subclones were ligated, transformed into *E. coli* TB1, and spread onto plates containing L agar and ampicillin. Small-scale plasmid isolation was performed on the resulting subclones which were sized to ensure that overlapping clones were produced in both orientations. A 7-deaza-dGTP Reagent Kit for DNA Sequencing with Sequenase (United States Biochemical Corp., Cleveland, Ohio) was used as described in manufacturer's directions to prevent compressions due to high G+C content of *Pseudomonas* DNA.

Analysis of nucleotide sequences. The sequence data were entered into the computer of the Oklahoma University Genetic Computer Group. DNA sequence was analyzed using the Genetics Computer Group's Sequence Analysis Software Package Version 6.0 (12) from the University of Wisconsin Biotechnology Center which is available through the Oklahoma University Genetic Computer Group. The sequence was saved, edited using SEQED, and translated using MAP and TRANSLATE. IFASTA was used to search amino acid data banks for homology. The N-terminal amino acid sequence of LPD-glc (7) was used to identify the start of the coding region. The programs ALIGN, GAP, and CONSENSUS were used to obtain the alignments of the amino acid sequences. STEMLOOP and REPEAT were used to determine whether any repeat sequences or rho-independent terminators were present in the nucleotide sequence.

Immunodiffusion assays. Double-diffusion assays were performed as previously described (32). The insert from plasmid pHP3 containing the complete *lpdG* gene was removed by digestion with *Hin*dIII and *Sac*I and inserted into the same sites of the promoterless broad-host-range vector pKT240, and the plasmid was inserted into *E. coli* D5Hα. The vector designated pHP4 was transferred from *E. coli* DH5α to *P. putida* by triparental mating (13). Samples of overnight cultures (100 μl each) of *E. coli* DH5α(pHP4) and *E. coli* DH5α(pRK2013) were mixed with 100 μl of *P. putida* JS348, and spun down and the pellet was resuspended in 50 μl of saline and transferred to a sterile filter disc and placed on Pseudomonas Isolation Agar with 2 mg of carbenicillin per ml. After 4 h at 37°C, the filter was suspended in 5 ml of physiological saline and a 100-μl aliquot was plated on the same medium. An isolated colony of *P. putida* JS348(pHP4) was then grown in 100 ml of L broth with 2 mg of carbenicillin per ml overnight at 37°C with aeration. Sonicated extracts were obtained and clarified by centrifugation at 90,000 × *g*.

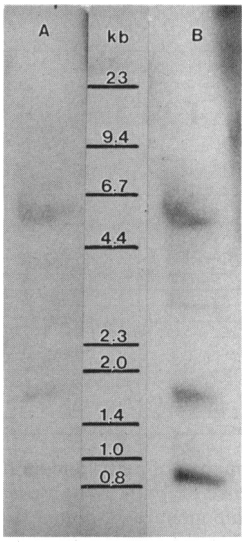

FIG. 2. Southern blots of *P. putida* genomic DNA and probes 1 and 2 produce three bands. *P. putida* DNA was hybridized to N-terminal probe 2 (lane A) or to probe 1 containing the redox-active site sequence (lane B). The three bands in lanes A and B at approximately 5, 1.4, and 0.9 kb correspond to DNA fragments encoding the three lipoamide dehydrogenases. The samples were analyzed by electrophoresis using a series of bacteriophage λ DNA fragments of known molecular sizes as markers (shown between the two lanes).

Nucleotide sequence accession number. The GenBank accession number for the *lpdG* sequence is M38421.

RESULTS

Demonstration of three lipoamide dehydrogenase structural genes in *P. putida*. Three bands resulted from hybridization of *P. putida* genomic DNA to probe 1 containing the nucleotide sequence encoding the highly conserved redox-active site of LPD-val (Fig. 2). The bands in the *Sph*I digest correspond to fragments encoding the redox-active sites of the three lipoamide dehydrogenase structural genes in *P. putida* PpG2. The largest fragment (approximately 5 kb) contained *lpdG*, the medium-size fragment (approximately 1.4 kb) contained *lpd3* (23a), and the smallest fragment (0.9 kb) contained part of the LPD-val gene (6). Identification of the genes in these fragments was confirmed by restriction enzyme analysis of their nucleotide sequences using the MAP program.

Cloning strategy. The strategy used to isolate *lpdG*, the gene encoding LPD-glc, was to use oligonucleotide probes 1 and 2 (Fig. 1) to screen genomic libraries of *P. putida* DNA. A clone which reacted positively to both probes, pHP1, was isolated. Double-strand sequencing (17) using probe 1 as a primer identified *lpdG* on the basis of its N-terminal amino acid sequence (14). Another positive colony which hybridized only to probe 1 contained an insert with the LPD-val structural gene (6).

Nucleotide sequence of *lpdG*. Figure 3 shows the strategy used to determine the nucleotide sequence of both strands of *lpdG*. This procedure yielded clones which provided over-

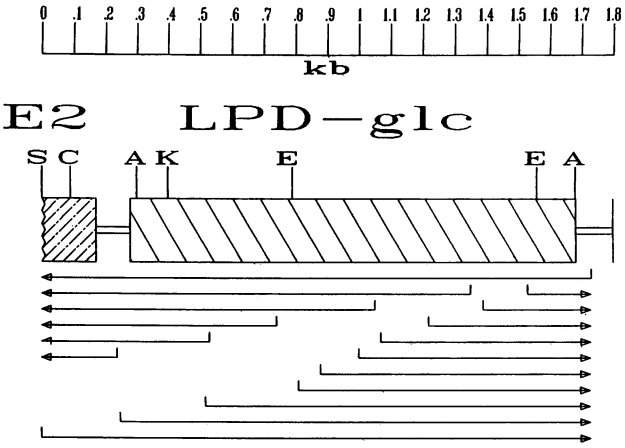

FIG. 3. Restriction map of pHP3 and strategy used to determine the nucleotide sequence of *lpdG*. E2 represents the partial gene for the presumed E2 component of *P. putida* 2-ketoglutarate dehydrogenase. LPD-glc is the gene product of *lpdG*. Numbering of nucleotides begins with the *Sph*I site 156 bases upstream from the termination of the E2 structural gene. Each arrow represents the length of a clone used for sequencing and the direction of reading of nucleotide sequence. Restriction enzyme site abbreviations: A, *Acc*I; C, *Cla*I; E, *Eco*RI; K, *Kpn*I; S, *Sph*I.

lapping sequences in both orientations and provided the sequence of both DNA strands. The nucleotide sequence of *lpdG* and the deduced amino acid sequence of LPD-glc are shown in Fig. 4.

The start of the clone consists of a partial open reading frame of 168 bases encoding 56 amino acids on the same strand as *lpdG*. This C-terminal region has 73% amino acid identity with the C-terminal region of E2 of the 2-ketoglutarate dehydrogenase from *E. coli* (34) but only 25% identity with the C-terminal region of E2 of pyruvate dehydrogenase from *E. coli* (37). There is only 30% amino acid identity with the corresponding region of E2 from branched-chain keto acid dehydrogenase of *P. putida* (5). Considering the above, there is a high probability that LPD-glc is the E3 component of the 2-ketoglutarate dehydrogenase operon of *P. putida* as are the lipoamide dehydrogenases of *A. vinelandii* (42) and *P. fluorescens* (2).

The E2 structural gene is followed by 94 bases of noncoding DNA. The *lpdG* coding region consists of 1,428 bases encoding 476 amino acids plus a TAA stop codon. The correct reading frame was identified with the aid of the partial amino acid sequence of LPD-glc. The sequence of the 10 N-terminal amino acids of LPD-glc is Met-Thr-Gln-Lys-Phe-Asp-Val-Val-Val-Ile (7). This sequence was found in the translation beginning with the ATG at base 266 and is indicated by a single underline in Fig. 4. A likely ribosome-binding site, AGGA, precedes the initiating ATG by 7 bases and is marked SD. The highly conserved redox-active site is indicated by a double underline in Fig. 4.

The noncoding region between E2 and *lpdG* and the region downstream of *lpdG* were examined for dyad symmetry and rho-independent terminators. The REPEAT program could not identify strong repeats larger than 7 bases or rho-independent terminator. The program STEMLOOP predicted a region of dyad symmetry, following the coding region, suggesting that there is a possible stem-and-loop structure that might serve as a transcript terminator. The free energy of formation of the stem-and-loop structure which could be formed in the mRNA transcript is −9.4 kcal. The stop codon is the less common form TAA, instead of TGA which is usually found in *Pseudomonas* genes (41).

Codon usage. The G+C content of *lpdG* is 60.6%, and 77% of the third codons are either G or C which is typical of organisms with a high chromosomal G+C content. In comparison, for 16 chromosomal genes of *P. putida* found in a computer search using Medline, the average G+C content was 62.3% and ranged from 56.6 to 66.3%.

Amino acid composition and molecular weight. The amino acid composition of LPD-glc predicted from the nucleotide sequence is shown in Table 2. The predicted composition agrees with that obtained by hydrolysis of LPD-glc (11). The M_r calculated from the amino acid composition in Table 2 is 49,955. When the M_r of flavin adenine dinucleotide is included, the total M_r is 50,740, which is somewhat lower than the value of 56,000 obtained by SDS-polyacrylamide gel electrophoresis (31).

Comparison of LPD-glc to other lipoamide dehydrogenases. The percent identities and similarities of LPD-glc to lipoamide dehydrogenases whose structural genes have been sequenced are shown in Table 3. The percent identity of LPD-glc to the indicated lipoamide dehydrogenases is shown. Percent similarity includes identities plus percent conserved substitution i.e., substitution of a charged amino acid for a charged amino acid.

The amino acid sequence of LPD-glc has very high amino acid identity to the lipoamide dehydrogenases of *P. fluorescens* (2) and *A. vinelandii* (42). These three genes are parts of operons in which the genes immediately upstream encode the E2 component of 2-ketoglutarate dehydrogenase. In contrast, the lipoamide dehydrogenase encoded by *lpd* of *E. coli* has low homology to LPD-glc and the *lpd* gene is a part of the pyruvate dehydrogenase operon (37) (Table 3). It is surprising that LPD-glc has comparatively low identity to LPD-val, considering that these lipoamide dehydrogenases are from the same organism.

All lipoamide dehydrogenases whose sequences have been reported have the same domain structure as glutathione reductase, whose structure was solved by X-ray crystallography (18). Glutathione reductase is composed of four domains, the flavin adenine dinucleotide and $NADP^+$ binding domains and central and interface domains (40). The computer programs ALIGN, GAP, and CONSENSUS were used to align the sequence of LPD-glc with that of glutathione reductase (data not shown), which confirmed that LPD-glc contains the four domains of glutathione reductase.

Separate expression of LPD-glc. *E. coli*(pHP1) and *E. coli*(pHP3) both expressed *lpdG* even though the *P. putida* DNA insert is in the orientation opposite that of the *lac* promoter in pHP1 (Table 1). This suggests that the 94-base noncoding region between E2 and E3 might contain a promoter for separate expression of *lpdG*, as is the case with *lpd* of *E. coli* (34). Plasmid pHP4 contains an insert which includes the C-terminal portion of the E2 structural gene plus the noncoding intergenic region and also contains the entire structural gene of *lpdG* in pKT240 (see Materials and Methods). pHP4 was inserted into *P. putida* JS348, a *lpdG* mutant (31), by triparental mating. An immunodiffusion assay was performed to detect the presence of LPD-glc in extracts of *P. putida* JS348(pHP4) and *P. putida* JS348(KT240) grown in L broth with carbenicillin (Fig. 5). The lines of immunoprecipitation clearly show expression of *lpdG* from pHP4. Since pKT240 does not contain a promoter, *P. putida* JS348(pHP4) must be using the noncoding region upstream of *lpdG* as a promoter. This result verifies that the lipoamide dehydroge-

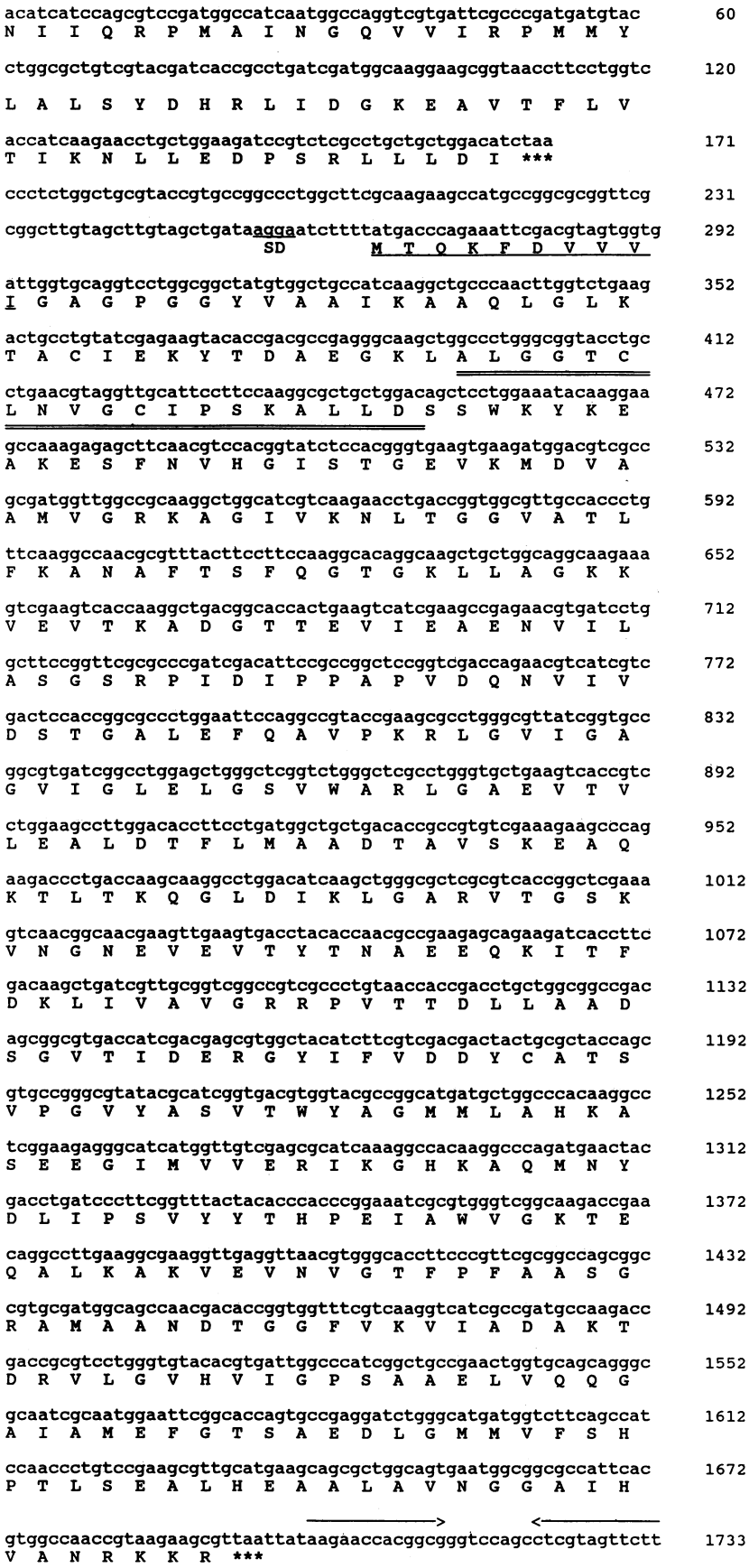

FIG. 4. Nucleotide sequence of pHP3 and deduced amino acid sequences. Bases 1 to 171 encode the C-terminal region of the presumed E2 (transsuccinylase) component. The underlined amino acids are the N-terminal amino acids of LPD-glc (7). The redox-active disulfide region is indicated by a double underline. A possible stem-and-loop terminator structure is indicated by opposing arrows. The underlined sequence marked SD is the Shine-Dalgarno ribosome-binding site. The termination codon (***) is also shown.

TABLE 2. Amino acid composition of LPD-glc predicted from nucleotide sequence and amino acid hydrolysis

Amino acid	No. of residues	
	From sequence	By hydrolysis[a]
Cysteine	4	3
Aspartic acid or asparagine	22	45
	14	
Threonine	34	32
Serine	22	22
Glutamic acid or glutamine	30	50
	12	
Proline	14	18
Glycine	50	56
Alanine	63	63
Valine	53	54
Methionine	12	8
Isoleucine	26	28
Leucine	35	38
Tyrosine	11	8
Phenylalanine	14	13
Lysine	35	33
Histidine	8	9
Arginine	13	16
Tryptophan	4	

[a] The original data were recalculated on the basis of M_r = 50,000 (11).

nase can be expressed separately from the rest of the operon.

DISCUSSION

Previous biochemical studies (7) and now genetic analysis confirms the presence of three lipoamide dehydrogenases in *P. putida*. Southern blots of *P. putida* PpG2 genomic DNA digested with *Sph*I hybridized to probes matching the coding sequences of the redox-active site and the N-terminal regions of LPD-glc. Probe 1 containing the sequence encoding the redox-active site hybridized to *P. putida* genomic DNA digested with *Sph*I, producing three bands corresponding to the active site of the three *lpd* genes. Identification of the three genes was verified by restriction mapping of the nucleotide sequences and restriction mapping of the clones. Probe 2 also hybridized to the fragments, but it hybridized with lower stringency to the 5- and 1.4-kb fragments and hybridized faintly to the 0.9-kb fragment. Since LPD-glc and LPD-3 have similar N-terminal amino acids, this was expected, but it was somewhat surprising that probe 2 also hybridized, even weakly, to the LPD-val gene fragment.

pHP1 contains 56 codons of a reading frame which encodes the C-terminal region of an E2 keto acid dehydrogenase component. This E2 component has 73% amino acid identity with the C-terminal region of 2-ketoglutarate dehydrogenase E2 component of *E. coli* but only 25% identity with E2 of *E. coli* pyruvate dehydrogenase (36). It appears that *lpdG* is a part of the 2-ketoglutarate dehydrogenase operon as are the structural genes for lipoamide dehydrogenases of *A. vinelandii* (42) and *P. fluorescens* (2).

TABLE 3. Percent amino acid identities and similarities of LPD-glc with other lipoamide dehydrogenases

Lipoamide dehydrogenase	% Identity	% Similarity	Reference
P. fluorescens	81.8	89.9	2
A. vinelandii	79.5	87.2	42
Pig	48.7	67.9	23
Human	45.9	67.7	23
Saccharomyces cerevisiae	45.6	67.9	26
E. coli	39.9	62.7	37
P. putida LPD-val	37.7	61.7	6

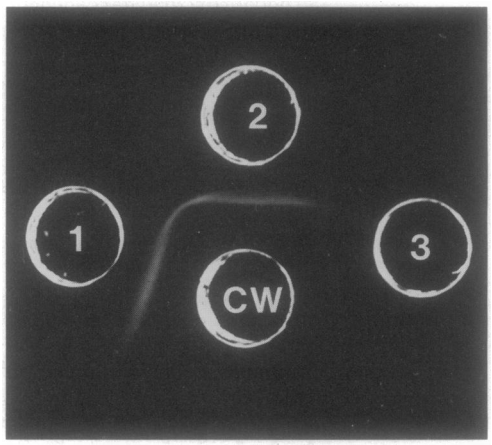

FIG. 5. Expression of *lpdG* independently of the rest of the operon. Wells: 1, 300 μg of protein from *P. putida* JS348(pHP4); 2, 2 μg of purified LPD-glc; 3, 300 μg of protein from *P. putida* JS348(pKT240); cw, 20 μl of a 1:1 dilution of anti-LPD-glc.

In *P. putida*, *P. fluorescens* and *A. vinelandii* lipoamide dehydrogenase structural genes are a part of the 2-ketoglutarate dehydrogenase operon. In *E. coli*, *lpd* is attached to the pyruvate dehydrogenase operon. In all three cases, it is likely that these genes provide lipoamide dehydrogenase for pyruvate and 2-ketoglutarate dehydrogenases. In *E. coli*, *lpd* can be expressed independently of the rest of the operon from a promoter 200 bp upstream from the start of *lpd* (35, 37). The *lpd* gene of *A. vinelandii* was strongly expressed in *E. coli* even though the insert was cloned in the orientation opposite that of the *lacZ* promoter, which also suggests independent expression of this gene (42). Independent expression was not detected from the 69-bp intracistronic region between E2 and E3 of *P. fluorescens* (2). We have shown in this study that *lpdG* can be expressed independently of the 2-ketoglutarate dehydrogenase operon, and it seems likely that there is a *lpdG* promoter in the 94 bases of noncoding DNA upstream of *lpdG*.

The extremely high amino acid sequence identity of LPD-glc with lipoamide dehydrogenases from *A. vinelandii* and *P. fluorescens* (Table 3) is interesting and suggests that these three proteins are closely related evolutionarily. It will be interesting to see whether the homology between the corresponding E1 and E2 components will be just as high. Recently, the structural genes for the E1 and E2 components of *A. vinelandii* 2-ketoglutarate dehydrogenase were cloned and their nucleotide sequences were determined (28, 43). For comparison, there is 59 and 63% amino acid identity between these proteins and the E1 and E2 components of the 2-ketoglutarate dehydrogenase of *E. coli* (10, 34).

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