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An Efficient Approach To Identify *ilvA* Mutations Reveals an Amino-Terminal Catalytic Domain in Biosynthetic Threonine Deaminase from *Escherichia coli*

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High-level expression of the regulatory enzyme threonine deaminase in *Escherichia coli* strains grown on minimal medium that are deficient in the activities of enzymes needed for branched-chain amino acid biosynthesis result in growth inhibition, possibly because of the accumulation of toxic levels of α-ketobutyrate, the product of the committed step in isoleucine biosynthesis. This condition affords a means for selecting genetic variants of threonine deaminase that are deficient in catalysis by suppression of growth inhibition. Strains harboring mutations in *ilvA* that decreased the catalytic activity of threonine deaminase were found to grow more rapidly than isogenic strains containing wild-type *ilvA*. Modification of the *ilvA* gene to introduce additional unique, evenly spaced restriction enzyme sites facilitated the identification of suppressor mutations by enabling small DNA fragments to be subcloned for sequencing. The 10 mutations identified in *ilvA* code for enzymes with significantly reduced activity relative to that of wild-type threonine deaminase. Values for their specific activities range from 40% of that displayed by wild-type enzyme to complete inactivation as evidenced by failure to complement an *ilvA* deletion strain to isoleucine prototrophy. Moreover, some mutant enzymes showed altered allosteric properties with respect to valine activation and isoleucine inhibition. The location of the 10 mutations in the 5′ two-thirds of the *ilvA* gene is consistent with suggestions that threonine deaminase is organized functionally with an amino-terminal domain that is involved in catalysis and a carboxy-terminal domain that is important for regulation.

The regulatory enzyme threonine deaminase [threonine dehydratase; L-threonine hydro-lyase (deaminating); EC 4.2.1.16] from *Escherichia coli* exerts enzymological control at the committed step of branched-chain amino acid biosynthesis as evidenced by a sigmoidal dependence of its initial velocity on threonine concentration (8, 34). In addition, the enzyme is allosterically regulated by the heterotropic effectors isoleucine and valine, which either inhibit or activate, respectively, the pyridoxal phosphate-dependent catalytic reaction at intermediate substrate concentrations. A molecular description of the structural and energetic changes that occur at the active sites when regulatory ligands bind to the effector sites is a key issue in the analysis of the cooperative behavior of threonine deaminase. A powerful strategy in studies focusing on the mechanism of communication between effector sites and active sites in other allosteric systems involves correlating the structural locations of genetic variants with the functional alterations that result in the mutant proteins (16, 32). At present, however, there is no high-resolution structure of threonine deaminase, and hence there is little information about the importance of specific amino acids for catalysis, feedback regulation, and the allosteric transition. In an effort to identify residues in the enzyme that are involved in catalysis, a method was developed to identify mutant strains which contain catalytically deficient variants of threonine deaminase that suppress the inhibition of growth seen on minimal medium for strains that express wild-type enzyme at high levels. Here we describe the isolation and identification of 10 unique mutations in *ilvA*, the structural gene for threonine deaminase, that result in catalytically deficient forms of the enzyme. The locations of these substitutions in the *ilvA* gene are consistent with suggestions that threonine deaminase is functionally organized with an amino-terminal domain that is responsible for catalysis (29, 35).

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MATERIALS AND METHODS

Reagents. Chemicals were of reagent grade purity. Tryptone, yeast extract, and agar were from Difco Laboratories. Restriction endonucleases, ligases, and polymerases were from New England BioLabs and were used as recommended by the supplier. Threonine, isoleucine, and valine were from Sigma. Oligonucleotides were synthesized on an Applied Biosystems model 380B synthesizer according to manufacturer's specifications and purified by reverse-phase high-pressure liquid chromatography.

Strains and plasmids. All strains were derived from *E. coli* K-12. Strain T31-4-452 [*thi-1 trpE*(Am)*9829 trpA*(Am)*9761 hisT76 ilvDAC115* Bgl$^+$] was kindly provided by G. W. Hatfield (22). Strain TG1 [*supE* Δ(*lac-proAB*) *thi hsd*Δ*5* F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)] was from Amersham. The previously described single-strand pUC vectors (37), kindly provided by J. Vieira, were propagated in JV30. pEE5 is a derivative of pUC120 (8), and pEE27 is a similar plasmid that has the *ilvA* nucleotide sequence engineered to incorporate eight additional, unique restriction enzyme sites (described below). pKK233-2 was from Pharmacia. pEE28 was constructed by subcloning the engineered *ilvA* gene from pEE27 into pKK233-2.

In order to determine whether inactive mutants were isoleucine auxotrophs, an *E. coli* strain with a deletion in only the

ilvA gene of the *ilvGMEDAYC* operon was constructed. Completely inactive mutants were scored by failure to complement the auxotrophic deletion strain to isoleucine prototrophy when plasmid-borne copies of mutant *ilvA* alleles were maintained. Construction of the strain was achieved by homologous recombination of a vector that contained the chloramphenicol acetyltransferase gene flanked by *ilvD* and *ilvY* sequences. This vector was constructed by first replacing the entire *ilvA* gene with the coding sequence from the chloramphenicol acetyltransferase gene (CAT GenBlock; Pharmacia) in a plasmid containing a 930-bp fragment of the *ilvD* gene from immediately upstream of *ilvA* and a 840-bp fragment of the *ilvY* gene located immediately downstream of *ilvA*. This resulted in a plasmid in which the 750-bp Cm^r gene was flanked by fragments of the *ilvD* and *ilvY* coding sequences in a manner identical to that for the *ilvA* gene in the *E. coli* chromosome (38). A 2,520-bp *ilvD*-Cm^r-*ilvY* fragment was excised by digestion with *Bam*HI and *Bgl*II, ligated into circular forms that lacked an origin of replication, added to $CaCl_2$-treated TG1, and then plated on medium containing chloramphenicol (15 μg/ml) and isoleucine (50 μg/ml). This approach was necessary to achieve recombination of the closed circle form with homologous *ilvD* and *ilvY* sequences in the chromosome, since strain TG1 is Rec^+. A transformant colony that lacked a copy of the *ilvA* gene was confirmed by several criteria. First, the strain was a chloramphenicol-resistant isoleucine auxotroph that could be complemented to prototrophy by expressing threonine deaminase from a plasmid-borne copy of wild-type *ilvA*. Second, the polymerase chain reaction was used to verify the presence in the strain of appropriately sized chromosomal *ilvD*-Cm^r DNA sequences that extended beyond the 5′ end of the *ilvD* fragment found in the original transforming sequence. Also, no DNA of any size that corresponded to an *ilvD-ilvA* collinear sequence could be detected by the polymerase chain reaction, and no amplification of any portion of the *ilvA* gene could be detected in the strain. Last, *ilvA* deletion strains containing plasmids expressing wild-type or inactive mutants of threonine deaminase showed no difference in growth rate under the selective growth conditions described below.

Media. L broth was used as a rich medium. The minimal medium used was M9 (24), and it was supplemented with 0.4% glucose, 0.001% vitamin B_1, 0.1 mM $CaCl_2$ and 2 mM $MgSO_4$. For selection of *ilvA* mutations that suppressed the growth inhibition caused by high-level expression of threonine deaminase in strain T31-4-452, minimal M9 medium was supplemented additionally with 0.01% vitamin B_6, 1 mM isopropyl 1-thio-β-D-galactoside (IPTG), and the amino acids isoleucine, leucine, valine, tryptophan, and threonine at 20 μg/ml each. This will subsequently be referred to as minimal selection medium. It was determined that these concentrations of amino acids were optimal to maintain a reasonable rate of cell growth and still provide colony discrimination for the selection. Minimal M9 medium supplemented with only ampicillin, vitamin B_6, IPTG, and 20 μg of L-isoleucine per ml was used to identify *ilvA* alleles that were unable to complement the *ilv* deletion strain to isoleucine prototrophy. Ampicillin was added at 100 μg/ml to maintain relevant plasmids.

Mutagenesis. Oligonucleotide-directed mutagenesis was performed as suggested by Kunkel et al. (17) with single-stranded pEE5 as a template (8). In some cases, site-directed mutations were introduced by using the oligonucleotide-directed in vitro mutagenesis system supplied with phosphorothioate nucleotide analogs (30) by Amersham International.

The vector that was ultimately used for random chemical mutagenesis, pEE27, is a pUC120 derivative that contains the *ilvA* gene with 13 unique restriction enzyme sites. It was constructed by site-directed mutagenesis of pEE5 (8), a similar vector that contains relatively few unique restriction enzyme sites. First, site-directed nucleotide changes were made in four regions of *ilvA* between the *Nco*I, *Sal*I, *Bst*XI, *Eco*RI, and *Hin*dIII restriction enzyme sites. Once all the desired sites were incorporated into each of the four regions, the segments were sequentially subcloned to assemble pEE27. (A cartoon of this vector, with the approximate locations of the newly introduced restriction enzyme sites, can be seen in Fig. 2, which depicts the mutagenesis strategy.) Finally, the DNA sequence of the entire vector was checked to verify that only the desired nucleotide changes were present and that the resulting amino acid sequence corresponded to that for wild-type threonine deaminase (5, 23).

Random mutagenesis of the *ilvA* coding sequence was achieved by using single-stranded pEE27 as a target for chemical mutagens that damage bases but do not cause strand breaks. Thus, formic acid, which has the potential to change A and G to all bases, and nitrous acid, which can potentially change C to T and A to G, were employed to modify three of the four bases in the DNA (25). The concentrations of these chemicals were adjusted empirically to achieve about one modification per 300 residues with a frequency of about 10%. Avian myeloblastosis virus reverse transcriptase was used to misincorporate deoxyribonucleotides opposite damaged bases by extending the 17-bp M13 sequencing primer (−40)5′-GTTTTCCCAGTCACGAC-3′ (New England BioLabs), which is complementary to the *lacZ* coding region in pEE27. The resulting reaction mixtures were phenol extracted, precipitated with ethanol, and then split into five aliquots to be cleaved by the following restriction enzyme pairs: *Nco*I and *Sal*I, *Sal*I and *Bgl*II, *Bgl*II and *Sac*I, *Sac*I and *Eco*RI, and *Eco*RI and *Hin*dIII (see Fig. 2). These restriction digests released approximately 300-bp fragments and together make up the entire *ilvA* coding sequence. The fragments were purified by electrophoresis in low-gelling temperature agarose (FMC BioProducts) and ligated with similarly digested and purified pEE27, and the five reactions were used to transform strain JV30 to ampicillin resistance.

Selection for threonine deaminase mutations that suppressed the inhibition of growth in strain T31-4-452 was achieved by subcloning DNA from potential clones containing the mutagenized 300-bp segments into the expression plasmid pKK233-2. The cloning and selection were performed in three steps. First, cells from each plate containing ampicillin-resistant transformants of JV30 from the five ligation mixtures described above were harvested by washing with 10 ml of rich medium. Plasmid DNA was then prepared by using alkaline sodium dodecyl sulfate (SDS) lysis (12). Next, the plasmid mixtures were digested with *Nco*I and *Hin*dIII to release a 1,545-bp *ilvA* "cassette" which was purified in low-melting-temperature agarose and ligated with similarly digested pKK233-2. Finally, the ligation mixes were used to transform strain T31-4-452 to ampicillin resistance by first washing the cells with M9 salts and then plating on minimal selection medium. Large colonies that appeared on a background of pinpoint colonies after growth at 37°C for 36 to 48 h were used to inoculate rich medium with ampicillin for storage and further analysis.

Colony immunoassay. An additional assay using antiserum to wild-type threonine deaminase was employed to identify selected clones that expressed enzyme at high levels. This procedure relies on normal cell lysis that occurs during growth, which facilitates intracellular protein binding to nitrocellulose. In general, the assay was performed as previously described (9) with the following modifications. Petri dishes containing min-

imal selection medium without threonine were overlaid first with 82-mm-diameter circular sheets of nitrocellulose (BA85; Schleicher and Schuell) and then with cellulose acetate (OE67; Schleicher and Schuell). The upper, cellulose acetate sheet was inoculated from overnight cultures grown in a 96-well microtiter dish with a 48-prong replica stamp. Colonies were grown at 37°C for 24 h, and the lower, nitrocellulose sheet was stained immunochemically at room temperature by a modification of the procedure of Hawkes et al. (11). The sheet was first coated with 3% bovine serum albumin in Tris-saline (10 mM Tris Cl, pH 7.4, containing 0.9% NaCl) and then incubated in Tris-saline containing a dilution (1:1,000) of rabbit antiserum (Hazelton Research Products) raised against purified wild-type threonine deaminase from *E. coli*. The nitrocellulose sheets were then incubated in Tris-saline containing a dilution (1:1,000) of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Cappel Laboratories). Color development of colonies that expressed threonine deaminase was achieved by incubating the sheets with 1 mM 4-chloro-1-naphthol in the presence of 10 mM hydrogen peroxide. Positive clones were purified and rescreened by the colony immunoassay.

Identification of mutations. The exact nucleotide change(s) in positive clones was determined by DNA sequencing of the 300-bp regions that corresponded to the initial mutagenized segments. The *ilvA* cassette was subcloned from pKK233-2 into M13mp18 that had been modified by site-directed mutagenesis to contain an *Nco*I restriction enzyme site at the ATG initiation codon of *lacZ*. Template was prepared with strain TG1, and sequencing was done by the chain-terminating method with Sequenase (U.S. Biochemicals). The 300-bp fragments containing the altered coding sequence were then subcloned into wild-type *ilvA* in pKK233-2 so that no other nucleotide changes were present in the mutant proteins that were analyzed.

Polymerase chain reaction. Verification of the chromosomal organization of the *ilvA* deletion strain was achieved with pairs of oligonucleotide primers to amplify intervening target DNA sequences with AmpliTaq DNA polymerase (Perkin Elmer Cetus) essentially as outlined by the supplier. Temperature control was achieved in a MicroCycler E 5465 (Eppendorf). The general procedure consisted of an initial denaturation step for 1 min at 97°C followed by 25 cycles of annealing for 1 min at 42°C, extension for 1.5 min at 72°C, and denaturation for 1 min at 94°C.

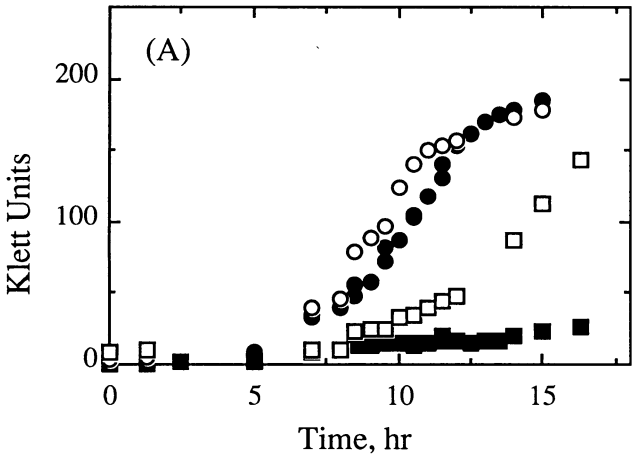
Preparation and analysis of cell extracts. A preliminary analysis of the newly identified genetic variants of threonine deaminase was achieved by expression of mutant proteins in the deletion strain T31-4-452. Cultures containing the various *ilvA* alleles, which contained the newly engineered restriction enzyme sites, were cloned downstream from the *trc* promoter in pKK233-2 (8) and grown in rich medium to mid-log phase before enzyme expression was induced by addition of IPTG to 1 mM. Cells were harvested after an additional 16 h of growth and were lysed in 0.1 M potassium phosphate buffer, pH 7.0, by incubation with 20 μg of lysozyme per ml for 10 min at 37°C followed by three cycles of freezing (3 min at −70°C) and thawing (3 min at 37°C). Relative levels of expression of threonine deaminase variants were estimated by scanning and relative digital integration of the intensities of the 56-kDa band after electrophoresis of cell lysates on 12% polyacrylamide gels in the presence of SDS (18). In this way a maximum difference of twofold was detected between the most intense band, corresponding to the D-266→Y (D266Y) threonine deaminase mutant shown in lane 8 of Fig. 4, and the least intense band, corresponding to the T251I-H303R double mutant in lane 2. The variation in the intensities for the other enzymes ranges from 65% of the maximum intensity for the S288F and G188C mutants in Fig. 4, lanes 6 and 7, respectively, to 92% for the K62E mutant and wild-type threonine deaminase in lanes 9 and 12, respectively. With the exception of the double mutant in lane 2, the variation in expression among the mutants is within the range normally seen for IPTG-induced expression of wild-type threonine deaminase from the *trc* promoter. The protein concentrations of the native lysates were determined by the Bradford (2) assay (Bio-Rad) with purified wild-type threonine deaminase as a standard.

The effects of the mutations on the catalytic and regulatory properties of threonine deaminase were estimated by determining threonine saturation curves by the enzyme assay of Davis (6) in the absence and presence of the heterotropic effectors isoleucine and valine. An estimate of 10^{-4} of the activity of wild-type threonine deaminase can be made for the upper limit of activity for the inactive mutants in native cell extracts on the basis of the sensitivity of the continuous spectrophotometric assay employed (6). Although other colorimetric assays based upon the detection of ketoacids with dinitrophenylhydrazine in acidic solutions show about 7.5-fold more sensitivity than the assay of Davis (6), they are too cumbersome and more time consuming for the small increase in sensitivity that is gained and were consequently not used in this study. Analysis of the kinetic data to determine the specific activity (maximal velocity in crude extracts), the $K_{0.5}$ for threonine (the threonine concentration at half-maximal velocity), and n_H (the Hill coefficient, an empirical measure of cooperativity in saturation curves) was performed as previously described for wild-type threonine deaminase (8).

RESULTS

High-level expression of threonine deaminase inhibits cell growth on minimal medium. Although a 100-fold increase in the level of expression of threonine deaminase from pEE6 in T31-4-452 in rich medium can be achieved by induction with IPTG (8) relative to that in *E. coli* TIR8 (4), which is constitutively derepressed for the synthesis of the enzymes of the *ilv* operon (28), negligible cell growth was observed when the same cultures were grown on minimal medium. As can be seen in Fig. 1, *E. coli* T31-4-452 containing pEE6 shows a marked inhibition of growth on minimal medium in the presence of IPTG compared with cells harboring a similar plasmid without the *ilvA* coding region. In the absence of IPTG, the time to reach mid-log phase for T31-4-452, an *ilv* deletion strain, carrying a control plasmid (pKK233-2) was about 8 h. Mid-log phase was reached at about the same time for cells grown in the presence of IPTG, as well as for the deletion strain T31-4-452 without any plasmid. By contrast, cells containing pEE28, which express threonine deaminase from the *trc* promoter when induced by IPTG, scarcely grew at all. In the absence of IPTG, however, mid-log phase was reached in about 14 h. This dramatic difference also was readily detected on minimal agar plates. As can be seen in Fig. 1B, there was a marked difference in the smaller average colony size seen for T31-4-452 containing pEE6 relative to that for strain T31-4-452 containing pKK233-2, which yielded larger colonies faster than cells that expressed active enzyme at high levels.

Rationale for selection of threonine deaminase mutations that affect catalytic activity. The observations that high-level expression of wild-type threonine deaminase led to an inhibition of cell growth on minimal medium but that this did not occur with control cultures that were either uninduced for enzyme synthesis or harbored control plasmids lacking *ilvA*



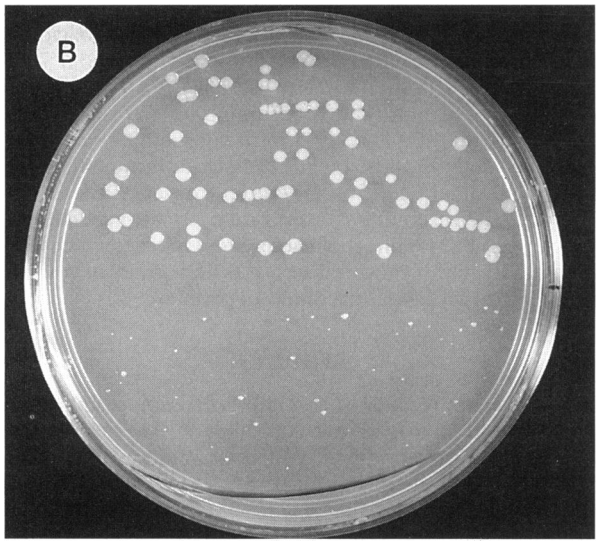


FIG. 1. Growth inhibition of *E. coli* T31-4-452 harboring a plasmid that highly expresses the *ilvA* gene. (A) Stationary-phase cultures of cells containing either pKK233-2 or pEE6 (*ilvA* driven by the *trc* promoter) were inoculated in liquid M9 medium supplemented with 0.2% glucose, 0.01% thiamine, 0.01% pyridoxine, 20 μg (each) of L-tryptophan, L-leucine, L-isoleucine, and L-valine per ml, 100 μg of ampicillin per ml, and 1 mM IPTG. Growth was measured by turbidity in a Klett meter with a green (no. 54) filter. Control cultures of cells containing pKK233-2 grown in the absence of IPTG were virtually identical to those of the parental strain without the plasmid. ○, pKK233-2; ●, pKK233-2 plus IPTG; □, pEE6; ■, pEE6 plus IPTG. (B) The difference in growth rates of T31-4-452 containing either pEE6 (bottom sector) or pKK233-2 (top sector) is readily seen on a minimal medium agar plate. Cells from stationary-phase liquid cultures were washed in minimal medium, spread at identical densities on the two sectors of the plate, and then grown for 36 to 48 h at 37°C.

suggested that mutations in *ilvA* that decreased threonine deaminase enzyme activity should suppress growth inhibition. We therefore proceeded with a two-part strategy to randomly introduce and readily identify mutations in the coding sequence of *ilvA* and to utilize defined growth conditions to select genetic variants that code for an enzyme less active than the wild type.

In order to execute this strategy, it was first necessary to modify *ilvA* to increase the relative ease of identifying randomly generated mutations in a protein the size of threonine deaminase (56,202-Da chains). The approach we adopted was to engineer *ilvA* such that the coding sequence was subdivided arbitrarily into smaller (200- to 300-bp) fragments which could be subcloned to facilitate the identification of mutations by minimizing the length of DNA that needed to be sequenced. This was accomplished by using oligonucleotide-directed mutagenesis to introduce eight new, unique restriction enzyme cleavage sites every 150 to 250 bp in the coding sequence of *ilvA*, without altering the amino acid sequence of threonine deaminase. The vector that was ultimately used as a target for chemical mutagenesis, pEE27, thus encodes wild-type threonine deaminase and has a total of 13 unique restriction enzyme sites within the structural gene. This version of the structural gene maintains the high level of expression of threonine deaminase when induced with IPTG from the *trc* promoter in pEE28, and the purified protein displays kinetic and biochemical properties indistinguishable from those seen for wild-type enzyme (8).

The second phase of our approach is illustrated in Fig. 2. The aim was to utilize the newly constructed *ilvA* vector as a target for mutagenesis, move small DNA segments into a nonmutagenized *ilvA* backbone, express high levels of the constructs in T31-4-452 by using pKK233-2, and identify clones that grew on minimal medium as a result of *ilvA* mutations that encoded an enzymically impaired threonine deaminase.

Suppression of threonine deaminase-promoted growth inhibition by *ilvA* mutations. Approximately 3,000 large transformant colonies (Fig. 1B) obtained as described in Materials and Methods were picked from minimal selection plates and were further screened for levels of threonine deaminase protein by colony immunoassay using polyclonal antiserum to purified wild-type enzyme. A total of 145 clones with immunologically detectable levels of enzyme were retested for their ability to grow on minimal medium and stored as 20% glycerol stocks at −80°C. As can be seen in Fig. 3, a comparison of the distribution of these clones with that of the five DNA segments that were treated with chemical mutagens revealed that the large majority of mutations (130 of 145) were located in the 5′ half of the *ilvA* gene, which corresponds to the first 231 amino acids in polypeptide chains of threonine deaminase. In contrast, only eight clones were localized to DNA fragments that correspond to the carboxy-terminal region of threonine deaminase.

Identification of *ilvA* mutations and their effect on threonine deaminase expression and activity. We first screened 40 of the 145 colony immunoassay-positive clones by SDS-polyacrylamide gel electrophoresis to identify variants that expressed mutant chains at levels comparable to wild-type enzyme. Of these 40 clones, 23 were chosen for DNA sequence determinations, including all 15 clones from the 3′ three-fifths of the *ilvA* gene (corresponding to the DNA fragments from *Bgl*II to *Hin*dIII [Fig. 3]). DNA sequencing of these alleles verified the 10 unique nucleotide substitutions in the *ilvA* structural gene that are summarized in Table 1. Nine of the mutations shown in Table 1 were single-base changes that led to a single amino acid substitution (two of them, P156S and S288F, were each obtained twice), and 1 of the 10 consisted of two nucleotide substitutions which resulted in two amino acid substitutions. Although no differences in nucleotide sequence compared with wild-type *ilvA* were detected in the remaining 11 clones, they all showed significant plasmid rearrangements outside the *ilvA* coding region.

Of the mutations that resulted in amino acid substitutions in threonine deaminase, five of the substitutions, including K62E, P156L, G188C, S288F, and the double mutation T251I-H303R, resulted in less than 10^{-4} of the activity of wild-type

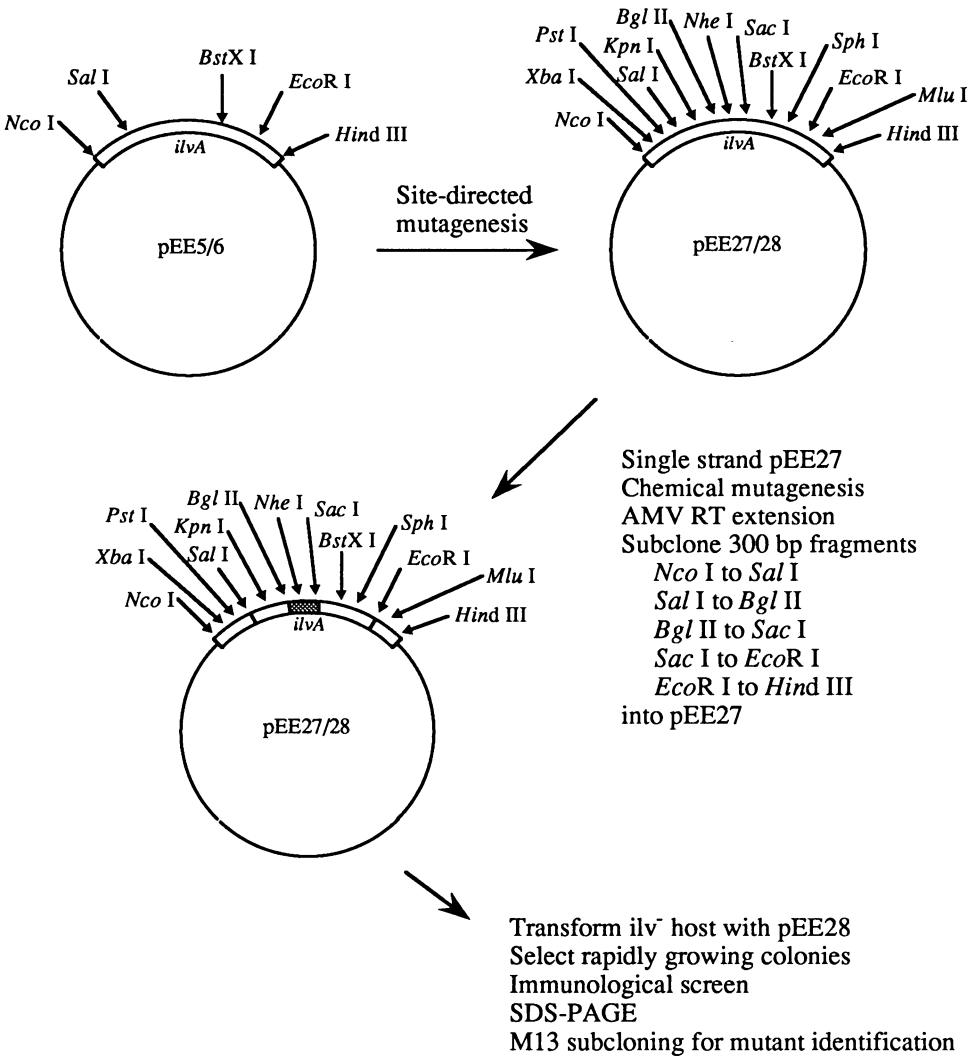


FIG. 2. Strategy for mutagenesis of *ilvA* and for selection of threonine deaminase variants that are less active than wild-type enzyme. The construction of pEE5 and pEE6, which contain the 1,545-bp *Nco*I-*Hin*dIII *ilvA* fragment cloned into pUC120 or pKK233-2, respectively, has been described (8). Site-directed mutagenesis was used as described in Materials and Methods to engineer eight new restriction enzyme sites throughout the coding region of *ilvA* to facilitate subcloning of relatively small (150- to 350-bp) fragments for DNA sequence determinations. The resulting vectors containing the modified *ilvA* sequence in either pUC120 or pKK233-2 were designated pEE27 and pEE28, respectively. Single-stranded pEE27 was used as a target for damage by nitrous acid and formic acid, and avian myeloblastosis virus reverse transcriptase was used to make a complementary copy of the damaged strand. Restriction enzyme digests that released small DNA fragments (for example, *Bgl*II to *Sac*I as shown) were ligated into similarly digested pEE27, and the ligation mixtures were used to transform strain JV30 to ampicillin resistance. Transformant colonies were pooled, and plasmid DNA was extracted to purify the 1,545-bp *ilvA* gene, which was subcloned into *Nco*I-*Hin*dIII-digested pKK233-2 to transform strain T31-4-452 to ampicillin resistance on minimal medium for evaluation of growth phenotype. Large colonies were picked and screened immunologically and by polyacrylamide gel electrophoresis in the presence of SDS for high-level synthesis of threonine deaminase. Identification of the specific base change was determined by DNA sequencing in M13 of the small fragment that initially had been exposed to the mutagenic agent.

threonine deaminase in crude cell lysates. Two of these inactive enzymes, those carrying K62E and P156L, were unable to complement an *ilvA* deletion strain to isoleucine prototrophy after 60 h. Thus, these two variants were completely inactive. As can be seen in Table 2, extracts of the other five mutant enzymes possessed various levels of activity, ranging up to 40% of that seen for the wild-type threonine deaminase.

Since it is possible that the nature of these mutations may influence catalytic activity indirectly by altering the in vivo stability of the enzyme relative to that of wild-type threonine deaminase, qualitative estimates of enzyme levels were made by examining polyacrylamide gels of cell lysates. As can be seen in Fig. 4, inspection of the 56-kDa polypeptide band corresponding to threonine deaminase chains reveals that in all cases except that of the T251I-H303R double mutant, which is present at only 50% of the level of wild-type chains, the mutants were expressed at comparably high levels, which varied between 65 and 110% of the level of the wild-type control.

It is also possible that some mutations which suppress the inability of cells to grow on minimal medium might have been due to altered allosteric properties. Isoleucine is a negative allosteric effector that decreases activity by increasing the $K_{0.5}$ for threonine; valine is a positive effector, which activates the

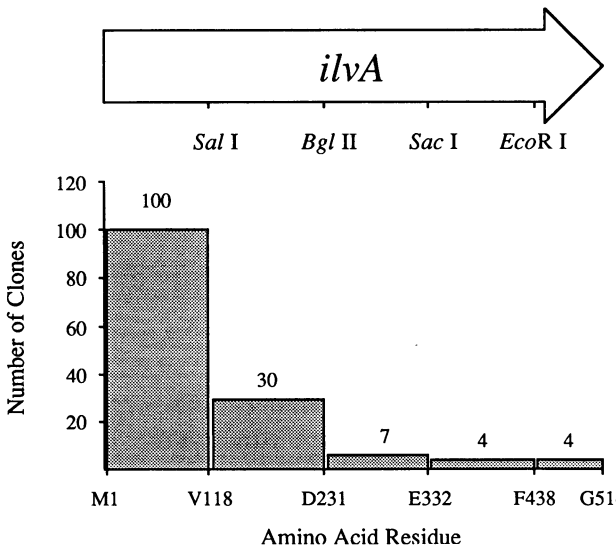


FIG. 3. Distribution of *ilvA* mutations that suppress growth inhibition. (Top) Schematic representation of the *Nco*I-*Hin*dIII *ilvA* cassette with the relative locations of the restriction enzyme sites that were used to arbitrarily divide the gene into smaller fragments to increase the relative ease of identifying mutations. (Bottom) Number of colony immunoassay-positive clones that were associated with each fragment and locations of the fragments with respect to the amino acid residue of the polypeptide chain for threonine deaminase.

enzyme by decreasing the $K_{0.5}$. Thus, it was of interest to determine the effects of isoleucine and valine on the kinetics of the active mutant enzymes. As can be seen in Table 2, four of the five active mutants, including the N46D, A66V, P156S, and G248C mutants, have an increased $K_{0.5}$ relative to that seen for wild-type threonine deaminase. The D266Y variant displays a $K_{0.5}$ virtually identical to that of wild-type enzyme. The addition of isoleucine to the assays results in mixed effects, however. Reliable estimates for the $K_{0.5}$ for the A66V and P156S mutants in the presence of isoleucine were unobtainable, as this ligand virtually completely inhibits these enzymes. The $K_{0.5}$ values for the N46D and D266Y mutants in the presence of isoleucine were not increased as greatly as seen for the wild type. The effect of valine on the mutants, which is difficult to determine in cell lysates since activation primarily affects the degree of cooperativity, suggests that the A66V and P156S mutants show measurably less cooperativity in the presence of the positive allosteric effector than do the others.

TABLE 1. Identities of *ilvA* mutations that suppress growth inhibition

Mutagen and nucleotide change[a]	Amino acid substitution[b]
Nitrous acid	
A136G	N46D
A184G	K62E
C197A	A66V
C466T	P156S
C467T	P156L
C863T	S288F
C752T-A908G	T251I-H303R
Formic acid	
G562T	G188C
G742T	G248C
G796T	D266Y

[a] Nucleotide numbering begins from A-1 of the ATG initiation codon for the *ilvA* gene.
[b] Single-letter abbreviations are used for substitutions, with the wild-type residue preceding the position number and the replacement amino acid following the number. The amino acid numbering system begins with methionine 1 encoded by the ATG initiation codon.

TABLE 2. Effects of isoleucine and valine on the kinetic parameters for active mutants of threonine deaminase[a]

Enzyme and addition	n_H	$K_{0.5}$ (mM)	Relative sp act
Wild type	2.3	9.0	1.0
Isoleucine	3.7	70	1.0
Valine	1.1	6.0	1.0
N46D mutant	1.7	13	0.33
Isoleucine	3.7	42	0.26
Valine	1.5	11	0.32
A66V mutant	1.1	130	0.10
Isoleucine	ND[b]	ND	ND
Valine	1.1	120	0.12
P156S mutant	1.2	52	0.30
Isoleucine	ND	ND	ND
Valine	1.0	55	0.32
G248C mutant	2.5	26	0.13
Isoleucine	3.1	93	0.13
Valine	1.4	23	0.14
D266Y mutant	2.6	9.0	0.16
Isoleucine	3.3	48	0.16
Valine	1.4	4.0	0.16

[a] Threonine deaminase activity was assayed by measuring spectrophotometrically the formation of α-ketobutyrate (6) in potassium phosphate buffer, pH 7.5, at 20°C. Assays were performed in the absence or presence of 50 μM isoleucine or 0.5 mM valine as previously described (8). Single-letter abbreviations are used for amino acid substitutions as described for Table 1. Detectable kinetic parameters, including n_H (the Hill coefficient), $K_{0.5}$ (the midpoint in threonine saturation curves), and relative specific activity in crude extracts (the specific activity of a particular variant relative to that of wild-type enzyme, which is approximately 25 to 35 μmol of product formed per mg of protein per min), are reported within 30% error as determined from three separate preparations.
[b] ND, not detectable.

DISCUSSION

In the course of establishing growth conditions for strains that highly express biosynthetic threonine deaminase in a background that is deficient in the activities of enzymes needed for isoleucine biosynthesis, we observed a marked decrease in growth in minimal medium relative to that of isogenic strains that did not contain plasmid-encoded *ilvA*. This can be seen in Fig. 1, which compares the growth of T31-4-452 containing a control plasmid lacking the *ilvA* gene with that of T31-4-452 containing a plasmid that contains the *ilvA* gene under the control of the inducible *trc* promoter. This observation was similar to that seen for *Salmonella typhimurium* treated with sulfometuron methyl (20), an herbicide which specifically inhibits the second enzyme of the isoleucine biosynthetic pathway (33), acetolactate synthase (EC 4.1.3.18) isozyme II (19), and has been attributed to the accumulation of toxic levels of α-ketobutyrate (21, 36). Because threonine deaminase catalyzes the pyridoxal phosphate-dependent dehydration/elimination of threonine to α-ketobutyrate, we sought to capitalize on these observations by devising an approach to

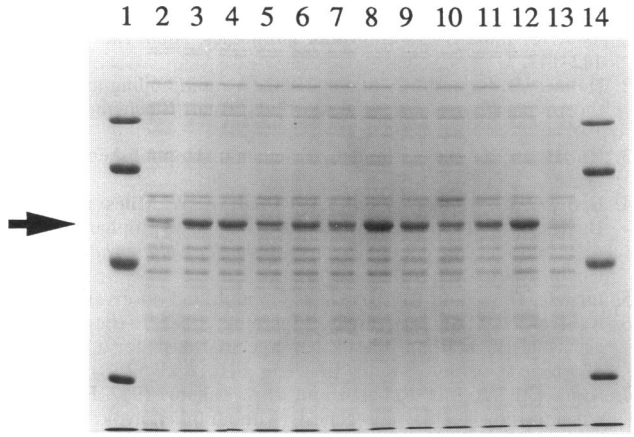


FIG. 4. Typical levels of expression for threonine deaminase mutants. Cells containing an *ilvA* mutation in pEE28 were inoculated in LB medium with 100 μg of ampicillin per ml, induced at mid-log phase with 1 mM IPTG, and then grown overnight. Native lysates were prepared and analyzed by polyacrylamide gel electrophoresis in the presence of SDS, using a 12% separating gel and a 4% stacking gel (18). Approximately 10 μg of total protein from the extracts was applied per lane; chains of threonine deaminase migrate with an apparent molecular weight of 56,000, as indicated by the arrow on the left. Lanes: 1 and 14, low-molecular-weight standards (Bio-Rad), including phosphorylase *b* (92,500), bovine serum albumin (66,200), ovalbumin (45,000), and carbonic anhydrase (31,000); 2, T251I-H303R; 3, P156L; 4, P156S; 5, G248C; 6, S288F; 7, G188C; 8, D266Y; 9, K62E; 10, N46D; 11, A66V; 12, wild-type threonine deaminase control; 13, pKK233-2 negative control.

select for genetic variants of threonine deaminase that possessed a lower level of activity than wild-type enzyme.

A limitation to the approach as it was employed was the requirement for high-level expression of threonine deaminase on the one hand and the need to determine the locations of the mutations in *ilvA* with relative ease on the other. Our attempts to use single-strand cloning and expression vectors in the selection (pEE5 or pEE27) were unsuccessful since the vectors themselves (without *ilvA*) caused growth inhibition of strain T31-4-452, possibly because of the deletion in this strain of the *rep* gene (7, 31), which is involved in phage and DNA replication (3, 27). Therefore, we modified the *ilvA* gene to introduce eight new restriction enzyme sites and, as can be seen in Fig. 2, subcloned this fragment into pKK233-2 to yield a plasmid vector, pEE28, that expresses threonine deaminase to a level of about 20% of the soluble protein when induced with IPTG. This enabled us to subclone 150- to 350-bp fragments from pEE28 into M13mp18 to determine by DNA sequencing the locations of the mutations that led to suppression of growth inhibition.

Another shortcoming concerns the high percentage of false-positive clones, identified as fast-growing colonies on minimal selection medium, which synthesized immunologically detectable levels of threonine deaminase but did not harbor any mutation in the *ilvA* structural gene. Restriction analysis of plasmids purified from these clones revealed anomalously sized fragments, suggesting that plasmid rearrangements leading to differences in copy number or in promoter structure may have led to spurious positive phenotypes in the selection.

The 10 unique mutants identified in this study displayed less activity in native cell lysates than wild-type threonine deaminase, despite being expressed at high levels that were comparable to that of the native enzyme (Fig. 4). An upper limit of only 10^{-4} of the activity of wild-type threonine deaminase was estimated for five of the mutations, whereas another five exhibit up to 40% of the activity seen for native enzyme. Preliminary steady-state kinetic results for the purified mutant enzymes have indicated that even the most active of the variants, the N46D mutant, displays a maximal velocity of 165 μmol/mg/min, which is only 70% of that obtained for the purified wild-type enzyme (235 μmol/mg/min) (13). The fact that the value for the relative activity of the purified N46D mutant differs from that determined in crude extracts demonstrates a pitfall of relying solely on assays of crude lysates and suggests that other factors may have contributed to the selection of these inactive variants of threonine deaminase. Two of the variants, the K62E and P156L mutants, were completely inactive as judged by their failure to complement an auxotrophic *ilvA* deletion strain. Support for a role of K-62 in catalysis by threonine deaminase comes from results of chemical modification and site-directed mutagenesis experiments (15) that identify this amino acid as the residue that forms the Schiff base with the pyridoxal phosphate cofactor in the active site. Although the three other inactive variants, those carrying G188C, S288F, and the double mutation T251I-H303R, were able to complement an *ilvA* deletion strain to isoleucine prototrophy, they exhibited such little activity in native cell lysates that it was impossible to measure their kinetic parameters with accuracy. As can be seen in Table 2, none of the five mutants that showed measurable levels of activity in native cell lysates were more than 40% as active as wild-type threonine deaminase, and the $K_{0.5}$ for threonine was dramatically increased for the variants in this class, except for the D266Y variant, whose $K_{0.5}$ for threonine was close to that of the wild type.

Since the level of activity of threonine deaminase in cells is doubtless controlled by the levels of isoleucine and valine in vivo and because mutations in other regulatory enzymes that affect catalysis can also give rise to unusual changes in regulatory properties (26), it was of interest to examine the effects of the feedback modifiers isoleucine and valine on the mutant enzymes. As can be seen in Table 2, the effect of isoleucine on the A66V and P156S mutants results in such dramatic inhibition of activity that a value for the $K_{0.5}$ for threonine could not be determined accurately for these mutants. It is possible, therefore, that these enzymes were selected not merely because of their reduced levels of activity in the absence of effectors but rather because of a hypersensitivity to isoleucine that led to almost complete inhibition. In contrast, the $K_{0.5}$ for threonine observed for the N46D and D266Y mutants was significantly lower than that for wild-type threonine deaminase, suggesting that these enzymes may be less sensitive to inhibition by this ligand. In addition, the allosteric activator valine has an enhanced effect on the A66V and P156S mutants, consistent with the interpretation that these two variants are markedly more sensitive to heterotropic effectors. Further analysis of the biochemical properties of these mutants may shed light on the nature of the effects of heterotropic ligands on wild-type threonine deaminase.

The locations of the mutations that affect catalytic activity in threonine deaminase provide potentially important clues about the structural organization of this regulatory enzyme. As can be seen in Fig. 3, the 145 clones isolated in this study are localized predominantly in the amino-terminal half of threonine deaminase, and no mutations that affected catalysis were identified in the carboxy-terminal third of the polypeptide chain. This distribution is consistent with several suggestions that the enzyme is organized functionally into an amino-

terminal catalytic domain and a carboxy-terminal regulatory domain (1, 29, 35).

The identification of striking sequence similarity within limited regions of a number of pyridoxal phosphate-dependent enzymes (1), including the β subunit of tryptophan synthase, whose three-dimensional structure has been solved by X-ray crystallography (14), has been taken as evidence for a limited number of protein folds compatible with binding the cofactor. It was of interest, therefore, to compare the locations of the amino-terminal mutations that affect catalysis in threonine deaminase with the structure of the β subunit of tryptophan synthase to deduce clues about the structural organization of the putative amino-terminal catalytic domain of the enzyme. The β subunit of tryptophan synthase is composed of two domains whose cores are structurally superimposable to 2.2-Å (0.22-nm) root-mean-square deviation for 73 structurally equivalent Cα pairs, despite a polypeptide sequence identity of only 8 of the 110 residues per domain. Sequence alignment of the core of the β subunit with threonine deaminase shows more than 30% similarity, which prompted us to construct a hypothetical model of a possible "catalytic" domain of threonine deaminase based on the atomic coordinates of the β chains of tryptophan synthase (10). This hypothetical model indicates that extensive similarity between the two sequences exists in a region that surrounds the pyridoxal phosphate cofactor and suggests that 8 of the 10 mutations, K62E, A66V, P156S, P156L, G188C, G248C, T251I, and S288F, which are within a 10-Å (1.0-nm) radius of the pyridoxal phosphate cofactor, may affect catalysis by altering the environment of the active site.

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REFERENCES

1. **Bork, P., and K. Rohde.** 1990. Sequence similarities between tryptophan synthase β subunit and other pyridoxal-phosphate-dependent enzymes. Biochem. Biophys. Res. Commun. **171:**1319–1325.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
3. **Calander, R., B. Lindqvist, G. Sironi, and A. J. Clark.** 1970. Characterization of REP$^-$ mutants and their interaction with P2 phage. Virology **40:**72–83.
4. **Calhoun, D. H., R. A. Rimerman, and G. W. Hatfield.** 1973. Threonine deaminase from Escherichia coli. I. Purification and properties. J. Biol. Chem. **248:**3511–3516.
5. **Cox, J. L., B. J. Cox, V. Fidanza, and D. H. Calhoun.** 1987. The complete nucleotide sequence of the *ilvGMEDA* cluster of *Escherichia coli* K-12. Gene **56:**185–198.
6. **Davis, L.** 1965. A spectrophotometric method for the assay of threonine dehydratase. Anal. Biochem. **12:**36–40.
7. **Driver, R. P., and R. P. Lawther.** 1985. Physical analysis of deletion mutations in the *ilvGEDA* operon of *Escherichia coli* K-12. J. Bacteriol. **162:**598–606.
8. **Eisenstein, E.** 1991. Cloning, expression, purification and characterization of biosynthetic threonine deaminase from *Escherichia coli*. J. Biol. Chem. **266:**5801–5807.
9. **Fahnestock, S. R., P. Alexander, J. Nagle, and D. Filpula.** 1986. Gene for an immunoglobulin-binding protein from a group G streptococcus. J. Bacteriol. **167:**870–880.
10. **Fidelis, K., J. Moult, and E. Eisenstein.** Unpublished observations.
11. **Hawkes, R., E. Niday, and J. Gordon.** 1982. A dot-immunobinding assay for monoclonal and other antibodies. Anal. Biochem. **119:**142–147.
12. **Holmes, D. S., and M. Quigley.** 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114:**193–197.
13. **Hu, H. D., K. Ducote, and E. Eisenstein.** Unpublished observations.
14. **Hyde, C. C., S. A. Ahmed, E. A. Padlan, E. W. Miles, and D. R. Davies.** 1988. Three-dimensional structure of tryptophan synthase $\alpha_2\beta_2$ multienzyme complex from *Salmonella typhimurium*. J. Biol. Chem. **263:**17857–17871.
15. **Iacuzio, D. A., and E. Eisenstein.** Unpublished observations.
16. **Kantrowitz, E. R., and W. N. Lipscomb.** 1990. Escherichia coli aspartate transcarbamoylase: the molecular basis for a concerted allosteric transition. Trends Biochem. Sci. **15:**53–59.
17. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154:**367–382.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
19. **LaRossa, R. A., and J. V. Schloss.** 1984. The sulfonylurea herbicide sulfometuron methyl is an extremely potent and selective inhibitor of acetolactate synthase in *Salmonella typhimurium*. J. Biol. Chem. **259:**8753–8757.
20. **LaRossa, R. A., T. V. VanDyk, and D. R. Smulski.** 1987. Toxic accumulation of α-ketobutyrate caused by inhibition of the branched-chain amino acid biosynthetic enzyme acetolactate synthase in *Salmonella typhimurium*. J. Bacteriol. **169:**1372–1378.
21. **LaRossa, R. A., T. K. Van Dyk, and D. R. Smulski.** 1990. A need for metabolic insulation: lessons from sulfonylurea genetics, p. 109–121. *In* Z. Barak, D. M. Chipman, and J. V. Schloss (ed.), Biosynthesis of branched chain amino acids. VCH Publishers, New York.
22. **Lawther, R. P., and G. W. Hatfield.** 1978. A site of action for tRNA mediated regulation of the ilvOEDA operon of *Escherichia coli* K-12. Mol. Gen. Genet. **167:**227–234.
23. **Lawther, R. P., R. C. Wek, J. M. Lopes, V. Perevia, B. E. Taillon, and G. W. Hatfield.** 1987. The complete nucleotide sequence of the *ilvGMEDA* operon of *Escherichia coli* K-12. Nucleic Acids Res. **15:**2137–2155.
24. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 431–435. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Myers, R. M., L. S. Lerman, and T. Maniatis.** 1985. A general method for saturation mutagenesis of cloned DNA fragments. Science **229:**242–247.
26. **Robey, E. A., S. R. Wente, D. W. Markby, A. Flint, Y. R. Yang, and H. K. Schachman.** 1986. Effect of amino acid substitutions on the catalytic and regulatory properties of aspartate transcarbamoylase. Proc. Natl. Acad. Sci. USA **83:**5934–5938.
27. **Scott, J. F., and A. Kornberg.** 1978. Purification of the rep protein of Escherichia coli. J. Biol. Chem. **253:**3293–3297.
28. **Szentirmai, A., M. Szentirmai, and H. E. Umbarger.** 1968. Isoleucine and valine metabolism of *Escherichia coli*. XV. Biochemical properties of mutants resistant to thiaisoleucine. J. Bacteriol. **95:**1672–1679.
29. **Taillon, B. E., R. Little, and R. P. Lawther.** 1988. Analysis of the functional domains of biosynthetic threonine deaminase by comparison of the amino acid sequences of three wild-type alleles to the amino acid sequence of biodegradative threonine deaminase. Gene **63:**245–252.
30. **Taylor, J. W., J. Ott, and F. Eckstein.** 1985. The rapid generation of oligonucleotide-directed mutations at high frequency using phosphorothioate-modified DNA. Nucleic Acids Res. **13:**8765–8785.
31. **Tessman, I., J. S. Fassler, and D. C. Bennett.** 1982. Relative map location of the *rep* and *rho* genes of *Escherichia coli*. J. Bacteriol. **151:**1637–1640.
32. **Turner, G. J. F. Galacteros, M. L. Doyle, B. Hedlund, D. W. Pettigrew, B. W. Turner, F. R. Smith, W. Moo-Penn, D. L. Rucknagel, and G. K. Ackers.** 1992. Mutagenic dissection of hemoglobin cooperativity: effects of amino acid alteration on

subunit assembly of oxy and deoxy tetramers. Protein Struct. Funct. Genet. **14**:333–350.

33. **Umbarger, H. E.** 1987. Biosynthesis of branched-chain amino acids, p. 352–367. *In* F. C. Neidhart, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.) *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
34. **Umbarger, H. E.** 1990. The study of branched-chain amino acid biosynthesis—its roots and fruits, p. 1–24. *In* Z. Barak, D. M. Chipman, and J. V. Schloss (ed.), Biosynthesis of branched chain amino acids. VCH Publishers, New York.
35. **Umbarger, H. E.** 1992. The origin of a useful concept—feedback inhibition. Protein Sci. **1**:1392–1395.
36. **Van Dyk, T. K., and R. A. LaRossa.** 1990. Prevention of endogenous 2-ketobutyrate toxicity in *Salmonella typhimurium*, p. 123–130. *In* Z. Barak, D. M. Chipman, and J. V. Schloss (ed.), Biosynthesis of branched chain amino acids. VCH Publishers, New York.
37. **Vieira, J., and J. Messing.** 1987. Production of single-stranded plasmid DNA. Methods Enzymol. **153**:3–11.
38. **Wek, R. C., and G. W. Hatfield.** 1986. Nucleotide sequence and *in vivo* expression of the *ilvY* and *ilvC* genes in Escherichia coli. J. Biol. Chem. **261**:2441–2450.