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Characterization of the Regulon Controlled by the Leucine-Responsive Regulatory Protein in *Escherichia coli*

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The leucine-responsive regulatory protein (Lrp) has been shown to regulate, either positively or negatively, the transcription of several *Escherichia coli* genes in response to leucine. We have used two-dimensional gel electrophoresis to analyze the patterns of polypeptide expression in isogenic *lrp*$^+$ and *lrp* mutant strains in the presence or absence of leucine. The absence of a functional Lrp protein alters the expression of at least 30 polypeptides. The expression of the majority of these polypeptides is not affected by the presence or absence of 10 mM exogenous leucine. Outer membrane porins OmpC and OmpF, glutamine synthetase (GlnA), the small subunit of glutamate synthase (GltD), lysyl-tRNA synthetase form II (LysU), a high-affinity periplasmic binding protein specific for branched-chain amino acids (LivJ), W protein, and the enzymes of the pathway converting threonine to glycine, namely, threonine dehydrogenase (Tdh) and 2-amino-3-ketobutyrate coenzyme A ligase (Kbl), were identified as members of the Lrp regulon by electrophoretic analysis. We have shown that Lrp is a positive regulator of glutamate synthase and glutamine synthetase and that exogenous leucine has little or no effect on the expression of these proteins. In strains carrying a *glnL* deletion and in strains carrying the *glnL2302* allele, which directs the synthesis of a GlnL protein that is constitutively active, expression of glutamine synthetase is no longer regulated by Lrp, demonstrating that the effect of Lrp on glutamine synthetase levels is indirect and requires an intact *glnL* gene. *lrp*::Tn*10* strains grow poorly when arginine or ornithine is present as the sole nitrogen source in the medium. On the bases of present studies and previous research, we propose that Lrp is involved in the adaptation of *E. coli* cells to major shifts in environment, such as those which occur when *E. coli* leaves the intestinal tract of its animal host. Several genes required for amino acid and peptide transport and catabolism are negatively regulated by Lrp, and other genes required for amino acid biosynthesis and ammonia assimilation in a nitrogen-poor environment are positively regulated by Lrp.

The leucine-responsive regulatory protein (Lrp) controls the transcription of a number of genes in *Escherichia coli*. The *lrp* gene was initially cloned and sequenced as *oppI*, a gene encoding a repressor of the oligopeptide permease operon that is responsible for the transport of tripeptides across the inner membrane (1). Lrp has subsequently been shown to regulate the expression of other genes and operons. The *ihb* gene, encoding a transcriptional activator of the *ilvIH* operon (32); *livR*, a repressor of the genes of the high-affinity transport system for branched-chain amino acids (14); and *rbl*, a locus encoding a regulator of several genes (18) were all shown to be identical to the *lrp* gene. Rex and coworkers have demonstrated that Lrp regulates the expression of the *tdh* and *serA* operons (33a).

LysU (lysyl-tRNA synthetase form II), a heat shock protein that is normally expressed only at elevated temperature, is expressed at 37°C when 10 mM leucine is added to the medium and is constitutively expressed in *metK* strains (16). Newman and coworkers have shown that *metK* strains grow very slowly and accumulate secondary *lrp* mutations that allow faster growth (18). Thus, *metK* strains rapidly become *metK lrp*. The expression of LysU at 37°C in *metK* strains has been shown to be associated with the secondary *lrp* mutation, and Lrp has been shown to lead to repression of the *lysU* gene by binding to the region upstream of this gene (19).

The diverse set of genes regulated by Lrp suggested a regulon controlled by Lrp, with the effect of Lrp on expression of the target genes modulated by leucine (18, 32). In order to determine the size and composition of the regulon and to learn more about its physiological function in *E. coli*, we examined the effects of several *lrp* alleles on the pattern of protein expression seen on two-dimensional gels. Two-dimensional gel electrophoresis is a useful technique for gathering simultaneous information about the net rates of synthesis of many of the most abundant cellular proteins. The results of our preliminary analysis indicate that Lrp controls an extensive regulon, including many polypeptides whose expression is unaffected by exogenous leucine. All genes previously identified as members of the Lrp regulon were responsive to exogenous leucine. Identification of some of the polypeptides of the Lrp regulon by their migration positions on a two-dimensional gel showed that a part of the regulon consists of proteins with prominent roles in the regulation of nitrogen metabolism. These proteins are among the group whose expression is not affected by exogenous leucine.

MATERIALS AND METHODS

Bacterial strains. The *E. coli* K-12 strains used in this work are described in Table 1.

Media and growth conditions. All cultures were grown aerobically in rotary action shakers at the indicated temperature. The growth of cells was monitored spectrophotometrically. Cells were grown in 3-(*N*-morpholino)propanesulfonic acid (MOPS) minimal medium (26) supplemented with 10 μM thiamine, 0.4% glucose as the carbon source, and amino acids as indicated, except for the glutamine synthetase

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TABLE 1. *E. coli* strains used in this work

Strain	Description	Source
YMC10	*thi-1 endA1 hsdR17 ΔlacU169 hutC*$_k$	B. Magasanik
YMC15	YMC10 *glnL2302*	B. Magasanik
TH16	YMC10 *glnA21*::Tn*5*	B. Magasanik
RB9132	YMC10 Δ*glnL2001*	B. Magasanik
W3110	F$^-$ prototroph	F. C. Neidhardt
MEW26	*ilvA lrp-201*::Tn*10*	E. B. Newman
CV1008	F$^-$ *ara thi* Δ(*lac-pro*) *ilvIH*::Mu dI1734 *lrp-35*::Tn*10*	J. M. Calvo
CV1011	F$^-$ *ara thi* Δ(*lac-pro*) *ilvIH*::Mu dI1734 *lrp-1 zca*::Tn*10*	J. M. Calvo
CV1014	CV1008 (pCV168, *lrp*$^+$)	J. M. Calvo
BE1	W3110 *lrp-201*::Tn*10*	This work
BE2	W3110 *lrp-35*::Tn*10*	This work
BE3	W3110 *lrp-1 zca*::Tn*10*	This work
WS7000	W3110 *glnA*::Tn*5*	This work
WS7001	BE1 *glnA*::Tn*5*	This work
WS7002	BE2 *glnA*::Tn*5*	This work
WS7011	W3110 *glnALG*$^+$ from YMC10	This work
WS7014	W3110 Δ*glnL2001*	This work
WS7017	W3110 *glnL2302*	This work
WS7023	BE1 *glnALG*$^+$ from YMC10	This work
WS7026	BE1 Δ*glnL2001*	This work
WS7029	BE1 *glnL2302*	This work
WS1035	BE2 *glnALG*$^+$ from YMC10	This work
WS7038	BE Δ*glnL2001*	This work
WS7041	BE2 *glnL2302*	This work

assays, for which W salts medium was used (31). The concentrations of amino acids were those used in defined rich medium (39) unless noted otherwise. Unless stated otherwise, the MOPS medium contained 9.52 mM ammonium chloride. Media lacking ammonium ion as the nitrogen source were supplemented with 0.2% glutamine, 0.2% arginine, or 0.2% ornithine. Media sometimes contained ampicillin (50 μg/ml), kanamycin (50 μg/ml), or tetracycline (20 μg/ml). Cultures were maintained on Luria-Bertani agar plates (35) supplemented with appropriate antibiotics.

Construction of strains. Generalized transduction mediated by P1 *vir* was carried out as described by Miller (24). Strains BE1 and BE2 were derived from strain W3110 and were isolated by selection for tetracycline resistance following transduction with P1 *vir* lysates of strains MEW26 (18) and CV1008 (32) respectively. Strains MEW26 and CV1008 carry independently constructed Tn*10* insertions in the *lrp* gene. Strain BE3 was constructed by transduction of strain W3110 with a P1 *vir* lysate of strain CV1011 and selection for tetracycline resistance. Strain CV1011 contains the *lrp-1* allele, which encodes a leucine-insensitive Lrp protein, and also contains a *zca*::Tn*10* insertion, which is close to but not within the *lrp* gene (32). Cotransduction of the *lrp-1* allele with *zca*::Tn*10* was confirmed by the ability to move the *lrp-1* allele from strain BE3 into strain CV975. The presence of the *lrp-1* allele in strain CV975 was determined by using the plate test for β-galactosidase activity described by Platko et al. (32).

Strains with altered *glnL* alleles were constructed by using generalized P1 transduction to introduce a *glnA*::Tn*5* allele from strain TH16 into strains W3110, BE1, and BE2. These strains were designated WS7000, WS7001, and WS7002 and were kanamycin-resistant glutamine auxotrophs. The *glnALG* operon in strains WS7000, WS7001, and WS7002 was then replaced, by using P1 transduction, with *glnALG* operons bearing three *glnL* alleles: a wild-type *glnALG* operon from strain YMC10 (2), a *glnL* deletion from strain RB9132 (7), and the *glnL2302* allele from strain YMC15 (9). In all cases, strains with altered *glnL* alleles were isolated by selection for glutamine prototrophy conferred by the introduction of a wild-type *glnA* allele. These strains are listed in Table 1.

Gel retardation assay. The gel retardation assay for binding of Lrp to the 406-bp fragment of the *ilvIH* promoter region from pCV112 was carried out as described by Ricca et al. (34). Cells were grown in glucose minimal MOPS medium until an A_{420} of 1.0 was reached and were collected by centrifugation and resuspended in buffer containing 10 mM Tris chloride (pH 8), 10% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 0.2 M NaCl. The cells were then sonicated and centrifuged at 16,000 × *g* for 15 min. The supernatant was used in the gel retardation assay.

Radioactive labeling of protein and resolution on two-dimensional gels for autoradiography. Samples (1 ml) of cultures of exponentially growing cells ($A_{420} \approx 0.3$) were transferred to prewarmed vials containing L-[^{35}S]methionine (60 μCi; 1,056 Ci/mmol) and incubated for 5 min. Unlabeled methionine (17 μmol) was added for a 3-min chase. Cultures to be labeled after a heat shock were shifted from 37 to 44°C at an $A_{420} \approx 0.3$ and pulse-labeled as described above from 5 to 10 min after the shift. Extracts were prepared and processed for resolution on two-dimensional polyacrylamide gels (29) as previously modified (3). Gels were fixed, stained with Coomassie blue, dried, and exposed to Kodak XAR film for 1 to 4 days.

Assays. Glutamate synthase activity was assayed by using the supernatant fraction from cells that had been grown to an $A_{420} \approx 0.3$, sonicated, and centrifuged at 16,000 × *g* for 30 min. The procedure for preparation and assay is based on that described by Pahel et al. (31). This procedure compares the rate of oxidation of NADPH at 340 nm in the presence of α-ketoglutarate and in the presence or the absence of glutamine. Glutamine synthetase assays were carried out as described by Pahel et al. (30). Cultures were grown in W salts medium (31) until an A_{600} of ≈1.0 was reached. Cells were then permeabilized, and either Mn^{2+} or Mg^{2+} was added as a cofactor. The γ-glutamyl transferase activity of glutamine synthetase was determined by monitoring the conversion of glutamine to glutamyl hydroxamate at 540 nm in the presence of ADP and hydroxylamine. Protein determinations were carried out by the method of Lowry et al. (20).

RESULTS

Construction and preliminary characterization of *lrp* mutant strains. To examine the regulation of polypeptide expression by Lrp, we wished to compare three *lrp* alleles in an isogenic background. The background chosen was that of strain W3110, which has been used in the construction of the gene-protein index (38) and the Kohara library (37). Strain W3110 is *lrp*$^+$. We compared it either to isogenic strains that lack a functional *lrp* gene or to a strain that produces a leucine-insensitive Lrp protein. For these purposes, we transduced strain W3110 with two *lrp* insertion mutations, *lrp-201*::Tn*10* and *lrp-35*::Tn*10*, constructed in the laboratories of Newman (18) and Calvo (32), respectively, and with an *lrp-1* allele that confers insensitivity to leucine on the Lrp protein but retains activity of Lrp as a transcriptional regulator (40). The construction of these strains is described in Materials and Methods, and the genotypes of these strains and their precursors are shown in Table 1.

TABLE 2. Doubling times of the isogenic strains W3110 (*lrp*$^+$), BE1 (*lrp*::Tn*10*), BE2 (*lrp*::Tn*10*), and BE3 (*lrp-1*) during exponential growth at 37°C in media of various compositions

Additions to minimal MOPS medium[a]	Doubling time (min) of strain			
	W3110 (*lrp*$^+$)	BE1 (*lrp*::Tn*10*)	BE2 (*lrp*::Tn*10*)	BE3 (*lrp-1*)
0.4% Acetate, thiamine	202	278	340	ND[b]
0.4% Glucose, thiamine	57	80	99	58
0.4% Glucose, thiamine, Ile, Val	55	74	84	55
0.4% Glucose, thiamine, Ile, Val, Leu[c]	64	72	80	58
0.4% Glucose, thiamine, 19 amino acids (no Met)	34	34	34	38

[a] The concentrations of amino acid supplements were those described by Wanner et al. (39).
[b] ND, not determined.
[c] Leucine was added at 9 final concentration of 10 mM.

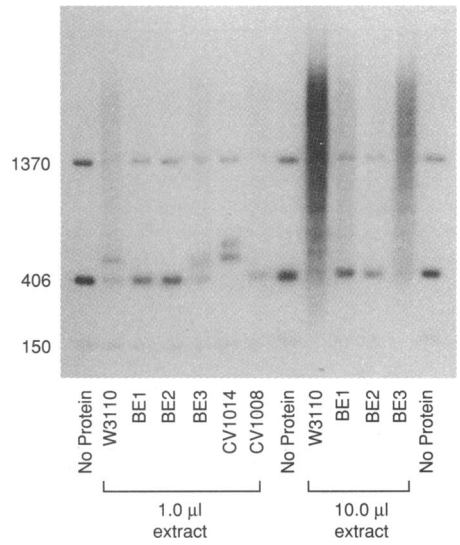

FIG. 1. Gel retardation assay for Lrp. Crude extracts (1 or 10 μl) of each of the indicated strains were mixed with the 1,370-, 406-, and 150-bp ^{32}P-labeled fragments of pCV112 (34), which were generated by digesting the plasmid with *Hin*dIII, *Eco*RI, and *Bgl*I. The 406-bp fragment contains the *ilvIH* promoter region and is the only fragment to show a mobility shift in this assay. Total protein concentrations (in micrograms per microliter) in each extract were as follows: W3110, 1.27; BE1, 0.53; BE2, 0.56; BE3, 0.79; CV1008, 1.03; CV1014, 0.73.

As a preliminary characterization of the growth properties of *lrp* strains, doubling times of strains BE1 (*lrp-201*::Tn*10*), BE2 (*lrp-35*::Tn*10*), and BE3 (*lrp-1*) were compared with the doubling times of strain W3110 in several media (Table 2). In a minimal medium supplemented with thiamine and all of the amino acids except methionine, strains BE1, BE2, and W3110 grew at the same rate. When cells were grown in glucose minimal MOPS medium lacking amino acids, strains BE1 and BE2 grew more slowly than strain W3110. The addition of isoleucine and valine to the growth medium supported slightly faster growth of both wild-type and mutant strains. The further addition of 10 mM leucine slowed the growth of strain W3110. This leucine concentration is much higher than the 0.8 mM concentration used in defined rich medium (39), and such high leucine concentrations are toxic to most strains of *E. coli*, especially in the absence of isoleucine and valine (13, 33). The addition of 10 mM leucine had the opposite effect on strains BE1 and BE2, supporting faster growth, as previously reported (18). The effect of leucine on *lrp*::Tn*10* strains suggests that the toxic effect of leucine on the wild-type strain is at least partly mediated by Lrp. When strain BE3, which produces a leucine-insensitive Lrp protein, was grown in glucose minimal medium, it grew at the same rate as did the wild-type strain W3110. Addition of 10 mM leucine did not significantly slow the growth of strain BE3, further supporting the hypothesis that leucine toxicity is mediated by Lrp. Strain BE3 did grow more slowly than strains W3110, BE1, and BE2 in medium supplemented with thiamine and all amino acids except methionine.

Differences in the growth rates of isogenic strains BE1 and BE2 might be attributed to a partially functional Lrp in strain BE1. In order to confirm that neither of these strains produces a functional Lrp, we assayed Lrp DNA binding activity in crude extracts (Fig. 1). Extracts prepared from strains W3110 (*lrp*$^+$) and BE3 (*lrp-1*) showed mobility shifts characteristic of the presence of a functional Lrp protein (32) when 1 μl of extract was used. An extract of strain CV1014, an Lrp overproducer, also showed a pattern of bands similar to that previously described for this strain, while an extract of strain CV1008 (*lrp-35*::Tn*10*), the parent of strain BE2, did not show detectable Lrp activity (32). Extracts of strains BE1 and BE2 did not show detectable Lrp activity, even when 10 μl of crude extract was included in the binding reaction.

Two-dimensional gel analysis of *E. coli lrp* mutants. To assess the size of the Lrp regulon and the patterns of regulation of its component genes, two-dimensional gel electrophoresis was used to determine the net rate of synthesis of cellular polypeptides in strains with and without a wild-type *lrp* gene. All differences in polypeptide expression were confirmed in two or more independent labelings. Strains BE1 (*lrp-201*::Tn*10*) and W3110 were grown in glucose minimal MOPS medium supplemented with thiamine, isoleucine, and valine in the presence or absence of 10 mM leucine. These cultures were pulse-labeled with [^{35}S]methionine. The levels of ^{35}S incorporation into trichloroacetic acid-precipitable material did not differ significantly in strains W3110 and BE1 (data not shown). Polypeptides were separated by two-dimensional gel electrophoresis and visualized by autoradiography. Visual comparison of the autoradiograms showed that at least 30 polypeptides were synthesized at different net rates in strain BE1 compared with strain W3110 in the presence or absence of exogenous leucine (Fig. 2).

To corroborate the results seen with strain BE1 (*lrp-201*::Tn*10*), we examined other *lrp* mutant strains. Strain BE2, containing an independently derived *lrp*::Tn*10* allele (*lrp-35*::Tn*10*) in the isogenic W3110 background, was labeled, and polypeptides were separated on two-dimensional gels. The pattern of polypeptide expression in strain BE2 was similar to that in strain BE1 under all conditions tested. The effect of the *lrp-35*::Tn*10* allele on the 30 polypeptides whose expression is affected by Lrp was the same as the effect of the *lrp-201*::Tn*10* allele, despite the difference in the growth rates of strains BE1 and BE2 in the labeling medium. The fact that strains BE1 and BE2 show the same patterns of regulation for all 30 polypeptides suggests that the differences in polypeptide expression between wild-type and *lrp* strains are not due exclusively to differences in growth rate.

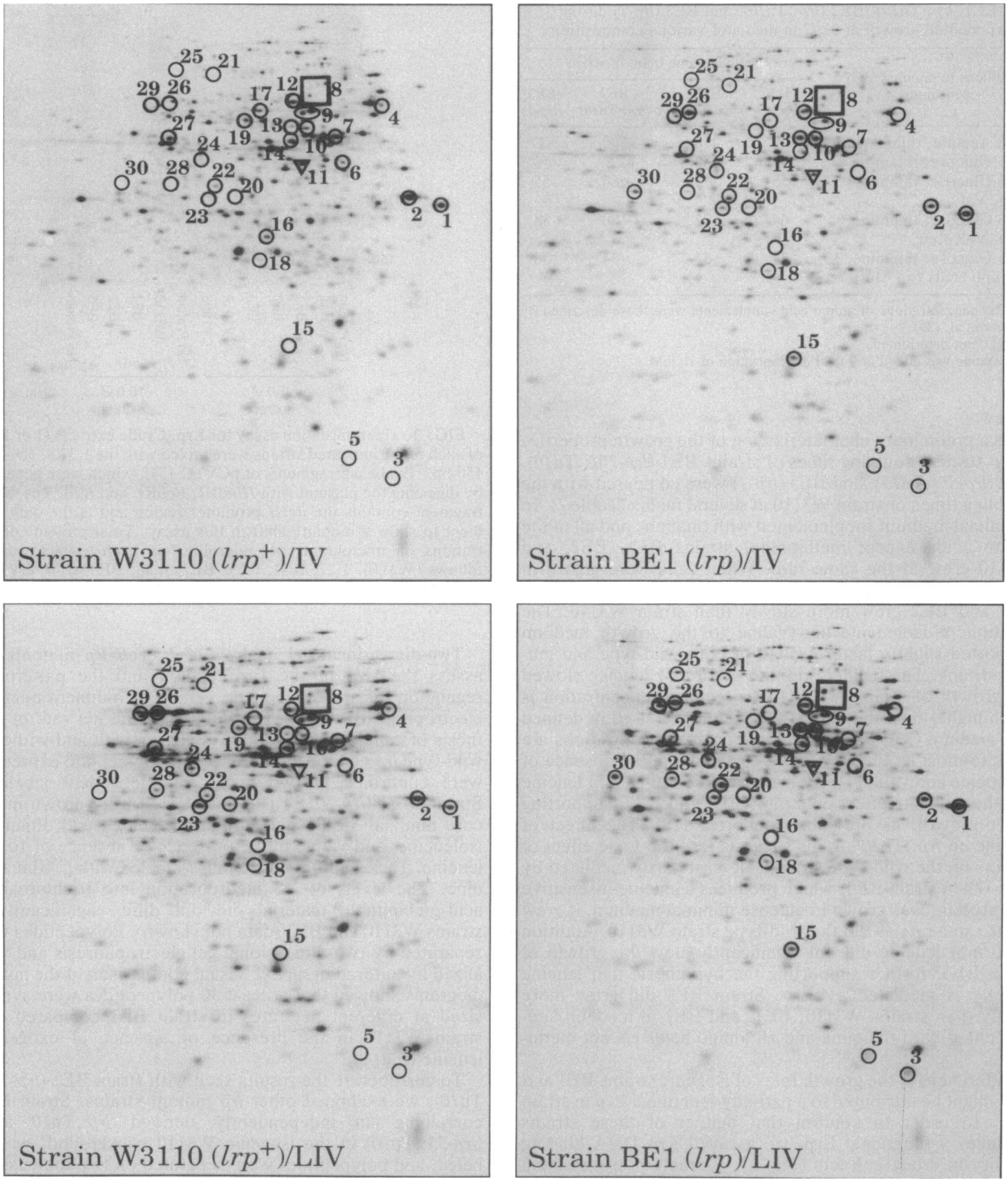

FIG. 2. Synthesis of individual polypeptides in strains W3110 and BE1 (W3110 *lrp*::Tn*10*) at 37°C. Cells were grown in glucose minimal MOPS medium supplemented with thiamine, isoleucine (0.4 mM), and valine (0.4 mM) plus or minus 10 mM leucine. Cultures were labeled for 5 min with [^{35}S]methionine during exponential growth. Polypeptides were separated by isoelectric focusing in the horizontal dimension (acidic polypeptides migrate to the right) and by electrophoresis in the presence of sodium dodecyl sulfate in the vertical dimension (small polypeptides migrate to the bottom). Circles, squares, and triangles indicate polypeptides whose synthesis is altered in strain BE1 under one or both labeling conditions. The squares enclose the LysU (upper left) and LysS (lower left) isoenzymes; the triangles enclose LivJ, the polypeptide whose expression is altered only in the presence of leucine; and the ovals enclose the adenylylated and unadenylylated forms of glutamine synthetase. Circles enclose other members of the regulon. The numbers next to each polypeptide correspond to the polypeptide numbers in Table 3.

It is possible, however, that some differences in expression are due to the fact that *lrp* strains grow more slowly than do wild-type strains.

Strain BE3, which carries the leucine-insensitive *lrp-1* allele, showed identical patterns of polypeptide expression whether or not leucine was included in the medium (data not shown). In both cases, the pattern of expression resembles that seen when strain W3110 is grown in the absence of leucine. These results are consistent with the description of the *lrp-1* allele as directing the expression of a leucine-insensitive Lrp protein (40). The leucine-insensitive Lrp protein in strain BE3 retains the ability to regulate all but one of the polypeptides regulated by the wild-type Lrp protein. The polypeptide whose expression is altered in comparisons of strains BE3 and W3110 is a polypeptide (LivJ) that is normally negatively regulated by Lrp only in the presence of leucine. In strain BE3, negative regulation of this polypeptide is not seen when leucine is present in the medium.

The 30 polypeptides whose expression was influenced by Lrp exhibited five modes of regulation (Table 3). Examples of these patterns of regulation are seen in Fig. 3. Lrp may act positively or negatively or have no effect, and Lrp in the presence of exogenous leucine may act in any of these ways. Ten of the 30 polypeptides are positively or negatively regulated by Lrp in the absence of added leucine, and the addition of 10 mM leucine to the medium abolishes regulation by Lrp. These patterns of regulation are those expected if Lrp were to act as an activator or repressor only in the absence of exogenous leucine. Most (19 of 30) of the polypeptides listed in Table 3 are regulated by Lrp in the same manner whether or not 10 mM leucine is added to the growth medium. This was the first indication that the majority of genes in the regulon are expressed at levels that are unaffected by the presence or absence of leucine in the medium.

If leucine does not modulate Lrp regulation of a subset of genes in the regulon, their regulation may nevertheless be modulated by another metabolite. Alanine is known to modulate the expression of several of the genes in the Lrp regulon. LysU is induced when 20 mM alanine is included in the growth medium (16). The enzymes required to convert threonine to glycine and acetyl coenzyme A (threonine dehydrogenase and 2-amino-3-ketobutyrate coenzyme A ligase) are induced by alanine (17), and alanine blocks positive regulation of the *ilvIH* promoter by Lrp (8). The expression of each of these genes is affected by leucine as well as by alanine. We wished to determine whether genes whose expression is not modulated by leucine might nevertheless be modulated by alanine. Two-dimensional gel analysis of strains W3310, BE1, and BE3 showed that none of the leucine-independent members of the regulon responded to alanine (data not shown).

Identification of the proteins of the Lrp regulon. Identification of the Lrp-regulated proteins was undertaken in order to understand which areas of metabolism are controlled by Lrp. The polypeptides of the Lrp regulon were assigned coordinates based on their migration during two-dimensional electrophoresis and comparison with a reference gel. Alphanumerics, consisting of a letter and a number (38), were then assigned (Table 3). To avoid inconsistencies, alphanumerics were assigned in consultation with R. A. VanBogelen and F. C. Neidhardt and are now incorporated into the gene-protein data base. The letter refers to the isoelectric point of the polypeptide as determined by equilibrium isoelectric focusing in 8 M urea, with A indicating an acidic isoelectric point and H indicating a basic isoelectric point. The number refers to the approximate molecular weight of the polypeptide. Our two-dimensional gel electrophoresis was performed under conditions identical to those used in the Neidhardt laboratory for assignment of coordinates. In some cases, our assigned coordinates corresponded to those of a previously identified protein. OmpC and OmpF (the major outer membrane porins) and W protein, which is required for the efficient translation of some mRNAs (10), are identified on the basis of gel position alone. Wherever possible, other methods of identification such as comigration with purified proteins and/or enzyme assay were used to confirm the identities of regulated polypeptides.

When an unlabeled pure protein is coelectrophoresed with a radiolabeled extract, the unlabeled protein dilutes out the corresponding labeled protein, and that spot becomes diffuse. Kbl (2-amino-3-ketobutyrate coenzyme A ligase), Tdh (threonine dehydrogenase), and glutamate synthase have been purified (4, 5, 25) and were confirmed to be members of the regulon by comigration of purified protein samples. The

TABLE 3. Polypeptides whose expression is regulated by Lrp

Polypeptide no.[a]	Coordinates[b]	Alphanumeric and gene name[b]	Mode of regulation by Lrp[c]	
			−Leu	+Leu
2	115 × 80	B36.0 *ompF*	+	+
4	104 × 102	B56.0	+	+
7	89 × 93	C43.5	+	+
9a	76 × 99	D49.9 *glnA* (unadenylylated)	+	+
9b	78 × 100	D50.0 *glnA* (adenylylated)	+	+
14	72 × 92	E42.5[d]	+	+
19	60 × 98	F50.4 *gltD*	+	+
27	40 × 93	G43.2 W protein	+	+
1	120 × 80	A35.5 *ompC*	−	−
3	115 × 13	A10.8[d]	−	−
5	93 × 18	C11.5	−	−
10	77 × 95	D48.4[e]	−	−
12	75 × 103	D58.0	−	−
13	73 × 96	E48.0[d]	−	−
15	70 × 42	E17.6[d]	−	−
18	66 × 64	F23.9	−	−
21	53 × 108	G70.1[d]	−	−
22	53 × 83	G38.1	−	−
25	43 × 109	G72.1[d]	−	−
30	22 × 84	H40.0[d]	−	−
6	92 × 88	C42.0[d]	+	0
16	67 × 68	F28.6	+	0
17	66 × 100	F56.1	+	0
8	78 × 105	D60.5 *lysU*	−	0
20	58 × 79	F32.7[d]	−	0
23	52 × 78	G35.0	−	0
24	50 × 88	G42.2 *kbl*[d]	−	0
26	42 × 102	G57.9	−	0
28	39 × 84	G38.8 *tdh*[d]	−	0
29	31 × 101	H55.0[d]	−	0
11	76 × 88	D40.7 *livJ*	0	−

[a] Numbers are those designating polypeptides in the Lrp regulon in Fig. 1.
[b] Coordinates and alphanumerics designate the position of the polypeptide on a reference two-dimensional gel as described by VanBogelen et al. (38).
[c] +, positive effect of Lrp; − negative effect of Lrp; 0, no apparent effect of Lrp.
[d] These alphanumerics will appear in a future edition of the gene-protein index (38).
[e] D48.4 migrates to the same position as does D48.5 and has been assigned the same coordinates, although the two proteins are different.

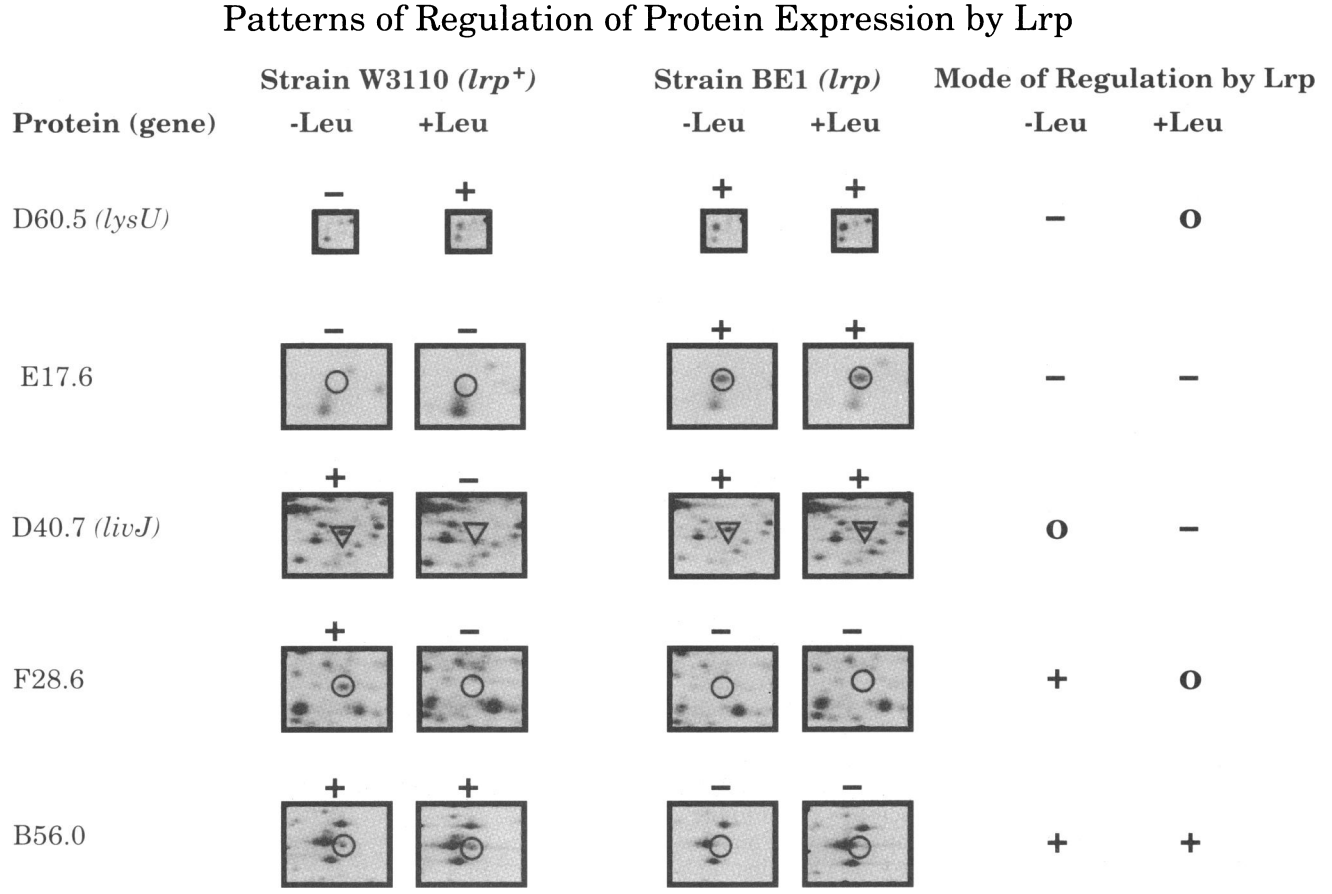

FIG. 3. Five modes of regulation of polypeptide expression by Lrp. One example of each mode of regulation is shown, with the proteins listed according to the alphanumerics from the gene-protein index. The boxes enclose small regions of the autoradiograms presented in Fig. 1. The mode of regulation of polypeptide synthesis by Lrp (positive [+] or negative [−]) was determined by comparing the net rates of synthesis in the presence and absence of a functional Lrp protein. Polypeptides that are positively regulated by Lrp show decreased rates of synthesis in strains lacking a functional Lrp protein, while polypeptides that are negatively regulated by Lrp show increased rates of synthesis in the absence of a functional Lrp protein. 0, no effect of Lrp was noted.

large subunit of glutamate synthase is encoded by the *gltB* gene, which is cocistronic with *gltD* (11). Because these two genes share the same promoter, the large subunit of glutamate synthase would be expected to be regulated in the same manner as the small subunit, but it was not resolved on these gels because of its large molecular mass of approximately 153 kDa and its basic isoelectric point (11, 22).

The list of regulated proteins shown in Table 3 does not include all proteins whose expression is known to be regulated by Lrp. IlvI and IlvH, subunits of an enzyme of the branched-chain amino acid biosynthetic pathway, have been shown to be regulated by Lrp at the level of transcription (32) but have not been detected in the two-dimensional electrophoretic analysis. The isoelectric points of IlvI and IlvH are too basic for these proteins to be detected on equilibrium two-dimensional gels. L-Serine deaminase and 3-phosphoglycerate dehydrogenase are also regulated by Lrp (18) but have not yet been identified by comigration.

Glutamate synthase and glutamine synthetase play important roles in the assimilation of ammonium ion by the cell and in the regulation of proteins required for the use of poor nitrogen sources under conditions in which the ammonium ion concentration is low (31). Since Lrp affected the synthesis of GltD and GlnA (Fig. 2 and Table 3), we examined the effects of Lrp on the corresponding enzymatic activities. Glutamate synthase activity was assayed in cells grown under the same conditions as were used for the two-dimensional electrophoretic analyses. The results of enzyme assays of glutamate synthase in isogenic *lrp*$^+$ and *lrp*::Tn*10* strains are shown in Table 4. Glutamate synthase activity is undetectable in strain BE1 (*lrp* mutant) and is 1.8 nmol s^{-1} mg^{-1} in strain W3110 grown in the absence of leucine. The addition of 10 mM leucine to the medium used for growth of

TABLE 4. Glutamate synthase activity in strains W3110 and BE1 grown in media with and without leucine

Additions to glucose minimal MOPS medium	Glutamate synthase activity (nmol s^{-1} mg^{-1}) in strain	
	W3110 (*lrp*$^+$)	BE1 (*lrp* mutant)
Thiamine, Ile, Val[a]	1.81 ± 0.53	0.002 ± 0.010
Thiamine, Ile, Val, Leu[b]	0.87 ± 0.13	0.002 ± 0.000

[a] The concentrations of amino acid supplements were those described by Wanner et al. (39).

[b] Leucine was added at a final concentration of 10 mM.

TABLE 5. Glutamine synthetase specific activity and adenylylation state

Growth medium and supplements	Specific activity[a] (nmol min^{-1} mg^{-1}) in strain (no. of adenylylates per dodecamer)[b]		
	W3110 (*lrp*$^+$)	BE1 (*lrp*::Tn*10*)	BE2 (*lrp*::Tn*10*)
Glucose minimal medium + thiamine			
+ Gln	1,465 (2.6)	338 (8.6)	447 (10.8)
+ Gln, Ile, Val, 10 mM Leu	1,486 (3.0)	315 (10.3)	413 (10.4)
+ Ammonia, Gln	197 (8.0)	103 (8.7)	103 (8.9)
+ Ammonia, Gln, Ile, Val, 10 mM Leu	166 (8.4)	112 (8.8)	111 (8.8)
Luria broth			
+ Gln	72 (9.8)	78 (10.1)	70 (10.1)
+ Gln, Ile, Val, 10 mM Leu	73 (9.0)	70 (10.0)	83 (10.2)

[a] Specific activities were determined as described in Materials and Methods, using Mn^{2+}. Under these conditions, both adenylylated and unadenylylated forms of glutamine synthetase are active.

[b] Adenylylates per dodecamer were determined from the ratio of specific activities in the presence of Mg^{2+} or Mn^{2+}. When Mg^{2+} is present, only the unadenylylated enzyme is active.

TABLE 6. Glutamine synthetase specific activity in strains with various *glnL* alleles

Strain and relevant genotype	Specific activity (nmol min^{-1} mg^{-1}) in growth medium		
	Glucose minimal medium + thiamine		Luria broth + Gln
	+ Gln	+ Ammonia, Gln	
WS7011 (*lrp*$^+$ *glnL*$^+$)	1,560	131	50
WS7014 (*lrp*$^+$ Δ*glnL*)	2,101	1,025	118
WS7017 (*lrp*$^+$ *glnL2302*)	1,473	851	555
WS7023 (*lrp-201*::Tn*10* *glnL*$^+$)	398	98	75
WS7026(*lrp-201*::Tn*10* Δ*glnL*)	1,915	886	142
WS7029 (*lrp-201*::Tn*10* *glnL2302*)	1,095	808	541
WS7035 (*lrp-35*::Tn*10* *glnL*$^+$)	367	96	86
WS7038 (*lrp-35*::Tn*10* Δ*glnL*)	2,000	1,175	116
WS7041 (*lrp-35*::Tn*10* *glnL2302*)	1,345	1,379	437

strain W3110 leads to a level of glutamate synthase activity about half that observed in the absence of leucine. In contrast, strain BE1 had several-hundredfold-lower activity, regardless of the presence or absence of leucine.

Glutamine synthetase is normally present in the cell in two forms, unadenylylated and adenylylated. The unadenylylated form is catalytically active, while the adenylylated form is inactive. These two forms differ in size and isoelectric point and can be separated on two-dimensional gels. When glutamine synthetase is assayed in the presence of Mg^{2+}, the physiological divalent ion required by this enzyme, only the unadenylylated form is active, but when the enzyme is assayed in the presence of Mn^{2+}, both the adenylylated and unadenylylated forms are active. By using these assays, it is possible to determine the total level of glutamine synthetase present in the cell and the degree of adenylylation of the enzyme (36). Table 5 shows the results of measurements of glutamine synthetase activity and adenylylation state in strains W3110, BE1, and BE2. Two differences in glutamine synthetase regulation were noted between the *lrp*$^+$ and *lrp*::Tn*10* strains. In nitrogen-limiting medium, with glutamine as the sole nitrogen source, glutamine synthetase activity is approximately fourfold lower in the *lrp* strains than in strain W3110, when measured under conditions in which both the adenylylated and unadenylylated enzymes are active. Under these conditions, most of the glutamine synthetase enzyme is unadenylylated in strain W3110, while most of the enzyme is adenylylated in the *lrp* strains. Thus *lrp*::Tn*10* strains have one-fourth the total level of glutamine synthetase seen in *lrp*$^+$ strains, and a larger proportion of the enzyme is in the adenylylated, inactive state. During growth in glucose minimal medium containing a high concentration of ammonia, the differences in total glutamine synthetase activity and adenylylation state between *lrp*$^+$ and *lrp*::Tn*10* strains are smaller but still significant. The effect of the *lrp* gene product on expression of glutamine synthetase activity appears to be completely independent of the presence or absence of leucine in the medium, in agreement with the patterns of expression deduced from two-dimensional electrophoretic analysis. When *lrp*$^+$ and *lrp*::Tn*10* strains are grown in Luria broth, the effect of Lrp on the level and adenylylation state of glutamate synthase is almost nonexistent.

Lrp regulates glutamine synthetase levels indirectly. The regulation of glutamine synthetase by Lrp can be explained as an effect secondary to the regulation of glutamate synthase activity. Strains that lack glutamate synthase activity are generally unable to induce proteins required for growth on many poor nitrogen sources (31), because glutamine cannot be converted to glutamate in these strains, and the intracellular ratio of glutamine to 2-ketoglutarate is consequently high. Glutamine synthetase activity is normally low in these strains, because activation and transcription of glutamine synthetase are initiated in response to a low ratio of glutamine to 2-ketoglutarate (7).

In order to rule out the possibility that the reduced level of glutamine synthetase in strains BE1 and BE2 was due to an abnormal *glnALG* operon, this region was replaced by a wild-type *glnALG* operon from strain YMC10, which was used in studies of the regulation of the glutamine synthetase promoter (2). As shown in Table 6, replacement of the *glnALG* region (strains WS7011, WS7023, and WS7035) did not alter the effect of Lrp on the glutamine synthetase level.

When a *glnL* deletion (7) was crossed into strains W3110, BE1, and BE2, the level of glutamine synthetase was no longer sensitive to the presence or absence of a functional Lrp protein (Table 6, strains WS7014, WS7026, and WS7038). The level of glutamine synthetase in strains carrying the *glnL2302* allele (9), which produces a GlnL protein that directs constitutive synthesis of glutamine synthetase, was also insensitive to the presence or absence of a functional Lrp protein (Table 6, strains WS7017, WS7029, and WS7041). These results indicate that the Lrp regulation of glutamine synthetase is not direct; rather, it is dependent on a functional GlnL protein. Furthermore, the constitutive kinase encoded by the *glnL2302* allele, which phosphorylates the transcriptional activator of *glnA*, also overrides the effect of *lrp* on the level of glutamine synthetase, suggesting that the effect of *lrp* is on the regulation of GlnL activity. Plate tests showed that strains BE1 and BE2 grow poorly on arginine or ornithine when no other nitrogen source is present, as do *gltBD* mutants (31). After 48 h of growth with arginine or ornithine as the sole nitrogen source at 37°C, strain W3110 had formed colonies but strains BE1 and BE2

had not. These results show that *lrp*::Tn*10* alleles confer an Ntr$^-$ phenotype and that a functional Lrp protein is required for the induction of the proteins of the Ntr regulon. Introduction of the *glnL2302* allele into strains BE1 and BE2 suppressed the Ntr$^-$ phenotype of these strains and allowed growth on arginine as the sole nitrogen source, showing that this effect of Lrp is also indirect and requires the participation of GlnL. Thus, the *lrp* mutation mimics the effects of a *gltBD* mutation (6, 31).

Regulation of heat shock polypeptides by Lrp. It has previously been shown that a *metK lrp* strain shows abnormalities in the expression of heat shock polypeptides. One heat shock protein, LysU, is expressed at 37°C (16), and two others, C14.7 and G13.5, are not induced following a shift from 37 to 44°C (23). When the *metK lrp* strain carried a plasmid expressing the wild-type *metK* gene product, induction of C14.7 and G13.5 was normal but LysU was still expressed at 37°C (23). In the present study, two-dimensional gel analysis of strain BE1 at 37 and 44°C showed that induction of C14.7 and G13.5 was normal upon a shift from 37 to 44°C, while LysU was expressed at 37°C. These results confirm that the abnormal expression of heat shock polypeptides in a *metK lrp* double mutant has complex origins. The failure to induce C14.7 and G13.5 is not associated with *lrp*, while the expression of LysU at 37°C is associated with the *lrp* locus.

DISCUSSION

In this study, we used two-dimensional gel electrophoretic analysis of the effect of Lrp on the expression of abundant cellular polypeptides in *E. coli* to investigate the size and composition of the Lrp regulon. In contrast with genetic methods previously used to identify genes in the Lrp regulon, this method does not require any knowledge of the phenotypic consequences of regulation by Lrp. Our results have demonstrated the existence of a large number of polypeptides whose expression is affected by Lrp but unaffected by the addition of leucine to the medium. Among this group of polypeptides, we have identified glutamine synthetase (GlnA), the small subunit of glutamate synthase (GltD), and the outer membrane porins OmpF and OmpC. For glutamine synthetase and glutamate synthase, we have confirmed the pattern of regulation seen on the two-dimensional gels by enzyme assays in crude extracts from isogenic *lrp*$^+$ and *lrp*::Tn*10* strains. We detected approximately twofold-lower glutamate synthase activity when *lrp*$^+$ strains were grown in the presence of exogenous leucine, but this stands in contrast to the several-hundredfold-lower glutamate synthase activity in an *lrp* strain. It is possible that other polypeptides apparently unaffected by the addition of leucine to the medium will show slightly altered levels of expression in response to leucine when more sensitive assays are carried out.

Where information has previously been published about the patterns of regulation of genes in the Lrp regulon, our results are in agreement with the observations of others. Haney et al. (14) have recently measured LivJ (the high-affinity branched-chain amino acid periplasmic binding protein) activity in strains W3110 and BE1, and their measurements show that LivJ activity is reduced in an *lrp*$^+$ strain when leucine is added to the medium. The addition of leucine has no effect on LivJ activity in *lrp*::Tn*10* strains. Their results are in complete agreement with our observations based on electrophoretic analysis. Lin et al. (19) report measurements of lysyl tRNA synthetase activity in isogenic strains MEW1 (*lrp*$^+$) and MEW26 (MEW26 contains the *lrp-201*:Tn*10* allele transduced into strain BE1). These activity measurements are consistent with repression of *lysU* by Lrp and with our observations of LysU expression by electrophoretic analysis. Lin and coworkers have also reported that threonine dehydrogenase activity is elevated in *lrp*::Tn*10* strains (18), and these results are again consistent with the results of our electrophoretic analysis.

It is important to note that for many of the polypeptides detected as members of the Lrp regulon, we do not yet know whether the regulation we observe is exerted by Lrp directly or indirectly. Regulation of several of the proteins of the Lrp regulon has been investigated by others and shown to occur at the level of transcription. We have established that the regulation of glutamine synthetase levels by Lrp is indirect. Transcription of the *glnA* gene requires the phosphorylated form of the *glnG* gene product, NR_I. The activity of NR_I as a transcriptional activator of *glnA* is regulated by a kinase, NR_{II}, which is the product of the *glnL* gene. The activity of the kinase is regulated in turn by a pathway that ultimately responds to the ratio of glutamine to α-ketoglutarate in the cell (for a review, see reference 21). This ratio also determines the degree of adenylylation and thus the activity of glutamine synthetase (36). Previous results have indicated that strains with a deletion in *glnL* are capable of expressing *glnA* and that the transcriptional regulation of *glnA* is less sensitive to ammonia in these strains (7, 28). Our results show that the deletion of the *glnL* gene abolishes the effect of Lrp on the level of glutamine synthetase, indicating that the effect of Lrp on GlnA expression requires a functional *glnL* gene product. Experiments with strains containing a constitutively active kinase, encoded by the *glnL2302* allele, indicated that the effects of Lrp on glutamine synthetase levels are also abolished in these strains. Thus Lrp appears to affect the level of glutamine synthetase by altering the regulation of kinase activity. We believe that the effect of Lrp on the level and activity of glutamine synthetase is secondary to the effect of Lrp on expression of glutamate synthase. It has been demonstrated that strains lacking glutamate synthase activity are unable to induce proteins of the Ntr regulon, including glutamine synthetase (6, 31). It is hypothesized that these strains show this Ntr$^-$ phenotype because they have a high ratio of glutamine to α-ketoglutarate because of the inability to convert glutamine to glutamate. The *glnL2302* allele suppresses the Ntr$^-$ phenotype of strains lacking glutamate synthase activity (9). In the absence of a functional Lrp protein, glutamate synthase activity is undetectable, and we postulate that this leads to the observed decrease in the expression and activity of glutamine synthetase. Since Lrp appears to affect glutamine synthetase through controls common to the Ntr regulon, we expected that other proteins of this regulon would also fail to be induced in strains lacking a functional Lrp protein. The very slow growth of *lrp* strains when arginine or ornithine is present as the sole nitrogen source indicates that *lrp* strains are Ntr$^-$. It will be of interest to determine how many of the polypeptides of the Lrp regulon are members of the Ntr regulon, and experiments to assess the overlap between the Ntr and Lrp regulons by electrophoretic analysis are in progress.

The indirect regulation of glutamine synthetase and other proteins of the Ntr regulon by Lrp provides a working model for the way in which a single regulatory protein can control the expression of a large number of gene products. In the Lrp regulon, glutamate synthase may serve as one of several lieutenants, each affecting its own platoon of target genes

and proteins. Many of the polypeptides whose expression is regulated by Lrp are also members of other regulons. LysU is a member of the heat shock regulon (27), while OmpF and OmpC are known to be regulated by the *ompR* and *envZ* regulatory genes (12). The fact that these proteins are members of other regulons suggests that regulation by Lrp may intersect with previously defined channels of regulation. In the case of LysU, Lrp regulation appears to be direct and not through signal transduction pathways mediating the heat shock response (19). Direct regulation of LysU by Lrp is consistent with the observation that other heat shock proteins are not regulated by Lrp.

The observation of a large class of polypeptides regulated by Lrp independently of exogenous leucine raises the question of how the effect of Lrp on these polypeptides is modulated. If this regulation is indeed modulated in response to altered physiological conditions, we can envisage several possibilities. The expression of Lrp itself might be modulated, although this has not yet been demonstrated. Alternatively, the activity of Lrp might be modulated, either by an unidentified metabolite or metabolites or by interaction with another regulatory protein whose expression or activity is modulated in response to changing physiological conditions. The interaction of the *Saccharomyces cerevisiae* transcriptional activator protein GAL4 with the accessory activator protein GAL11 provides one model for a system of transcriptional activation requiring two proteins (15).

Our results, combined with those of other researchers, provide some insight into the physiological role of the regulon controlled by Lrp. Genes that are positively regulated by Lrp, either directly or indirectly, encode proteins involved in the assimilation of nitrogen under nitrogen-limiting conditions (GltD and GlnA), proteins involved in the biosynthesis of amino acids (IlvIH and SerA), and proteins involved in the adaptation of the cell to media of low osmolarity and low temperature (OmpF). A number of genes that are negatively regulated by Lrp are involved in the transport and catabolism of amino acids and peptides (the proteins of the oligopeptide permease operon, Tdh, Kbl, and SdaA) and in the adaptation of the cell to media of high osmolarity and high temperature (OmpC and LysU). Thus there is a correlation between genes that are positively regulated by Lrp and genes that are needed for survival in an environment that is nitrogen limited, cold, and of low osmolarity; such an environment may be encountered during the growth of *E. coli* outside of its animal host. A similar correlation is apparent between genes that are negatively regulated by Lrp and genes that are needed during growth in an environment that is warm and of high osmolarity and in which nitrogen is provided by peptides and amino acids, i.e., conditions encountered in the intestinal tract of the host. The presence of leucine in the external medium is one, but presumably not the only, means by which *E. coli* senses the intestinal environment. On the basis of these observations, we propose that Lrp is involved in the adaptation of *E. coli* to major shifts in the environment, such as those which occur when *E. coli* leaves the host intestinal tract. Further testing of this hypothesis will require an understanding of the factors that modulate the effect of Lrp on genes that do not respond to leucine in the medium, and it will require the identification of additional genes in the Lrp regulon and the characterization of the phenotypic differences between *lrp*$^+$ and *lrp* mutant strains of both pathogenic and nonpathogenic *E. coli*.

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