Biosynthesis of Lipoic Acid: Characterization of the Lipoic Acid Auxotrophs *Escherichia coli* W1485-lip2 and JRG33-lip9†

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ABSTRACT: The abilities of the *Escherichia coli* lipoic acid auxotrophs W1485-lip2 and JRG33-lip9 to grow on succinate medium in the presence of octanoate, 8-mercaptooctanoate, or 6-mercaptooctanoate have been determined. Both organisms are mutated in *lipA*. Neither organism can use octanoate or 6-mercaptooctanoate for production of lipoate, but the lip2 allele can use 8-mercaptooctanoate. Chromosomal DNA from the auxotrophs was amplified by PCR using primers derived from the DNA sequence of wild-type *lipA* and then sequenced. Both mutants contain single G/C to A/T mutations in *lipA*, resulting in conversion of Ser_{307} into Phe in W1485-lip2 and Glu_{195} into Lys in JRG33-lip9. These results support the hypothesis that *lipA* is involved in the sulfur insertion step(s) of lipoate biosynthesis and indicate that it is possible to selectively block formation of the C_8–S bond through suitable mutation in *lipA*.

The terminal biosynthetic stages of the enzyme cofactor lipoic acid involve insertion of sulfur into unactivated carbon–hydrogen bonds. Lipoate is formed from octanoate (Reed et al., 1964; Parry, 1977) by insertion of two sulfur atoms, with feeding studies (White, 1980) suggesting that the C_8–S bond is formed first (Scheme I). The chemistry of these steps is not understood, despite a long history of research in the area. Unlike the conceptually related hydroxylase enzymes, sulfur insertion enzymes have proven difficult to isolate in active form (Parry, 1983; Ohsawa et al., 1989). To date, only isopenicillin N synthase has been available for investigation (Baldwin, 1990), but the absence of sequence similarities between this enzyme and those thought to be involved in lipoate and biotin C–S bond forming steps makes extrapolation of mechanistic studies difficult.

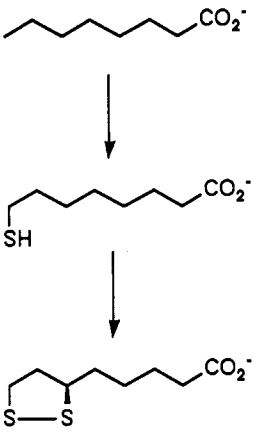
We recently reported the cloning and sequence of the *lipA* gene, which codes for a protein having some sequence similarities with the sulfur-insertion enzyme biotin synthase (Hayden et al., 1992). The localization and cloning of *lipA* was made possible by the existence of two lipoate auxotrophs, *Escherichia coli* W1485-lip2 and JRG33-lip9 (Herbert & Guest, 1968). Complementation experiments performed during the cloning indicated that both auxotrophs are mutated in *lipA*. We report here the sequences of the mutant *lipA* genes and a more detailed picture of the resulting phenotypes based on feeding experiments with potential intermediates in the conversion of octanoate to lipoate.

MATERIALS AND METHODS

Bacterial Strains. *E. coli* W1485-lip2 (F^+ *lip-2*) was obtained from the American Type Culture Collection (ATCC 25645), and JRG33-lip9 (F^- Δ(*gpt–proA*)*62 lacY1 lip-9 supE44*? *galK2 purB15 hisG4 rpsL35 xyl-5 mtl-1 thi-1* λ^-) was a gift of Dr. Barbara Bachmann (*E. coli* Genetic Stock Center, Yale University). JM109 (e14$^-$(*mcrA*) *recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^-,m_K^+) *supE44 relA1* Δ(*lac–proAB*) [F′ *traD36 proAB lacI*^{q}ZΔM15]) was from Stratagene.

8-Mercaptooctanoic Acid. 8-Mercaptooctanoic acid was prepared from 8-bromooctanoic acid (Aldrich Chemical Co.) according to Bruner and Richards (1980) and purified by distillation.

Scheme I: Proposed Order of C–S Bond Formation in Lipoate Biosynthesis



6-Mercaptooctanoic Acid. A solution of 6-hydroxyoctanoic acid (5.50 g, 33 mmol), prepared by $NaBH_4$ reduction of 6-oxooctanoic acid (Hünig et al., 1963), in 100 mL of CH_3-OH under N_2 at −10 °C was treated with thionyl chloride (4.76 g, 40 mmol) and kept for 2 h. After addition of H_2O, the mixture was extracted with $CHCl_3$ and the organic phase was dried over $MgSO_4$, filtered, and evaporated. Bulb-to-bulb distillation (95 °C, 0.5 mmHg) yielded 2.96 g of methyl 6-hydroxyoctanoate as a colorless oil. This was dissolved in 16 mL of pyridine and treated with *p*-toluenesulfonyl chloride (4.58 g, 24 mmol) at 4 °C for 18 h. After addition of H_2O, the mixture was extracted with ether and the organic phase was dried with $MgSO_4$, filtered, and evaporated, yielding 5.00 g of methyl 6-(*p*-toluenesulfonyloxy)octanoate as a pale yellow oil. The crude tosylate was dissolved in 65 mL of acetone and treated with potassium *O*-ethylxanthate (3.37 g, 21 mmol) at reflux for 1 h. The mixture was diluted with ether and washed with H_2O. The organic phase was dried over $MgSO_4$, filtered, and evaporated to give 3.87 g of methyl 6-(*O*-ethylxanthyl)-octanoate as a yellow, unpleasant-smelling oil. The xanthate was hydrolyzed by refluxing in 75 mL of 1 N NaOH in 1:9 H_2O/ethanol for 1 h under N_2. The reaction mixture was washed with $CHCl_3$ and then acidified with concentrated HCl.

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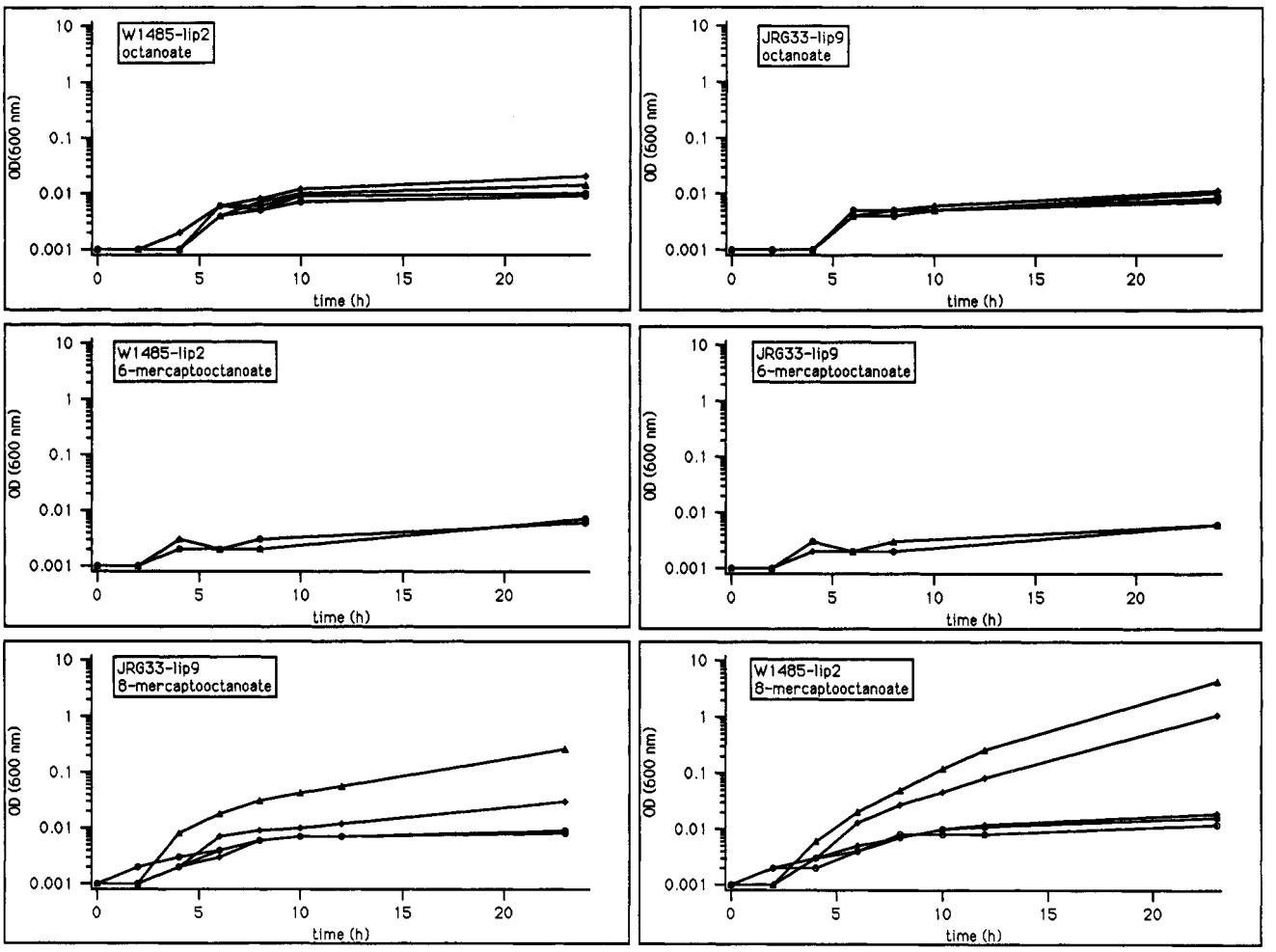


FIGURE 1: Growth of *E. coli* lipoate auxotrophs on lipoate-deficient medium in the presence of potential lipoate precursors: (left) W1485-lip2; (right) JRG33-lip9. Liquid cultures were grown aerobically on succinate medium supplemented with (top panels) (○) 0, (●) 0.25, (◇) 2.5, (◆) 25, or (△) 250 μM octanoate; (middle panels) (○) 0, (●) 0.5, (◇) 5, (◆) 50, or (△) 500 μM 6-mercaptooctanoate; or (bottom panels) (○) 0, (●), 0.25, (◇) 2.5, (◆) 25, or (△) 250 μM 8-mercaptooctanoate. Growth was measured by turbidity at 600 nm.

The acidified mixture was extracted with $CHCl_3$, and the organic phase was dried with $MgSO_4$, filtered, and evaporated to a yellow oil. Bulb-to-bulb distillation (110 °C, 0.5 mmHg) gave 2.14 g of 6-mercaptooctanoic acid as a colorless, unpleasant-smelling oil.

Feeding Studies. Colonies of *E. coli* W1485-lip2 or JRG33-lip9 were grown to saturation in 5 mL of LB medium (Maniatis et al., 1982). These cultures were used to inoculate 25 mL of lipoate-deficient medium (Herbert & Guest, 1970) supplemented with 50 μg/mL adenine, 1 μg/mL thiamine hydrochloride, and either potassium octanoate, sodium 6-mercaptooctanoate, sodium 8-mercaptooctanoate, or sodium (D,L)-lipoate at the desired concentration. All JRG33-lip9 cultures were also supplemented with 100 μg/mL streptomycin sulfate. Growth of these cultures was followed by measuring turbidity at 600 nm. At the end of the study, the cultures were streaked onto lipoate-deficient agar to check for revertants.

Chromosomal DNA Isolation. A 1-L culture of cells was grown overnight in LB medium containing 1 ng/mL (D,L)-lipoic acid at 37 °C. The medium for JRG33-lip9 was supplemented with 100 μg/mL streptomycin sulfate. After centrifugation, the cell paste was washed with 50 mM Tris-HCl, 50 mM EDTA, and 20% sucrose, pH 7.6 (SET buffer), and centrifuged. The cell pellet was then flash-frozen for 2 min in a dry ice/ethanol bath. The cells were thawed and suspended in 25 mL of SET buffer, cooled on ice, and treated with 2.5 mL of 5 mg/mL hen egg white lysozyme and 0.625 mL of 10 mg/mL heat-treated ribonuclease A for 15 min. A 1.5-mL portion of 10% sodium dodecyl sulfate was added, and the mixture was kept for 3 h at 37 °C with gentle shaking. A 1.0-mL portion of 4 mg/mL proteinase K and 18 mL of $CHCl_3$ were added and the mixture was kept overnight at 37 °C with gentle shaking. The aqueous phase was subjected to chloroform–phenol extraction twice, and then the DNA was precipitated with cold ethanol. The DNA was isolated by spooling onto a glass rod, washing with 70% ethanol, drying briefly, and then dissolving in 10 mM Tris-HCl, 1 mM EDTA, and 10 mM NaCl, pH 7.6. It was necessary to repeat the chloroform–phenol extraction on the DNA from JRG33-lip9 to completely remove contaminating proteins. Both mutants provided DNA having $A(260\ nm)/A(280\ nm)$ ratios of 1.9.

PCR Amplification. PCR reagents and equipment were from Perkin Elmer–Cetus. Each amplification reaction contained 50 mM KCl, 10 mM Tris-HCl, pH 8.4, 1.5 mM $MgCl_2$, 0.1 mg/mL gelatin, 100 μM dATP, 100 μM dCTP, 100 μM dGTP, 100 μM dTTP, 0.5 μM primer 1, 0.5 μM primer 2, 0.025 unit/μL *Taq* DNA polymerase, and 0.5 μg/mL of template DNA in a total volume of 2.0 mL. Aliquots of 200 μL were placed in the reaction tubes, covered with 150 μL of light mineral oil, and subjected to 35 cycles of PCR. The reactions were analyzed by agarose gel electrophoresis, staining with ethidium bromide. The contents of the reaction tubes were pooled for further manipulations, yielding 180 μg of amplified DNA.

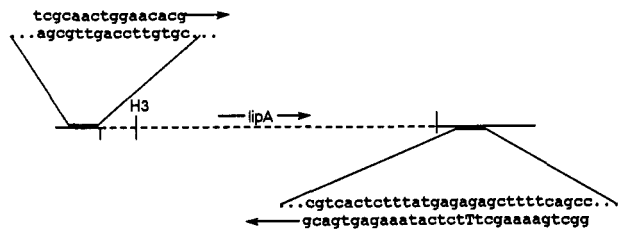


FIGURE 2: PCR primers. The extent of *lipA* is indicated by a dashed line. H3 shows the location of the internal *Hin*dIII site in *lipA*. The location of the G/T mismatch introduced for construction of a second *Hin*dIII site is indicated by a capital T.

Cloning of lipA Alleles. The PCR-amplified DNA was cut with *Hin*dIII, purified on an agarose gel, and ligated into pUC-18 cut with *Hin*dIII. The ligation mixture was transformed into competent JM109 cells, which were then plated onto LB medium containing X-gal. White colonies were selected and screened for plasmid insert by *Hin*dIII restriction analysis of miniprep DNA.

Sequencing. Primers were removed from the PCR-amplified DNA using the PrimErase kit (Stratagene). Both strands of the DNA were then sequenced using the CircumVent (New England Biolabs) thermal cycler sequencing protocol.

RESULTS

Feeding Studies. The ability of *E. coli* W1485-lip2 and JRG33-lip9 to grow on lipoate-deficient succinate medium in the presence of the potential lipoate biosynthetic precursors octanoate, 6-mercaptooctanoate, and 8-mercaptooctanoate was studied. Neither auxotroph will grow in this selective medium when either unsupplemented or supplemented with octanoate or 6-mercaptooctanoate up to 250 μM (Figure 1). W1485-lip2 grows well on medium supplemented with 8-mercaptooctanoate, while JRG33-lip9 does not. A small amount of growth is noted with JRG33-lip9 at the highest concentration of 8-mercaptooctanoate studied, yet this is still 20-fold lower than that observed for W1485-lip2, and it is not certain that this small growth is in fact due to conversion of 8-mercaptooctanoate into lipoate. Measured growth was not due to contamination or reversion to wild-type as shown by the inability of the final cultures to grow on unsupplemented selective medium agar. The 6-mercaptooctanoate was provided at twice the concentration of 8-mercaptooctanoate as it is a racemic mixture. Growth response to (D,L)-lipoate is maximal at 5 nM, whereas much higher (micromolar) concentrations of 8-mercaptooctanoate are needed to achieve maximal growth.

PCR of lipA Alleles. Primers for PCR were derived from the known sequence of *lipA* (Hayden et al., 1992). In order to allow facile cloning, the sequence at one primer was altered so as to introduce a *Hin*dIII site (Figure 2). There is a unique *Hin*dIII site near the 5′-end of wild-type *lipA*, and while use of this site for cloning removes the 5′-terminal segment of the gene, complementation experiments have demonstrated that the functionally important mutations in W1485-lip2 and JRG33-lip9 lie in the 3′-terminal portion of the gene (Hayden et al., 1992). PCR amplification of W1485-lip2 and JRG33-lip9 chromosomal DNA using these primers gave a DNA fragment of the expected 1-kb size based on the wild-type *lipA* sequence. These fragments were cut with *Hin*dIII and cloned into pUC-18 for further manipulation.

DNA Sequencing. The DNA sequences were read from both strands over the region of *lipA* known to be required for complementation of the two auxotrophs (Hayden et al., 1992). The *lipA* gene of W1485-lip2 contains a single G/C to A/T

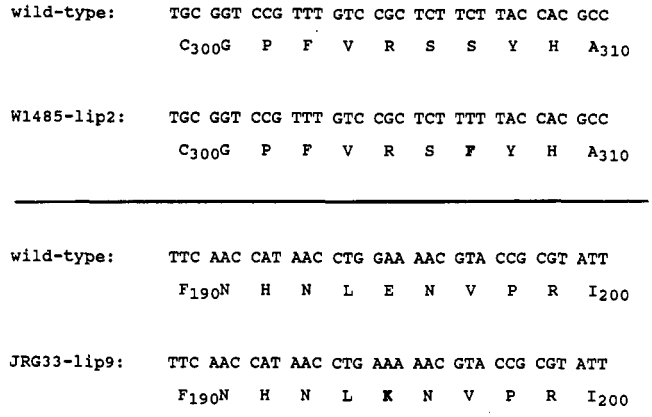


FIGURE 3: DNA sequences and translations of wild-type (upper line) and mutant (lower line) *lipA* sequences in the regions of the lip2 (top) and lip9 (bottom) point mutations (boldface).

mutation resulting in replacement of Ser_{307} (TCT) by Phe (TTT) (Figure 3). The *lipA* gene from JRG33-lip9 contains a single G-to-A mutation on the coding strand, resulting in a change of Glu_{195} (GAA) to Lys (AAA). No other differences from wild-type were found. Such G/C to A/T transversions are typical of nitrosamine-induced mutagenesis, which is caused by mispairing of T with O^6-alkylated G residues during replication (Loechler et al., 1984).

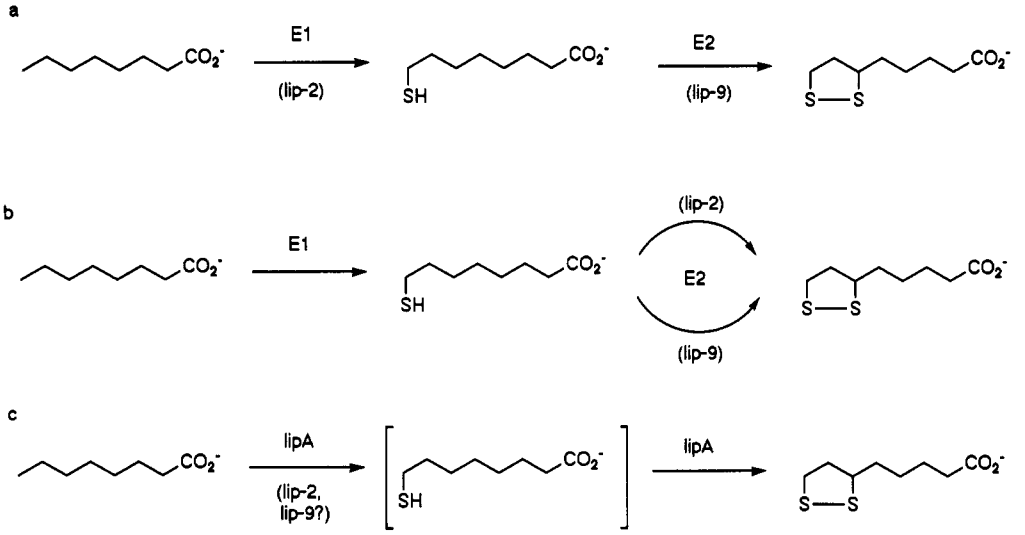
DISCUSSION

E. coli W1485-lip2 and JRG33-lip9 cannot biosynthesize lipoate and so do not grow on lipoate-free succinate medium in the absence of acetate due to a lack of 2-oxoacid dehydrogenase activity (Herbert & Guest, 1968). Previous work has demonstrated that both auxotrophs are mutated in *lipA* and that complementation with wild-type *lipA* restores lipoate prototrophy (Hayden et al., 1992; Vanden Boom et al., 1991). The different abilities of the lip2 and lip9 alleles of *lipA* to support use of 8-mercaptooctanoate for the biosynthesis of lipoate is thus of considerable interest for understanding the lipoate biosynthetic pathway. The inability of the auxotrophs to grow in the presence of octanoate suggests that the organisms are not simply using the precursors as sources of acetate.

The stable-label incorporation studies of White (1980) resulted in the proposal of Scheme I for lipoate biosynthesis, in which C_8–S bond formation precedes C_6–S bond formation. This was based upon the observation of fairly efficient (19%) incorporation of deuterated 8-mercaptooctanoate into lipoate in a prototrophic strain. The incorporation of 6-mercaptooctanoate observed in those studies (2%) was higher than the apparent background observed with hydroxylated octanoates (<0.5%) and suggests some ability to introduce the C_8–S bond after formation of the C_6–S bond. It cannot be ascertained from these studies whether there is a single enzyme activity responsible for formation of both C–S bonds in lipoate and having a definite order of reactions or whether there are two separate enzymes involved, one converting octanoate into 8-mercaptooctanoate and the other converting 8-mercaptooctanoate into lipoate.

Several scenarios for lipoate biosynthesis may thus be envisioned. In the first (Scheme IIa), there are separate enzymes responsible for conversion of octanoate into 8-mercaptooctanoate (E1) and for conversion of 8-mercaptooctanoate into lipoate (E2). The lip2 mutation would affect E1 while the lip9 mutation would affect E2. E2 would correspond to *lipA*. The mapping of both alleles to *lipA* and the complete

Scheme II



complementation of both auxotrophs by transformation with wild-type *lipA* rules out this possibility.

The second scenario (Scheme IIb) is similar, except that both mutations occur in E2 (*lipA*), with lip2 representing a binding mutation which requires intracellular levels of 8-mercaptooctanoate to provide sufficient lipoate for growth higher than can be produced by fully functional E1. The lip9 mutation is a catalytic mutant essentially incapable of converting 8-mercaptooctanoate at any level into lipoate. This scenario postulates that both lip2 and lip9 mutations affect formation of the C_6–S bond but not the C_8–S bond, such that the results of White (1980) predict that both auxotrophs should be rescued by 6-mercaptooctanoate. They are not, making this possibility unlikely.

A third scenario (Scheme IIc) seems more likely given the present data. In this case, a single enzyme performs both C–S bond formations, and the lip2 and lip9 alleles represent differential effects on the activity of this enzyme. The auxotroph feeding studies presented here suggest that the lip2 mutation prevents formation of the C_8–S bond but allows the subsequent formation of the C_6–S bond. This could arise from partial inactivation of the enzyme responsible for formation of both C_8–S and C_6–S bonds. The lip9 mutation blocks essentially all C–S bond formation by producing a more seriously damaged protein. The simplest interpretation of these feeding study results, when combined with the results of White (1980), is that there is a single enzyme, coded by *lipA*, which is responsible for both C–S bond-forming steps in lipoate biosynthesis. This is analogous to the two C–S bond-formation steps involved in biotin biosynthesis, which are apparently catalyzed by a single enzyme, biotin synthase. Combined with the small similarities between the *lipA* and biotin synthase sequences (Hayden et al., 1992), these results suggest that the chemistry of sulfur introduction into lipoate may be quite similar to that for sulfur introduction into biotin, despite clear differences in the number of sulfur atoms introduced and the stereochemistry of C_6 functionalization (Parry, 1983).

The differential phenotype observed for the two *lipA* mutants makes knowledge of the exact sites of mutation extremely important for understanding the chemistry of C–S bond formation in lipoate biosynthesis. The lip2 gene product contains a Ser-to-Phe mutation near the C-terminus at position 307, while that from lip9 contains a Glu-to-Lys mutation near the center of the peptide at position 195. The connection

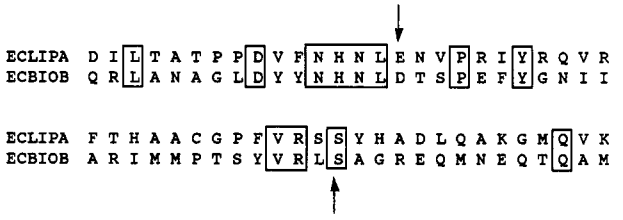


FIGURE 4: Comparison of *E. coli lipA* and *bioB* translations in the regions of the mutations observed in JRG33-lip9 (top) and W1485-lip2 (bottom). The sites of mutation are marked by arrows. Identities are boxed. No gaps have been introduced to optimize alignment.

between these mutations and the observed phenotypes is unclear. Sequence alignments with various *bioB* gene products (biotin synthase) do not give strong evidence for conservation of Ser_{307} between the two types of enzymes as the C-termini are quite different in the two proteins (Figure 4), although there is some potential similarity. Glu_{195} on the other hand is a closely related residue in both enzymes, either Asp (*E. coli*) or Asn (*Bacillus subtilis*) in the known biotin synthase sequences, and occurs within the major stretch of sequence similarity between the two proteins. As both observed mutations involve substantial changes in residue size, the effects of the mutations may well be more structural than mechanistic. This point will be better addressed through the use of site-directed mutagenesis having more conservative replacements.

In summary, evidence has been presented in support of *lipA* encoding the sulfur-introducing enzyme in lipoate biosynthesis. The genetic mutations leading to the lip2 and lip9 phenotypes have been described.

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