Expression Cloning and Demonstration of *Enterococcus faecalis* Lipoamidase (Pyruvate Dehydrogenase Inactivase) as a Ser-Ser-Lys Triad Amidohydrolase.

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Summary

*Enterococcus faecalis* lipoamidase was discovered almost fifty years ago (Reed, L. J., Koike, M., Levitch, M. E., and Leach, F. R. (1958) *J Biol Chem* 232, 143-158) as an enzyme activity that cleaved lipoic acid from small lipoylated molecules and from pyruvate dehydrogenase thereby inactivating the enzyme. Although the partially purified enzyme was a key reagent in proving the crucial role of protein-bound lipoic acid in the reaction mechanism of the 2-oxoacid dehydrogenases, the identity of the lipoamidase protein and the encoding gene remained unknown. We report isolation of the lipoamidase gene by screening an expression library made in an unusual cosmid vector in which the copy number of the vector is readily varied from 1-2 to 40-80 in an appropriate *Escherichia coli* host. Although designed for manipulation of large genome segments, the vector was also ideally suited to isolation of the gene encoding the extremely toxic lipoamidase. The gene encoding lipoamidase was isolated by screening for expression in *E. coli* and proved to encode an unexpectedly large protein (80 kDa) that contained the sequence signature of the Ser-Ser-Lys triad amidohydrolase family. The hexa-histidine tagged protein was expressed in *E. coli* and purified to near homogeneity. The purified enzyme was found to cleave both small molecule lipoylated and biotinylated substrates as well as lipoic acid from two 2-oxoacid dehydrogenases and an isolated lipoylated lipoyl domain derived from the pyruvate dehydrogenase E2 subunit. Lipoamidase mediated inactivation of the 2-oxoacid dehydrogenases was observed both *in vivo* and *in vitro*. Mutagenesis studies showed that the residues of the Ser-Ser-Lys triad were required for activity on both small molecule and
protein substrates and confirmed that lipoamidase is a member of the Ser-Ser-Lys triad amidohydrolase family
Lipoic acid (6,8-thioctic acid or 1,2-dithiolane-3-pentanoic acid) is a sulfur-containing cofactor found in most prokaryotic and eukaryotic organisms (1,2). The cofactor is essential for function of several key enzymes involved in oxidative metabolism including pyruvate dehydrogenase (PDH), 2-oxoglutarate dehydrogenase, the branched-chain 2-oxoacid dehydrogenases, and the glycine cleavage system (1,2). In each enzyme a specific subunit is modified by lipoic acid attachment and the sites of attachment are specific lysine residues within conserved domains of these subunits, called lipoyl domains. An amide linkage is formed between the carboxyl group of lipoic acid and the lysine residue ε-amino group of the enzyme subunit. In the 2-oxoacid dehydrogenases the lipoyl domains are found at the amino termini of the E2 subunits. During catalysis, the protein-bound lipoamide moieties serve as carriers of activated acyl groups between the active sites of these multi-enzyme complexes (1,2). Recent work from this laboratory and others have outlined the mechanisms whereby lipoic acid is synthesized and attached to its cognate enzymes (3-9). However, the removal of lipoic acid from lipoylated proteins, a reaction catalyzed by lipoamidase (Lpa), an Enterococcus faecalis enzyme described many years ago (10-12), has remained an unexplored aspect of lipoic acid metabolism.

Lpa was originally described in E. faecalis (then Streptococcus faecalis) strain 10C1 in the late 1950s (10-12). The enzyme cleaves the amide bond that links lipoic acid to the lipoylated lysine ε-amino groups, the cleavage products being free lipoic acid plus the unmodified protein (10-15). Lpa was discovered during studies of the E. faecalis pyruvate dehydrogenase. An ATP-requiring enzyme that is now called lipoate protein ligase was discovered that catalyzed attachment of lipoic acid to the apo form of pyruvate dehydrogenase and thereby activated the enzyme (10-12,14,16). Several amides of lipoic acid were synthesized for testing as possible
inhibitors of the ligase activity. These amides failed to inhibit activation of the cell-free extract pyruvate dehydrogenase by lipoic acid and unexpectedly were found to replace lipoic acid in the ATP-requiring activation (ligase) reaction (10). These observations indicated that \textit{E. faecalis} contained an enzyme that hydrolyzed the amides thereby freeing the lipoate carboxyl group for use by lipoate protein ligase. The hydrolytic enzyme activity, first designated lipoyl X-hydrolase, was purified about 100-fold and was also found to inactivate the 2-oxoacid dehydrogenases by removal of lipoic acid (10-12). Inactivation was shown to be due only to removal of lipoic acid because dehydrogenase activity returned upon treatment of inactivated preparations with lipoate-protein ligase. These data provided a rigorous proof of the key role of protein-bound lipoic acid in the 2-oxoacid dehydrogenase reaction mechanism (10,12).

Although Lpa had not received recent study, partially purified enzyme preparations have been used in studies of several 2-oxoacid dehydrogenases (13-15).

In bacteria Lpa activity has thus far been detected only in \textit{E. faecalis}. \textit{Escherichia coli}, the organism in which lipoic acid metabolism is best understood, lacks Lpa activity (11) as does \textit{Bacillus subtilis} (unpublished data). Lipoic acid metabolism in \textit{E. coli} involves three enzymes; the lipoic acid biosynthetic enzyme LipA (17) and two lipoate-protein attachment enzymes, LplA (6,18) and LipB (4,7), which are responsible for forming the amide bond that links lipoic acid to apo proteins. \textit{E. faecalis} lipoic acid metabolism is distinctly different. This organism is a naturally occurring lipoic acid auxotroph when grown on pyruvate (19,20) and the genome lacks sequences encoding LipA and LipB homologues. The \textit{E. faecalis} genome encodes two LplA homologues, the biochemical functions of which have not yet been demonstrated. In order to begin to understand the role of Lpa in \textit{E. faecalis} and to facilitate the use of the enzyme as a
reagent for studies of lipoic acid metabolism we have isolated the gene encoding Lpa by expression cloning and have purified the enzyme to homogeneity. The purified Lpa showed activity towards both high molecular weight protein substrates such as a lipoyl domain and intact 2-oxoacid dehydrogenases as well as small molecule substrates such as lipoyl-lysine. Both activities were shown to be dependent on the same active site residues, the Ser-Ser-Lys triad of the amidase signature family.
EXPERIMENTAL PROCEDURES

Materials — The CopyControl Fosmid Library Production Kit, End-Repair Enzyme Mix, Colony Fast-Screen Kit (Size Screen) and MaxPlax Lambda Packaging Extracts were purchased from Epicentre (Madison, WI). The Large-Construct Kit for isolation of cosmid DNA free of genomic DNA was purchased from Qiagen (Valencia, CA). Phage λCE6 and its host strain LE392 were purchased from Novagen (Madison, WI). [1-14C]Octanoate was purchased from American Radiolabeled Chemicals, Inc (St. Louis, MO). The ε-lipoyl-α-acetyl derivative of lysine methyl ester, lip-K(A-Me), was a gift from Wacker Chemie (Munich, Germany). Lipoyl-p-aminobenzoic acid (biotinyl-PABA) was synthesized according to Tate (21). Biotinyl-lysine and biotinyl-p-aminobenzoic acid (biotinyl-PABA) were purchased from Sigma (St. Louis, MO). The genomic DNA of E. faecalis 10C1 was prepared using the Genome DNA Kit from QBIOgene (Carlsbad, CA). The Mass Spectrometry Laboratory of the University of Illinois at Urbana-Champaign performed Mass Spectrometry.

Bacterial strains and plasmids—The bacterial stains and plasmids used in this work are listed in Table 1. All E. coli strains were K-12 derivatives. The 10C1 strain of E. faecalis and E. faecalis V583 genomic DNA were from the American Type Culture Collection. E. faecalis V583 is a pathogenic strain of known genome sequence (22). Strain YFJ115 (EP1300/pCY598) was the host strain for the cosmid library construction. The parental strain, EP1300, and cosmid pCC1FOS were purchased from Epicentre (as components of the CopyControl Fosmid Library Production Kit). Plasmid pCY598 was constructed by ligation of the 4 kb fragment of pDLK30
(containing the replication origin plus the kanamycin determinant) to the 5.1 kb *araC-pBAD*
fragment of pTara (23).

Plasmid pYFJ16 containing a 1212 bp fragment encoding *E. coli* LplA was constructed by PCR
amplification from pTM61-4 (6) with primers LplA 3 and LplA 2 (Table 2) and the resulting
*lplA* PCR product was cloned into the pCR2.1-TOPO vector to give pYFJ13. pYFJ13 was
digested with NdeI and HindIII and the 1183 bp DNA fragment was ligated into vector pQE-2
(Qiagen) digested with the same enzymes resulting in pYFJ16 in which expression of an N-
terminus His-tagged LplA was under the control of an IPTG-controlled phage T5 promoter.
Plasmid pYFJ45, a high copy number expression vector with blue-white screening, was
constructed by ligating the 810 bp AvrII-BspLU11I digestion fragment of pETBlue-1 to the 2080
bp Nhel-BspLU11I fragment of pK18 (24). Plasmids pYFJ55 and pYFJ56 were constructed by
PCR amplification from the genomic DNA preparations from strains10C1 and V583,
respectively, using primers EF1033 NdeI and EF1033 BamHI (Table 2) and the PCR products
were cloned into pCR2.1-TOPO. The plasmids were then digested with NdeI and BamHI and
the 2.2 Kb EF1033 fragments were gel purified and ligated into pET16b digested with the same
enzymes to give plasmids pYFJ61 (strain10C1) and pYFJ62 (strain V583). All the PCR
reactions were performed with *PfuTurbo* DNA polymerase (Stratagene) which gives blunt-ended
PCR products. Thus for cloning into pCR2.1-TOPO vector, the products were purified (Qiagen
QIAPrep spin columns) and a 3’-A nucleotide was added by *Taq* DNA polymerase.

Plasmids pYFJ66, pYFJ68, and pYFJ70 which encoded three mutant derivatives (S259A, S235A
and K159A respectively) of *E. faecalis* V583 Lpa in vector pET16b were obtained by used of the
QuikChange Site-Directed Mutagenesis Kit (Stratagene). The DNA template was pYFJ62 and the primers (all beginning with EF1033 as in Table 2) used were the S259A, S235A, and K159A primers for pYFJ66, pYFJ68, and pYFJ70, respectively. The PCR products were transformed into strain TOP10F’ after DpnI digestion. The presence of the expected mutations were confirmed by DNA sequencing. The primers (Table 2) used in PCR reactions were synthesized by the Biotechnology Center, University of Illinois at Urbana-Champaign (Urbana, IL) and Integrated DNA Technologies, Inc. (Coralville, IA).

Culture Media and Growth Conditions—Enterococcus faecalis 10C1 was cultured in the lipoic acid-deficient synthetic medium of Gunsalus (25) with vigorous aeration at 37°C. E. coli strain TM245 was cultured in LB medium supplemented with 10 mg/L of lipoic acid for lipoylated-lipoyl domain preparation whereas for preparation of apo lipoyl domain E. coli strain TM250 was grown in 2XYT medium supplemented with 0.4% glucose, 5 mM sodium succinate and 5 mM sodium acetate. To prepare octanoylated lipoyl domain E. coli strain YFJ38 was grown in minimal E supplemented with 0.4% glucose, 5 mM sodium succinate, 5 mM sodium acetate and 5 mM octanoic acid. E. coli strain YFJ37 was cultured in LB medium supplemented with 50 mg/l lipoic acid. The screening medium used for E. faecalis cosmid expression library construction and screening was LB medium supplemented with medium E, 0.1% glucose, 5 mM sodium succinate, 5 mM sodium acetate, 30 µg/ml kanamycin and 12.5 µg/ml chloramphenicol. The medium E components were the same concentrations as those use in the defined minimal medium.
Partial Purification of Lpa from *E. faecalis*—The method used was a modification of that of Suzuki and Reed (11). This partially purified preparation was used as the source of Lpa activity prior to the isolation of the Lpa gene. *E. faecalis* 10C1 cells were washed and resuspended in 20 mM potassium phosphate buffer, pH 7.0 and disrupted by sonication. The cell-free supernatant obtained by centrifugation at 12,000 x g for 30 min was subjected to a three-step ammonium sulfate fractionation (11). The final precipitate was dissolved in 20 mM potassium phosphate buffer (pH 7.0) and dialyzed against two changes of the same buffer overnight at 4°C. The enzyme preparation was stored at –20°C for several years without significant loss of activity.

Expression and Purification of Apo and Holo Lipoyl Domains—The lipoylated and apo forms of the *E. coli* lipoyl domain were purified from *E. coli* strains TM245 and TM250, respectively, cultured as described above. Domain expression was induced by addition of IPTG to 1 mM and the purification procedure was simplified from the prior protocol (26). Harvested cells were washed and resuspended in lipoyl domain buffer (20 mM sodium phosphate buffer, 2 mM EDTA and 0.02% NaN₃, pH 7.0) and disrupted by two passages through a French Press cell at 20,000 psi and then centrifuged at 40,000 x g for 1 h. The cell-free crude extracts were adjusted to pH 4.0 with 1 M HCl and insoluble material was removed by centrifugation at 40,000 x g for 30 min. The pH of the resulting supernatants was then adjusted to 7.0 with 1 M NaOH and the neutralized supernatant was dialyzed against freshly prepared 10 mM ammonium acetate, pH 5.0. This solution was loaded on a POROS QE anion exchange chromatography column (column volume of 0.831 ml) and eluted with 30 column volumes of a 10 to 600 mM ammonium acetate (pH 5.0) gradient. Protein fractions were analyzed on a the 20% non-denaturing PAGE gel system previously described (26) in which the apo and holo forms of the domains readily
separate. The appropriate fractions were pooled and dialyzed against 2 mM ammonium acetate buffer and the modification status of the purified domains (apo, octanoylated or lipoylated) was confirmed by mass spectroscopy (8). The domain preparations were stored at –20°C.
Assays of Lpa Activity — The gel shift assay was conducted at 37°C in a 100 µl total volume containing purified holo lipoyl domain (2-3 µM), 20 mM potassium phosphate buffer (pH 7.0), 0.3 mM DTT, and an Lpa preparation. The reaction was allowed to proceed for various time periods (0.5-12 h) depending on the concentration and the activity of the Lpa preparation. Part or all of the reaction mixtures were then analyzed together with standards of purified holo and apo lipoyl domains on 20% non-denaturing PAGE gels (26). When the extent of conversion of holo domain to apo domain was very low, the entire reaction mixture was concentrated by trichloroacetic acid precipitation and loaded on the gel. The radioactive Lpa assay was performed in the same reaction mixture except that [1-14C]octanoylated lipoyl domain was the substrate. The reactions were terminated by boiling for 10 min and reaction mixtures were spotted on Whatman 3MM filter discs that had been pre-wetted with 50% TCA. The filter disks were dried and then washed three times with chloroform/methanol/acetic acid (3:6:1, by volume) and twice with 95% ethanol. Each wash was for 5 min with 10 ml of solvent per disc. The filter disks were then dried and immersed in 5 ml of scintillant cocktail and counted for radioactivity in a Beckman LS6500 scintillation counter. A control reaction that lacked Lpa treatment was always performed and the counts were subtracted from those of the Lpa samples and the corrected values were used to calculate the amount of product formed. One unit of Lpa activity was defined as the amount of Lpa required for generation of 1 nmol of apo domain from holo domain in 1 min at 37°C.

Expression and Purification of N-terminal His-tagged Lipoate-Protein Ligase (LplA)—His-tagged LplA was purified from strain YFJ51 grown in LB medium and induced with 1 mM IPTG. The cells were suspended in buffer A (50 mM sodium phosphate, 0.3 M NaCl, 10 mM...
imidazole, pH 8.0) and disrupted by two passages through a French Pressure cell. The resulting lysate was cleared by centrifugation at 38,000 x g for 1 h. The His-tagged LplA was purified from the cell free supernatant by Ni-NTA agarose chromatography under native conditions with the following imidazole concentrations in buffer A: loading buffer, 10 mM; wash buffer, 20 mM; and elution buffer, 250 mM. The protein fractions were analyzed on 12% SDS-PAGE and the purified His-tagged LplA was dialyzed against LplA buffer (20 mM sodium phosphate (pH 7.6) 2 mM EDTA and 5% glycerol) and stored at –80°C. The LplA assay was similar to that of Green et al. (18). In a 50 µl reaction volume, 10 µM apo lipoyl domain, 10 mM sodium phosphate (pH 7.0), 5 mM ATP, 5 mM MgCl2, 0.3 mM DTT, 1 mM lipoic acid (or fatty acid) and LplA enzyme (crude or purified) were mixed. The reactions were usually allowed to proceed at 37°C for 2 h. Modification of the domain was analyzed on 20% non-denaturing PAGE gels. The [1-14C]octanoylated domain was synthesized with apo lipoyl domain and a five-fold excess of [1-14C]octanoic acid using purified His-tagged LplA which resulted in complete conversion of the to the holo domain. LplA was inactivating by heating the reaction mixture to 100°C.

Construction and Screening of the Cosmid Expression Library—A cosmid expression library of E. faecalis genomic DNA fragment was constructed by using the CopyControl Fosmid library Production Kit with some modifications. Genomic DNA prepared from strain10C1 was sheared into fragments of approximately 40 Kb by repeatedly pipetting the DNA with a 200 µl pipette tip. The sheared DNA was end-repaired to give blunt ends and then analyzed by electrophoreses on a 1% gel of SeaPlaque low melting temperature agarose. Fragments that migrated with T7 DNA genomic DNA (39.9 Kb) were selected and cut from the gel. The E. faecalis DNA
fragments were not exposed to UV light. Instead, the marker lanes were cut from the gel, stained, and marked under UV light and the gel was then reassembled and the desired DNA band was excised from the gel. The DNA fragments were recovered from the gel slice and ligated to cosmid pCC1FOS (purchased as an Eco72I361 digest). The ligation mixture was packaged into phage λ particles that were titered by transfection of a host strain to chloramphenicol resistance. Infected cells of strain YFJ115 were plated on the screening medium (LB medium supplemented with full-strength medium E, 0.1% glucose, 5 mM sodium succinate, 5 mM sodium acetate, 30 µg/ml kanamycin, and 12.5 µg/ml chloramphenicol). The plates were incubated at 37°C for 48 hours to allow very small colonies to appear. Colonies were picked from these plates according to colony size (large, medium or small) and fifty colonies patched onto duplicate plates of screening medium. A total of 400 colonies were screened (4 small-colony pools, 3 medium-colony pools, and one large-colony pool). In the first round of screening each of the 50 colonies of a colony pool was inoculated into a single flask of screening medium. The eight cultures were grown at 37°C overnight and then diluted 5-fold into 500 ml of the same medium. These cultures were grown at 37°C until the OD₆₀₀ reached 0.6. Arabinose was then added to a final concentration of 0.01% and the cells were harvested 4 h later. The resulting cell pellets were washed with 20 mM potassium phosphate buffer (pH 7.0) and stored at −20°C before assay. The cell pellets were thawed at 4°C and the cells were resuspended in 10 ml of 20 mM potassium phosphate buffer (pH 7.0) and passed twice through a French Pressure cell at 18,000 psi and the lysate was cleared by centrifugation at 40,000 x g for 1 h. The resulting supernatants were assayed for Lpa activity by both the gel-shift and radioactive assays. Supernatants that showed detectable Lpa activity were concentrated by ammonium sulfate fractionation (see above) and again assayed for Lpa activity by the gel shift assay. In the second round of screening, the 50
colonies of the pool showing activity were divided into ten pools of five colonies each that were processed as in the first round screening, but ammonium sulfate fractionation was omitted. In the final round of screening each of the five colonies of the active pools was separately grown, induced and the supernatants were assayed by the gel shift assay. Two strains YFJ137 and YFJ139 carrying Lpa-encoding cosmid clones were twice colony-purified on the screening medium and single colonies were used to inoculate liquid screening medium. The cultures were induced with 0.01% arabinose and the presence of Lpa activity in the cultures was confirmed by assay of cell-free extracts. The remainders of the induced cultures were used to prepare mixtures of cosmid DNA and pCY598 plasmid DNA. The cosmid DNAs in the DNA preparations, pYFJ46 and pYFJ47 were packed into phage lambda particles as above (the lack of a cos site prevented packaging of pCY598 DNA). The resulting lambda particles were used to infect strain EP1300 followed by plating on screening medium lacking kanamycin to give strains YFJ141 and YFJ143, respectively. Large-scale preparations of pYFJ46 and pYFJ47 cosmid DNA were obtained from these strains following arabinose induction with the Qiagen Large-Construct Kit. Subclones were made by sonication of the cosmid DNAs (pYFJ46) or by partial restriction enzyme digestion (pYFJ47). Sonication fragmentation was done by dissolving 20 μg of cosmid DNA in 500 μl of TE buffer sonicating at the lowest power output for 1 to 2 seconds. A portion of the sonicated DNA was analyzed on 0.8% agarose gels. If most of the DNA fragments were in the desired size range the remaining sonicated DNA was precipitated and dissolved in 20 μl of TE buffer and end-repaired to blunt-ended DNA fragments. The end-repaired DNA was then loaded on an 0.8% SeaPlaque low melting temperature agarose gel which was run slowly to maximize resolution of large fragments. DNA fragments of 1.6-3 Kb or 3-5 Kb were recovered from the gel as described above and ligated to cloning vector pYFJ45 digested with Smal and
dephosphorylated with shrimp alkaline phosphatase (Roche Applied Science, Indianapolis, IN). The ligation mixtures were transformed into TOP10 chemically competent cells (Invitrogen, Carlsbad, CA) and plated on plating medium lacking chloramphenicol and containing 80 µg/ml X-Gal. The cosmid pYFJ47 DNA was fragmented by partial digestion with ApoI and fragments of 3-4 Kb or 4-5 Kb were isolated and cloned as described above. White colonies were examined by the Colony Fast-Screen Size Screen Kit (Epicentre, Madison, WI). Plasmids containing inserts of the desired size were randomly picked for sequencing using the pETBlueDown primer. White colonies were also screened for Lpa activity as pools of ten colonies grown at 37°C overnight in LB medium supplemented with 10 mM Tris-HCl (pH 7.5), 0.2% maltose, 5 mM sodium succinate, 5 mM sodium acetate, and 50 µg/ml kanamycin. The overnight cultures were then subcultured into 100 ml of the same medium and grown at 37°C until mid-log phase (about 2 h) at which time 4 x 10⁹ pfu/ml final concentration of λ CE6 and MgSO₄ (10 mM final concentration) were added into the cultures. The cultures were harvested 3 h after infection and cell extracts were assayed for Lpa activity by the gel assay.

Expression and Purification of N-terminal His-tagged Lpa—A single colony of strain YFJ202 was inoculated into 10 ml of LB medium supplemented with 5 mM sodium succinate, 5 mM sodium acetate, 10 µg/ml lipoic acid and 100 µg/ml ampicillin. The culture was grown at 37°C overnight and added to 500 ml of the same medium plus 0.2% maltose. This culture was shaken vigorously at 30°C for about 4 h (until the OD₆₀₀ reached 0.6) and then MgSO₄ (10 mM final concentration) and a λ CE6 stock (final concentration about 2 x 10⁹ pfu/ml to give a multiplicity of infection of about 5). After 3 h of infection the cells were harvested and washed extensively with His-tag purification lysis buffer (see below) to remove any contaminating lipoic acid. The
His-tagged Lpa was purified from the cell-free crude extract utilizing Ni-NTA agarose (Qiagen, Valencia, CA) under native conditions. The imidazole concentrations used in the buffers were: lysis buffer, 10 mM; wash buffer, 40 mM; and elution buffer, 200 mM. The protein fractions were analyzed on 8% SDS-PAGE gels and fractions of purified Lpa were combined and dialyzed against 20 mM potassium phosphate, pH 7.0 and 5% glycerol with three buffer changes. The dialyzed enzyme was aliquoted and stored at -80°C. Thorough washing of the cell pellet and the extensive dialysis of the purified enzyme were sufficient to remove lipoic acid contamination that might interfere with subsequent assays of Lpa activity.

*Lpa Inactivation of PDH and KGDH*—Strains YFJ206 and YFJ207 were cultured in LB medium plus 5 mM sodium succinate, 5 mM sodium acetate, 10 µg/ml lipoic acid and 100 µg/ml ampicillin at 37°C overnight. The overnight cultures were then diluted 50-fold into 500 ml of the same medium plus 0.2% maltose. Both cultures were grown at 30°C for about 4 h until OD$_{600}$ reached 0.6. Half of each culture was removed and harvested as the uninduced samples. To the second half of each culture was added 10 mM MgSO$_4$ and 25 ml λ CE6 phage stock (2 x $10^{10}$ pfu/ml). The infection of the cultures proceeded for 3 h. After harvest the uninduced and induced cells were washed and then resuspended in 20 mM potassium phosphate buffer (pH 7.0) and disrupted by passing through a French Press cell. The lysates were cleared by centrifugation and the cell free supernatants were used for PDH and KGDH assays (8,27).

For the *in vitro* experiment strain JK1 was cultured in LB medium plus 30 µg/ml streptomycin to stationary phase. A cell-free crude extract of JK1 made in 20 mM potassium phosphate buffer, pH 7.0 was the source of PDH and KGDH. The crude extract (970 µl) was treated with 2 µg
(20 μl) of purified His-tagged Lpa in the presence of 0.3 mM DTT (10 μl) in a 1 ml reaction. Control reactions lacking Lpa were also performed. The reactions were incubated at room temperature for 20 minutes before assaying PDH and KGDH activities. The PDH and KGDH activities of the JK1 crude extracts without room temperature incubation were also assayed.

$Lpa$ cleavage of lipoylated small molecule substrates—$Lpa$ assays using small molecule substrates were performed using reaction conditions similar to those used for protein substrates. The small substrates tested were lip-K(A-Me), lipoyl-PABA, biotinyl-lysine and biotinyl-PABA. Lipoyl-PABA was dissolved in dimethylformamide or dimethylsulfoxide and other substrates were dissolved in water. The reaction mixtures contained the substrate at 60-100 μM, 20 mM potassium phosphate (pH 7.0) 0.3 mM DTT plus appropriate amount of Lpa (crude or purified) in a 50 μl volume. The reactions were allowed to proceed at 37°C for times ranging from several hours to overnight. Released lipoic acid or biotin was assayed by lipoic acid or biotin bioassays. Ten μl of each reaction from the 50 μl system was loaded onto the bioassay plate. The unavoidable lipoic acid or biotin contaminations of the substrates and enzyme preparations were monitored by control reactions with the substrates alone or the enzyme preparations alone in the bioassays.

Bioassay of lipoic acid was performed as described before (28) except that amide-linked species were cleaved by Lpa treatment (see above) rather than acid hydrolysis. The indicator strain KER176 was grown without lipoic acid supplementation in medium A containing with 0.4% glucose, 0.1% Casamino Acids, 5 mM sodium succinate, 5 mM sodium acetate and 1 mM MgSO$_4$ and 10 μl of each sample to be assayed was loaded on the assay plates. The Lpa
preparation and samples without Lpa treatment were also loaded on the plates to detect any contamination with free lipoic acid. The biotin bioassay was similar the lipoate bioassay. Strain NRD25 was used as the indicator strain and inocula of varying numbers of cells were picked from an LB plate and were cultured in 20 ml of medium A supplemented with 0.4% glucose, 0.1% vitamin assay Casamino acids, 0.01% thiamine-HCl, 1 mM MgSO₄ and 25 µg/ml chloramphenicol and grown at 37°C overnight to deplete the supplies of both extracellular and intracellular biotin. The final OD₆₀₀ of the biotin-depleted cultures largely depended on the initial inoculum and generally was 0.1 to 0.2. These cultures were then harvested and the pelleted cells washed twice with 10 ml of medium and resuspended in 5 ml of medium A and mixed with 95 ml of top agar. The compositions of the basal agar were: medium A containing 1.5% agar, 0.4% glucose, 0.1% vitamin assay Casamino acids, 0.01% vitamin B1, 1 mM MgSO₄, 0.01% 2, 3, 5-triphenyltetrazolium chloride and 25 µg/ml chloramphenicol. Twenty-five ml of basal agar was poured on each plate. The compositions of the top agar were basically the same as the basal agar except that vitamin assay Casamino acids and chloramphenicol were omitted. Five ml per plate of top agar was poured over the base agar.

Gel Filtration Chromatography—Gel filtration chromatography was performed a column of Superdex 200 (Amersham Biosciences, Piscataway, NJ). Since Lpa showed some aggregation at the high protein concentrations required for detection by absorbance, we specifically labeled the His-tagged protein by incorporation of ³⁵S-methionine in the presence of rifampicin (29). The protein was then purified by nickel cheleate chromatography as described above. Lpa (25 µg) was loaded on the column and its elution was monitored by scintillation counting. The column was run in PBS buffer (20 mM sodium phosphate (pH 7.0), 150 mM NaCl). The molecular
weight standards used were from the Gel Filtration Calibration Kit HMW (Amersham Biosciences, Piscataway, NJ).
Results

*Rationale of the Lpa Assays Utilized*—The objective of our work was the isolation of an enzyme activity that catalyzed hydrolysis of lipoic acid from lipoylated protein domains and intact 2-oxoacid dehydrogenases. We intended to screen cultures of *E. coli* that harbored plasmids carrying *E. faecalis* genome fragments for Lpa activity. A possible shortcut was to screen for an activity that cleaved a chromogenic lipoylated small molecule. However, although the original literature discussed Lpa as a single enzyme activity that utilized both intact 2-oxoacid dehydrogenases and small lipoate-containing molecules such as lipoamide as substrates (11), this was based on enzyme preparations that had been purified only ca. 100-fold from crude extracts. Hence, it seemed possible that the early Lpa preparations might have contained several different lipoate-releasing activities. Indeed, later workers reported that ion exchange chromatography resulted in two peaks of small molecule substrate activity, only one of which was active on lipoylated proteins (13,21). Moreover, the early literature had reported a second *E. faecalis* enzyme removed during Lpa purification that also cleaved a lipoylated small molecule (10) and Tate (21) purified Lpa >800-fold from *E. faecalis* crude extracts by assay of cleavage of a lipoylated small molecule, but was unable to demonstrate a unique protein to possess Lpa activity. Finally, two hydrolyases of mammalian origin know to be physiologically active on other substrates have been reported to cleave small lipoyl substrates (30,31). For these reasons it appeared prudent to use a lipoylated protein rather than a lipoylated small molecule as the screening substrate.
The classical lipoamidase assay in the literature, inactivation of a 2-oxoacid dehydrogenase (10,12), could be used to directly screen for clones encoding a lipoylated protein cleavage activity, but proteolytic inactivation was a possible artifact. This plus the lack of sensitivity due to indirect detection made this assay seem a poor choice for library screening. Another assay was essentially the reversal of the assay we and others have used to assay lipoylation or biotinylation of apo domain proteins (3,4,32,33). The apo form of a small (60-90 residues) stable protein domain that carries the post-translational modification in its holo form is obtained by expression of the appropriate recombinant clones. Both the lipoyl and biotinoyl domains are highly acidic proteins and migrate rapidly on non-denaturing PAGE gels. Since on such gels protein motilities depend on both size and charge, the holo domains (octanoylated and lipoylated in case of the lipoyl domain) migrate faster than the apo forms of the domains due to loss of the positive charge of the unmodified lysine residue. Thus, the detection of Lpa activity can be visualized by conversion of the fast moving holo domain band to the slower migrating apo domain band. This assay has the advantage that proteolytic artifacts are readily ruled out since the protein products of the hydrolytic reaction are directly observed. The assay has the additional advantage that when a tightly linked gel is used for non-denaturing PAGE, the highly acidic nature and small size of the domains results in their ready resolution from the bulk of bacterial proteins thereby allowing domain modification to be assayed in crude E. coli cell extracts (32). However, since an appreciable fraction of the holo domain has to be cleaved before an apo domain band becomes visible, the gel shift assay is not particularly sensitive. A more sensitive assay was a radioactive assay that measured the release of label from [1-14C]octanoate-labeled octanoylated domain and is based on the prior report that octanoylated domains are good substrates for Lpa (13). This assay complemented the gel shift assay and
provided a quantitative measurement of Lpa activity. In our work the gel shift assay was generally used because it was robust and readily interpreted. It should be noted that upon storage the holo form of the lipoyl domain accumulated a faster migrating species which is presumably the product of deamidation during storage. However, since Lpa also cleaved this species, the only consequence was a slight increase in complexity of the protein band patterns. We validated the two assays using a partially purified Lpa preparation from *E. faecalis* and included this Lpa preparation as an internal control in our screening protocols (see below).

*Construction of an Cosmid E. faecalis Expression Library*—Expression cloning of *E. faecalis* Lpa seemed problematic for two reasons. First, *E. faecalis* promoters were expected to be inactive in *E. coli*, although *E. faecalis* ribosome binding sites seemed likely to function. Second, expression of Lpa seemed likely to be toxic in that it should inhibit aerobic growth of *E. coli* through inactivation of the host 2-oxoacid dehydrogenases. The expected toxicity precluded use of the usual high copy number vectors as cloning vehicles and mandated use of low copy number vectors. However, expression from a low copy number plasmid could make detection of Lpa in a screening protocol problematic. To avoid these issues we chose a system in which the plasmid copy number could be varied from 1-2 to 40-80 by arabinose addition (34). This system uses a cosmid vector that contains two different replication origins. The *E. coli* F factor partitioning and single copy replication origin (*ori*), *ori2*, is responsible for maintaining the cosmid clones as single copy plasmids under non-induced conditions thereby providing replicational stability of the constructs and avoiding toxicity due to high levels of protein expression. The *ori2* of the vector readily replicates the large DNA inserts required for packaging into *λ* phage particles. The second origin is the high copy *oriV*, the function of which
depends on the TrfA protein. A trfA gene under control of a pBAD promoter is integrated on the chromosome of a special host strain (34) and encodes a mutant TrfA that gives higher copy numbers than does wild type TrfA. Therefore, upon addition of arabinose the mutant TrfA is expressed allowing utilization of oriV and a 40 to 80-fold increase in cosmid clone copy number thereby facilitating detection of cloned gene products and subsequent DNA manipulations. The phage λ cos site of the vector allows plasmids with inserts of sufficient size (ca. 40 kb) to be readily packaged into λ phage particles either in vitro or in vivo and thus the cosmids can be very efficiently moved among host strains. We used a commercial (Epicentre) version of this system in which the vector pCC1FOS carries a T7 promoter that reads into the cloning site. The phage T7 promoter located upstream of the vector cloning site provided the means for high-level gene expression. Transcription by phage T7 RNA polymerase is unaffected by most bacterial transcription terminators and is very processive and thus could potentially transcribe the length of a cosmid (35,36). Therefore, we supplied T7 RNA polymerase by arabinose-induced expression of the T7-RNA polymerase gene resident on a compatible plasmid. Hence, addition of arabinose increased both the copy number and transcription of the library cosmids. The problem of growth inhibition due to Lpa expression was mitigated by use of a medium supplemented with glucose, succinate and acetate. Addition of succinate and acetate was expected to bypass the PDH and KGDH deficiency as it does under lipoate starvation conditions (6,7,26). Glucose was supplied both as the major carbon source and to repress basal level expression from pBAD promoters thereby decreasing oriV and T7 promoter function and hence increase the stability of the desired clones. Finally, the medium was heavily buffered to counter the acid produced by sugar metabolism. Fragments (~40 Kb) of E. faecalis genomic DNA were ligated into pCC1FOS vector and a ligation mixture gave about $10^4$ phage particles following in
vitro packaging. These phage particles were used to transfect strain YFJ115 and the transfected cells were plated for single colonies. The plates were incubated at 37°C for 48 hours to allow small colonies to appear. (Clones that contained the Lpa gene were expected to form very small colonies due to the Lpa toxicity since succinate-acetate supported growth of lipoate auxotrophic strains is much slower than when lipoate is the supplement.)

Screening for Clones Encoding Lpa Activity—Given the equation of Clarke and Carbon (37) 366 clones should ensure a 99% probability of finding the Lpa gene in the 3.2 Mb E. faecalis genome. Therefore, we picked 400 recombinant colonies in pools of 50. One pool included large colonies (L1), three pools contained colonies of medium size (M2-M4) and four pools consisted of colonies of small size (S1-S4). Each pool was grown and induced as described in Experimental Procedures. Crude extract supernatants were assayed for Lpa activity by both the gel shift assay and the radioactive assay. Because each supernatant was derived from 50 clones the level of Lpa expressed by a single positive clone seemed likely to be difficult to detect given the small amounts of protein that could be assayed. Thus, although both assays detected signals for some pools, the signals were close to background. To confirm that Lpa activity was indeed present the supernatants of pools, S2, S3, S4 and M4, were subjected to ammonium sulfate fractionation to enrich and concentrate any Lpa present (Experimental Procedures). These preparations were then assayed for Lpa activity by the gel shift assay and all but pool S3 showed obvious Lpa activity (Fig. 1A). (Note that accumulation of the apo domain increased during overnight incubations at 37°C indicating that Lpa remained active under these conditions.) The fifty colonies of pool S2 were divided into ten pools of five colonies each and crude extracts were again screened. Two of the sub-pools contained Lpa activity. Screening cultures derived
from the individual colonies of these sub-pools gave two strains, S2-6 and S2-17 (renamed strains YFJ137 and YFJ139, respectively) that expressed cosmid-encoded Lpa activity (Fig. 1B). The cosmids of these strains (pYFJ46 and pYFJ47, respectively) were packaged into phage λ particles. Infection of strain EP1300 with these λ particles preparations gave strains YFJ141 and YFJ143, respectively. This packaging step was done to resolve the cosmids from plasmid pCY598.

Identification of the Gene Encoding Lpa—We initially attempted to isolate the Lpa-encoding gene of pYFJ46 and pYFJ47 by constructing and screening subclone libraries of different insert sizes in a high copy number vector. Although about 200 colonies of each subclone library were screened, no subclone expressing Lpa was found. In theory (37) screening of 100 colonies having an average insert size of 2 Kb should give 99% probability of finding the desired clone. We hypothesized that the failure to find a subclone was due to acerbation of Lpa toxicity due to the high copy number of the vector. This suspicion was later confirmed by the finding that purified Lpa inactivated PDH and KGDH both in vivo and in vitro and making Lpa very difficult to clone and express in E. coli (see below). The failure to find an active subclone could also be due to the fact that the Lpa-encoding gene proved unexpectedly large relative to the insert sizes chosen for some of the subclone libraries and thus those libraries would have contained only fragments of the lpa gene. We therefore decided to sequence the subclones to identify the segment of the then newly released E. faecalis V583 genome sequence (22) in which the Lpa gene was located. Although the genomic DNA used for cosmid expression library construction was prepared from E. faecalis 10C1, we expected that the sequences would be sufficiently similar to those of strain V583 that we could take advantage of the V583 genome sequence.
Hence, several sub-clones from both pYFJ46 and pYFJ47 were chosen at random and sequenced. BLAST searches of the resulting sequences against the *E. faecalis* V583 genomic sequence (www.tigr.org) showed that all matched a single segment of the V583 genome and virtually all of the 10C1 sequences were >90% identical to the V583 sequence. The pYFJ46 sub-clones covered a 32.58 Kb segment of the V583 chromosome spanning from nucleotide 960,820 (within open reading frame EF1002) to nucleotide 993,400 (within open reading frame EF1032) whereas sequences of the pYFJ47 subclones covered a 35.508 Kb region from nucleotide 966,981 (within open reading frame EF1008) to nucleotide 1,002,489 (within open reading frame EF1040). Sequencing showed that V583 nucleotide 1,002,489 was the junction with the pCC1FOS vector, thereby giving the orientation of the pYFJ47 insert (Fig. 2). To determine the lengths of the two inserts PCR primers were designed to amplify the gap regions between the inserts and the vector. Since the orientation of the insert had been determined for pYFJ47, two primers, one complementary to a vector sequence vector (pCC1for) and the other complementary to a sequence within open reading frame EF 1008, were used to prime PCR reactions and a 1.5 Kb product was obtained. Thus, the pYFJ47 insert corresponded to ca. nucleotide 965,500 (in open reading frame EF1006) to 1,002,489 bp (in open reading frame EF1040) of the *E. faecalis* V583 chromosome. In the case of pYFJ46 we failed to isolate a clone carrying the junction between the insert and the vector and thus the orientation of insert was determined by PCR using two primer sets. As expected one primer set (pCC1rev plus EF 1032) failed and the other (pCC1for plus EF1032) gave a 4.5 Kb PCR product. Thus, the orientation of the pYFJ46 insert was opposite that of pYFJ47 and the known pYFJ46 insert extended from nucleotide 960,820 (in open reading frame 1002) to ca. nucleotide 998,000 (in open reading frame EF1036) of the *E. faecalis* chromosome. The length of the unknown DNA segment at the
other end of the pYFJ46 insert remained undetermined, but was irrelevant since the pYFJ46-pYFJ47 overlap region (the DNA segment between open reading frames EF1006 and EF1036) defined the location of the Lpa-encoding gene. The fact that the two inserts had opposite orientations indicated that the Lpa gene was expressed from a promoter other than the vector T7 promoter. This was confirmed by expression in a host lacking the T7 RNA polymerase plasmid (data not shown). Expression of the Lpa-encoding gene seems likely to be from a serendipitous promoter arising from the low GC (37.5%) content of the *E. faecalis* genome (although use of the native promoter remains possible).

Examination of the open reading frames encoded between open reading frames EF1006 and EF1036, one open reading frame, EF1033, annotated as a putative 6-aminohexanoate-cyclic-dimer hydrolyase (a bacterial amidase involved in degradation of a by-product of nylon manufacture), seemed the strongest candidate to encode Lpa. To test if EF1033 indeed encoded Lpa, this open reading frame together with its upstream region (presumably containing the sequence acting as a promoter in *E. coli*) was PCR-amplified from *E. faecalis* v583 genomic DNA and cloned into vector pCR2.1-TOPO. Ten white colonies were randomly picked and grown together as a pool. A pool rather than a culture from a single colony was assayed to avoid the possibility of PCR-engendered mutations inactivating a single clone. (Clones having PCR-engendered mutations seemed likely to be a problem since EF1033 is a 2.2 kb open reading frame and the assumed toxicity of Lpa would select for those clones encoding an inactive protein). Lpa activity was detected in the crude extract of this pool indicating that EF1033 encoded Lpa (Fig. 3). Another PCR product comprising the region upstream of EF1033 to the end of the downstream open reading frame, EF1034, was also obtained and cloned into vector
pCR2.1-TOPO. Surprisingly, a pool of ten clones carrying this PCR product failed to show any Lpa activity (Fig. 3). However, it should be noted that the sequences of these PCR products was not validated and thus the absence of Lpa activity in the second construct could be due to PCR-engendered mutations within EF1033 open reading frame.

The cloned Lpa genes of strains 10C1 and V583 were sequenced. It should be noted that PCR amplification and the subsequent cloning of Lpa genes were prone to a high frequency of mutations presumably because of the toxicity of the enzyme and the relatively large size of the gene. Hence, the sequence of the strain 10C1 Lpa-encoding gene we have reported to Genbank\textsuperscript{2} is based on the consensus sequence obtained from several independent clones. Our consensus sequence for the V583 Lpa gene matched that reported for EF1033 (22). We believe that the EF1033 sequence in the database is correct since that sequence was based on sequencing small (1.5–2.5 kb) clones (22) derived directly from the genomic DNA such that very few (or none) of the clones sequenced should have carried the complete open reading frame. Therefore, no selection for inactivating mutations should have occurred. The lipoamidase-encoding gene of strain 10C1 is 12 bp shorter than that of strain V583 and encodes a protein of 77,516.20 D. The two proteins are 96.6% identical and most of the residues that differ are near the C-termini of the proteins. Database searches with the protein sequences deduced from these genes indicated Lpa to be a member of the amidase signature super-family. These enzymes have an active site Ser-Ser-Lys triad in contrast to the Ser-His-Asp triad of the classical serine proteases. The alignments predict that the Lpa catalytic triad is composed of Ser259 (the nucleophile), Ser235, and Lys159 and this was shown to be the case (see below).
Expression and Purification of His-tagged Lpa—The E. faecalis V583 Lpa gene (EF1033) was inserted into vector pET16b to obtain pYFJ62 which encodes an N-terminally His-tagged Lpa. Attempts to express Lpa from pYFJ62 in E. coli strain BL21 DE3 carrying pLysS were unsuccessful despite use of different growth temperatures, isopropyl-β-D-thiogalactoside concentrations, time of induction, and supply of succinate and acetate (to bypass the PDH and KGDH functions). No expression of a soluble or insoluble protein of the appropriate size was visible either on stained SDS-PAGE gels or by Western blotting with anti-His tag antibody (data not shown). However, Lpa activity was detectable in the cell-free supernatants of the uninduced and induced cultures. Thus, Lpa was expressed even when expression from the T7 promoter was repressed and thus inactive clones would be readily selected.

Based upon these observations Lpa was expressed by providing T7 RNA polymerase via infection of the host strain carrying pYFJ62 with phage λCE6. Use of λCE6 phage to introduce T7 RNA polymerase has been shown to be an excellent means to express very toxic proteins (36). The growth medium (Experimental Procedures) contained succinate and acetate plus a high concentration of lipoic acid (10 µg/L, a 2000-fold excess over that required for the growth of an E. coli lipoate auxotroph) to maximize function of the host lipoate protein ligase (LplA). A variety of expression conditions were tested including different λCE6 multiplicities of infection and times of infection and the presence or absence of isopropyl-β-D-thiogalactoside. Although only modest levels of Lpa expression were seen, the His-tagged protein was readily purified (Fig. 4). Surprisingly, addition of isopropyl-β-D-thiogalactoside had little effect despite LacI-mediated repression of the T7 promoter. This might be due to the physiology of phage infection, perhaps nonspecific binding of LacI by the large amount of λ NA. The His-tagged version of
strain V583 Lpa has a calculated molecular weight of 80,597.21 Da, although the protein behaved as a somewhat larger protein (ca. 97 Kda) in SDS-PAGE. Western blotting with an anti-His-tag antibody demonstrated that the Lpa band carried a His-tag and that the other bands on the gel were products of proteolytic degradation of Lpa (data not shown). As determined by the radioactive assay the \( K_m \) of the purified His-tagged Lpa for octanoylated-domain was 80 \( \mu M \) and the \( V_{\text{max}} \) was 67 pmol/min. The His-tagged Lpa was specifically labeled with \(^{35}\)S-methionine (29) and analyzed by gel filtration. The protein eluted in a manner very similar to an aldolase standard as a 158 KDa protein (data not shown) suggesting that Lpa is a dimer in solution as previously observed for malonamidase E2, another soluble AS amidase, (38). The Lpa gene of \( E. faecalis \) 10C1 was also cloned and Lpa activity was obtained upon expression of the gene in \( E. coli \).

**Lpa Utilizes Intact PDH and KGDH as Substrates Both In Vivo and In Vitro**—As mentioned earlier Lpa was discovered as an activity that inactivated pyruvate dehydrogenase suggesting that Lpa expression should be a toxic to cell growth. Indeed, our difficulties in cloning and expressing the encoding gene in \( E. coli \) indicated this was the case and this was readily attributed to dehydrogenase inactivation. To test for inactivation *in vivo* the host PDH and KGDH activities were assayed in two strains on containing the Lpa expression plasmid (strain YFJ206) and strain YFJ207, a control strain containing vector pET16b. The strains were infected with phage \( \lambda \) CE6 (Experimental Procedures) and Lpa expression in strain YFJ206 was confirmed by SDS-PAGE (data not shown). infection of the strain YFJ206 resulted in complete loss of both PDH and KGDH activities whereas the vector control strain retained both activities as did a culture of strain YFJ206 strain that was not infected (Fig. 5A). This last result indicated that
basal level expression of Lpa did not significantly decrease PDH or KGDH function, although we detected residual expression of Lpa in the absence of T7 polymerase presumably due to recognition of an adventitious promoter by *E. coli* RNA polymerase. Decreased 2-oxoacid dehydrogenase activities following infection of the vector control strain was also seen and is presumably due to the replication of λCE6 phage.

We confirmed the activity of Lpa on PDH and KGDH *in vitro*. A cell free extract of the wild type strain JK1 was treated with purified His-tagged Lpa and assayed for PDH and KGDH activities (Fig. 5B). Lpa treatment decreased PDH activity dramatically whereas KGDH activity was completely lost. This differential result was expected since PDH is more resistant to Lpa inactivation than KGDH due to its greater number of lipoyl domains. The E2 subunit of *E. coli* PDH has three lipoyl domains, although one domain is sufficient for full PDH activity in vitro (39,40) whereas the E2 subunit of KGDH has only a single lipoyl domain. Note that the PDH and KGDH activities of the crude extract declined during the 20 min room temperature incubation in the absence of Lpa indicating that PDH and KGDH were heat-sensitive. This heat sensitivity precluded restoration of these activities by treatment with LplA. However, LplA readily attached lipoic acid to a preparation of the isolated lipoyl domain that had been converted to the apo form by Lpa treatment (Fig. 6). As expected from prior reports (10) bioassay showed a large Lpa-dependent increase in free lipoic acid upon treatment of the dehydrogenase preparations (data not shown).

*Activity of Lpa on small-molecule substrates*—Lipoic acid-releasing activities from numerous biological sources have been assayed with small molecule substrates such as lip-K(A-Me). We used lip-K(A-Me), a form of lipoyl-lysine, in which the α-amino and carboxyl group were
blocked in order to more closely mimic a lipoyl moiety bound to a protein or peptide. As mentioned in the introduction it was unclear if the same *E. faecalis* protein cleaved lipoate from both the small molecule and protein substrates. Therefore, we tested both the purified recombinant Lpa and the partially purified preparation from *E. faecalis* for activity on small molecule substrates (Fig. 7). Both Lpa preparations efficiently released lipoate from lip-K(A-Me) and also released biotin from biotinyl-lysine. The partially purified *E. faecalis* crude extract demonstrated a much higher biotinyl-lysine hydrolyzing (biotinidase) activity than did purified Lpa consistent with presence of a *E. faecalis* enzyme specific for biotinyl-lysine cleavage (41). In contrast, all three sources of Lpa had similar levels of lip-K(A-Me) hydrolyzing activity indicating that Lpa is the major (and perhaps the sole) lip-K(A-Me) hydrolyzing enzyme of *E. faecalis* (Fig. 7).

*Mutagenesis of the Putative Lpa Active Site Residues*—The purified His-tagged Lpa cleaved lipoic acid from both small molecule and protein-bound substrates and it seemed likely that both substrates were cleaved by the active site identified by sequence alignment with proteins of the amidase signature family (see below). To test this hypothesis and to functionally test the annotation of Lpa as an amidase signature family member, we constructed and expressed three mutant proteins, S259A, S235A and K159A and tested their enzymatic activities towards both lipoylated protein and small molecule substrates. SDS-PAGE analyses showed that all three mutant proteins were soluble and expressed at the same level as wild type Lpa (data not shown). The S259A and S235A proteins had no detectable activity on the lipoyl domain substrate whereas K159A had a dramatically decreased activity (Fig. 8A). Similar results for the three
mutant proteins when assayed with the lipoylated small molecule substrates tested lip-(A-Me) and lipoyl-PABA (Fig. 8B). Cleavage of the biotinylated small molecules, biotinyl-lysine and biotinyl-PABA, was similarly decreased by the mutants of the catalytic triad (data not shown). Note, however, that we cannot preclude traces of activity of the S259A and S235A proteins on the small molecules due to the inherent imprecision of bioassays when the nutrient levels approach the assay background. It should also be noted that as seen with Lpa, the residual enzyme activity of malonamidase E2 mutant proteins in which the triad lysine had been changed to other residues was much greater than that of mutant proteins having alterations of the other active site residues (42).

It should be noted that rather than with the purified forms of the proteins both the protein substrate and small molecule substrate assays were performed with cell-free crude extracts of strains expressing either the wild type or mutant proteins. Although low levels of activity are difficult to quantitate with both the gel-shift and biological assays, the finding that all three mutant proteins had little or no activity towards both the protein-bound and the small molecule substrates indicated that the Ser-Ser-Lys triad is indeed the Lpa active site and that this site cleaves lipoate from both from lipoylated proteins and lipoylated small molecule substrates. Moreover, the vector control strain did not show any activity in either assay. This provided further evidence that Lpa activity directed towards the small molecule substrates (Fig. 8) cannot be attributed to an E. coli hydrolase that co-purified with Lpa.
DISCUSSION

The isolation of the gene encoding *E. faecalis* Lpa plus the purification of the protein to homogeneity provides a well-defined reagent for the study of lipoic acid metabolism. Given the extreme toxicity of the protein in *E. coli* and the likelihood of adventitious promoters due to the low GC content of *E. faecalis* DNA, it seems likely that virtually all other expression cloning approaches would have failed. A strategy that might have succeeded would have involved cloning *E. faecalis* DNA segments into phage λ. This approach would have avoided carrying the gene in a host cell, but might also have failed since the gene would have been at very high copy number during phage replication. Moreover, to achieve full coverage of the *E. faecalis* genome would have required production and titer of almost 1000 phage stocks. Indeed, although not designed for this purpose, the pBAC/oriV copy number control plasmids (34) seem to provide an ideal system for expression cloning in *E. coli*.

*E. faecalis* Lpa contains all of the hallmarks of an AS signature amidase, the Ser235 Ser259-Lys159 triad plus G234, the glycine residue that allows Ser235 to be maintained in the cis conformation plus the conserved stretch of ~130 residues rich in serine and glycine residues. Indeed, our mutagenesis studies show that each of the triad residues is required for activity. Proteins containing AS sequences have been found in a wide range of organisms including bacteria, archaea, fungi, plants, and mammals (43). The physiological functions of the AS enzymes are quite diverse with the functions of many annotated members remaining unknown. However, the common biochemical reaction catalyzed by most AS enzymes is amide bond hydrolysis such as that catalyzed by Lpa. When aligned with the AS signature amidases of
known structure, fatty acid amide hydrolase (FAAH) (44) malonamidase E2 (MAE2) from *Bradyrhizobium japonicum* (38,42) and peptide amidase (Pam) from *Stenotrophomonas maltophilia*, Lpa aligned most closely with FAAH, a membrane-bound protein. The two proteins can be aligned with a gap of only a single residue over residues 150-275 (Lpa numbering), the segment that contains the active site residues. Also, relative to most of the AS family members Lpa and FAAH proteins have N-terminal extensions that show some sequence similarities. The relationship between Lpa and FAAH seems likely to reflect the fact that the two enzymes recognize similar aliphatic substrates given that lipoic acid is a modified fatty acid. Indeed, during comparison of the FAAH structure with that of MAE2, Bracey and coworkers (44) noted that many of the hydrophobic residues of FAAH that line the tunnel containing the acyl chain of the fatty acid amide substrate arise from sequence insertions relative to MAE2 and we find that many of analogous positions in Lpa are also occupied by hydrophobic residues. Therefore, it seems likely that Lpa and FAAH bind the acyl moieties of their substrates in a similar manner. Substrate structural similarities probably also explain the alignment with the 6-aminohexanoate-cyclic-dimer hydrolyases that led to the current erroneous annotation of EF1033 as encoding an enzyme that would degrade a synthetic substrate most unlikely to be present in an environment where *E. faecalis* is found.

Search of the extant databases suggest that Lpa is a rare enzyme that is confined to close relatives of *E. faecalis*. Good quality alignments (40-45% identical residues) are seen only for *Streptococcus pyogenes* and *Streptococcus agalactiae*. Other sequences align with Lpa at about 30% identity, but these proteins are 30-40% smaller than Lpa. Note that enzyme activities that cleave amide-linked lipoic acid have also been reported in eukaryotes such as yeast (45) and
various mammalian sources including human serum and breast milk (30,31,46,47). However, the literature provides conflicting and confusing information about the substrate specificities of these enzyme activities, although all have been called lipoamidases. In most cases the putative lipoamidase activity was detected by using small molecule substrates with amide-linked lipoyl moieties, such as lip-K(A-Me) and lipoyl-PABA and in some cases the activity was shown to be due to a known enzyme such as biotinidase (30) or cholesterol esterase (31). When tested with a large molecule substrate such as intact pyruvate dehydrogenase the eukaryotic enzyme activities were unable to catalyze lipoic acid release (e.g., (47). The other enzyme activities reported to act on intact proteins can be explained by proteolysis followed by release of lipoic acid from lipoyl-lysine or small peptides. To date *E. faecalis* Lpa is only enzyme that has been rigorously shown to release lipoic acid from intact proteins modified with lipoic acid. In our hands the recombinant Lpa has properties very similar to those reported many years ago using partially purified preparations from the native bacterium (11). Therefore it seems that the early preparations from *E. faecalis* were substantially free of proteases and other hydrolyases active on the substrates tested.

The fact that Lpa has similar levels of activity on lip-K(A-Me) and lipoylated proteins (11) suggests that the physiologically important substrates are lipoyl-lysine and small peptides containing lipoyl-lysine. *E. faecalis* requires lipoic acid or acetate for growth and hence is a natural lipoate auxotroph (19,25). Indeed, lipoic acid was discovered as a growth factor that replaced acetate in allowing growth of *E. faecalis* on a synthetic medium (48). *E. faecalis* lives in the mammalian digestive system where amidated forms of lipoic acid from the host diet and from other flora are expected to be present. Therefore, Lpa seems likely to enable this bacterium
to utilize amide-linked forms of lipoic acid that otherwise could not be assimilated. Since Lpa is a cytosolic enzyme, protease digestion products of lipoylated proteins rather than the intact proteins seem most likely to be the relevant substrates. Moreover, the level of Lpa activity in *E. faecalis* is increased by starvation for lipoic acid (11) consistent with a salvage role for Lpa. Increased Lpa activity would be of obvious advantage when lipoic acid must be scavanged from the environment, but when the cofactor has been acquired and has been attached to the 2-oxoacid dehydrogenase proteins, Lpa becomes a liability because of its ability to cleave lipoyl-proteins. Three solutions to this dilemma are suggested. First, accumulation of salvaged lipoic acid could shut down the increased Lpa gene expression resulting from lipoate starvation and, if Lpa is an unstable protein in *E. faecalis*, a return to basal Lpa levels might rapidly occur. Another possibility is that lipoate protein ligase activity (of which *E. faecalis* encodes two homologues) might be coregulated with Lpa and hence cleavage of lipoylated proteins would be canceled by increased rates of lipoate attachment. A third possibility is an inhibitor of Lpa action that is synthesized only when the lipoic acid supply is adequate. A combination of these scenarios also seems plausible.

If Lpa is a lipoate salvage enzyme, why then is it able to release lipoate from intact lipoylated proteins, an activity that should work at cross-purposes to the objective of activating the 2-oxoacid dehydrogenases? One possibility is that the unusually exposed nature of the modified lysine residues of lipoylated proteins allows Lpa access to the amide bond. The lipoylated lysine is at the tip of an exposed tight β-turn that markedly protrudes from the surface of the lipoyl domain (1). Therefore, neither protein secondary structure nor neighboring side chains would sterically hinder attack of the lipoyl-lysine amide bond by Lpa. Indeed, it is difficult to imagine
a more exposed post-translational modification excepting those of protein termini. Testing of the hypothesis that steric exposure plays a key role in Lpa action on proteins will require assay of lipoate release from lysine residues located in different protein structural contexts made by chemical lipoylation of proteins of known structure.
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Footnotes

1. The abbreviations used are: Lip-K(A-Me), \(\varepsilon\)-lipoyl-\(\alpha\)-acetyl derivative of lysine methyl ester; lipoyl-PABA, lipoyl-\(p\)-aminobenzoic acid; biotinyl-PABA, biotinyl-\(p\)-aminobenzoic acid.

2. The sequence of the \textit{E. faecalis} 10C1 Lpa gene has been entered in Genbank as GenBank Accession Number AY735444.
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| Strains or Plasmids | Relevant Characteristics or Derivation | Source or Reference |
|---------------------|----------------------------------------|---------------------|
| **E. faecalis 10C1** | Strain in which Lpa was originally detected | ATCC 11700 |
| JK1                 | Wild type                              | (27) |
| DH5α                | \(\phi^{80d} \text{lacZ} \Delta M15 \text{ endA1 recA1 hsdR17}\) (m\(\Delta\), \(m^+\)) | Cronan Lab Collection |
| TOP10               | \(mc\text{rA} \Delta(mrr-hsdRMS-mcrBC) \phi^{80d} \text{lacZ} \Delta M15 \Delta \text{lac}X74 \text{ recA1 araD139 } \Delta(\text{ara-leu})7697\) | Invitrogen |
| TOP10F’             | \(F^{lacI^8} \text{Tn10/mcrA} \Delta(mrr-hsdRMS-mcrBC) \phi^{80d} \text{lacZ} \Delta M15 \Delta \text{lac}X74 \text{ recA1 endA1 araD139 } \Delta(\text{ara-leu})\) | Invitrogen |
| BL21(\(\lambda\text{DE3})\) | \(h\text{sdS} \lambda\text{DE3}\) | (36) |
| EP1300              | \(mc\text{rA} \Delta(mrr-hsdRMS-mcrBC) \phi^{80d} \text{lacZ} \Delta M15 \Delta \text{lac}X74 \text{ recA1 endA1 araD139 } \Delta(\text{ara, leu})\)7697\(\lambda^-\) | Epicentre |
| LE392               | \(h\text{sdR514} (r\(\Delta\), m\(\Delta\)) \text{mcR}\text{A}\) | Novagen |
| YFJ115              | EP1300/pCY598                          | This work |
| YFJ51               | DH5α/pYFJ16                           | This work |
| TM202               | BL21(\(\lambda\text{DE3})\)/pTM70      | (6) |
| YFJ37               | BL21(\(\lambda\text{DE3})\)/pCY499     | |
| TM245               | JK1/pGS331                             | (7) |
| TM250               | \(lipB182::Tn1000dKn\)                | (7) |
**Plasmids**

- **pCR2.1-TOPO**: TOPO TA cloning vector
  - Invitrogen
- **pQE-2**: Expression vector
  - Qiagen
- **pETBlue-1**: Expression vector with blue-white screening
  - Novagen
- **pK18**: kanamycin resistant cloning vector
  - (24)
| Vector ID | Description |
|-----------|-------------|
| **pTM61-4** | LplA under the control of the tac promoter in pKK223-3 backbone |
| **pCC1FOS** | Cosmid cloning vector |
| **pYFJ13** | LplA coding sequence PCR amplified from pTM61-4 and cloned into pCR2.1-TOPO |
| **pYFJ16** | 1183bp NdeI and HindIII digestion fragment from pYFJ13 cloned into pQE-2 digested with the same enzymes |
| **pCY598** | Ori RSF1030, T7 RNA polymerase under the control of a pBAD promoter |
| **pYFJ45** | Promoter region and multiple cloning sites of pETBlue-1 in the backbone of pK18. |
| **pGS331** | A hybrid lipoamid domain under the control of the tac promoter |
| **pYFJ46** | Cosmid clone from YFJ137, contained lipoamidase coding sequence |
| **pYFJ47** | Cosmid clone from YFJ139, contained lipoamidase coding sequence |
| **pYFJ55** | *E. faecalis* 10C1 lipoamidase gene PCR amplified and cloned into pCR2.1-TOPO |
| **pYFJ56** | *E. faecalis* V583 lipoamidase gene (EF1033) PCR amplified and cloned into pCR2.1-TOPO |
| **pYFJ61** | 2.2 Kb NdeI and BamHI fragment from pYFJ55 ligated into pET16b digested with the same enzymes |
| Construct       | Description                                                                 | Source       |
|-----------------|-----------------------------------------------------------------------------|--------------|
| pYFJ62          | 2.2 Kb NdeI and BamHI fragment from pYFJ55 ligated into pET16b digested with the same enzymes | This work    |
| pYFJ66          | pYFJ62 with Lpa mutation S259A                                              | This work    |
| pYFJ68          | pYFJ62 with Lpa mutation S235A                                              | This work    |
| pYFJ70          | pYFJ62 with Lpa mutation K159A                                              | This work    |
| pYFJ55          | *E. faecalis* 10C1 lipoamidase gene PCR amplified and cloned into pCR2.1-TOPO | This work    |
| pYFJ56          | *E. faecalis* V583 lipoamidase gene (EF1033) PCR amplified and cloned into pCR2.1-TOPO | This Work    |
| pYFJ61          | 2.2 Kb NdeI and BamHI digestion fragment from pYFJ55 ligated into pET16b digested with the same enzymes | This work    |
| pYFJ62          | 2.2 Kb NdeI and BamHI digestion fragment from pYFJ56 ligated into pET16b digested with the same enzymes | This work    |
| pYFJ66          | pYFJ62 with Lpa mutation S259A                                              | This work    |
| pYFJ68          | pYFJ62 with Lpa mutation S235A                                              | This work    |
| pYFJ70          | pYFJ62 with Lpa mutation K159A                                              | This work    |
Figure legends.

Fig. 1. A First round screening of the *E. faecalis* cosmid expression library. Crude extracts of four pools of fifty colonies each, S2, S3, S4 and M4, were fractionated by ammonium sulfate precipitation (Experimental Procedures) and the resulting preparations were assayed for Lpa activity by the gel-shift method after overnight incubation at 37°C. B Final round screening of the *E. faecalis* cosmid expression library. The individual colonies of pool S2 16-20 were each grown as a separate culture which was induced and assayed for lipoamidase activity as described in Experimental Procedures. Pool S2 6-10 gave a similar result (data not shown). The + signs denote assays performed with the partially purified *E. faecalis* crude extract as a positive control whereas the – signs denote assays containing only the holo domain. Marker proteins consisting of purified apo and holo lipoyl domains plus a mixture of apo and holo domains (denoted as Stds) were loaded on each gel. The band (labeled D) that migrated faster than the holo domain (below the holo band) was presumably the product formed by deamidation of a holo domain amino acid amide residue during storage. This band is converted to its apo form by Lpa. This apo form can in turn be converted back to the holo form by LplA treatment (see Fig. 6).

Fig. 2. Sequence analysis of two cosmid DNA that contained the Lpa-encoding gene, pYFJ46 and pYFJ47. The black line denotes the backbone of pCC1FOS vector with the position and orientation of T7 promoter as shown. The regions identified by DNA sequencing of the subclones are shown in purple and the regions detected by PCR amplifications are shown in yellow. The green line indicates the unidentified gap region of pYFJ46. All the primers used in PCR reactions are shown as thin blue arrows. Primer #1 is pCC1 for, #2 is EF1008, #3 is EF1032 and #4 is pCC1 rev (Table 2). open reading frame EF1033 is shown...
in red arrow, with the arrow indicating the orientation of the open reading frame. The nucleotide numbers and the open reading frame numbers are from www.tigr.org.

Fig. 3  *E. faecalis* open reading frame EF1033 encodes Lpa. Open reading frame EF1033 together with its upstream region was PCR amplified and cloned into pCR2.1-TOPO vector with primers EF1033 UP and EF1033 DOWN. A second PCR product that contained the sequence from upstream of EF1033 to the end of the downstream open reading frame (EF1034) was cloned into pCR2.1-TOPO with primers EF1033 UP and EF1034 DOWN. Lipoamidase activity was assayed in both the constructs in pools of ten clones. EF1033 denotes the clone pool containing the PCR product that contained the EF1033 open reading frame and its upstream region. EF1033 & EF1034 denotes the clone pool containing the PCR product that contained both the EF1033 and EF1034 open reading frames. Other designations as in Fig. 1.

Fig 4. Purification of His-tagged Lpa. Lanes UI and I are the crude extracts of the uninduced and induced cultures, respectively. Lanes FT, W, F1 and F2 are the flow through, wash, and combined elution fractions, respectively (Experimental Procedures). Each lane received five µg of protein was loaded except lanes F1 and F2 where 1 µg of protein was loaded. Molecular weight markers are shown at the right.

Fig. 5. Inactivation of PDH and KGDH in vivo and in vitro. Upper panel. In vivo effects of Lpa expression in *E. coli*. The activities of PDH (shaded columns) and KGDH (black columns) are given. The Lpa expression strain YFJ206 and strain YFJ207 (the pET16b vector control strain) were cultured and infected with λ CE6 (Experimental Procedures) or left uninfected as given. Cell-free crude extracts were prepared and assayed for PDH and KGDH activity. Lower panel.
Inactivation of PDH and KGDH in vitro by Lpa. A crude extract of strain JK1 was treated with purified lipoamidase or left untreated. Both samples were incubated at room temperature for 20 minutes. The strain JK1 crude extract without incubation (0 T) was also assayed for PDH and KGDH activities. In both panels, PDH and KGDH activities are given as μmol of APAD reduced per minute per mg total protein in the crude extract.

Fig. 6. Removal of lipoic acid from holo lipoyl domain by Lpa treatment and its restoration by LplA. Lipoylated lipoyl domain was either treated (at 37°C for 2 h) or not treated with purified His-tagged lipoamidase. After removing the His-tagged lipoamidase by passage through Ni-NTA spin columns (Qiagen, Valencia, CA, equilibrated with 20 mM potassium phosphate, pH 7.0) the reaction mixtures were divided into two halves. One half was treated with LplA lipoate protein ligase (Experimental Procedures) at 37°C for 2 h whereas the other half was left untreated. The samples were analyzed on a 20% non-denaturing gel.

Treatments with Lpa or LplA are denoted by + or – signs as given.

Fig. 7. Lpa cleaves lip-K(A-Me) and biotinyl-lysine. In the left series of Petri plates lip-K(A-Me) (60 μM final concentration) was the substrate and the lipoic acid released was detected by bioassay (Experimental Procedures). The sources of lipoamidase activity were purified His-tagged lipoamidase (400 ng), partially purified E. faecalis crude extract, or the ammonium sulfate fractionated crude extract of the cosmid library screening S2 pool. Lpa reactions were incubated at 37°C for 2 before bioassay. Abbreviations: Lip-K, lip-K(A-Me); Btn-K, biotinyl-lysine. In the right series of Petri plates the assays were conducted in the same manner except that biotinoyl-lysine (60 μM final concentration) was the substrate, the biotin bioassay (Experimental Procedures) was used, and two different Lpa concentrations (400 ng or 4000 ng) were tested. The higher concentration is denoted as 10X. The single
plate at the right of each set is the control containing only the substrate. The plates at the left of each set are control for lipoate or biotin contamination of the protein preparations. The dark zone is due to reduction of the triphenyltetrazolium chloride in the medium by bacterial respiration. The areas of the dark zones are proportional to the extents of cell growth. The proper name of biotinyl-lysine is N-ε-(biotinyl)lysine.

Fig. 8. Activities of the mutant Lpa proteins. Upper panel. Activity towards the holo lipoyl domain. Crude extracts of strains YFJ206 (WT Lpa), YFJ207 (pET16b vector control), YFJ220 (S259A Lpa), YFJ222 (S235A Lpa) and YFJ224 (K159A Lpa) were assayed as given in Experimental Procedures (the same amount of extract protein was utilized in each assay). The reactions were allowed to proceed at 37°C for 2 h before loading onto a 20% non-denaturing gel. The holo protein and its deamidated derivative are seen in all lanes 1-4 whereas lane 5 shows some formation of the apo forms of these protein which migrate more slowly due to the increased positive charge of the freed lysine ε-amino group. The source of the extract assay is given at the top of each lane. The lower panel shows the lipoyl-lysine (Lip-K) and lipoyl-PABA (Lip-PABA) hydrolyzing activities of same crude extracts used in panel A. The reactions were incubated at 37°C for 2 h before loading onto the bioassay plates as in Fig. 7. The bottom row of plates contained the crude extracts without substrate. The right hand plates are lipoyl-lysine and lipoyl-PABA without added protein (the controls for contamination of the samples with free lipoate).

Fig. 9. Clustal W alignment (identical residues mode) of Lpa with the amidase signature enzymes of known structure. Identical residues are boxed. The Ser235 and Lys159 active site residues are marked with asterisks whereas the third member of the triad, the Ser259 nucleophile is marked with an X. Note that only the protein segments that align with first
460 residues of the 729 Lpa residues are shown since the other proteins are significantly smaller and thus the downstream alignments are poor.
Fig. 1
Fig. 2.
Fig. 3
Fig 4.
Fig. 5.
Fig. 6.
| Enzyme Preparation | Additions | Additions |
|---------------------|-----------|-----------|
|                     | Lpa       | Lip-K + Lpa | Lip-K |
| Pure Lipoamidase   |           |            |       |
| E. faecalis Prep.  |           |            |       |
| Pool S2 Crude      |           |            |       |

Fig. 7.
Fig. 8

### A.

|        | WT | Vector | S259A | S235A | K159A |
|--------|----|--------|-------|-------|--------|
| 1      |    |        |       |       |        |
| 2      |    |        |       |       |        |
| 3      |    |        |       |       |        |
| 4      |    |        |       |       |        |
| 5      |    |        |       |       |        |

### B.

| Substrate | Extract |
|-----------|---------|
| Lip-K     |         |
| Lip-PABA  |         |
| None      |         |

|          | WT | Vector | S259A | S235A | K159A | No extract |
|----------|----|--------|-------|-------|--------|------------|
| Lip-K    |    |        |       |       |        |            |
| Lip-PABA |    |        |       |       |        |            |
| None     |    |        |       |       |        |            |
Expression cloning and demonstration of enterococcus faecalis lipoamidase (pyruvate dehydrogenase inactivase) as a Ser-Ser-Lys triad amidohydrolase
Yanfang Jiang and John E. Cronan

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