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Two Roles for the Leucine-Responsive Regulatory Protein in Expression of the Alanine Catabolic Operon (*dadAB*) in *Klebsiella aerogenes*

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The *lrp* gene, which codes for the leucine-responsive regulatory protein (Lrp), was cloned from *Klebsiella aerogenes* W70. The DNA sequence was determined, and the clone was used to create a disruption of the *lrp* gene. The lack of functional Lrp led to an increased expression of the alanine catabolic operon (*dad*) in the absence of the inducer L-alanine but also to a decreased expression of the operon in the presence of L-alanine. Thus, Lrp is both a repressor and activator of *dad* expression. Lrp is also necessary for glutamate synthase formation but not for the formation of two other enzymes controlled by the nitrogen regulatory (Ntr) system, glutamate dehydrogenase and histidase.

Many bacteria use the amino acid alanine as a carbon and nitrogen source by converting the D-stereoisomer to pyruvate and ammonia. The two enzymes in this catabolic pathway, alanine racemase and D-amino acid dehydrogenase, have been studied for *Klebsiella aerogenes*, *Escherichia coli*, and *Salmonella typhimurium* (14, 15, 20, 26). The levels of both enzymes increase under conditions of carbon limitation and when L-alanine is present in the growth medium. In *K. aerogenes* enzyme levels are increased by nitrogen limitation as well. The *dad* operon (*dadAB* in *S. typhimurium* and *K. aerogenes* and *dadAX* in *E. coli*) contains a gene that codes for one subunit of the dehydrogenase and another gene that codes for the racemase. This operon is controlled by the catabolite activator protein charged with cyclic AMP, the leucine-responsive regulatory protein (Lrp), and, in the case of *K. aerogenes*, the nitrogen assimilation control protein (NAC) (15, 17, 19, 31). Catabolic activator protein-cyclic AMP and NAC regulate *dad* by binding to the *dad* promoter and increasing transcription of the operon. The regulatory role of Lrp is more complex. Lrp is a repressor of *dad* in *E. coli*, suggesting that the induction by alanine results from overcoming the Lrp-mediated repression of *dad* (19).

Lrp binds to the *dad* promoter of *K. aerogenes* in vitro, but the addition of L-alanine or L-leucine does not completely abolish the ability of Lrp to bind (15). We therefore studied the role of Lrp in the alanine-mediated induction of *dad*. The results presented here suggest two roles for Lrp in the alanine-dependent induction of the *dad* operon, a role as a repressor and a role as an activator.

Cloning *lrp* from *K. aerogenes* W70. In order to clone the *lrp* gene from *K. aerogenes* W70 we took advantage of the fact that *lrp* from *K. aerogenes* RT48 had already been sequenced (12). Primers that contained a recognition sequence for a restriction endonuclease (*Eco*RI for the upstream primer and *Bam*HI for the downstream primer) and the 5′- or 3′-terminal 25 nucleotides of the RT48 *lrp* gene were used to amplify a DNA fragment approximately 500 bp in length from chromosomal DNA isolated from KC2668 (which is derived from strain W70) by standard methods of PCR. The exact conditions for the PCR have been described elsewhere (24). This fragment was cloned into the expression vector pQE70, creating pCB1074, so that an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter would drive the expression of any gene in the cloned fragment. pCB1074 was introduced into an *E. coli lrp* mutant, BE2 (*lrp-35*; a complete list of strains and plasmids used in this work is included in Table 1), and tested for its ability to complement the *lrp-35* allele. The presence of pCB1074 restored two phenotypes of the *lrp-35* allele to the wild-type state. *lrp* mutants grow slowly on glucose minimal medium and are unable to use L-glycine as a sole source of nitrogen (1, 16, 29). BE2 carrying pCB1074 grew well on glucose minimal medium and was able to use L-glycine as the sole nitrogen source. It therefore seemed likely that pCB1074 contained a functional *lrp* gene, and it complemented the *lrp-35* allele in the presence or absence of IPTG. DNA sequence analysis of the plasmid identified a gene highly similar, but not 100% identical, to the *lrp* gene of RT48. Thus, the gene isolated from W70 was different from the *lrp* gene of RT48, as well as from all other known *lrp* sequences.

As a result of the cloning strategy, pCB1074 contains a hybrid *lrp* gene. Most of it is from W70, but the 25 terminal nucleotides (both 5′ and 3′) are from RT48. In order to determine the sequences of the termini of the W70 *lrp* gene we cloned a larger fragment of DNA that contained the *lrp* gene. Southern blot analysis with pCB1074 as a probe of chromosomal DNA from strain KC2668 cut with various restriction enzymes indicated that the *lrp* gene would be contained within a 5.5-kb *Bam*HI fragment (data not shown). Chromosomal DNA from KC2668 was digested with *Bam*HI and cloned into pUC19. The ligation mixture was introduced directly into EB4516 (created by moving the *lrp-35* allele into the restriction-deficient strain YMC9) and plated on glucose minimal medium with L-glycine as the sole nitrogen source. A single colony which gave rise to strain EB4517 was isolated after 4 days of incubation at 37°C. This strain was shown to contain a plasmid of the expected size (pCB1075). When introduced into strain DH5α and the *lrp* mutant EB4516, plasmid pCB1075 caused the strains to grow poorly, and in the latter strain it did not allow growth with glycine as the sole nitrogen source. Subsequent DNA sequence analysis identified an *lrp* gene in pCB1075 indistinguishable from that in pCB1074 (except for three silent substitutions in the 3′-terminal 25 nucleotides).

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TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Genotype or relevant characteristics	Source or reference
Strains		
K. aerogenes		
KC2148	*hutC515 dadA1 srl-7013*::Tn*5-132*	This laboratory
KC2668	*hutC515 dadA1* Δ[*bla*]-2	15
KC2671	*hutC515 dadA1* Δ[*bla*]-2 *recA3011*	This laboratory
KC3346	*hutC515* Δ[*bla*]-2	15
KC4562	*hutC515 dadA1* Δ[*bla*]-2 *lrp-101*	This work
KC4596	*hutC515 dadA1* Δ[*bla*]-2 *recA3011*/pGE82	Transformation of KC2671 with pGE82
KC4600	*hutC515* Δ[*bla*]-2 *lrp-101*	P1 · KC4562 × KC3346
KC4601	*hutC515* Δ[*bla*]-2 *lrp-101 srl-7013*::Tn*5-132*	P1 · KC2148 × KC4600
KC4602	*hutC515* Δ[*bla*]-2 *lrp-101 recA3011*	P1 · KC4596 × KC4601
E. coli		
DH5α	F$^-$ ϕ80*dlacZ*Δ*M15* Δ*lacU169 endA1 recA1 hsdR17 deoR thi-1 supE44* λ$^-$ *gyrA96 relA1*	Gibco-BRL
W3110	Prototroph	R. G. Matthews
BE2	W3110 *lrp-35*::Tn*10*	10
YMC9	*endA1 thi-1 hsdR17 supE44* Δ*lacU169*	2
BW20767	RP4-2-*tet*::Mu-1*kan*::Tn*7* integrant *leu-63*::IS*10 recA1 creC510 hsdR17 endA1 zbf-5 uidA*(Δ*Mlu*I)::*pir*$^+$ *thi*	21
EB4516	*endA1 thi-1 hsdR17 supE44* Δ*lacU169 lrp-35*::Tn*10*	P1 · BE2 × YMC9
EB4517	EB4516/pCB1075	This work
EB4594	BW20767/pCB1093	This work
Plasmids		
pUC19	Cloning plasmid	Gibco-BRL
pQE70	Expression plasmid	Qiagen
pGE82	*E. coli recA*	G. Weinstock
pWW-84	Kanamycin resistance cassette	22
pAH34	Suicide vector	21
pCB1074	PCR clone of KC2668 *lrp* cloned into pQE70	This work
pCB1075	5.5-kb *Bam*HI chromosomal DNA fragment from KC2668 containing *lrp* cloned into pUC19	This work
pCB1076	Kanamycin resistance cassette from pWW-84 cloned into *lrp* in pCB1075	This work
pCB1093	*Bgl*II-*Xmn*I internal fragment of *lrp* from pCB1074 cloned into pAH34	This work

Thus, this *lrp* gene appears to be fully functional. It has been shown that high levels of Lrp are toxic to the cell (5, 7). Therefore, it seemed likely that pCB1075, which contained the wild-type *lrp* gene in a high copy number, was toxic to the cell and that the original host had developed an additional mutation that compensated for the toxic effects of the high level of Lrp. Inactivating the *lrp* gene provided further evidence that the overexpression of *lrp* was toxic. Plasmid pCB1076 was constructed by cloning a kanamycin resistance gene (from pWW-84) into a unique *Bgl*II site contained in the *lrp* gene. This construct disrupted *lrp* at codon 10, and therefore no functional Lrp was provided by the plasmid. Transformants of DH5α that contained this plasmid grew normally.

Isolation of pCB1075 allowed us to obtain the complete sequence of the *lrp* gene from W70. At the nucleotide level, *lrp* from W70 is 95, 94, 94, 90, and 88% identical to *lrp* from RT48, *Enterobacter aerogenes*, *Serratia marcescens*, *E. coli*, and *S. typhimurium*, respectively. At the amino acid level, Lrp is identical among these organisms except at position 3 (glycine in *S. marcescens* and serine in the others) and at position 95 (alanine in *K. aerogenes* W70 and *S. typhimurium*, serine in *K. aerogenes* RT48, and threonine in the others).

Isolation of an *lrp* mutant of *K. aerogenes* W70. We isolated an *lrp* mutant of *K. aerogenes*, KC4562, by reverse genetics with the ampicillin-resistant suicide plasmid pAH34 (21). We cloned an internal fragment of the *lrp* gene from pCB1075 into pAH34, resulting in pCB1093, and transferred this plasmid into KC2668 by conjugation. Since pAH34 cannot replicate in the absence of the *pir* gene, ampicillin-resistant transconjugants were expected to result from the integration of the plasmid into the chromosomal *lrp* gene. This integration would result in a partially diploid strain with one *lrp* allele that was 3′ truncated at codon 104 (of 164) and another allele that was 5′ truncated at codon 10. The plasmid pAH34, containing the *bla* gene, would separate the two nonfunctional alleles. Ampicillin-resistant transconjugants were isolated after mating EB4594 with KC2668. PCR analysis of several of these integrants revealed that the wild-type *lrp* gene was not present and that the pAH34 plasmid had integrated into the chromosome. Southern blot analysis also revealed that the *lrp* gene had been disrupted (data not shown). One of these strains was designated KC4562 (*dadA1 lrp-101*). Since the *lrp-101* allele contains a duplication of part of the *lrp* sequence, it should be unstable and could revert to the wild type by RecA-mediated excision of pCB1093. Therefore, we used consecutive P1 transductions to create KC4602, a *dad*$^+$ *recA* strain carrying the *lrp-101* allele. KC4602 (*lrp-101*) grew slowly on minimal medium compared to KC3346 (*lrp*$^+$). The growth rates of these two strains were indistinguishable on minimal medium supplemented with the branched-chain amino acids (leucine, isoleucine, and valine [0.005% each]) and serine and 0.2% glutamine.

The addition of the branched-chain amino acids and serine had a negative effect on the growth of KC4602 on minimal medium. It was necessary to supplement the medium with glutamine in order to restore wild-type growth. When the medium was supplemented only with glutamine the cells still grew at a lower rate than did the wild type. Under these conditions, the addition of branched-chain amino acids and serine was beneficial.

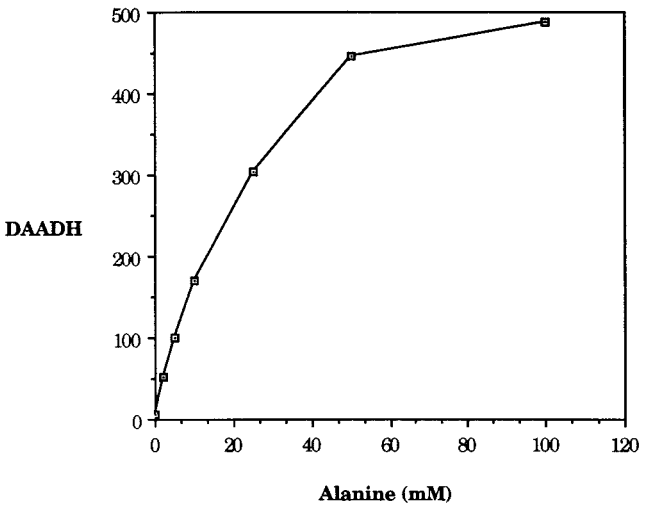

FIG. 1. Induction of D-amino acid dehydrogenase (DAADH) by increasing amounts of L-alanine. Strain KC3346 was grown overnight in glucose-ammonium minimal medium and used to inoculate fresh cultures that included L-alanine at increasing concentrations. Cells were grown to mid-log phase and assayed for DAADH as described previously (15). DAADH is reported as specific activity (units per milligram of protein), and the values are the means of three independent experiments.

Effect of *lrp-101* on *dad* operon expression. The presence of L-alanine in the growth medium leads to an induction of D-amino acid dehydrogenase and alanine racemase. Levels as low as 2 mM have been used to study the alanine-dependent induction of *dad* (19). We suspected that these levels might not saturate the induction system, and therefore we determined to what extent *dad* could be expressed in the presence of L-alanine. Figure 1 shows that the dehydrogenase levels continued to increase until L-alanine was present at levels between 50 and 100 mM. We therefore chose to use 100 mM L-alanine in our subsequent characterization of *lrp-101* to ensure that *dad* expression was maximized. We have shown previously that L-alanine present in the medium at 22 mM (0.2%) derepresses the nitrogen-regulatory (Ntr) system by inhibiting glutamine synthetase (15). Then the Ntr system activates *dad* expression through NAC. In order to circumvent any role of NAC in the alanine-dependent activation of *dad* in our study of *lrp-101*, we included 0.2% L-glutamine in the medium. L-glutamine at these levels has been shown to prevent the derepression of the Ntr system by alanine (15).

Table 2 shows the effect of the *lrp-101* allele on the expression of the *dad* operon and illustrates the two observed roles for Lrp in the alanine-dependent induction of *dad* (although it should be noted that the two strains are not completely isogenic, as KC4502 also carries the *recA-3011* allele). In the absence of alanine, the level of dehydrogenase is four times higher in the *lrp* mutant than in the wild type. This suggests that Lrp is a repressor of *dad* expression in *K. aerogenes*. However, in the presence of alanine, the level of dehydrogenase is four times lower in the mutant than in the wild type. This suggests that Lrp is an activator (either direct or indirect) of *dad*. The addition of alanine leads to a 25-fold increase of *dad* expression in the wild type. While the addition of alanine has little or no effect on *dad* expression in the mutant, the level of dehydrogenase is much higher than the repressed level of the wild type. Thus, it appears that half of the alanine-dependent induction of *dad* can be explained by the repression of the operon by Lrp, while the other half requires the activation of the operon by Lrp. This pattern repeats in *E. coli*, although the evidence of Lrp as a repressor of transcription is not so clear (see results obtained for W3110 and BE2 without alanine in Table 2).

TABLE 2. D-Amino acid dehydrogenase levels in wild-type and *lrp* mutants of *K. aerogenes* and *E. coli*

Strain[a]	Relevant genotype	Dehydrogenase level obtained with[b]:	
		0 mM L-alanine	100 mM L-alanine
KC3346	Wild type	9 ± 2	222 ± 9
KC4602	*lrp-101*	40 ± 6	53 ± 5
W3110	Wild type	24 ± 4	292 ± 24
BE2	*lrp-35*::Tn*10*	31 ± 1	63 ± 3

[a] KC3346 and KC4602 are *K. aerogenes* strains derived from strain W70. W3110 and BE2 are *E. coli* strains derived from strain K-12.

[b] The growth medium was comprised of W4 salts (4) supplemented with glucose (0.4%), ammonium sulfate (0.2%), L-glutamine (0.2%), L-leucine, L-isoleucine, L-valine, L-serine (0.005%), and L-alanine as indicated. Cultures of KC4602 also contained ampicillin at 100 μg/ml to maintain the *lrp-101* allele, while those of BE2 contained tetracycline at 30 μg/ml to maintain the *lrp-35*::Tn*10* allele. Enzyme activity is reported as specific activity (units per milligram of protein), and values are the means and standard errors of the means of at least three independent experiments. The assays were performed as described previously (15).

Effect of *lrp-101* on glutamate synthase expression. The lack of functional Lrp has effects on the expression of enzymes other than those of alanine catabolism. Glutamate synthase (the product of the *gltBD* operon) has been well characterized as an Lrp-dependent operon in *E. coli* (5, 9, 10, 30). The levels of glutamate synthase were reduced eightfold or more (Table 3) in the *K. aerogenes lrp-101* strain, consistent with the observation that a functional Lrp is also necessary for the full expression of *gltBD* in *K. aerogenes*. Glutamate synthase plays a role in maintaining a functional Ntr system, in that strains unable to produce functional glutamate synthase also fail to derepress the Ntr system normally (26). However, any role that the Ntr system plays in the expression of the enzymes studied here has been circumvented by the addition of glutamine to the medium. Therefore, Lrp must play a direct role in the loss of glutamate synthase formation observed in the mutant. Other enzymes of the Ntr regulon do not require functional Lrp for their formation, i.e., histidase and glutamate dehydrogenase (Table 3).

Conclusions and implications. In this work we have characterized an *lrp* mutant of *K. aerogenes* W70. A strain that lacks functional Lrp grows poorly on minimal medium, although the addition of glutamine, serine, and the branched-chain amino acids allows the strain to grow in a manner indistinguishable from that of the wild type. At the genetic level, Lrp is necessary

TABLE 3. Role of Lrp in the regulation of various operons of the Ntr regulon

Strain[a]	Relevant genotype	Sp act (U/mg of protein) of[b]:		
		Glutamate synthase	Glutamate dehydrogenase	Histidase
KC3346	Wild type	117 ± 11	427 ± 66	41 ± 4
KC4602	*lrp-101*	≤15 ± 0.3	225 ± 23	53 ± 6

[a] The growth medium was comprised of W4 salts (4) supplemented with glucose (0.4%), ammonium sulfate, L-glutamine (0.2%), L-leucine, L-isoleucine, L-valine, and L-serine (0.005%). KC4602 also contained ampicillin at 100 μg/ml to maintain the *lrp-101* allele.

[b] Means ± standard errors of the means of at least three independent experiments. The assays were performed as described previously (18).

to activate *gltBD* and is both an activator and repressor of the *dad* operon. The role of Lrp in *gltBD* expression and as a repressor of the *dad* operon is analogous to well-studied systems in *E. coli* (6, 19, 23, 31). The growth phenotypes observed are similar to those seen in *E. coli lrp* mutants, although to restore a wild-type growth rate to *K. aerogenes* it was necessary to add glutamine to minimal medium containing branched-chain amino acids and serine. The role of Lrp as an activator of the *dad* operon was unanticipated.

There are several possible explanations for how Lrp acts as both a repressor and an activator of the *dad* operon. The simplest is that in the absence of alanine Lrp acts as a repressor of the operon, but when alanine is bound to Lrp, a conformational change occurs while Lrp is bound to the DNA that allows the protein to activate transcription. Since Lrp has been shown to be either an activator or a repressor of several leucine-responsive operons, and alanine can mimic the effects seen with leucine, this model does not suggest a new function for Lrp (6). There are several Lrp binding sites present in the *dad* promoter, as is the case for many Lrp-dependent operons (6). Whether the hypothesized conformational change resulting from alanine being bound to Lrp would result in different interactions between two or more Lrp molecules, between an Lrp complex and RNA polymerase, or between Lrp molecules and the DNA of the promoter is not clear.

A recent study by Roesch and Blomfield (28) has suggested how Lrp could both activate and repress an operon. They show that for the *fim* switch in *E. coli*, a single Lrp binding site in a three-binding site complex is sensitive to leucine-bound Lrp. In the absence of leucine, three Lrp molecules bind to the DNA region and recombination is inhibited. When leucine is present, one binding site can no longer keep Lrp bound, and recombination is stimulated by the remaining two Lrp molecules. It is possible that a similar interaction occurs at *dad* in which an Lrp molecule binding alanine can no longer bind to the DNA, and the absence of Lrp at this site is the cause of the derepression seen in *lrp* mutants but not in the wild type. However, other Lrp molecules bound to the promoter do not exhibit this sensitivity and are necessary to activate transcription.

Lrp could also be indirectly involved in the alanine-dependent activation of *dad* if there is a separate (but Lrp-dependent) positive activator of *dad* expression. In this scenario the presence of alanine would lead to the derepression of *dad* by releasing the Lrp-mediated repression at the *dad* promoter and lead to the activation of *dad* through the proposed Lrp-dependent activator. However, this model requires the existence of yet another regulator, possibly the *dadQ* allele identified by others (3, 11).

Other explanations for the dual role of Lrp in the alanine-dependent activation of *dad*, including Lrp-dependent alanine transport and Lrp's effect on the gene that codes for the other dehydrogenase subunit, are possible but seem even less likely. At least two systems have been identified that transport alanine in *E. coli*, the *cycA* system and the LIV-1 system (8, 20, 25, 27). It is not known whether Lrp plays a role in the *cycA* system, but the lack of functional Lrp leads to higher levels of expression of the LIV-1 system (13). Therefore, transport of alanine via LIV-1 should not be reduced in an *lrp* mutant strain. In addition, KC4602 can use alanine as its sole nitrogen source, although its growth rate under this condition is severely reduced compared to that of the wild type (data not shown). Although we have no data to address the regulation of the gene that codes for the other dehydrogenase subunit, the pattern of expression for alanine racemase mimics that of the dehydrogenase (data not shown). Therefore, no matter how the other gene is regulated, Lrp plays two roles in regulating *dadAB*.

Our results lead us to favor the idea that the *dad* promoter can be in three states with respect to Lrp: it can be free of Lrp or it can be occupied by inducer-free Lrp or inducer-bound Lrp. Inducer-free Lrp represses transcription below basal level, the absence of Lrp leads to basal-level expression, and inducer-bound Lrp leads to activation of transcription above basal level. These three hypothetical states and the existence of two possible inducers (leucine and alanine) suggest that the role played by Lrp in genetic expression may be quite complex. This complexity is reflected in the large number of cellular responses in which Lrp plays a role.

Nucleotide sequence accession number. The DNA sequence of *lrp* from *K. aerogenes* W70 has been submitted to GenBank and has been assigned accession no. AF090144.

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