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# The *tdh* and *serA* Operons of *Escherichia coli*: Mutational Analysis of the Regulatory Elements of Leucine-Responsive Genes†

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**The *tdh* promoter of *Escherichia coli* is induced seven- to eightfold when cells are grown in the presence of exogenous leucine. A scheme was devised to select mutants that exhibited high constitutive expression of the *tdh* promoter. The mutations in these strains were shown to lie within a previously identified gene (*lrp*) that encodes Lrp (leucine-responsive regulatory protein). By deletion analysis, the site of action of Lrp was localized to a 25-bp region between coordinates −69 and −44 of the *tdh* promoter. Disruption of a 12-bp presumptive target sequence found in this region of *tdh* resulted in constitutively derepressed expression from the *tdh* promoter. Similar DNA segments (consensus, TTTATTCtNaAT) were also identified in a number of other promoters, including each of the Lrp-regulated promoters whose nucleotide sequence is known. The sequence of the promoter region of *serA*, an Lrp-regulated gene, was determined. No Lrp consensus target sequence was present upstream of *serA*, suggesting that Lrp acts indirectly on the *serA* promoter. A previously described mutation in a leucine-responsive *trans*-acting factor, LivR (J. J. Anderson, S. C. Quay, and D. L. Oxender, J. Bacteriol. 126:80–90, 1976), resulted in constitutively repressed expression from the *tdh* promoter and constitutively induced expression from the *serA* promoter. The possibility that LivR and Lrp are allelic is discussed.**

A two-step pathway initiated by threonine dehydrogenase (TDH) constitutes a major route of threonine degradation in *Escherichia coli*. Via this pathway, L-threonine is converted to glycine and acetyl coenzyme A. Under certain circumstances, the TDH pathway can provide an important alternate route for the biosynthesis of glycine and serine (13, 30, 35, 36). Among *E. coli* mutants that utilize threonine as a carbon source ($Tuc^+$), a large number exhibit high constitutive levels of the *tdh* operon gene products. Many, but not all, $Tuc^+$ strains contain an IS*3* insertion at coordinate −19 of the *tdh* promoter (5). Such IS*3* insertions simultaneously replace an inefficient −35 hexamer with one of superior potency and disengage any *tdh*-specific *cis*-acting upstream regulatory site(s).

TDH levels rise about sevenfold when cells are grown in the presence of leucine (5, 30, 32). Other genes of enteric bacteria known to be regulated by leucine are the *leu* operon (40), the *opp* operon (3), *ilvIH* (10), *livJ* and *livK* (34), *sdaA* (41), *serA* (27), and *lysU* (18). The *leu* operon is regulated by transcriptional attenuation, whereas the other leucine-regulated genes seem to respond to a *trans*-acting factor(s). In addition, a serine transport system (16) and the glycine cleavage pathway (13) are controlled by leucine. The latter regulated systems have not yet been fully characterized.

Results from several laboratories indicate that a common *trans*-acting factor, encoded by a single regulatory gene, controls the expression of *ilvIH* (31), *opp* (40a), *sdaA*, *serA*, and the enzymatic activity of TDH (43). This gene, designated *lrp* (leucine-responsive regulatory protein), is situated at the 20-min region of the *E. coli* chromosome. A previously studied regulatory gene for the leucine transport systems, *livR*, was also localized in the 20-min region (1). Whether *lrp* and *livR* are alleles is unknown. Ricca et al. (37) and Platko et al. (31) have shown that Lrp binds to the *ilvIH* promoter and activates the expression of the *ilvIH* operon. Leucine interferes with the assembly or stability of this protein-DNA complex. Leucine represses *ilvIH* and *serA*, whereas it activates *sdaA*, the *opp* operon, and the *tdh* operon. Therefore, Lrp may function in different systems either as a repressor or as an activator. Alternatively, there may be additional uncharacterized factors important to the regulation of leucine-responsive genes.

A role for Lrp in the induction by leucine of the *tdh* operon has been proposed (43). This report describes a new scheme for the isolation of mutations in a gene that encodes a *trans*-acting factor involved in the leucine induction of the *tdh* promoter. These mutants have a lesion in *lrp*. The previously described *livR* mutation was also found to affect the expression of *tdh* and *serA*. The possible allelism of *lrp* and *livR* is discussed. By computer analysis, it was found that many of the genes in the leucine regulon contain a common 12-bp sequence within or near their promoters. Disruption of this 12-bp sequence in the *tdh* promoter abolished the leucine-Lrp induction. Based on this analysis, a *cis*-acting target for *lrp* action is proposed. The sequence of the *serA* promoter region was determined. Because the proposed target site for Lrp was absent from this promoter and because the effect of leucine-Lrp on *serA* expression was relatively small, *serA* regulation may be mechanistically distinct from other leucine-regulated systems.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and bacteriophages.** The bacterial strains, bacteriophages, and plasmids used are described in Table 1. The P1 transduction protocol used in strain constructions was that of Miller (29).

**Media.** Minimal liquid medium contained glycerol (0.25%), vitamin $B_1$ (1 mg/liter), biotin (0.1 mg/liter), and salt mix E of Vogel and Bonner (44). Solid medium contained 1.5% Bacto Agar (Difco Laboratories) and glucose (0.2%) instead of glycerol. The following compounds were included

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TABLE 1. Bacterial strains, phage, and plasmids

| Strain, phage, or plasmid | Relevant genotype or description | Source or reference |
|---|---|---|
| *E. coli* | | |
| BW3912 | Δ*lacZ169 pho510 thi* | B. Wanner |
| SP1234(λpdr1) | As BW3912(λpdr1) but *lrp-101* | This work |
| SP1235(λpdr1) | As BW3912(λpdr1) but *zcb*::Tn*10kan* | This work |
| SP1236(λpdr1) | As SP1235(λpdr1) but *lrp-101* | This work |
| YS57 | F⁻ *his pro* Δ(*trpEDCBA tonB*) *rpsL* | R. Somerville |
| SP1274 | As YS57 but *zcb*::Tn*10kan lrp-101* | This work |
| CSH26 | *ara* Δ(*lac-pro*) *thi* | 29 |
| CV1008 | As CSH26 but *ilvIH-lacZ lrp*::Tn*10* | 31 |
| SP1300(λpdr1) | As BW3912(λpdr1) but *lrp*::Tn*10* | This work |
| AE84064 | *trp his arg livR* | 2 |
| SP1314(λpdr1) | As SP1235(λpdr1) but *livR* | This work |
| SP1357 | As BW3912 but *zcb*::Tn*10kan* | This work |
| SP1358 | As SP1357 but *livR* | This work |
| SP1359 | As BW3912 but *lrp*::Tn*10* | This work |
| JM101 | Δ(*lac-pro*) *thi rpsL hsdR4 endA sbcB supE44* F′ *traD36 proA⁺B⁺ lacI^q* Δ(*lacZ*)M15 | 28 |
| λ phage | | |
| λRZ11 | *c*I857 *S*am7 promoterless *lacZ* | 49 |
| λpdr1 | As λRZ11 but *tdh-lacZ* | P. Ravnikar |
| λRZ11 p-121 | As λRZ11 but *tdh-lacZ* | 5 |
| λRZ11 -122 | As λRZ11 p-121 but 5′ deletion in *tdh* to position −122 | This work |
| λRZ11 -69 | As λRZ11 p-121 but 5′ deletion in *tdh* to position −69 | This work |
| λRZ11 ΔMlu | As λRZ11 p-121 but 5′ deletion in *tdh* to position −44 | This work |
| λΔ15 | As λRZ11 p-121 but deleted from −75 to −61 | This work |
| λ*Apa*I | As λRZ11 p-121 with GGGC at −68 to −71 | This work |
| λ*serA* | As λRZ11 but *serA-lacZ* | This work |
| λΔ*serA* | As λ*serA* but only 456 bp present upstream of *serA* | This work |
| λ1105 | p*tac* + mini-*kan* | 46 |
| Plasmids | | |
| pJAC4 | Kan^r promoterless *ampC* | 20 |
| pJR4 | As pJAC4 with *tdh*p-*ampC* | This work |
| pCV168 | As pBluescript II KS− but *lrp⁺* | 31 |
| pBR322 | | 7 |
| pJR7 | As pBR322 but *lrp⁺* | This work |
| pMLB1034 | Amp^r promoterless *lacZ* | 6 |
| pGTS17 | *serA⁺*, derivative of pGT17 | 42 |
| pDR121 | Amp^r *kbl⁺ tdh⁺* | 36 |
| pDR123 | Amp^r *kbl tdh⁺* | 36 |
| pUC19 | | 48 |
| M13 phage | | |
| M13mp18/p | Promoterless *lacZ*α | 5 |
| M13mp18/p p-121 | *tdh-lacZ*α | 5 |
| M13mp18/p(+1) | Frameshift in *lacZ*α; promoterless | B. Hagewood and B. Aronson |
| M13mp18/p(+1)*serA* | *serA-lacZ*α | This work |

when appropriate: valine (0.5 mg/liter), DL-4-azaleucine (100 mg/liter), L-leucine (100 mg/liter), acid-hydrolyzed casein (0.2%), ampicillin (25 mg/liter), kanamycin (25 mg/liter), and tetracycline (15 mg/liter). M13 phage were propagated in cells grown in 2× yeast tryptone liquid medium (29). The susceptibility of cells to λ infection was enhanced by propagating them in λym broth (46) or tryptone-maltose broth (14). Either L broth (25) (with 0.1% glucose) or nutrient agar (31 g/liter; Difco) was used as complete medium.

**DNA preparations.** Plasmid and M13 replicative-form DNAs were isolated by the alkaline lysis procedure of Ish-Horowicz and Burke (19). Small preparations of plasmid and replicative-form DNA were made by a scaled-down version of the alkaline lysis procedure. Cells were transformed by the method of Cohen et al. (9). Single-stranded M13 templates were prepared for sequence analysis by the procedure of Sanger et al. (38).

**Chemicals and reagents.** Restriction endonucleases were purchased from New England BioLabs, Promega, and Stratagene and used according to the manufacturers' directions. T4 DNA ligase was purchased from Promega. DNA polymerase I large fragment (Klenow) and −20 and −40 oligonucleotide sequencing primers were purchased from New England BioLabs. The Taquence (*Taq* polymerase) DNA sequencing kit was purchased from U.S. Biochemicals. [α-$^{35}$S]dATP (1,000 Ci/mmol) was purchased from Amersham. Acid-hydrolyzed casein was purchased from ICN Biochemicals. Special-purpose oligodeoxynucleotides used for DNA sequencing or for mutant construction were synthesized on an Applied Biosystems machine.

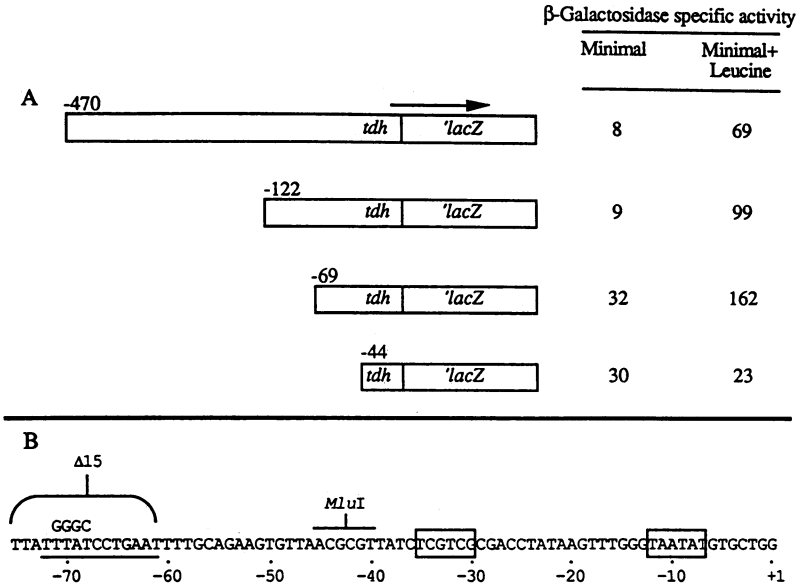


FIG. 1. Analysis of 5′ *tdh* regulatory sequences. (A) Deletions were made by using restriction endonuclease sites present upstream of the *tdh* promoter. All constructs have identical 3′ junctions with *lacZ*. The −69 construct was unstable as a λ lysogen and was therefore assayed on a multicopy plasmid and normalized to the −470 construct (see text). BW3912 was used as the host strain in all assays. (B) Nucleotide sequence of the promoter region. The boxed nucleotides are the −10 and −35 hexamer promoter sequences. The nucleotide designated as +1 is the transcription start site (5). The *Mlu*I site used in construction of the deletions is overlined. The underlined sequence designates the region of similarity with other *lrp*-regulated genes. The bracket above the sequence indicates the 15 nucleotides deleted in the λΔ15 construct. The four nucleotides replaced in the λ*Apa*I construct are indicated above −68 to −71.

**Construction of plasmids. (i) pJR4.** A 428-bp *Sau*3AI fragment containing the *tdh* promoter was excised from pDR121 and ligated into the *Bgl*II site of an intermediate vector, pCB182 (39). From this construct, the *tdh* promoter was subcloned as a *Bam*HI-*Xba*I fragment into pJAC4 (20) to make pJR4.

**(ii) pJR7.** A 2.5-kb fragment that contained *lrp* was subcloned from pCV168 into pBR322 by using the restriction endonucleases *Eco*RI and *Hin*dIII.

**Construction of λ phage derivatives.** Promoter fragments were subcloned into pMLB1034 (6) to create in-frame fusions with *lacZ*. The resulting Lac$^+$ plasmid derivatives were transformed into strain CSH26(λRZ11). Lac$^+$ λ recombinants were isolated as described by Yu and Reznikoff (49). Candidate Lac$^+$ phage were purified, lysogenized, and assayed as described previously (5).

**(i) λpdr1.** An *Eco*RI fragment containing the *tdh* promoter was subcloned from pDR123 (36) into pMLB1034. Recombination with λRZ11 was done as described above.

The 5′ deletion derivatives (Fig. 1) were made as follows. The −122 construct was made by cloning the *Rsa*I-*Bam*HI (190-bp) fragment from M13mp18/p p-121 (5) into pUC19 (48). An *Eco*RI-*Bam*HI fragment from the pUC derivative was then inserted into similarly cleaved M13mp18/p. The −69 construct was made by cleaving M13mp18/p p-121 with *Eco*RI and *Mlu*I. The cleaved vector was then ligated in the presence of an *Eco*RI-*Mlu*I cassette. This cassette was made by annealing two oligonucleotides (5′ CGCGTTAACACTT CTGCAAAATTCAGGATG 3′ and 5′ AATTCATCCTGAA TTTTGC 3′). The sequence at the vector/*tdh* junction is GAATTCATCCT. The −44 construct was made by cleaving M13mp18/p p-121 with *Eco*RI and *Mlu*I, filling in the 3′ overhangs with Klenow enzyme and deoxynucleoside triphosphates, and religating. The modified *tdh* promoter fragments were inserted into pMLB1034 by using restriction endonucleases *Eco*RI and *Bam*HI.

The λRZ11 derivative of the −69 construct was genetically unstable as a lysogen under all conditions tested. Therefore, the β-galactosidase assays were done in host strain BW3912 with the pMLB1034 plasmid derivative. Assays for the wild-type *tdh-lacZ* pMLB1034 derivative were done in parallel. These β-galactosidase activities were normalized by using β-lactamase assay values, thereby correcting for variations in plasmid copy number. A second correction factor was derived by dividing the wild-type *tdh-lacZ* lysogen value by the wild-type *tdh-lacZ* plasmid value when strains having either one or the other fusion were assayed under uninduced conditions. Using these correction factors, the plasmid-specified activities were converted to values that allowed direct comparisons with assays on single-copy lysogens.

**(ii) λΔ15.** A 30-residue oligodeoxynucleotide (GTTAACA CTTCTGCAAACGTGTAACTTGAT) was annealed to 1 μg of M13mp18/p p-121 single-stranded DNA template at 85°C for 5 min in 1× Klenow buffer (10 mM Tris, 5 mM $Mg^{2+}$ [pH 8.0]). The mixture was allowed to cool slowly to room temperature. The oligonucleotide was extended in the presence of 0.125 mM deoxynucleotide triphosphates, 4 mM dithiothreitol, and 2.5 U of DNA polymerase (Klenow) for 15 min at room temperature. The reaction mixture was directly transformed into JM101. Phage from darker blue plaques were isolated, and their DNA was sequenced to verify that the correct mutation had taken place. The modified *tdh* promoter fragment was subcloned into pMLB1034 by using restriction endonucleases *Eco*RI and *Bam*HI and then recombined into λRZ11 as before.

**(iii) λ*Apa*I.** A 30-residue oligodeoxynucleotide (TGCAA AATTCAGGGCCCATAACGTGTAACT) was used to modify the *tdh* promoter region as described above for λΔ15.

**(iv) λ*serA*.** A 1.1-kb *Eco*RI-*Pvu*II fragment containing the *serA* promoter was excised from pGTS17. This fragment was ligated into M13mp18/p(+1) cut with *Eco*RI and *Sma*I to construct M13mp18/p(+1)*serA*. The 1.1-kb *serA* promoter fragment was excised from this construct with *Hin*cII, inserted into the *Sma*I site of pMLB1034, and then recombined into λRZ11 as before.

**(v) λΔ*serA*.** M13mp18/p(+1)*serA* was cut with *Sma*I and *Bsp*EI, and the 5′ overhang was filled in with Klenow fragment and religated. The 555-bp *Eco*RI-*Hin*cII promoter fragment was excised from this construct and inserted into pMLB1034 cut with *Eco*RI and *Sma*I. The fusion was recombined into λRZ11 as before.

**Isolation of mutants affecting a *tdh*-specific *trans*-acting factor.** BW3912pJR4(λpdr1) contains three copies of the *tdh* promoter driving *ampC* from a plasmid, *lacZ* from a λ phage, and the *tdh* operon from the chromosome. Any mutation in a *trans*-acting factor that regulates the *tdh* promoter should affect the expression of all three promoters in parallel fashion. Approximately $10^6$ cells of BW3912pJR4(λpdr1) were plated at 30°C on minimal glucose medium containing kanamycin and ampicillin (10 mg/liter). Ampicillin-resistant cells arose at a frequency of $10^{-5}$. The plasmid was allowed to segregate from isolated mutants by propagating cells in minimal liquid medium without antibiotic selection. The resulting isolates were retransformed with pJR4 and screened on minimal glucose plates containing kanamycin and ampicillin (10 mg/liter). Four plasmid-free mutants that were ampicillin resistant upon retransformation were retained for further study.

**Marking the 20-min region.** Strain BW3912 was infected with λ1105, a λ phage transposon delivery vehicle carrying the mini-*kan* element. This variant form of Tn*10* was allowed to hop randomly into the chromosome according to published procedures (46). A 100-μl sample from the hop mixture was used to inoculate 10 ml of L broth containing 1.25 mM sodium $PP_i$ and kanamycin (50 mg/liter). The resulting culture was grown to saturation at 42°C with shaking. A P1 lysate made on this mixed culture was used to transfer the hopped kanamycin cassettes to one of the four plasmid-free mutants, SP1234(λpdr1). A series of transductants were isolated that were both kanamycin and valine (0.5 mg/liter) resistant, as determined by streak tests on solid medium, indicating the simultaneous recombination of a kanamycin cassette with the wild-type allele. The linkage relationships of 17 candidates were determined by P1 transduction. One isolate (*zcb*::Tn*10kan*) that contained a Tn*10kan* cassette 20% linked to the valine resistance phenotype was kept for further study.

**Mapping of mutation.** The approximate map location of *zcb*::Tn*10kan* was determined by conjugation with a collection of strains with Tn*10* insertions approximately 10 min distal to their Hfr (45). Fine mapping was done by P1 transduction with known markers in the 20-min region.

**Enzyme assays.** β-Galactosidase was assayed by the procedure of Miller (29), using whole cells grown to the mid-log phase with glycerol as the carbon source. β-Lactamase was assayed by the procedure of Kelly et al. (22).

**Strain constructions.** *lrp-101* was transferred by P1 transduction from strain SP1236(λpdr1) by selecting kanamycin resistance and scoring valine or azaleucine sensitivity. *lrp*::Tn*10* was transferred from strain CV1008 by selecting tetracycline resistance.

The *livR* mutation was received in a background strain (AE84064) that carried markers incompatible with an azaleucine sensitivity test. To circumvent this problem, we transduced *zcb*::Tn*10kan* into AE84064. A pool of kanamycin-resistant transductants were used as hosts for the preparation of a P1 lysate. This lysate was used to transduce BW3912(λpdr1). A kanamycin-resistant azaleucine-sensitive transductant was purified and designated SP1314(λpdr1).

**DNA sequence analysis.** DNA sequences were determined by the method of Sanger et al. (38) modified for use with [α-$^{35}$S]dATP as the labeling nucleotide. Recombinant M13 phage were used as templates. *Taq* polymerase and termination mixes containing either dGTP or 7-deaza-dGTP were used in the sequencing reactions. Commercially available −20 and −40 universal primers and custom-made oligodeoxynucleotide primers were used as dictated by the available M13 clones.

**Computer analysis.** All computer analyses were done with the University of Wisconsin Genetics Computer Group programs (11).

**Identification of Lrp consensus target.** Visual observation and the program Bestfit were used to find sequence similarities between the −44 to −69 region of *tdh* and the upstream regions of *ilvIH* and *sda*. These regions were aligned and a consensus table was constructed by using the program Consensus. Fitconsensus was used to scan the *ilvIH*, *tdh*, and *sda* promoters to find the sequences that were most similar to those in the consensus table. The table was then modified to incorporate the highest-quality sequence similarities or "hits" into the table, while the worst hits were deleted. This process was repeated until a consensus table was constructed that identified the sequences in the table as the best hits in the *tdh*, *sda*, and *ilvIH* promoters.

As other operons were implicated in the leucine regulon, and their sequences became known, Fitconsensus was used to match these sequences against the consensus table. The highest-scoring hits were incorporated into the table, and the sequences were reexamined. This process was repeated until the best hits in the promoter regions of the eight operons were the hits of the table. The Fitconsensus program was modified so that the final consensus table could scan all the *E. coli* sequences in the GenBank and EMBL data bases.

**Nucleotide sequence accession number.** The nucleotide sequence reported in this article has been submitted to GenBank (accession number M64630).

## RESULTS

**Isolation of mutations affecting a *trans*-acting regulatory factor controlling the *tdh* operon.** TDH activity increases about sevenfold when cells are grown in the presence of leucine (5, 30, 32). This induction is at the level of transcription (5). It was reasoned that if a *trans*-acting factor was critical for leucine induction of the *tdh* operon, a lesion in the gene for such a factor might result in constitutive levels of expression. To select for such mutants, we designed a system based on pJR4. In this construct, the *tdh* promoter drives the expression of *ampC*. In terms of colony-forming ability, the 50% lethal doses of ampicillin for BW3912(pJR4) on minimal medium with and without casein hydrolysate were found to be 4.4 and 11.6 μg/ml, respectively (data not shown). To screen out plasmid copy number mutations, we lysogenized BW3912(pJR4) with λpdr1, on the expectation that mutations affecting a *trans*-acting factor should simultaneously alter the expression of both *ampC* and *lacZ*. Spontaneous mutants were selected on minimal medium containing 10 μg of ampicillin per ml and 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside per ml. Four independent plasmid-free segregants derived from these mutants

TABLE 2. β-Galactosidase activities of strains harboring λpdr1[a] under various growth conditions

| Strain (λpdr1)[b] | Relevant genotype | β-Galactosidase activity[c] with: | | | Induction ratio for leucine | Induction ratio for casein hydrolysate |
|---|---|---|---|---|---|---|
| | | Minimal medium | Minimal + leucine | Minimal + casein hydrolysate | | |
| BW3912 | *lrp*$^+$ | 9.6 | 86.7 | 106 | 9.0 | 11 |
| pBR322 | *lrp*$^+$ | 9.2 | 146 | 70.6 | 16 | 7.7 |
| pJR7 | *lrp*$^+$/*lrp*$^+$ | 3.9 | 13.4 | 6.4 | 3.4 | 1.6 |
| SP1234 | *lrp-101* | 207 | 104 | 201 | 0.5 | 1.0 |
| pBR322 | *lrp-101* | 144 | 301 | 180 | 2.1 | 1.3 |
| pJR7 | *lrp-101*/*lrp*$^+$ | 11.5 | 14.2 | 6.5 | 1.2 | 0.6 |
| SP1300 | *lrp*::Tn*10* | 194 | 120 | 186 | 0.6 | 1.0 |
| pBR322 | *lrp*::Tn*10* | 138 | 281 | 210 | 2.0 | 1.5 |
| pJR7 | *lrp*::Tn*10*/*lrp*$^+$ | 12.2 | 12.6 | 7.0 | 1.0 | 0.6 |
| SP1314 | *livR* | 9.7 | 9.3 | 48.5 | 1.0 | 5.0 |
| pBR322 | *livR* | 11.5 | 7.7 | 47.3 | 0.7 | 4.1 |
| pJR7 | *livR*/*lrp*$^+$ | 3.0 | 7.2 | 5.5 | 2.4 | 1.8 |

[a] λpdr1 contains the *tdh* promoter driving the expression of an in-frame fusion of *kbl* with *lacZ*.
[b] All strains are lysogenic for λpdr1.
[c] β-Galactosidase values are in Miller units and are the means of at least three independent experiments ($\sigma < 18\%$).

were assayed for β-galactosidase. All mutants [represented by SP1234(λpdr1)] exhibited more than a 20-fold increase in β-galactosidase expression in minimal medium when compared with BW3912(λpdr1) (Table 2). The presence of leucine or casein hydrolysate in the growth medium had little effect on *lacZ* expression in the mutants, whereas wild-type cells exhibited a 9-fold induction with leucine and an 11-fold induction with casein hydrolysate (Table 2).

Since the mutants exhibited constitutive expression from the *tdh* promoters driving both reporter genes, it was reasonable to presume that the chromosomal *tdh* operon was also constitutively expressed. In growth studies, the four isolated mutants proved to be hypersensitive to valine (0.5 μg/ml), as expected if the enzymes of the *tdh* operon were depleting the threonine and isoleucine pools (see Discussion). Valine hypersensitivity was used as a phenotype to map the locus for the presumed *trans*-acting factor in one of the mutants, SP1234. By allowing a Tn*10kan* element to hop randomly in the chromosome, a strain was isolated that had a kanamycin resistance cassette 20% linked by P1 transduction to the mutant gene that imposed the valine-hypersensitive phenotype. The mutation imposing the valine-sensitive phenotype in the other three mutants was also 20% linked to this kanamycin resistance cassette. β-Galactosidase assays on valine-resistant and valine-hypersensitive transductants [SP1235(λpdr1) and SP1236(λpdr1), respectively] gave values identical to those of wild-type cells [BW3912(λpdr1)] and mutants [SP1234(λpdr1)], respectively. By this criterion, the Tn*10kan* element was judged to be 20% linked to a gene in which mutations affect the expression of the *tdh* operon.

The Tn*10kan* element was used as a selectable marker to locate the gene that conferred valine supersensitivity. Conjugation experiments with different Hfr-mapping strains indicated that the Tn*10kan* element that was linked to the mutation of interest was situated approximately between 10 and 20 min on the *E. coli* chromosome. Using P1-mediated transduction, the Tn*10kan* element was found to be 64% cotransducible with *aroA*. Thus, Tn*10kan* and the mutation of interest are located near the 20-min region of the chromosome.

***lrp* regulates the *tdh* promoter.** As this work was in progress, Lrp (leucine-regulatory protein) was shown to be involved in the regulation of several leucine-regulated genes (31, 40a, 43). The gene that encodes Lrp, *lrp*, was also shown to lie in the 20-min region of the *E. coli* chromosome. To test whether the mutation in SP1234(λpdr1) affected *lrp*, we transduced an *lrp*::Tn*10* mutation (kindly supplied by J. Calvo via M. Levinthal) into BW3912(λpdr1). This strain [SP1300(λpdr1)] proved to be sensitive to 0.5 μg of valine per ml. β-Galactosidase assays of SP1300(λpdr1) gave a set of values nearly identical to those of SP1234(λpdr1) grown under three different conditions (Table 2). A multicopy plasmid containing an insert that expresses only *lrp*$^+$ (pJR7) was introduced into wild-type, SP1234, or *lrp*::Tn*10* strains. Under all growth conditions tested, β-galactosidase expression from the *tdh* promoter was strongly reduced in comparison with that in control strains carrying pBR322. It was inferred from these results that SP1234 contained a mutation in *lrp* that accounts for both the valine hypersensitivity phenotype and the constitutive transcription from the *tdh* promoter. On these grounds, Lrp is judged to be a regulatory element of the *tdh* operon, and the mutation in SP1234 was given the designation *lrp-101*.

***livR* affects *tdh* expression.** An independently described mutation in the 20-min region, *livR*, affects the transport of several branched-chain amino acids (1). A well-characterized phenotype of the *livR* mutant is sensitivity to azaleucine (100 μg/ml). Both SP1234(λpdr1) and SP1300(λpdr1) exhibited an azaleucine-sensitive phenotype when streaked on solid medium containing 100 μg of azaleucine per ml. To determine whether *livR* is an allele of *lrp*, we constructed a strain [SP1314(λpdr1)] that contained a *zca*::Tn*10kan* element 20% linked to a mutation extracted from a *livR* strain that conferred azaleucine sensitivity. SP1314(λpdr1) was also found to exhibit valine supersensitivity. β-Galactosidase assays on SP1314(λpdr1) indicated that the *tdh* operon was uninducible (Table 2). Therefore, strain AE84064 contains a mutation in the 20-min region that exhibits valine and azaleucine supersensitivity and imposes low-level constitutive expression on the *tdh* operon. It is possible that *livR* is an allele of *lrp* that binds to DNA but is unresponsive to leucine. Even though this model seems inconsistent with the

TABLE 3. Sequence similarities detected in the 5′ upstream areas of genes that belong to the leucine regulon

| Gene | Sequence | Location | Score[c] |
|---|---|---|---|
| *tdh* | T T T A T C C T G A A T | −61 to −72 | 72.17 |
| *ilvIH* | T T T A T T C T G C A T[a] | −242 to −253 | 77.08 |
| | T T T A T T C T G A A T[a] | −211 to −222 | 79.17 |
| | T T T A T T A T C A A T | −140 to −129 | 73.67 |
| | T T T A T T C T T A T T[a] | −66 to −77 | 74.33 |
| *sdaA* | T C T A T T C G A C A T | Over the initiating ATG | 66.08 |
| *lysU* | T T T A T T C A T T A C[b] | 95 bp upstream of the putative −35 hexamer | 69.42 |
| | T T T A T T A G T G A T | 15 bp upstream of the putative −35 hexamer | 68.08 |
| *oppA* | T T T A T T C T G G T G[b] | 15 bp upstream of the putative −35 hexamer | 65.33 |
| *oppBDCF* | T T T A T T C T A C G T | 10 bp downstream of the initiating ATG | 70.17 |
| *livJ* | T T T A T T A A C A A T[b] | −50 to −61 | 70.17 |
| *livKHMGF* | T C T G T T C T T A A T | +1 to +12 | 66.67 |
| Consensus | T T T A T T C t N a A T | | |

[a] Within the regions recognized by the Lrp protein (37).
[b] These sequences are found on the complementary strand.
[c] Quality score obtained by using the program Fitconsensus with this table as the consensus table.

supersensitivity phenotypes of both *lrp*::Tn*10* and *livR* strains, these phenotypes can be explained physiologically (see Discussion).

**Identification of *tdh* operator.** To delineate the 5′ boundary of the *tdh* operator sequence, we constructed a series of deletion mutants lacking progressively greater amounts of DNA (Fig. 1A). An *Rsa*I site at coordinate −122 was used to make a construct that contained 122 bp upstream from the transcription start site. An *Mlu*I site at position −44 was utilized to install a cassette containing *tdh* sequences up to 69 bp upstream from the transcription start site. A different deletion, constructed by using an *Mlu*I restriction site, contained *tdh* sequences up to 44 bp upstream from the transcription start site. The reporter enzyme data (Fig. 1A) showed that the leucine inducibility of the *tdh* promoter is only slightly affected by the loss of DNA segments upstream of coordinate −69. The deletion of DNA segments between −69 and −44 led to full promoter constitutivity. This result implicates a segment of DNA between coordinates −44 and −69 in the leucine regulation of the *tdh* operon. The *tdh* sequence from −75 to +1 is shown in Fig. 1B.

Band shift assays with *ilvIH* promoter DNA had indicated that two regions were recognized by the Lrp protein (37). When the DNA sequence of these regions of *ilvIH* was compared with the sequence of the *tdh* promoter region from −44 to −69 (see Materials and Methods), some similarities were found (Table 3). The promoter sequences for five of the six loci that are regulated by Lrp are known. The program, Fitconsensus, was used to find a similar 12-bp sequence in all of them (Table 3). Fitconsensus uses a consensus table as a probe to find the best examples of the consensus in a DNA sequence. For each example, the program calculates a statistical measure of its quality in terms of a numerical score. Using Table 3 as the consensus table, the highest and lowest quality scores possible are 79.17 and 15.00, respectively, with a mean score of 24.7. The quality score of each sequence is shown (Table 3). The 12-bp sequence was also found in *lysU*, *livJ*, and *livKHMGF*, a set of genes that are also regulated by leucine (18, 34).

To test whether this 12-bp segment of DNA is involved in the regulation of the *tdh* operon, we deleted the sequence from an M13 construct containing the *tdh* promoter region. The new construct was used to make a recombinant λ phage (λΔ15) containing the same protein fusion as λpdr1 but missing the 12-bp sequence and 3 bp adjacent to it upstream of the *tdh* promoter. λΔ15 was inserted as a lysogen into wild-type, *lrp*::Tn*10*, and *livR* strains. β-Galactosidase assays on these strains indicated that the *tdh* promoter of λΔ15 had lost the ability to respond to leucine (Table 4). To pinpoint the presumptive operator more precisely, nucleotides 2 through 5 of the proposed operator (TTAT) were replaced with four nucleotides (GGGC), an operation that simultaneously created an *Apa*I restriction site. This construct was transferred to a phage λ derivative which was used to lysogenize the previously mentioned strains. β-Galactosidase assays on this series of lysogens likewise indi-

TABLE 4. β-Galactosidase activities of strains harboring λΔ15[a] and λ*Apa*I[b] under various growth conditions

| Strain | Relevant genotype | β-Galactosidase activity[c] with: | | | Induction ratio for leucine | Induction ratio for casein hydrolysate |
|---|---|---|---|---|---|---|
| | | Minimal medium | Minimal + leucine | Minimal + casein hydrolysate | | |
| BW3912(λΔ15) | Wild type | 78.1 | 73.2 | 78.0 | 0.9 | 1.0 |
| SP1359(λΔ15) | *lrp*::Tn*10* | 99.2 | 88.7 | 96.8 | 0.9 | 1.0 |
| SP1358(λΔ15) | *livR* | 88.3 | 104 | 75.9 | 1.2 | 0.9 |
| BW3912(λ*Apa*I) | Wild type | 159 | 122 | 144 | 0.8 | 0.9 |
| SP1359(λ*Apa*I) | *lrp*::Tn*10* | 169 | 146 | 190 | 0.9 | 1.1 |
| SP1358(λ*Apa*I) | *livR* | 145 | 116 | 127 | 0.8 | 0.9 |

[a] λΔ15 contains a *tdh-lacZ* fusion with the putative operator sequence deleted.
[b] λ*Apa*I contains a *tdh-lacZ* fusion with a 4-bp substitution in the putative operator sequence.
[c] β-Galactosidase values are in Miller units and are the means of at least three independent experiments ($\sigma < 11\%$).

TABLE 5. β-Galactosidase activities of strains harboring λ*serA*[a] and λΔ*serA*[b] under various growth conditions

| Strain | Relevant genotype | β-Galactosidase activity[c] with: | | | Repression ratio for leucine | Repression ratio for casein hydrolysate |
|---|---|---|---|---|---|---|
| | | Minimal medium | Minimal + leucine | Minimal + casein hydrolysate | | |
| BW3912(λ*serA*) | Wild type | 446 | 189 | 153 | 2.4 | 2.9 |
| SP1359(λ*serA*) | *lrp*::Tn*10* | 104 | 88.8 | 67.3 | 1.2 | 1.5 |
| SP1358(λ*serA*) | *livR* | 1,280 | 1,130 | 716 | 1.1 | 1.8 |
| BW3912(λΔ*serA*) | Wild type | 1,010 | 430 | 239 | 2.3 | 4.2 |
| SP1359(λΔ*serA*) | *lrp*::Tn*10* | 76.9 | 76.7 | 40.3 | 1.0 | 1.9 |
| SP1358(λΔ*serA*) | *livR* | 1,360 | 1,280 | 605 | 1.1 | 2.2 |

[a] λ*serA* contains a *serA-lacZ* fusion including approximately 1 kb of upstream DNA.
[b] λΔ*serA* contains a *serA-lacZ* fusion including 457 bp of upstream DNA.
[c] β-Galactosidase values are in Miller units and are the means of at least three independent experiments ($\sigma < 15\%$).

cated a complete loss of leucine regulation at the *tdh* promoter (Table 4). These results suggest that the 12-bp sequence identified by computer analysis is important in the regulation of the *tdh* operon.

**Sequence of the *serA* promoter.** The sequence of the *serA* promoter has not been reported. Tuan et al. (43) have shown that *serA* is regulated by Lrp and leucine. A segment of DNA containing *serA* and its natural promoter, along with a preliminary sequence of the promoter region, was obtained from G. Grant. A recombinant λ phage containing a *serA-lacZ* protein fusion was constructed and inserted as a lysogen into wild-type, *lrp*::Tn*10*, and *livR* strains. β-Galactosidase assays on these strains showed that the *serA* promoter is present on this DNA fragment (Table 5). As shown previously (43), *serA* was constitutively repressed in an *lrp*::Tn*10* strain. Interestingly, the *serA* promoter was constitutively induced in the *livR* strain (Table 5). These results suggest that both Lrp and LivR affect *serA* expression but in a manner that is the opposite of the way they both affect *tdh* expression. Similar β-galactosidase values were obtained in studies of a construct (λΔ*serA*) that contained only 457 bp of DNA upstream of the *serA-lacZ* fusion (Table 5). Although the Δ*serA* promoter was about twice as strong in the wild-type background, the ability of leucine to regulate β-galactosidase expression was unaltered. Therefore, the site of action of Lrp-LivR must still be present on this 457-bp promoter fragment. The sequence of this DNA fragment is presented in Fig. 2. There is no obvious Lrp consensus sequence in the 457-bp promoter fragment. Since the amplitude of leucine regulation of *serA* is relatively small, it is possible that the observed effect of leucine-Lrp is indirect or that this promoter has a target for Lrp that cannot be identified by the computer algorithm that was employed.

## DISCUSSION

The *tdh* operon encodes two genes necessary for the conversion of threonine to glycine and acetyl coenzyme A. Levels of the gene products of the *tdh* operon rise seven- to eightfold when cells are grown in the presence of leucine. A novel selection scheme was utilized to acquire mutants in a putative *trans*-acting factor that is responsible for this leucine induction. One such mutant, SP1234(λpdr1), was shown to contain a single mutation in the 20-min region that was responsible for high leucine-independent expression of β-galactosidase from the *tdh* promoter. An isogenic strain containing an *lrp*::Tn*10* allele [SP1300(λpdr1)] exhibited identical leucine-independent expression from the *tdh* promoter. There are some small effects (twofold) of leucine on the *lrp* strains with and without pBR322. It is possible that there are other minor factors involved in the regulation of the *tdh* promoter. Overexpression of Lrp from a high-copy-number plasmid repressed expression from the *tdh* promoters of both SP1234(λpdr1) and SP1300(λpdr1) under all growth conditions tested. These results support the proposal (43) that Lrp is a transcriptional regulator of the *tdh* operon.

Lrp is known to regulate the expression of at least four other operons in *E. coli*: *ilvIH* (31), *oppA* (40a), *sdaA*, and *serA* (43). It is thought that Lrp acts differently on these operons in that it represses expression of *sdaA*, *oppA*, and *tdh* and induces expression of *ilvIH* and *serA*. In the case of *ilvIH*, leucine has been shown to interfere with the ability of Lrp to bind to the promoter DNA, possibly inhibiting the promoter activation (37). This model can be applied to all Lrp-regulated promoters in that Lrp can bind to the promoter DNA and either repress or activate transcription. Leucine may inhibit the Lrp effect by removing Lrp from the DNA. If this model is accurate, the Lrp-responsive promoter sequences may contain a similar consensus sequence for Lrp binding.

To delineate the 5′ boundary of the *tdh* operator, a series

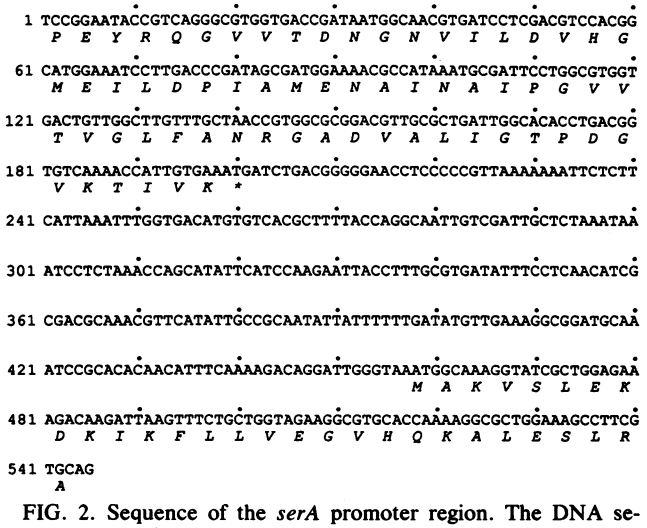


FIG. 2. Sequence of the *serA* promoter region. The DNA sequence starts with the *Bsp*EI site used to make λΔ*serA* and ends with the site of fusion with *lacZ*. The amino acid sequence of both the unidentified upstream open reading frame and *serA* are indicated below the DNA sequence. The *serA* coding region begins with the ATG at 458 (42).

of deletion mutants lacking progressively greater amounts of DNA from the region upstream of the *tdh* promoter was fused to *lacZ*. The −69 fusion exhibited near-normal induction by leucine. Leucine inducibility of the *tdh* promoter was abolished when a segment between −69 and −44 was removed. A similar 12-bp nonpalindromic sequence was found once in this region of the *tdh* promoter and in the promoter regions of the four Lrp-regulated operons which have been sequenced. In the −69 fusion, the first 3 bases of this 12-bp sequence are replaced by 3 bases from the vector. By chance, the first two of these three bases are identical to the original sequence, leaving only a T-to-C change at −70. This conservative change could explain the diminished induction by leucine of the −69 fusion compared with the −122 fusion.

This presumptive operator is also present within the promoter regions of other operons that are known to be regulated by leucine: *lysU* (18), *livJ*, and *livKHMGF* (34). In the two transport systems (*opp* and *liv*), the sequence was found in the promoters of both the periplasmic binding proteins (*oppA*, *livJ*) as well as the other genes necessary for transport (*oppBCDF*, *livKHMGF*). The sequence was found at various distances from the RNA polymerase-binding site, either upstream or downstream, and sometimes on the complementary strand. There does not seem to be a simple correlation between the placement of the potential operator relative to the promoter and the mode of regulation. Disruption of this 12-bp sequence in the *tdh* promoter either by deletion or by a 4-bp substitution abolished the effect of leucine-Lrp on expression. This 12-bp sequence was found four times upstream of the *ilvIH* promoter. Lrp protects the DNA from DNase I and λ exonuclease at three of these four sites (37). This evidence is consistent with the notion that this 12-bp sequence plays an important role in regulation.

Lrp is thought to be evolutionarily related to AsnC, a regulatory protein that can positively stimulate the expression of one operon and negatively affect its own promoter (47). AsnC stimulates expression from the *asnA* promoter. Stimulation is reduced by asparagine (12, 23). Kolling and Lother (23) proposed a consensus sequence for AsnC binding as TT(A/T)TT(T/G)(A/C)ATG. Although this sequence is not similar to the proposed Lrp-binding site, it is a short, nonpalindromic, A+T-rich sequence that occurs in both orientations and at various distances from the promoters it regulates. It is conceivable that two evolutionarily related proteins have retained similar mechanisms of regulation yet have distinct binding site specificities.

The sequence of the *serA* promoter region has not been reported. A DNA fragment of 1.1 kb containing the *serA* promoter was incorporated into a *lacZ* reporter system. β-Galactosidase assays showed that external leucine and an *lrp*::Tn*10* mutation had identical effects on *serA* expression, corresponding in magnitude to previously reported effects (43). A DNA fragment containing 456 bp upstream of the *serA* coding region was sequenced. When the Lrp consensus table (Table 3) was used to scan the *serA* promoter sequence, the highest score for any 12-bp sequence was 57.58. This score is significantly lower than the worst score of the possible Lrp sites (Table 3). When the *serA* promoter within this DNA fragment was used to drive *lacZ*, the effect of leucine and of the *lrp*::Tn*10* mutation was unaltered, indicating that the site of action of Lrp, or of a factor that responds to Lrp, is still present in this construct. The effects of leucine and *lrp*::Tn*10* on *serA* expression were relatively small compared with their effects on the other genes or operons of the leucine regulon (Table 6). Among the possible models that might explain this result is the possibility that Lrp is regulating an intermediate effector that in turn regulates *serA*.

TABLE 6. Effect of leucine and the *lrp*::Tn*10* allele on expression of operons in the leucine regulon

| Gene | Function | Fold effect on expression[a] | | Reference |
|---|---|---|---|---|
| | | +Leu | *lrp*::Tn*10* | |
| *tdh* | TDH | 8–10×↑ | 20×↑ | This work, 5 |
| *sdaA* | Serine deaminase | 4×↑ | 8×↑ | 43 |
| *oppA* | Oligopeptide transport | 5×↑ | 8×↑ | 3, 40a |
| *ilvIH* | Acetohydroxy acid synthase III | 5–10×↓ | 30×↓ | 31 |
| *serA* | Phosphoglycerate dehydrogenase | 2×↓ | 4–5×↓ | This work, 43 |

[a] As determined by β-galactosidase activity from transcriptional-translational *lacZ* fusions.

LivR has been considered to be a leucine-responsive repressor of the genes responsible for leucine transport (*livJ*, *livKHMGF*) (4). A mutation in *livR*, the gene that encodes LivR, leads to high-level constitutive expression of *livJ* and *livK* (1). It is possible that the previously described *livR* mutation is in fact an allele of Lrp that renders the protein unresponsive to leucine. In a *livR* background, the *tdh* promoter was found to be constitutively repressed, while the *serA* promoter was constitutively activated. Disruption of the putative Lrp-binding site abolishes repression at the *tdh* promoter. A potential Lrp target consensus sequence was found near the LivR-regulated promoters of *livJ* and *livKHMGF*. These results are consistent with a model that a *livR* mutation is one in which leucine is unable to negate the binding of Lrp to its target site. Such a mutation in Lrp has been independently isolated (31, 37). However, one cannot yet rule out the possibility that the function of LivR is to modify leucine or another cofactor so that it can act on Lrp. The possible allelism of Lrp and LivR is currently being addressed.

Both *livR* strains and *lrp*::Tn*10* strains are valine (0.5 μg/ml) and azaleucine (100 μg/ml) sensitive. Both of these phenotypes have been shown previously for a *livR* mutation (1, 33). In the *lrp*::Tn*10* strain, *ilvIH* is expressed at very low levels, imposing a partial starvation for the branched-chain amino acids. Therefore, addition of a smaller amount of valine would tend to impose starvation for isoleucine. The levels of the branched-chain amino acid biosynthetic enzymes and of the aromatic amino acid permease (AroP) can affect the sensitivity of the cell to azaleucine (17). Lrp regulates the expression of *ilvIH* and possibly *ilvGMEDA* and *aroP* (see below). Therefore, the common phenotypes of these two strains can be explained even though the mutations they harbor affect leucine-regulated promoters differently.

The computer program Fitconsensus was modified so that the consensus table for the Lrp recognition sequence (Table 3) could be used to scan the nucleic acid data bases in search of other potential Lrp-regulated promoters. This program assigns a numerical quality score for every 12-bp sequence and displays those sequences that are above an arbitrary quality score. Since the lowest quality score of a probable authentic target in the consensus table was 65.3 (*oppA*) (Table 3), only those sequences with a score above 65.3 were considered a hit (36a). Among all the *E. coli* K-12 sequences in the GenBank and EMBL data bases, there were 420 hits.

Since over 1.3 megabases of chromosomal *E. coli* K-12 have been sequenced (24), a hit occurs every 3,000 bases in the chromosome. Therefore, without any suggestive experimental evidence, many of these hits are most likely artifacts. However, 165 of these hits occur in 123 promoter regions. A promoter region was defined as the segment of DNA extending from 300 bp upstream of the translation start (or transcription start, if known) to 100 bp downstream of the translation start. Hits were found in the promoter regions of many genes for which regulation by leucine has been observed or suggested. Included in this group are the genes for adenylate cyclase (8), serine hydroxymethyl transferase (15), methionine adenosyltransferase (15), and tyrosine permease (21), in addition to those already incorporated into the consensus table.

There is experimental evidence that Lrp functions as a transcriptional regulator for *glyA*, *metK*, and *lysU*. Tuan et al. (43) have shown that strain RG62, a commonly studied *metK* strain, actually is a double mutant containing an *lrp* mutation. Previously, it was shown that in strain RG62, *lysU* is constitutively expressed and *glyA* and residual *metK* activity become leucine inducible (15, 26). The effect of Lrp-LivR on *glyA* expression is currently under investigation. The 12-bp consensus sequence was also found near the promoters of *ilvGMEDA* and *aroP*, operons whose gene products could be potentially important in mediating the azaleucine-sensitive phenotype of the *lrp* strain.

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