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Use of an Inducible Regulatory Protein To Identify Members of a Regulon: Application to the Regulon Controlled by the Leucine-Responsive Regulatory Protein (Lrp) in *Escherichia coli*

SAMIR P. BHAGWAT,[1] MANDA R. RICE,[1] ROWENA G. MATTHEWS,[2] AND ROBERT M. BLUMENTHAL[1]*

Department of Microbiology and Immunology, Medical College of Ohio, Toledo, Ohio 43614-5806,[1] and Biophysics Research Division and Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109-1055[2]

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Procedures were developed to facilitate the identification of genes that belong to a given regulon and characterization of their responses to the regulator. The regulon controlled by the *Escherichia coli* leucine-responsive regulatory protein (Lrp) was studied by isolating random transcriptional fusions to *lacZ*, using λp*lac*Mu53 and a strain in which *lrp* is under isopropylthio-β-D-galactopyranoside (IPTG)-inducible control. Fusions exhibiting IPTG-responsive β-galactosidase activity were cloned by integrating the suicide vector pIVET1 via homologous recombination at *lacZ*, followed by self-ligating digested chromosomal DNA. We verified the patterns of *lacZ* expression after using the plasmid clones to generate merodiploid strains with interrupted and uninterrupted copies of the same sequence. If the merodiploid expression pattern was unchanged from that shown by the original fusion strain, then the cloned fusion was responsible for the regulatory pattern of interest; a difference in the expression pattern could indicate that the original strain carried multiple fusions or that there were autogenous effects of having interrupted the fused gene. Using these procedures, we generated a fusion library of ~5×10^6 strains; ~3,000 of these strains were screened, yielding 84 Lrp-responsive fusions, and 10 of the 84 were phenotypically stable and were characterized. The responses of different fusions in a given operon to in vivo Lrp titrations revealed variations in expression with the position of insertion. Among the newly identified members of the regulon is an open reading frame (*orf3*) between *rpiA* and *serA*. Also, expression of a fusion just downstream of *dinF* was found to be Lrp dependent only in stationary phase.

Our understanding of cell physiology is changing from the extremely reductionist viewpoint of cells as collections of individual macromolecules specified by strings of self-sufficient genes toward the more complex view of interacting proteins and RNAs specified by cross-signaling global regulons. Regulons are sets of genes controlled by a common regulator, and since many genes are controlled by multiple regulators, there can be considerable overlap between regulons (11, 47). We are interested in the roles played by global regulons in integrating a cell's responses to its environment.

Escherichia coli is perhaps the best-studied organism in the biosphere, yet it was not until 1991 that the leucine-responsive regulatory protein (Lrp) was recognized as being a global regulator. It had been suggested previously that L-leucine might be an important regulatory signal, since it was seen to affect the expression of several unrelated operons (24, 53). A single gene, *lrp*, was found to be required for most of these leucine effects; *lrp* is located at 20 min on the *E. coli* chromosome and specifies an 18.8-kDa polypeptide that forms a homodimer (64). Lrp is a sequence-specific DNA-binding protein that contains a predicted helix-turn-helix motif (51, 64). Lrp binds and bends regulatory sequences upstream of several operons in order to exert its effect on expression (3, 13, 16, 17, 20, 25, 26, 39, 55, 56, 61–63). In this regard, it is a classical gene regulator. The abundance of Lrp in the cell (~3,000 copies/haploid genome) (34, 64) and its degree of DNA sequence specificity (17) are intermediate between specific DNA regulators like the *lac* repressor and general chromosomal organizers like H-NS and HU (33, 49).

Targets of Lrp regulation include genes involved in amino acid biosynthesis, such as *ilvIH* (52), *ilvGMEDA* (55), *leuABCD* (38), *serA* (40, 54), *glnALG* (19), and *gltBDF* (19); genes involved in amino acid catabolism, such as *gcv* (38), *tdh* (40, 54), *kbl* (40, 54), *dad* (28), and *sdaA* (40); and genes required for (or regulating) the transport of metabolites, such as *livJ* (27), *livKHMGF* (27), *oppABCDF* (5–7), *ompC* (21), and *micF* (21). As a broad generalization, Lrp activates genes that are required for amino acid biosynthesis and represses genes that are required for amino acid catabolism (15, 49). This insight, along with the fact that expression of *lrp* itself is low in a nutritionally rich medium like Luria-Bertani (LB) and higher in a minimal medium (34, 38), led to the hypothesis that Lrp allows the cell to adapt to changes in the nutrient content of the growth medium (19).

A most intriguing aspect of Lrp regulation is the variety of ways in which leucine, the effector, can modulate the regulatory effect of Lrp. For operons that are activated by Lrp, leucine may antagonize the activation (e.g., *ilvIH* [52]), it may be required for the activation (e.g., *fimB*- and *fimE*-promoted switching [25]), or it may have relatively little effect on activation (e.g., *gltBDF* [19, 20]). These same three patterns of leucine responsiveness are seen for operons that are repressed by Lrp, as exemplified by *tdh* (40, 54), *livJ* (27), and *ompC* (21). Lrp thus stands in sharp contrast to gene regulators such as the *lac* repressor, where the coregulator modulates the effect of the regulator in only one way. In a comparison of two Lrp-regulated operons, leucine antagonized the activation of *ilvIH* more strongly than that of *gltBDF* (20). Gel mobility shift assays

* Corresponding author. Phone: (419) 383-5422. Fax: (419) 383-3002. E-mail: Blumenthal@opus.mco.edu.

TABLE 1. *E. coli* strains, plasmid, and phages used in this work

Name	Description[a]	Source and/or reference
Strains		
AAEC546	W3110 Δ*lacZYA lrp-35*::Tn*10* p_{lacUV5}::*lrp*	I. C. Blomfield (10)
BE10.2	W3110 *lrp-35*::Tn*10*	19
DB3	AAEC546 *gltB1594*(*psiQ32*)::*lacZ* (Mud1-1734)	D. Borst (12)
DH5α · λpir	Lysogen that supports replication of pIVET1	J. Mekalanos (41)
SPB101 and -102	AAEC546 *gltB4527*::*lacZ* (λp*lac*Mu fusion, merodiploid)	This work
SPB103 and -104	AAEC546 *gltB4702*::*lacZ* (λp*lac*Mu fusion, merodiploid)	This work
SPB105 and -106	AAEC546 *livG6919*::*lacZ* (λp*lac*Mu fusion, merodiploid)	This work
SPB107 and -108	AAEC546 *livK3663*::*lacZ* (λp*lac*Mu fusion, merodiploid)	This work
SPB109 and -110	AAEC546 *orf3-397*::*lacZ* (λp*lac*Mu fusion, merodiploid)	This work
SPB111	AAEC546 *zzz*::*lacZ*	This work
SPB113	AAEC546 *yyy*::*lacZ*	This work
SPB115 and -116	AAEC546 *dinF+103*::*lacZ* (λp*lac*Mu fusion, merodiploid)	This work
W3110	F^- prototrophic *E. coli* K-12 strain	F. C. Neidhardt
Plasmid		
pIVET1	Ap^r, promoterless *lacZ*, replication depends on pi protein	J. Mekalanos (41)
Bacteriophages		
P1*vir*	Used for generalized transduction	F. C. Neidhardt
λp*lac*Mu53	*lacZ* operon fusion λ phage with Mu ends, Km^r	G. Weinstock (14)
λp*lac*Mu507	Helper phage supplying Mu B	G. Weinstock (14)

[a] The allele number assigned to each of the various *lacZ* fusions corresponds to the nucleotide position of the fusion, using the numbering from an appropriate GenBank or EMBL entry. The *dinf+103* allele has a fusion 103 bp past the 3′ end of *dinf*.

indicate that the affinity of Lrp for the upstream region of *gltBDF* is greater than for *ilvIH*, with leucine reducing the amount of DNA binding in both cases; however, at an Lrp concentration matching the effective in vivo level, the higher-affinity *gltBDF* promoter region is nearly saturated and leucine shows little effect, while the *ilvIH* promoter region is not saturated and leucine has a profound effect (20). Based on these results, Ernsting et al. (20) proposed that the leucine sensitivity of positively regulated operons is a function of the intrinsic affinity of Lrp for the target operator, with higher-affinity operators showing lower sensitivity to leucine. This model was supported by in vivo studies that monitored the effects of leucine and varied Lrp levels on a *gltB*::*lacZ* operon fusion (12). To further test this model, as well as to understand the links between the Lrp regulon and other global regulons, we sought to obtain a battery of Lrp-controlled operons varying in leucine sensitivity and Lrp affinity.

Initially members of the Lrp regulon were identified by comparing two-dimensional gels of cell extracts of wild-type and *lrp* mutants (19) and by screening a λp*lac*Mu9 translational fusion library in the presence or absence of leucine (38). Our interest in leucine-insensitive operons, which were originally discovered by using two-dimensional electrophoresis of cell proteins (19), appeared to rule out our using fusion responses to leucine as a primary screening method. Furthermore, we are interested in identifying members of the Lrp regulon that range in affinity for Lrp. Thus, we made our fusion library by using a strain in which the *lrp* gene itself was inducible. We also developed approaches to cloning, confirming, and sequencing λp*lac*Mu53 fusions. Using these approaches, fusions could be subjected to an in vivo titration with Lrp, providing extensive information on the response of the targeted gene. Interestingly, the majority of fusions that we isolated did in fact respond to changes in the leucine concentration when subsequently tested, as predicted by the hypothesis (20) that all Lrp-controlled genes are leucine sensitive to some extent, with the degree of sensitivity a function of the affinity of Lrp for the relevant control region.

MATERIALS AND METHODS

Bacterial strains, plasmids, phages, and growth media. All bacterial strains, plasmids, and phages used in this study are listed in Table 1. Cultures were grown in LB broth or agar (58) or 3-(*N*-morpholino)propanesulfonic acid (MOPS) minimal glucose medium (46). The following antibiotics were used as needed at the given concentrations: carbenicillin, 20 and 120 μg/ml for stringent and relaxed replicons, respectively; tetracycline, 10 μg/ml; kanamycin, 10 μg/ml; and chloramphenicol, 25 μg/ml. In agar plates, where used, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and isopropylthio-β-D-galactopyranoside (IPTG) were added to final concentrations of 40 μg/ml and 0.2 mM, respectively. All cultures were incubated at 37°C.

Construction and screening of a *lacZ* transcriptional fusion library. A promoterless *lacZ* gene was randomly fused into various locations of the chromosome of strain AAEC546 (p_{lacUV5}::*lrp*) (10) by using λp*lac*Mu (14). The phage used to construct the transcriptional fusions, λp*lac*Mu53, carries the c end of phage Mu (including the genes Mu*cI*^{ts62}, *ner* [*c*II], and *A*) and the terminal sequences from the Mu S end (β end) but lacks a functional *B* gene (Fig. 1). In the absence of a helper phage supplying B protein, λp*lac*Mu53 transposes at a very low frequency and thus generates extremely stable and temperature-resistant lysogens (14). The presence of *kan* in λp*lac*Mu53 allows positive selection for integration into the chromosome independent of the Lac phenotype, allowing isolation of *lacZ* fusions under conditions where the expression of the fused gene is too low to give a detectably Lac^+ phenotype. There are translation termination codons in all three reading frames between the S end and the translation initiator for *lacZ*.

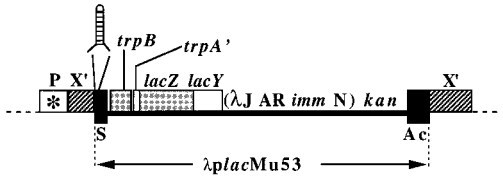

FIG. 1. Chromosomally integrated bacteriophage λp*lac*Mu53. The fusion library was generated by using λp*lac*Mu53 and λp*lac*Mu507 (helper phage) (14) as described in Materials and Methods. Random integration of λp*lac*Mu53 is achieved by using its bacteriophage Mu integration sequences (S and c), and Mu B function is provided by the helper phage. In the correct orientation, *lacZ* expression is driven from the promoter of the unknown gene (*); X′ is part of the unknown gene X that has been disrupted by integration of λp*lac*Mu53. The *lacZ* open reading frame is fused to a short portion of *trpA* and preceded by *trpB*. Not all λp*lac*Mu53 genes are shown, and genes derived from bacteriophage λ (in parentheses) are not to scale. At the S-end fusion junction, a 48-bp inverted repeat is indicated by the hairpin structure.

Strain AAEC546 was grown overnight in LB medium supplemented with tetracycline and 0.2% maltose, and the cells in a 1-ml portion were infected with 10^8 PFU of λp*lac*Mu53 and 10^9 PFU of λp*lac*Mu507 (helper phage). After incubation for 30 min at room temperature, the cells were washed three times with LB and brought up in 1 ml of λdil (8 mM $MgSO_4$, 10 mM Tris-HCl [pH 7.5]). Portions were spread, undiluted, onto LB agar plates containing tetracycline and kanamycin to yield dense but not confluent growth; at the same time, dilutions were plated to allow an estimate of CFU/milliliter in the infected culture. After overnight incubation of the plates, 1 to 2 ml of LB was added, the plates were scraped, and the suspended cells were pooled and frozen without further growth to generate a library of $\sim 5 \times 10^6$ fusion strains.

This library was screened for IPTG-dependent *lacZ* expression by replica plating onto six different agar media, each containing X-Gal: LB, LB-IPTG, minimal glucose, minimal glucose-IPTG, minimal glucose-isoleucine-valine-leucine, and minimal glucose-isoleucine-valine-leucine-IPTG. No antibiotics were included in the screening plates, as antibiotics can interfere with β-galactosidase activity (31). Lrp-responsive fusions were screened for stability by streaking out a frozen culture and retesting 10 independent colonies on each of the six agar media.

In vivo titrations with Lrp. Cultures were grown in glucose MOPS minimal medium (46) supplemented with isoleucine, valine, leucine (where indicated), and varied concentrations of IPTG. Samples were removed at various times from cultures in logarithmic growth. β-Galactosidase activity was determined by measuring *o*-nitrophenyl-β-D-galactopyranoside (ONPG) hydrolysis in permeabilized cells (44, 52). While the cultures were growing logarithmically (optical density at 600 nm [OD_{600}] of between 0.15 and 0.5), a series of 0.25-ml samples were removed and permeabilized in an equal volume of aqueous cetyl trimethylammonium bromide (200 μg/ml) and sodium deoxycholate (100 μg/ml) by incubation at 4°C overnight. Permeabilized cells (0.5 ml) were mixed with 0.5 ml of assay buffer (0.1 M sodium phosphate [pH 7.0], 1 mM $MgSO_4$, 2 mM $MnSO_4$, 50 mM β-mercaptoethanol) and 0.15 ml of ONPG stock solution (4 mg/ml). The assay mixtures were then incubated at 25°C until visibly yellow, at which point the reactions were terminated by adding 0.325 ml of 1 M Na_2CO_3. Cells were removed by centrifugation, and the A_{420} of the supernatant was measured. β-Galactosidase activity was calculated by dividing the A_{420} by the time allowed for the reaction and by the volume of the permeabilized cells used in the reaction. To determine fusion activity, the slope from linear regression of a plot of activity versus A_{600} of the culture was determined; this slope is expressed in arbitrary units: $(1{,}000 \times A_{420}\ \text{min}^{-1}\ \text{ml}^{-1})/\text{OD}_{600}$ of the culture.

Cloning and sequencing of fusions. The cloning strategy is illustrated in Fig. 2. The fusion strains were transformed with pIVET1 by electroporation. Since the fusion strains do not produce the pi protein (22), which is required for replication of pIVET1, stable expression of β-lactamase requires pIVET1 integration into the chromosome by homologous recombination. To clone each fusion, overnight cultures were grown from five different integrant colonies (to increase the probability that an integrant via *lacZ* was present), and chromosomal DNA was isolated by using a Purgene DNA isolation kit (Gentra Systems, Inc.). This DNA was digested with *Bgl*II and self-ligated at a concentration of 0.025 μg/μl for 16 h at 16°C in the presence of 1 U of T4 DNA ligase (Life Technologies). The ligation mix was used to transform strain DH5α · λ*pir*. Plasmid DNA from the carbenicillin-resistant (Cb^r) transformants was screened for putative clones by restriction mapping. Desired clones should lack an ~1-kb *Bgl*II-*Eco*RI fragment and show increased overall size as assessed by *Eco*RI digestion (Fig. 2). DNA from selected clones was used to transform AAEC546; integration would result from homologous recombination between the plasmid and chromosomal copies of the promoter regions upstream of the reporter *lacZ* gene. Such transformants, now merodiploid for the fused gene, were retested for effects of IPTG on expression by streaking onto medium containing or lacking IPTG as described above.

Clones were sequenced by using a RapidWell DNA sequencing kit (U.S. Biochemicals) and primers L19 and U173 (Table 2; Fig. 2C). The S end of Mud1, the progenitor of λp*lac*Mu53, contains a 48-bp hairpin presumably generated by illegitimate transposition (43). When primer L19 was used, progression of DNA polymerases through this hairpin structure was found to be inefficient and yielded strong bands in all lanes through the hairpin and faint (but readable) bands beyond the fusion junction. This was true even when we used thermostable polymerases and elevated temperatures (not shown). The sequence obtained from both primers was sufficient to allow unambiguous identification of the fusion junction, and in some cases this identification was confirmed using insert-specific primers.

RESULTS

Construction and screening of a transcriptional fusion library. To screen fusions for responsiveness to Lrp without depending on use of the coregulator leucine, we prepared a *lacZ* transcriptional (operon) fusion library in *E. coli* AAEC546. This strain, constructed by Blomfield et al. (10), carries the *lrp* gene under the control of a p_{lacUV5} promoter. Strain AAEC546 also has its native *lrp* gene disrupted by a Tn*10* insertion, and the *lac* operon has been deleted. In this strain, *lrp* expression is repressed by LacI and is induced by IPTG. It has been found that the Lrp levels in this strain are undetectable when IPTG is omitted from the medium and can be titrated up to a maximum of two-thirds of the level in wild-type strains when increasing amounts of IPTG are added to the medium (12).

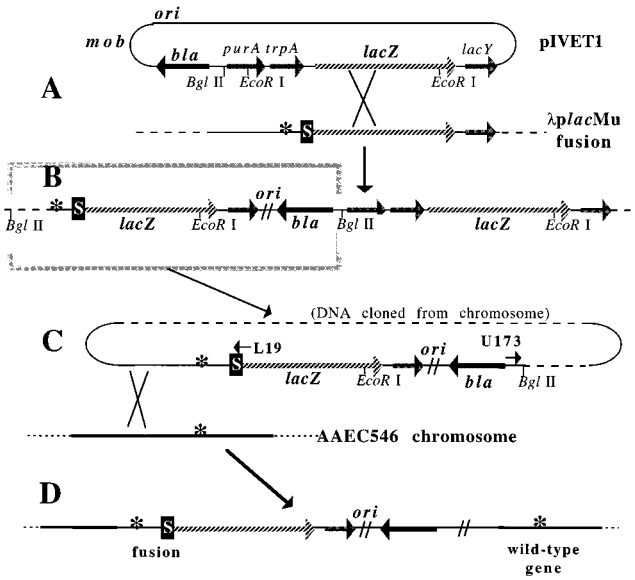

FIG. 2. Outline of the strategy for cloning and sequencing λp*lac*Mu53 fusions. The suicide vector pIVET1 (41) replicates autonomously only in a host that produces pi protein (22). S, bacteriophage Mu-derived S integration sequence; *, promoter for the fused gene. (A) Integration of pIVET1 into the chromosome of a λp*lac*Mu fusion strain. When pIVET1 is introduced into a *lacZ* fusion strain, stable carbenicillin resistance results from integration via homologous recombination. (B) Genetic map of the integrant strain generated by the recombination shown in panel A. Chromosomal DNA was isolated from a pool of several Cb^r integrants, cut with *Bgl*II, circularized by self-ligation, and introduced into strain DH5α · λ*pir* to allow autonomous replication. After carbenicillin selection, putative plasmid clones were isolated. (C) Genetic map of a plasmid containing the cloned fusion. Clones that yielded appropriate restriction maps and phenotypes were sequenced by using primers U173 and L19. (D) The plasmid shown in panel C was introduced into strain AAEC546, and the genetic map of the expected tandem merodiploid strain is shown. This strain was used to confirm the expression pattern of the cloned fusion.

To increase the sensitivity of our screen, the fusion library was screened by replica plating onto three media that each contained or lacked 0.2 mM IPTG: rich medium and minimal glucose medium containing or lacking leucine. Fusion strains that showed IPTG-responsive expression on one or more of these three media were subjected to further study. To test for phenotypic stability, frozen stocks of individual fusion strains were streaked onto a rich medium containing kanamycin (to select for λp*lac*Mu53), and isolated colonies were tested on all six plates for the original pattern of expression. Only fusions for which 10 of 10 tested colonies showed the original regulatory pattern on all six plates were studied further. Of more than 3,000 fusions initially screened by replica plating, 84 (~3%) showed Lrp-responsive regulatory patterns in primary screening; some of these could have been siblings. Of the 84 fusions tested, 10 were found to be phenotypically stable by our criteria.

In vivo titrations with Lrp to characterize regulatory patterns of fusions of interest. For the fusion strains showing stable Lrp-responsive expression, varying the amount of IPTG in the medium allowed an in vivo analysis of the effect of

TABLE 2. Oligonucleotide primers used in this study

GenBank accession no.	Database match	Primer used	
		Name	Sequence
M33723	140–122	L19	5′-GGGGTTAAGTAATGTTGTC-3′ (complementary to *trpB′* at S end of λp*lac*Mu53)
J01749	4254–4270	U173	5′-CCGCGCACATTTCCCCG-3′ (complementary to the region between the *Bgl*II site and *bla* gene of pIVET1)
J05516	3587–3606	liv577	5′-GCCGTTGTCGGCGCGATGTC-3′ (complementary to *livK*)

changing Lrp concentrations on fusion expression. In strain DB3, which differs from AAEC546 only by the presence of a *gltB*::*lacZ* fusion, the concentration of Lrp has been quantitated on Western blots as a function of the concentration of IPTG in glucose minimal medium; near-maximal expression of *lrp* is achieved in the presence of 50 μM IPTG (12). For each concentration of IPTG used, we measured fusion activity versus OD_{600} of the culture, as shown in Fig. 3. The linearity of plots such as this one confirm that the cultures were in pseudo-steady state (stable exponential) growth (1, 23), and the slopes provide an accurate measure of fusion expression. In Fig. 4, these slopes were plotted against the IPTG concentration.

Figure 4 summarizes the expression patterns of seven of the stable Lrp-responsive transcriptional fusions. Of the three fusions not shown, one yielded a pattern similar to that shown in Fig. 4G, and two gave responses similar to those shown in Fig. 4C to E. Transcription of the fusions shown in Fig. 4A and B is stimulated by Lrp, and leucine antagonizes the stimulation. These two fusions were found to be in the same gene (*gltB* [see below]), and while they exhibit similar regulatory patterns, they differ significantly in the level of transcription at a given IPTG concentration. Transcription of the fusions shown in Fig. 4C to E is stimulated by Lrp in the absence of leucine and depressed by Lrp in the presence of leucine; the leucine-dependent reduction in expression is shown more clearly in Fig. 4F, where the "+Leu" data from Fig. 4C to E have been replotted against an expanded *y* axis. Surprisingly, given the results of the original plate screening, Lrp and leucine had no discernible effects on transcription of the fusion shown in Fig. 4G (but see below). Transcription of the fusion shown in Fig. 4H is depressed by Lrp, and leucine antagonizes the depression.

It is possible that the internal cellular concentration of IPTG in these experiments was affected by varying expression of *lacY* (the lactose permease gene that is downstream of *lacZ* in λp*lac*Mu53). While IPTG diffuses across the bacterial membrane without the need for a permease, LacY can concentrate IPTG (44a). Any LacY-dependent effects would be most pronounced in those fusions having the highest levels of expression, and then only under conditions giving rise to high levels of expression. LacY concentrations will not affect the measured levels of fusion expression in the presence of a saturating concentration of IPTG or in the absence of IPTG. In sum, the presence of LacY could affect the apparent concentrations of IPTG required for half-maximal effects on fusion expression but will not affect the general patterns shown in Fig. 4.

Cloning and identification of the Lrp-responsive fusions. While methods have been developed to sequence λp*lac*Mu fusions without intermediate cloning steps (57, 59), cloning allows phenotypic verification that the fusion being sequenced is actually the desired Lrp-responsive fusion. It provides more information than, and obviates the need for, P1 transduction of the fusion into a clean background. The cloning method that we devised makes use of the suicide vector pIVET1 (Fig. 2), a plasmid that replicates only in the presence of the pi protein, provided in host strain DH5α · λ*pir* by a λ*pir*$^+$ prophage. In a *recA*$^+$ *pir* background, such as strain AAEC546, Cb^r transformants can be obtained by pIVET1 integration into the chromosome via homologous recombination (Fig. 2A). In the case of λp*lac*Mu53 fusion strains, such homologous recombination is most likely to take place via *lacZ* (as shown in Fig. 2A and B). For brevity, we refer to the resulting strains as integrants and to the original λp*lac*Mu53 strains as fusion strains. Plasmid pIVET1 contains unique restriction sites, such that digestion of the integrant chromosomal DNA followed by circularization with T4 DNA ligase can yield a functional Cb^r plasmid carrying *lacZ* and some portion of the chromosome upstream of the fusion point (Fig. 2C). Eight of ten stable fusions were successfully cloned in this manner, and the resulting plasmids were used as templates for DNA sequencing to identify the site of the fusion.

It was important to verify that the cloned fusion exhibits the expected pattern of regulation in response to Lrp and leucine. Plasmid DNA from positive clones was used to transform AAEC546, which is Lac$^-$ and lacks λ*pir*. Cb^r integrants resulting from homologous recombination at the location of the original fusion are tandem fused/wild-type merodiploids (Fig. 2D). These merodiploid strains were tested for the expression pattern shown by the original fusion. If the merodiploid pattern of expression was unchanged from that shown by the original fusion strain, then the cloned fusion is directly responsible for the regulatory patterns of interest; a difference in the expression pattern could indicate that the original strain carried multiple fusions or that there were autogenous effects of having interrupted the fused gene. Figure 4D shows a detailed comparison of the expression patterns of one fusion strain and its corresponding merodiploid; they yield virtually identical patterns and levels of expression. The results for five of the eight fusion/merodiploid pairs are shown in Table 3, and the merodiploids and corresponding fusion strains have closely matching activities. The three strain pairs not shown in Table

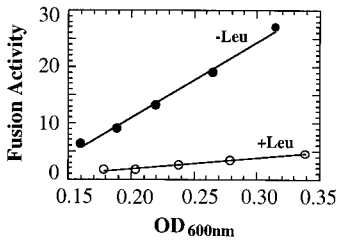

FIG. 3. Measurements of β-galactosidase activity of fusions generated by λp*lac*Mu insertions. Shown are data from a representative fusion strain, plotted as β-galactosidase activity versus optical density of the culture. Samples were removed at various times from cultures growing exponentially in glucose minimal MOPS medium (18) containing isoleucine, valine, and, where indicated, leucine. The data shown are from fusion strain SPB105 grown with 0.01 mM IPTG in the medium. Points were fitted to a straight line by linear regression to obtain the slope (fusion activity/OD_{600} of the culture).

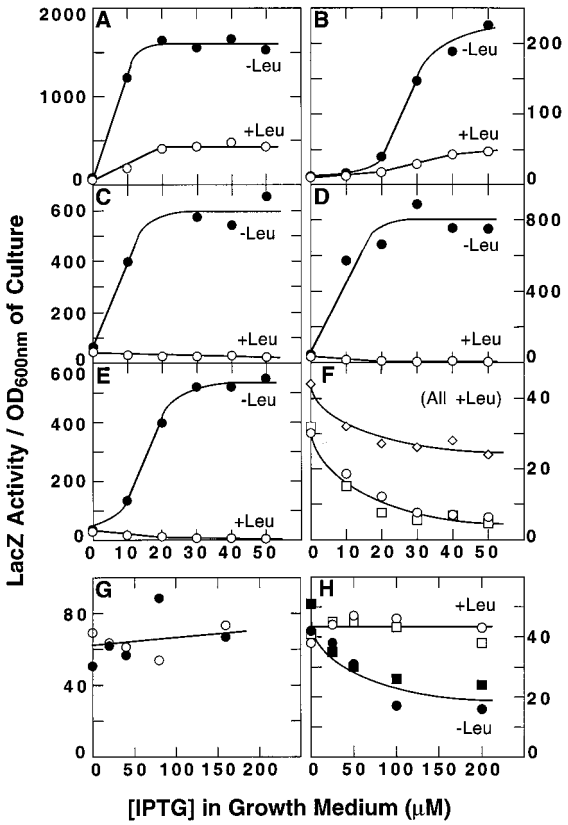

FIG. 4. In vivo titrations with Lrp to characterize the regulatory patterns of selected fusions. (A to G) Regulatory patterns of various fusions. For each fusion grown at each of several IPTG concentrations, the slopes of lines similar to those shown in Fig. 3 were plotted against the IPTG concentration in the growth medium to give the regulatory patterns shown. Thus, each point results from linear regression of at least five points, and all *r* values were greater than or equal to 0.9. Six fusions which were subsequently sequenced and identified are shown, along with one of the unidentified fusions. Open symbols indicate the presence of 10 mM leucine; closed symbols indicate its absence from the medium. The intracellular Lrp concentration varies fairly linearly with the IPTG concentration in the growth medium between 0 and 0.05 mM IPTG (actually measured in strain DB3, a *gltB*::*lacZ* derivative of strain AAEC546), ranging from undetectable up to 0.35 ng of Lrp per μl of cell extract (roughly equivalent to an intracellular concentration of 1.5 μM) (12). The regulatory patterns shown are for the following fusions: SPB101 (*gltB*) (A), SPB103 (further downstream in *gltB*) (B), SPB111 (an unidentified fusion) (C), SPB107 (*livK*) (D), and SPB105 (*livG*) (E). (F) Regulatory patterns of SPB105, SPB107, and SPB111 in the presence of leucine are shown on an expanded scale. Circles, SPB105; squares, SPB107; diamonds, SPB111. (G) SPB115 (between *dinF* and *o69*), with the line representing linear regression of all points. (H) SPB109 (in *orf3*, between *rpiA* and *serA*; circles represent the original fusion, and squares represent a merodiploid strain). Note that panels G and H go to higher IPTG concentrations than panels A to F.

3 have activities that differ substantially between merodiploid and fusion strains, and these are discussed below.

We next determined the sequence of the DNA flanking the insertions. We used a primer (L19 [Fig. 2C]) complementary to *trpB* (in λp*lac*Mu53, *lacZ* is actually fused to a short segment of *trpA* corresponding to the amino terminus, preceded by *trpB* [Fig. 1]). This strategy worked, though it provided only a relatively short sequence across the fusion point for reasons described in Materials and Methods. We also sequenced the opposite end of the cloned fragment by using a primer (U173 [Fig. 2C]) that corresponds to the region upstream of the *bla* gene. Sequences obtained by using each primer were used to search the GenBank and EMBL databases, using the program BLAST (2). As shown in Fig. 5A, sequences from six of the eight clones matched database entries. For a given clone, comparing the corresponding sequences from primers L19 and U173 confirmed these matches in three ways (not shown): they match the same regions, the U173 sequence matches are opposite in polarity to the L19 matches, and the U173 and L19 matches are separated by the distance predicted by restriction analysis of the plasmid clones. In one case, the quality of the match led us to confirm the identification with a specific sequencing primer (Fig. 5B). In another case (not shown), the sequence obtained from a clone matched the *E. coli trp* operon. However, the merodiploid strain gave a regulatory pattern completely different from that of the original fusion, and others have reported recombination between chromosomal and λp*lac*Mu-carried *trp* DNA (9); we thus have to reclone the original fusion in this case.

The fusions that have been identified fall into three categories: fusions to genes known to be in the Lrp regulon and showing expected patterns of expression, fusions to genes known to be in the regulon but showing unexpected patterns of expression, and fusions to new loci including open reading frames of unknown function. Two of our fusions, *gltB4527*::*lacZ* and *gltB4702*::*lacZ*, were found to be in the *gltBDF* operon, and their expression is stimulated by Lrp (Table 3). *gltB* specifies the large subunit of glutamate synthase and is known to be activated by Lrp in a relatively leucine-insensitive manner (19, 20). Thus, our strategy for construction and cloning of fusions yields known members of the Lrp regulon that are regulated in the expected manner. Two other fusions, *livG6919*::*lacZ* and *livK3663*::*lacZ*, were found to be in the *livKHMGF* operon, which is also known to be part of the Lrp regulon (27, 38, 59). *livG* codes for a membrane-associated subunit of the leucine transport system, while *livK* specifies a periplasmic binding protein associated with this transport system. As previously noted for certain *livJ* and *livH* translational fusions (59), fusions in *livK* (Fig. 4D and F) and *livG* (Fig. 4E and F) show an unusual regulatory pattern; expression is activated by Lrp in the absence of leucine and decreased by Lrp in

TABLE 3. Comparison of LacZ expression patterns of selected fusions and their respective merodiploid strains

Strain[a]	Fused gene	β-Galactosidase activity (U)[b]			
		−Lrp (0 mM IPTG)		+Lrp (0.05 mM IPTG)	
		−Leu	+Leu	−Leu	+Leu
DB3	*gltB1594*::*lacZ*	49	34	1,117	298
SPB101/102	*gltB4527*::*lacZ*	47, 33	74, 29	1,097, 771	342, 191
SPB103/104	*gltB4702*::*lacZ*	13, 15	12, 14	268, 277	63, 81
SPB105/106	*livG6919*::*lacZ*	36, 55	30, 32	520, 515	8, 13
SPB107/108	*livK3663*::*lacZ*	47, 54	32, 41	750, 692	5, 17
SPB109/110[c]	*orf3-397*::*lacZ*	42, 51	38, 41	16, 24	43, 38

[a] The *E. coli* AAEC546 strains carrying the original fusion are indicated, and the numbers after the slashes indicate strains carrying the corresponding merodiploids (obtained by transforming *E. coli* AAEC546 with pIVET clones of original fusions). Strain DB3, described in Table 1, is from another study and has no corresponding merodiploid strain.

[b] In each case, the first number indicates the units of LacZ activity from the original λp*lac*Mu fusion strain, and the second number represents units of LacZ activity from the corresponding merodiploid strain. Assays and activity units are described in Materials and Methods. Each activity value is the slope from linear regression of activity versus culture density; at least five points define each slope, and the correlation coefficients were in all cases greater than 0.9. Cultures were grown in MOPS minimal glucose medium containing isoleucine, valine, and, where indicated, 0.05 mM IPTG and/or 10 mM leucine.

[c] In this case the concentration of IPTG, when added, was 0.2 mM.

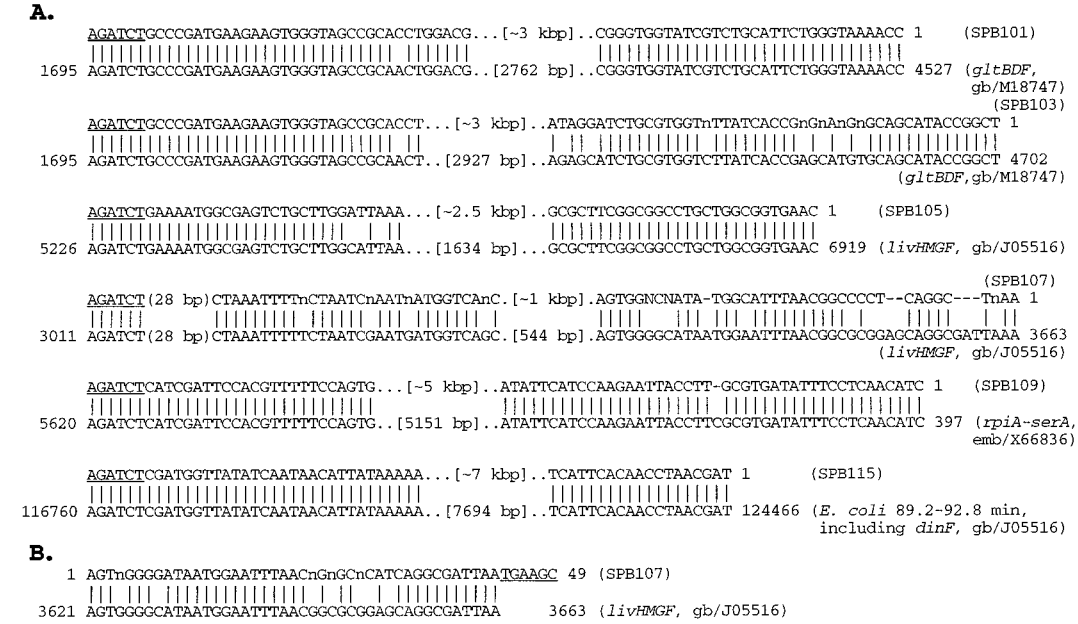

FIG. 5. Alignment of fusion clone sequences to known DNA sequences. (A) Sequences across the λp*lac*Mu53 fusion junction (primer L19) and across the pIVET1 *Bgl*II site (primer U173 [Fig. 2C]) were independently compared to GenBank database entries by using the program BLAST (2). Alignments are shown for the six phenotypically confirmed fusion clones which were sequenced and identified. The *Bgl*II site, to the left as represented, is underlined. For each clone, the distance between the two sequences is indicated either as an estimate from the restriction map (clone; upper strand) or as the exact nucleotide count (matched sequence; lower strand). (B) Confirmatory sequence obtained by using the primer liv577, aligned as in panel A to define the fusion location from strain SPB107. The sequence reveals the fusion junction to be in *livK* at position 3663 (numbering according to GenBank entry J05516). Underlined nucleotides correspond to the S end of λp*lac*Mu53.

the presence of leucine. Two newly identified fusions occur in *orf3* (EMBL accession no. X66836) and downstream of *dinF* (GenBank accession no. U00006). *orf3* lies between *rpiA*, the gene for the constitutive form of ribose phosphate isomerase, and *serA*, the gene for 3-phosphoglycerate dehydrogenase. Two groups have shown that *serA* transcription is activated by Lrp (40, 54); we find that transcription of *orf3* is depressed by Lrp (Fig. 4H). *dinF* is not known to be controlled by Lrp but is a member of the SOS regulon; the role of *dinF* has not yet been determined (60).

Regulation of a fusion downstream of *dinF* by Lrp. We further explored regulation of the fusion that yielded the data in Fig. 4G, because the effects of Lrp on β-galactosidase expression were so minimal (at least during logarithmic growth) that we wondered how this fusion had been identified by our screen. Since screening colonies on plates is essentially an analysis of stationary-phase cells, we decided to determine the expression of this fusion in liquid culture during the transition to stationary phase. We confirmed that while the culture is actively growing, expression of fusion *dinF+103*::*lacZ* is essentially the same whether or not IPTG or leucine is present in the medium (Fig. 6). However, as the cultures enter stationary phase, expression of this fusion is substantially lower when IPTG (Lrp) is present; this depression of expression is antagonized by leucine (Fig. 6).

A surprising and yet unexplained observation is that the patterns of expression of the original fusion strain and the merodiploid strain are essentially identical, while the amounts of expression in each case differ nearly 10-fold (compare Fig. 6C and D). As mentioned above, one possible explanation for this type of discrepancy is that the fusion interrupts an autogenous regulatory circuit.

Because this fusion is downstream of a member of the SOS regulon, we also tested its response to the DNA-damaging agent mitomycin. The results (not shown) reveal that fusion expression increases somewhat following mitomycin addition, whether or not IPTG or leucine is present, but that expression of a control fusion (to *gltB*) does not show this increase. This modest induction is similar to that for the Lrp-regulated L-

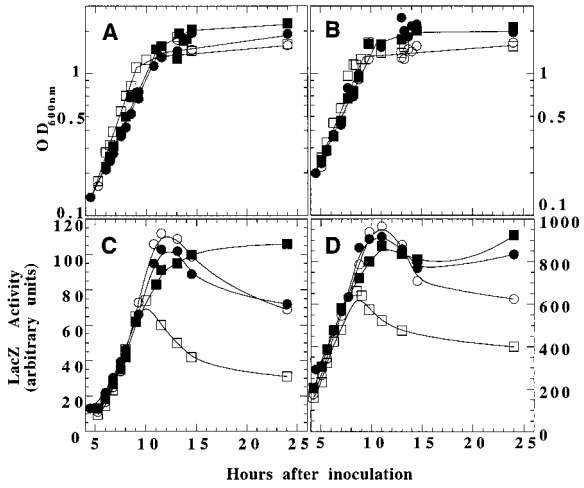

FIG. 6. Expression of a fusion downstream of *dinF* during transition to stationary phase. Strain SPB115 (A and C) carries a λp*lac*Mu fusion to a point between *dinF* and *o69*, and SPB116 (B and D) is the corresponding merodiploid strain (*o69* is an uncharacterized open reading frame). (A and B) Culture density versus time; (C and D) LacZ activity corrected for sample volume but not for culture density. Circles represent cultures with no added IPTG (and thus no Lrp), and squares represent cultures to which 0.2 mM IPTG was added. The cultures were grown in glucose minimal MOPS medium containing isoleucine, valine, and either no leucine (open symbols) or 10 mM leucine (closed symbols). The doubling times for these cultures (without and with IPTG) were 112 and 87 min for SPB115 and 107 and 85 min for SPB116.

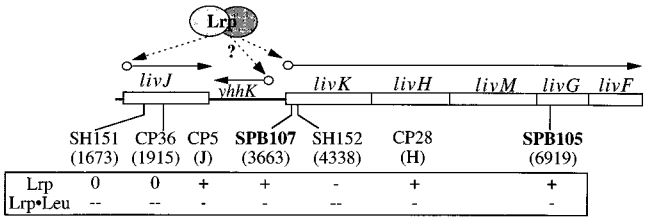

FIG. 7. The *liv* operon. A genetic map of the *livJ-livKHMGF* region (35) is shown with base pair locations (where known) of gene fusions from this study (shown in bold) and from earlier studies (27, 59). For two fusions, the affected gene was reported but insertion coordinates were not published (59). The chart indicates effects of Lrp alone or of Lrp in the presence of exogenous leucine, relative to expression in an Lrp$^-$ (*lrp*::Tn*10*) background; + indicates stimulation of expression, 0 indicates no effect, and − and −− indicate partial and profound reduction of expression. The simplest model consistent with the available information would involve three promoters (circles): one promoter for *livJ*, one for the *livKHMGF* cluster, and one for the oppositely oriented *yhhK*. Deletion of *yhhK* (originally called *orf-19*) results in pantothenate auxotrophy (35). Two *lacZ* fusions (CP5 and SH152) show expression patterns that are dissonant with this three-promoter model (27).

serine deaminase (*sdaA*) gene when cells were treated with mitomycin, nalidixic acid, or UV irradiation (48).

Positional effects of insertions into the *gltBDF* operon. Even in cases of genes already known to be regulated by Lrp, important features have become apparent from having multiple fusions at various locations within the gene or operon. *gltBDF* expression is stimulated by Lrp, and the stimulation is antagonized by leucine (19, 20). The *gltB4527*::*lacZ* and *gltB4702*::*lacZ* fusions both showed the expected regulatory patterns, though the highest observed level of expression of *gltB4702*::*lacZ* was ~4-fold lower than that of *gltB4527*::*lacZ* (Table 3). This difference could result from the extents to which the responses are sigmoidal: in 20 μM IPTG, expression of *gltB4527*::*lacZ* is already maximal (Fig. 4A) whereas that of *gltB4702*::*lacZ* is only slightly above its baseline level (Fig. 4B). The results shown in Fig. 4B also suggest that *gltB4702*::*lacZ* expression might increase further with further increases in Lrp concentration (not achievable in strain AAEC546). To further explore the differences between these two fusions, we studied an additional *gltB*::*lacZ* fusion located near the beginning of *gltB*. Strain DB3, an AAEC546 derivative, contains a Mud1 (*kan*, *lac*) fusion in *gltB* at position 1594 (12, 43). The results for *gltB1594*::*lacZ* (DB3) were similar to those for *gltB4527*::*lacZ* and ~4-fold higher than those for *gltB4702*::*lacZ* (Table 3), implying a drop in expression associated with the 175 bp of DNA between positions 4527 and 4702. The sequence between the two fusion points contains no obvious transcription terminators. It appears that something within this 175-bp region either attenuates transcription under the influence of Lrp or affects Lrp-mediated transcript initiation at the *gltBDF* promoter. This observation illustrates the value of examining multiple fusions even within a single gene.

Positional effects of adjacent insertions into operon clusters. Operons with related functions sometimes occur in clusters; an example is the adjacent *livJ* and *livKHMGF* operons that specify the high-affinity branched-chain amino acid transport system (Fig. 7) (35). As described above, we observed marked effects on expression with the position of an insertion within a single gene. In addition, we and others (27, 59) have observed that multiple insertions into the same operon cluster can reveal differences in both the patterns of regulation and the extent of expression.

(i) Positional effects of insertions into the *liv* operons. Two of our fusions were found to involve the *livK* and *livG* genes (Fig. 7). Expression of both *livK3663*::*lacZ* and *livG6919*::*lacZ* was strongly stimulated by Lrp in the absence of leucine but depressed by Lrp in the presence of leucine (Table 3; Fig. 4D to F). The *liv* genes appear to be organized into two operons. The *livJ* gene was previously shown to be repressed by Lrp, with leucine being required for repression (27), which differs in part from the regulatory pattern shown by our fusions and by a *livH*::*lacZ* fusion (coordinates not reported) cited in another study (59): all fusions from all three studies show depression of expression mediated by Lrp in the presence of leucine, but results differ in the absence of leucine (no effect versus stimulation). It should be noted that only Haney et al. confirmed their results by assays of leucine transport activity (27). The three characterized fusions in the *livKHMGF* operon (Fig. 7) are thus far the only reported examples of Lrp both stimulating and depressing expression dependent on whether leucine is present. A possible physiological rationale for *livJ* having a distinct regulatory pattern from *livKHMGF* is suggested by the facts that *livK* specifies a leucine-binding protein, while *livJ* codes for a protein that binds all three branched-chain amino acids (the *livHMGF* genes specify the transport complex with which both LivJ and LivK interact) (4, 35, 45).

(ii) Regulation of *orf3* by Lrp. One of our fusions, which shows Lrp-dependent repression (Fig. 4H), lies within *orf3* about 100 bp upstream of the translational start of *serA* (Fig. 8A). *serA* is an Lrp-controlled gene (40, 50, 54) transcribed from two promoters, P1 (activated by Lrp) and P2 (repressed by Lrp) (50, 65); our fusion lies between the two promoters. However, *orf3* begins upstream of both identified *serA* promoters and appears to be transcribed from a third Lrp-repressed promoter (labeled P3 in Fig. 8) (9a). Just upstream of *orf3* and in the same orientation is the gene for the constitutive form of ribose phosphate isomerase, *rpiA* (30). The possible effects of transcriptional readthrough from *rpiA* on *orf3* expression have not been determined.

DISCUSSION

A set of procedures for characterizing regulons. Several approaches have been used to identify members of the Lrp regulon, including two-dimensional gel electrophoresis of extracts from Lrp$^+$ and Lrp$^-$ strains (19) and screening *lacZ* fusion libraries in the presence or absence of leucine (38). Both approaches identified important members of the regulon but suffered some limitations. In the two-dimensional gel approach, most Lrp-responsive polypeptides could not be identified and polypeptides with pI values above ~8 could not easily be studied. The leucine screening approach was also limited in its ability to identify genes whose regulation by Lrp is relatively insensitive to leucine.

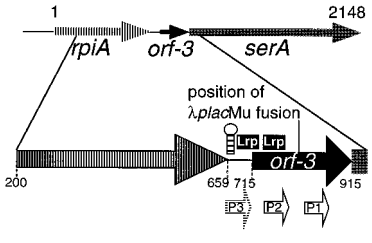

FIG. 8. Genetic map of the region including the *rpiA* (ribose phosphate isomerase) and *serA* (3-phosphoglycerate dehydrogenase) genes. The amplified region of the map shows the transcription terminator following *rpiA* (30) (shown as a hairpin), reported Lrp-binding sites (50, 65), and the *serA* promoters P1 and P2 (50, 65). The apparent promoter identified in this study, P3, is indicated by a dashed arrow.

We are particularly interested in identifying genes whose regulation by Lrp is relatively insensitive to leucine and in obtaining genes that range in the degree of their responsiveness to Lrp. Hence, we constructed a *lacZ* transcriptional (operon) fusion library in a strain in which the *lrp* gene itself is inducible by IPTG. We screened this library in media either containing or lacking IPTG and looked for members which showed differential expression of *lacZ* under these conditions. Selected fusions were tested for stability of the Lrp effect, and the most stable ones were selected for further study.

We next sought to clone and identify the genes that yielded Lrp-responsive fusions. We developed an approach to clone and sequence chromosomal λp*lac*Mu53 fusions that offers some advantages over recently published methods. One published method involves directly sequencing phage DNA prepared from UV-induced λp*lac*Mu lysogens, using a primer specific to the Mu c end (57). Another method involves inverse PCR amplification of the upstream region of a λp*lac*Mu insertion followed by sequencing of the PCR product by using a primer specific to the Mu S end (59). Both of these methods are straightforward and useful; however, neither yields plasmid clones of the fusions and thus neither can rule out misidentifications due to DNA rearrangements or to multiple fusions having occurred in the same cell. Further, neither method provides for a way to distinguish between normal regulator-mediated effects and possible additional effects of having disrupted the target gene. Our method has the advantages of confirming that the DNA that was cloned and sequenced actually contains a gene regulated by Lrp and revealing effects of the gene disruption on expression of that gene.

Differences between the expression patterns shown by corresponding merodiploid and nonmerodiploid fusion strains would be expected for autogenously regulated genes (for example), since in the latter case there may be no intact copy of the fused autoregulatory gene. For most of the fusions that we characterized, the regulatory patterns of the merodiploids and the corresponding fusion strains were similar or identical, and the effects of Lrp and leucine were also very close in most cases (Table 3; Fig. 4H). One pronounced difference that we did see between a fusion strain and its corresponding merodiploid involved the fusion downstream of *dinF*; the effects of Lrp, leucine, and growth phase are virtually identical in the two strains, but there is a ~10-fold difference in the total amount of LacZ activity (Fig. 6). A second case of fusion/merodiploid difference in expression revealed the occurrence of a DNA rearrangement (recombination between the λp*lac*Mu and host *trp* sequences, as has been seen by others [9]).

Sampling the membership of the Lrp regulon. While we have found some interesting new regulon members, many of our fusions were in genes or operons previously known to be regulated by Lrp. The *gltBDF* and *liv* operons were shown by electrophoretic and genetic means, respectively, to be Lrp regulated (19, 27) and were also detected in an earlier study using a variant of λp*lac*Mu that yields translational fusions (38). There are several possible explanations for the fact that so many of the same genes are found in various surveys of the Lrp regulon. The simplest explanation is that perhaps this regulon is already well defined. The alternative and, in our view, more likely explanation is that the survey methods are biased in some way. One possible source of bias is that λp*lac*Mu may have some site preference for integration (57), but we have not observed any obvious sequence preference at our fusion junctions. Second, the genes that are most readily detected in such screenings are presumably those that show the strongest effects of a particular regulator, and both *gltBDF* and the *liv* operon fall into this category. Third, many genes regulated by Lrp may be essential or physiologically important, such that their disruption is lethal or greatly slows growth. In contrast, while mutation of *gltBDF* results in an Ntr^- phenotype (an inability to grow with very low concentrations of inorganic nitrogen as the sole source of nitrogen), our libraries were constructed in a rich medium; and while mutation of the *liv* operon eliminates a high-affinity transport system for branched-chain amino acids, there is still another low-affinity system as well as endogenous biosynthesis.

A fourth possible source of bias in the various surveys of the Lrp regulon involves the preference for genetically stable fusions. Of 3,000 clones initially tested, 84 showed Lrp-dependent differential expression in the initial round of screening, which is about 3% of the total. This number of fusions was about what we expected given the frequency of Lrp responsiveness among *E. coli* polypeptides (19), considering that over half of the fusions would be in the wrong orientation or in untranscribed regions. Of these 84, only 10 were found to give consistent Lrp-dependent regulatory patterns. In some cases, such genetic instability may be instructive: mutations in *metK*, which codes for an adenosylmethionine synthetase, lead to much lower growth rates, and *metK* strains accumulate secondary mutations in *lrp* that restore the growth rates (39). *metK* is not known to be regulated by Lrp, and there is no satisfactory explanation for the secondary mutations in *lrp*, but now that we have a good method to clone and characterize these fusions and generate merodiploids, characterization of the unstable fusions could well identify genes that previous screens have missed.

One of our original goals was to find additional leucine-independent operons (defined as showing a leucine effect on expression of less than twofold). We found none among the set of fusions described here. However, strain AAEC546, even when maximally induced, produces only about 70% of the wild-type level of Lrp (12). Reduced levels of Lrp are expected to increase the leucine sensitivity of expression of Lrp-regulated operons (12, 20). Thus, our having used a strain with reduced Lrp levels decreased the likelihood that what are normally leucine-independent fusions would appear so in our screens. Our results are consistent with the model that all Lrp-regulated genes are leucine responsive at sufficiently low Lrp concentrations (20).

Growth phase-dependent regulation by Lrp. A fusion downstream of *dinF* showed an unusual pattern of expression, being depressed by Lrp only on the transition to stationary phase (Fig. 6). In other words, this fusion is not simply responding to a transition to stationary phase, but rather the transition to stationary phase is required to reveal Lrp dependence of expression. In strain AAEC546, *lrp* is under the control of p_{lac}, but in a wild-type strain, Lrp levels are reported to rise in stationary phase (34); thus, the stationary-phase effects that we observed with the fusion in strain AAEC546 might be more dramatic in a background wild type for *lrp*. Lrp has previously been shown to affect the expression of some other genes preferentially expressed in stationary phase: *otsBA*, *bolA*, *osmY*, and *csiD* (29) and *aidB* (36). Lrp is reported to stimulate stationary-phase expression of *csiD*, to depress expression of *osmY* in all growth phases in a minimal medium, and to prevent induction of *osmY* in rich medium prior to stationary phase (29, 37); also, Lrp represses RpoS-dependent transcription of *aidB* (36). Regardless of exactly how Lrp is acting, the pattern shown by the *dinF+103*::*lacZ* fusion is consistent with the role that Lrp plays in controlling *osmY* in rich medium, a role that Hengge-Aronis et al. refer to as transition state regulator, controlling the time of expression rather than the extent

of expression (29, 37). This result underscores the importance of screening fusions under a variety of growth conditions.

Summary. There are two main advantages provided by our approach to characterizing members of a regulon. First, this approach provides phenotypic confirmation of the relevance of the DNA being sequenced. Second, the identified fusions can be immediately characterized by an in vivo titration with the regulatory protein (Lrp, in this case), not only revealing whether positive or negative effects are being seen but also providing an indication of the relative affinity of various control regions for the regulator. Such information is important for any regulatory protein that has different concentrations under different growth conditions, for example, Lrp (34), Fis (8), integration host factor (42), H-NS (18), and cyclic AMP receptor protein (32). Combining many of these in vivo titration results, to give a more extensive version of Fig. 4, would provide a useful and interesting snapshot of cell physiology as it is modulated by a regulator. We have identified some previously unknown members of the regulon, but it remains to be determined whether their modulation by Lrp is direct. Even in operons previously shown to belong to the Lrp regulon, the new fusions have revealed apparent complexities that remain to be investigated.

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