Identification of *Actinobacillus pleuropneumoniae* leucine-responsive regulatory protein (Lrp) and its involvement in the regulation of *in vivo* induced genes.

*A. pleuropneumoniae* Lrp

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Abstract

*Actinobacillus pleuropneumoniae* is a Gram-negative bacterial pathogen that causes a severe hemorrhagic pneumonia in swine. We have previously shown that the limitation of branched-chain amino acids (BCAAs) is a cue that induces the expression of a subset of *A. pleuropneumoniae* genes identified as specifically induced during infection of the natural host animal using an *in vivo* expression technology screen. Leucine-responsive regulatory protein (Lrp) is a global regulator and has been shown in *Escherichia coli* to regulate many genes including genes involved in BCAA biosynthesis. We hypothesized that *A. pleuropneumoniae* contains a regulator similar to Lrp and that this protein is involved in the regulation of a subset of genes important during infection and recently shown to have increased expression in the absence of BCAAs. We report the identification of an *A. pleuropneumoniae* serotype 1 gene encoding a protein with similarity to amino acid sequence and functional domains of other reported Lrp proteins. We further show purified *A. pleuropneumoniae* His6-Lrp binds *in vitro* to the *A. pleuropneumoniae* promoter regions for *ilvI*, antisense *cps1AB*, *lrp*, and *nqr*. A genetically-defined *A. pleuropneumoniae lrp* mutant was constructed using an allelic replacement and sucrose counter-selection method. Analysis of expression from the *ilvI* and antisense *cps1AB* promoters in wild-type, *lrp* mutant, and complemented *lrp* mutant indicated that Lrp is required for induction of expression of *ilvI* under BCAA limitation.
Introduction

*Actinobacillus pleuropneumoniae* is a bacterial pathogen that causes both acute and chronic forms of necrotizing hemorrhagic pleuropneumonia in swine (16, 28, 42, 51). The severe economic effect of this disease on the swine industry has been ameliorated by improvements in detection and prevention of the disease and in management practices. However, the methods by which *A. pleuropneumoniae* infects and causes disease in swine are still not fully understood. While a variety of virulence factors have been reported to contribute to the pathogenesis of *A. pleuropneumoniae* (1, 3, 4, 9, 13, 39, 48, 56, 62, 63), little is known about what signals induce expression of these virulence factors during infection. Certain environmental cues such as iron limitation, heat shock, oxidative stress, and osmotic stress have been shown to play a part in the regulation of virulence genes in other organisms. We have recently shown that the limitation of branched-chain amino acids (BCAAs), which include leucine, isoleucine, and valine, is an additional cue that induces in vitro the expression of a subset of *A. pleuropneumoniae* promoters that were previously identified by in vivo expression technology as in vivo-induced (*ivi*) (19, 59). However, the mechanism or mechanisms by which these *ivi* genes are regulated in response to BCAA limitation in *A. pleuropneumoniae* is unknown.

The study of gene regulators in *A. pleuropneumoniae* has been limited and has yielded the identification of only two to date. These include HlyX (33, 35), a homologue of the *E. coli* global regulator FNR, and the ferric uptake regulator protein, Fur (27). To elucidate how genes are regulated in response to BCAA limitation, a better understanding of potential regulators in *A. pleuropneumoniae* is needed.
One mechanism known to regulate genes in response to BCAA limitation is leucine-responsive regulatory protein (Lrp). Lrp was first identified in *Escherichia coli* as the positive regulator of *ilvI* (44, 46), a gene whose protein product is involved in BCAA biosynthesis. Other genes, both activated and repressed by Lrp, have been subsequently identified (reviewed in references 6, 7, 14, 40, 41). A DNA microarray study by Tani et al. showed Lrp to be involved in the regulation of up to 10% of all *E. coli* genes either directly or indirectly (54). In general, Lrp positively regulates genes involved in biosynthesis of amino acids and negatively regulates genes involved in catabolism of amino acids in *E. coli*. However, Lrp has been shown to regulate, either directly or indirectly, genes associated with virulence, such as fimbriae in *E. coli* (21, 24, 57, 65, 66) and the *hpmBA* haemolysin operon of *Proteus mirabilis* (17). Recently, Lrp was shown to positively regulate the XhlA haemolysin of *Xenorhabdus nematophila* (10), which is required for virulence in insects.

Genes either directly or indirectly regulated by Lrp may respond to Lrp differently depending upon availability of BCAAs in the environment. Lrp can be a positive or negative regulator with leucine antagonizing the effect of Lrp, potentiating the effect of Lrp, or having no effect on Lrp (34, 54). For example, Lrp positively regulates the *E. coli* *ilvI* gene in the absence of leucine, but the effect is antagonized in the presence of leucine (46). In contrast, the *livJ* gene, involved in BCAA transport, is repressed by both Lrp and leucine together, but repression is not achieved by either individually (34).

The presence of Lrp and its role in gene expression in *A. pleuropneumoniae* has not been investigated. We hypothesized that *A. pleuropneumoniae* contains an Lrp homologue and that this protein is involved in the regulation of a subset of genes.
expressed during infection and recently shown to have increased in vitro expression in
the absence of BCAAs (59). In this study, we have identified an *A. pleuropneumoniae*
serotype 1 gene with similarity to the *lrp* gene of *E. coli*. The *A. pleuropneumoniae*
serotype 1 *lrp* gene was cloned, sequenced, expressed in a protein expression vector, and
hexahistidine (His$_6$)-tagged protein purified. We report that *A. pleuropneumoniae* His$_6$-
Lrp binds to two in vivo-induced promoters, the *iviI* promoter, which has been identified
as the promoter for the *ilvIH* operon, and the *iviG* promoter, which expresses a transcript
antisense to the *A. pleuropneumoniae cps1AB* capsule biosynthetic genes, as well as to
the *nqr* promoter and to its own *lrp* promoter. Furthermore, we report the construction
and confirmation of an *A. pleuropneumoniae* *lrp* mutant and show through
complementation assays that *A. pleuropneumoniae* Lrp regulates expression of *ilvI* in *A.
pleuropneumoniae*. 
Materials and Methods

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *A. pleuropneumoniae* strains were cultured in brain heart infusion (BHI; Difco Laboratories, Detroit, MI) or chemically defined medium (CDM) incubated at either 35°C with 5% CO₂ for agar media or 35°C and 150 rpm for broth media. For growth rate experiments, heart infusion broth (HI; Difco) was also used. Media were supplemented with nicotinamide adenine dinucleotide (NAD, also designated V factor; Sigma Chemical Company, St. Louis, MO) to a final concentration of 10 µg/ml and riboflavin as needed (Sigma) to a final concentration of 200 µg/ml. Ampicillin and kanamycin, when required, were added to 50 µg/ml for plasmid selection in *A. pleuropneumoniae*. When investigating the response of *A. pleuropneumoniae* to the limitation of BCAAs, the amino acids isoleucine, leucine, and valine were excluded (-ILV) from the complete (+ILV) CDM. For analysis of gene expression in CDM broth, bacterial strains grown for 18 h on BHI agar were inoculated into 5 ml of CDM broth to an optical density of ~0.1 at 520 nm (OD₅₂₀).

*E. coli* XL1-Blue mRF’ (Stratagene, La Jolla, Calif.) and *E. coli* S17-1 (λpir) (53) were cultured in Luria-Bertani (LB) medium and used for cloning and mating, respectively. Ampicillin and kanamycin, when required, were added to 100 µg/ml and chloramphenicol was added to 10 µg/ml for plasmid selection in *E. coli*.

The pIvi plasmids listed in Table 1 were constructed by cloning Sau3A-digested *A. pleuropneumoniae* serotype 1 genomic DNA fragments into the promoter trap IVET plasmid pTF86 (19) and were identified as containing promoters that were induced
during infection in a swine animal model (19, 30). pTF86 contains promoterless luciferase genes, \textit{luxAB}; promoterless riboflavin genes, \textit{ribBAH}; and a unique \textit{Bam}HI cloning site. When a functional promoter is cloned into pTF86 in the proper orientation, both the luciferase and riboflavin genes are expressed when the promoter is active. Expression of the riboflavin genes complements an attenuating mutation in the host \textit{A. pleuropneumoniae} strain used for \textit{in vivo} expression studies and restores virulence. Expression of luciferase activity from the promoter::Lux fusions can be used to measure the promoter activity.

Molecular manipulations. Genomic DNA from \textit{A. pleuropneumoniae} was isolated using a QIAGEN-tip 500 according to the QIAGEN Genomic DNA Handbook (QIAGEN Inc., Valencia, CA). Plasmid DNA was purified using QIAprep Spin-columns (QIAGEN). DNA-modifying enzymes were obtained from Roche (Roche Applied Science, Indianapolis, IN) and New England Biolabs (New England Biolabs, Inc., Beverly, MA) and used according to the respective manufacturer’s specifications. Electrocompetent AP225 was prepared and electroporated as previously described (20). \textit{E. coli} XL1-Blue mRF’ was electroporated using the same conditions as those for \textit{A. pleuropneumoniae}.

Luciferase assays. For quantitative measurement of luciferase activity, a Turner model 20e luminometer (Turner Designs, Sunnyvale, CA) was utilized as previously described (19). Briefly, 20 µl of broth culture was added to 20 µl of luciferase substrate and mixed
for 10 s. The substrate was made by dissolving 20 mg/ml Essentially Fatty acid Free bovine serum albumin (BSA; Sigma) and 1 µl of N-decyl aldehyde in 1 ml of H₂O and sonicating the solution. The luminometer was set to a delay of 10 s, integration of 30 s, and a sensitivity of 39.9%. The luminometer relative light unit (RLU) readings were normalized to the optical density units of the culture at 520 nm (OD₅₂₀).

**Induction, purification, and quantification of *A. pleuropneumoniae* and *E. coli* His₆-Lrp.** The *A. pleuropneumoniae* *lrp* gene was amplified by polymerase chain reaction (PCR) using AP100 genomic DNA, *Pfu* turbo DNA polymerase (Stratagene), and *A. pleuropneumoniae* *lrp* specific primers, MM379-*Sal*I and MM430-*Bam*HI and ligated in frame into pQE30 (QIAGEN) to generate pTW313.

*E. coli* XL1-Blue mRF'/pTW313 and XL1-Blue mRF'/pCV294, the *E. coli* His₆-Lrp protein expression vector, were grown in LB medium at 35°C and 150 rpm for 4.5 h. One mM isopropyl-β-D-thiogalactoside (IPTG) was added to each culture and incubated for an additional 3 h to an optical density at 600 nm (OD₆₀₀) of 0.4, at which time cell pellets were harvested and frozen. Frozen pellets were resuspended in 4 ml ice-cold binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). Samples were sonicated for 1 min until not viscous and then centrifuged at 14,000xg for 20 min at 4°C to remove cellular debris. His-tagged proteins were purified using Novagen His-Bind Quick 900 Cartridges (EMD Biosciences, Inc., Madison, WI) according to the manufacturer’s instructions. The cartridges were washed with 20 ml of binding buffer, 10 ml of wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9), and eluted with 4 ml of elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH
Eluted proteins were dialyzed into 20 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, 0.2 mM dithiothreitol, 0.4 M NaCl, using a Centricon 10 (Millipore Co., Bedford, MA) and an equal volume of 100% ultrapure glycerol was added to each sample. Bio-Rad protein micro assays were performed using BSA as a standard to determine final protein concentrations. The purified *E. coli* His$_6$-Lrp and *A. pleuropneumoniae* His$_6$-Lrp protein samples were stored at -80°C until use.

**SDS-PAGE.** Protein samples were resuspended in an equal volume of 2x SDS-PAGE sample buffer (125 mM Tris-HCl, pH 6.8, 25% glycerol, 2.5% SDS, 2.5% B-mercaptoethanol, 1.25% bromophenol blue) and loaded on a 12% polyacrylamide gel, as described by Laemmli (31). Gels were stained with Coomassie blue (22).

**Electrophoretic gel mobility shift assays.** *A. pleuropneumoniae* DNA fragments for electrophoretic gel mobility shift assays (EMSA) were isolated by PCR using AP100 genomic DNA and gene specific primers, or vector specific primers in the case of *ivi* fragments. The 623-bp genomic fragment in pIviI was cloned into pUC19 to generate the *iviI* PCR template, pTW286. Primers specific to the pUC19 vector, MM531 and MM532, were used in this case to generate the *iviI* fragment. For all other *ivi* fragments, primers specific to pTF86, MM478-*lux* and MM533-T4, were used.

For an internal control, an *E. coli* *ilvIH* promoter DNA fragment was PCR-amplified from pTW328 using primers MM478-*lux* and MM533-T4. To generate pTW328, the *ilvIH* promoter was PCR-amplified from a colony of CV975 using primers MM362-*BamHI* and MM363-*BamHI*. The PCR product was digested with *BamHI* and
ligated to *BamHI* digested pUC19 to generate pTW296. The ~300-bp *BamHI* fragment from pTW296 was ligated to *BamHI* digested pTF86 to generate pTW328.

PCR products were gel extracted and purified using the QIAEX II system (QIAGEN). For radiolabeling of DNA fragments, 1 pmole of purified DNA fragment was combined with 10 units T4 polynucleotide kinase (Roche), 50 uCi $^{32}$P γ-ATP (Amersham Biosciences, Piscataway, N.J.), and 1x polynucleotide kinase buffer in a final volume of 20 µl and incubated at 37°C for 1 h. Completed reactions were inactivated by heating to 68°C for 10 min and cleaned by centrifuging the reaction volume through a quick spin column for radiolabeled DNA purification (Roche). Purified His$_6$-Lrp was diluted to 5 ng/µl in binding buffer (20 mM Tris-HCl, pH 8.0, 75 mM NaCl, 5 mM MgCl$_2$, 1 mM dithiothreitol, 12.5% glycerol, 0.1 mg/ml BSA, 25 µg/ml poly-(dI-dC)). Binding reactions containing 0, 5, 30, or 60 ng of His$_6$-Lrp were incubated with 0.05 pmole of radio labeled DNA fragment and 0.5 µg poly-(dI-dC) brought to a final volume of 20 µl with binding buffer (8). Binding reactions were incubated at room temperature for 20 min and then stopped by adding 5 µl of STOP solution (USB Co., Cleveland, OH). The entire reaction volume was loaded onto a 5% non-denaturing polyacrylamide gel prepared in 1x TBE, pH 8.0, and electrophoresed at 200 volts for 2-3 h. Gels were dried at 80°C for 40 min and exposed to Amersham Biosciences Hyperfilm MP film. For quantitative analysis, gels were scanned on a Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and images were analyzed using ImageQuant-TL v2005 software from Amersham BioSciences (Piscataway, NJ).
Primer extension analysis. Total RNA was isolated from the cell pellets of cultures of AP225/pTW338 and AP225/pKB11 grown in CDM-ILV to an OD$_{520}$ of 0.8 using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Primer extension analysis was performed as previously described (59) to identify the transcriptional start points for lrp and nqr, using gene specific primers. Primer extension analysis of the iviI and iviG promoters was previously published (59).

Construction of an *A. pleuropneumoniae* lrp mutant. Primers MM459-PstI and MM460-PstI, both designed with internal PstI restriction sites, were used with an inverse PCR technique to amplify around the pTW338 construct in opposite directions. The 4.5-kb linear product from the inverse PCR was digested with PstI and ligated to itself to generate the reconstituted plasmid, pTW355. The annealing position of the primers during the inverse PCR resulted in a ~100-bp deletion from the center of lrp. An 0.9-kb PstI-digested chloramphenicol acetyl transferase resistance (CAT) cassette, from a PCR reaction using pER187 as template and MM489-PstI and MM511-PstI as primers, was ligated to the newly generated PstI site of pTW355 to generate pTW401. The 3.4-kb sacR-sacB-nptI BamHI fragment from pUM24Cm (47) was ligated to partially BamHI-digested pTW401 to generate pTW402. The sacR and sacB genes confer sucrose sensitivity in the presence of sucrose and allow for future selection of double-crossover events. The nptI gene confers resistance to kanamycin. The SphI/SacI insert from pTW402 was ligated to similarly digested pGP704 (37) to generate the knock-out construct, pTW404. The pTW404 construct was electroporated into *E. coli* S17-1 (λpir) and this strain was filter-mated with AP225, a nalidixic acid resistant derivative of
AP100, according to the protocol of Mulks and Buysse (38). Transconjugants were
isolated on BHV agar containing 2 µg/ml chloramphenicol and 50 µg/ml nalidixic acid
after 48 h and screened by PCR for single or double-crossover events at the lrp locus. A
single-crossover transconjugant, designated APTW405, was selected and grown
overnight at 35°C and 5% CO₂ on BHV agar medium supplemented with 2 µg/ml
chloramphenicol. The following day, the single-crossover mutant was inoculated into 1
ml of BHV supplemented with 5 µg/ml chloramphenicol and grown at 37°C and 220
rpm for 2 h until slightly turbid. At this point, 1 ml of BHV broth medium
supplemented with 20% sucrose and 10 µg/ml chloramphenicol was added to the single-
crossover mutant culture to achieve a final concentration of 10% sucrose and 7.5 µg/ml
chloramphenicol. This culture was incubated at 37°C and 220 rpm for 5 h to select for
chloramphenicol resistance and sucrose insensitivity. Dilutions of the chloramphenicol
selection/sucrose counter-selection culture were plated on BHV agar supplemented with
5 µg/ml chloramphenicol and 10% sucrose and incubated overnight at 35°C and 5% CO₂.
This chloramphenicol selection/sucrose counter-selection on APTW405 resulted in
chloramphenicol-resistant and sucrose-insensitive bacteria at a density of 6.7x10⁷
CFU/ml, suggesting the single-crossover event in APTW405 was forced into a double-
crossover event to generate an lrp mutant.

**Southern blot analysis.** Chromosomal DNA and plasmid controls were digested with
the restriction enzyme EcoRI and the DNA fragments were separated on an 0.8%
ultrapure agarose gel in Tris-acetate-EDTA (TAE) buffer. Southern blots were
performed as described by Sambrook et al. (50). DNA probes were labeled with
digoxigenin using either the PCR DIG probe synthesis or the DIG DNA labeling kit (Roche Applied Science, Indianapolis, IN). Probes included an 0.5-kb lrp PCR fragment, an 0.8-kb chloramphenicol acetyl-transferase (CAT) cassette fragment, and a 3.7-kb pGP704 fragment. The lrp fragment was generated by PCR using the MM430-BamHI and MM379-SalI primers with AP100 genomic DNA as a template. The CAT cassette fragment was generated by PCR using the MM508 and MM509 primers with pER187 as a template. The pGP704 fragment was generated by digesting the plasmid with BglII. Hybridizations, washes, and developing was performed as by Fuller et al (20). Hybridizations were carried out at 42°C for 18 h in 50% formamide, 2% Blocking Solution (Roche), 5X SSC, 0.1% Sarkosyl detergent, and 0.02% SDS. Blots were washed 3 times in 2X SSC (1X SSC is 0.15 M NaCl, 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate (SDS) for 15 min at room temperature and twice in 0.1X SSC-0.1% SDS for 60 min at 68°C. Blots were developed with an alkaline phosphatase-conjugated anti-digoxigenin and CDP-Star substrate kit (Roche) according to the manufacturer’s instructions.

Complementation induction assays. The ~1.9-kb SphI/SacI restriction digest fragment from pTW338 was ligated to similarly digested pGZRS39 to generate the lrp complementation plasmid, pTW415. Wild-type (AP225/pGZRS39), lrp mutant (AP359/pGZRS39), and complemented mutant (AP359/pTW415) strains containing pIviI or pIviG as a second plasmid were grown overnight on BHIV agar supplemented with 50 µg/ml kanamycin and 50 µg/ml ampicillin for maintenance of both plasmids. The pIviI and pIviG plasmids contain previously identified (19) in vivo induced promoters fused to
luciferase. A sterile cotton tipped swab was used to resuspend each bacterial strain in 1.2 ml of CDM –ILV broth medium and 100 µl was used to inoculate 5 ml cultures of CDM –ILV and CDM +ILV broth medium for each strain. Cultures were grown at 37°C for 8 h at 220 rpm with samples taken every 1-2 h and analyzed for luciferase expression by quantitative luciferase assays. Assays were performed in triplicate at each time point in each experiment, and experiments were repeated a minimum of three times.

Nucleotide sequence accession number. A sequence of the *A. pleuropneumoniae lrp* gene has been deposited in the GenBank database under accession number DQ370064. Sequences of the inserts in the pIvi plasmids used in this study have been previously submitted to GenBank under the following accession numbers: clone *ivi*A, DQ370062; *ivi*G, DQ370063; *ivi*I, DQ370055; *ivi*P, DQ370061; *ivi*S, DQ370056; *ivi*U, DQ370060; *ivi*X, DQ370059; *ivi*Y, DQ370058; *ivi17b*, DQ667682; and *ivi17g*, DQ370057. Sequence data for the *nqr* promoter has been submitted to GenBank as an amendment to accession number U24492, which contains the sequence for the *A. pleuropneumoniae nqr* (previously designated *aopA*) gene (11).
Results

Cloning of A. pleuropneumoniae lrp. We have previously identified the A. pleuropneumoniae ilvI gene as an in vivo induced (ivi) gene (19), and have shown that expression of a luciferase reporter is induced from the ilvI promoter under BCAA limiting conditions (59). IlvI has been extensively studied in E. coli and shown to be positively regulated by Lrp. To investigate the role of Lrp in the regulation of ilvI and other A. pleuropneumoniae ilvi genes, we identified a gene with similarity to lrp in the unfinished A. pleuropneumoniae serotype 5 genome by homology to known lrp genes. Primers, MM450-SphI and MM451-SalI, were designed from A. pleuropneumoniae serotype 5 sequence and used to clone a ~1.9-kb region from A. pleuropneumoniae serotype 1 into pUC19 (58), which included the 3’ end of the upstream rnd gene, the complete lrp gene, and the 5’ end of the downstream ftsK gene, to generate pTW338. The insert from pTW338 was sequenced and the translated lrp sequence from pTW338 was aligned with Lrp sequences from eight different bacterial species, including four members of the family Pasteurellaceae to which A. pleuropneumoniae belongs (Fig. 1). The translated protein sequence of A. pleuropneumoniae is 71% identical to that of E. coli Lrp (Fig. 1). The alignment showed an overall amino acid sequence conservation, including within the domains for DNA binding, transcriptional activation, and leucine response identified in Lrp from E. coli (43) and Pyrococcus furiosis (32). The conservation of the domains within A. pleuropneumoniae Lrp suggests the domains may have similar functions to those characterized in E. coli Lrp.
Purification of *A. pleuropneumoniae* and *E. coli* His$_6$-Lrp. To obtain a better understanding of the function of *A. pleuropneumoniae* Lrp, both *E. coli* and *A. pleuropneumoniae* His$_6$-Lrp were purified. Induction of both *A. pleuropneumoniae* (Fig. 2A) and *E. coli* (data not shown) His$_6$-Lrp resulted in a dominantly expressed ~23 kDa protein, as shown by SDS-PAGE. This was ~4.7 kDa larger than the expected size of 18.3 kDa as predicted from the translated *A. pleuropneumoniae* *lrp* gene and ~4.2 kDa larger than what has been reported for *E. coli* Lrp. The addition of the (His$_6$)-tag could account for this difference. Purification of *A. pleuropneumoniae* (Fig. 2B) and *E. coli* His$_6$-Lrp (data not shown) was analyzed by SDS-PAGE.

His$_6$-Lrp binding to *A. pleuropneumoniae* promoters. To investigate whether the *A. pleuropneumoniae* Lrp directly regulates expression of *ivi* genes, we analyzed whether purified *A. pleuropneumoniae* His$_6$-Lrp would bind *in vitro* to the purified DNA inserts of *A. pleuropneumoniae* *ivi* promoter clones. To confirm that the electrophoretic gel mobility shift assays (EMSA) were functioning as designed, the *E. coli* *ilvIH* promoter fragment from pTW328 was analyzed by EMSA. The mobility of the *E. coli* *ilvIH* promoter fragment was retarded in an EMSA reaction when either *A. pleuropneumoniae* or *E. coli* His$_6$-Lrp was present (data not shown). Furthermore, *E. coli* His$_6$-Lrp bound to the *A. pleuropneumoniae* *iviI* insert (data not shown).

We then analyzed eight *ivi* promoter clones that had previously been shown to be induced under BCAA limitation. These included: *iviG, iviI, iviP, iviS, iviU, iviX, iviY,* and *ivi17g* (Table 1) (59). *IviA* was used as a negative control because the *iviA* clone did not respond to limitation of BCAAs (59) and the pIviA insert DNA sequence did not
have any similarity to published Lrp consensus binding sites (12, 60). Binding of *A. pleuropneumoniae* His$_6$-Lrp to the *iviG* and *iviI* inserts, but not to the *iviA* control, was demonstrated by EMSA (Fig. 3A). The presence of *A. pleuropneumoniae* His$_6$-Lrp retarded the migration of both the 623-bp *iviI* and the 211-bp *iviG* fragments in a dose-dependent manner. The presence of two separate retarded bands with the *iviI* insert suggests *iviI* has multiple *A. pleuropneumoniae* His$_6$-Lrp binding sites. In contrast, *A. pleuropneumoniae* His$_6$-Lrp did not bind to the *iviP*, *iviS*, *iviU*, *iviX*, *iviY*, and *ivi17g* fragments under these assay conditions (data not shown).

The DNA sequences of the inserts to pIviI and pIviG have been identified (59). The pIviI insert sequence has similarity to the 5' end of *ilvI*, a gene involved in the biosynthesis of BCAAs. The pIviG insert sequence has sequence similarity to, but is in an antisense orientation to, the *cps1AB* genes identified to be involved in the biosynthesis of A. *pleuropneumoniae* serotype 1 capsule.

To obtain a better understanding of the Lrp regulon of *A. pleuropneumoniae*, we extended this analysis to include the promoter regions of other genes. The *A. pleuropneumoniae* putative promoter regions of the Apx toxin genes *apxI*, *apxII*, and *apxIV*, the *apf* type IV fimbriae cluster, the *flp-rcp-tad* locus for type IV fimbriae involved in biofilm formation, the *ilvG* gene involved in BCAA biosynthesis, the *lrp* gene, the *nqr* operon encoding the Na$^+$-translocating NADH:ubiquinone oxidoreductase, and the *serA* gene involved in serine biosynthesis were isolated by PCR using gene specific primers (Table 2) and analyzed by EMSA. The migration of the 778-bp *nqr* and 751-bp *lrp* promoter fragments were retarded when *A. pleuropneumoniae* His$_6$-Lrp was added to the reaction (Fig. 3B). In contrast, *A. pleuropneumoniae* His$_6$-Lrp was not
observed to bind to the *apxI, apxII, apxIV, apf, flp-rcp-tad, ilvG, or serA* promoter fragments under these assay conditions (data not shown).

Accurate calculation of binding affinities of Lrp for each of these genes will require more extensive EMSA with a greater range of Lrp concentrations. However, using the band intensities seen with *iviI* (Fig. 3A), as measured using a Storm PhosphorImager, we can calculate an estimated binding constant for the binding of Lrp to the *iviIH* promoter encoded in the *iviI* insert to be 125 nM.

**Identification of transcriptional start sites and analysis of putative promoter regions.** Primer extension analysis was used to determine the transcriptional start sites of *iviI, iviG, lrp, and nqr*. Figure 4 shows the DNA sequences of the regions upstream from the transcriptional start sites for these four promoters. The initiating nucleotide in all 4 transcriptional start sites identified was predicted to be T. The regions immediately upstream of the transcriptional start sites displayed a conserved -10 region with the sequence TATA(A/T)T as well as a conserved -35 region with the consensus sequence TAGACA, with spacings of 17-20 bp between the putative -35 and -10 regions.

Using the *E. coli* consensus binding site for Lrp of YAGHAWATTWTDCTR, where Y = C or T; H = C, A, or T; W = A or T; D = G, T, or A; and R = G or A, we identified potential binding sites for Lrp in all 4 of these promoter regions (Fig. 4). All 4 contained a potential Lrp binding site overlapping the -10 region. There were also potential binding sites overlapping or close to the -35 region in the *iviI, iviG, and lrp* promoters, as well as an additional site further upstream in *iviI and lrp*. 
Construction and confirmation of an *A. pleuropneumoniae* *lrp* mutant. To further investigate the role of Lrp in the regulation of *ivi* genes responding to limitation of BCAAs, an *lrp* mutant was constructed (Fig. 5). To confirm that the chloramphenicol selection/sucrose counter selection was successful in producing an *lrp* mutant, twenty potential *lrp* mutants were screened by PCR using *lrp* specific primers, MM480 and MM481. Fifteen of the 20 colonies displayed a single 1-kb product that corresponded to a mutant *lrp* allele. Five colonies displayed both the wild-type 200-bp and mutant 1-kb band predicted for a single-crossover event (data not shown).

Seven of the 15 *lrp* mutants were further characterized and were confirmed as Gram-negative coccobacilli, NAD dependent, chloramphenicol-resistant, sucrose-insensitive, kanamycin-sensitive, and nalidixic acid-resistant. These characteristics were as predicted for a double-crossover *lrp* mutant. A single representative mutant, AP359, was selected for more thorough validation experiments.

A colony PCR on AP359 using MM480 and MM481 resulted in a 1-kb product as predicted for an *lrp* mutant (Fig. 6B). In comparison, a predicted 200-bp product was observed for wild-type AP225, and a 1-kb product for *E. coli* S17-1 (λpir)/pTW404 (Fig. 6B). The PCR screen supported AP359 as an *lrp* mutant.

Southern blot analyses of genomic DNA prepared from wild-type AP100 and the *lrp* mutant, AP359, are shown in figure 6C, D, and E. In AP100, the *lrp* probe hybridized to a 5.2-kb *EcoRI* fragment (Fig. 6C, lane 1), but there was no reaction with either the CAT (Fig. 6D, lane 1) or the pGP704 (Fig. 6E, lane 1) probes. In the *lrp* mutant, the *lrp* probe hybridized to 3.4-kb and 2.6-kb *EcoRI* fragments (Fig. 6C, lane 2), while the CAT probe hybridized to a 3.4-kb *EcoRI* fragment (Fig. 6D, lane 2); there was no reaction with
the pGP704 probe (Fig. 6E, lane 2). The hybridization pattern seen with AP359 genomic DNA is the pattern predicted in transconjugants where the wild-type \textit{lrp} locus has been replaced by the mutated \textit{lrp}:CAT locus by a double-crossover event (Figure 6A). All 3 probes bound to \textit{EcoRI} digested pTW404 (Fig. 6C, D, and E, lane3). These data confirm AP359 as an \textit{A. pleuropneumoniae lrp} deletion disruption mutant.

**Growth of wild-type, \textit{lrp} mutant, and complemented mutant in CDM+ILV and CDM-ILV.** Exponential growth rates (µ) of wild-type \textit{A. pleuropneumoniae}, the \textit{lrp} mutant, and a complemented mutant constructed by cloning pTW415 into the \textit{lrp} mutant, were compared in both complex and chemically defined growth media (Table 3). Specific growth rates for wild-type and \textit{lrp} mutant \textit{A. pleuropneumoniae} were similar in complex media and similar although much lower in complete chemically defined medium. However, while the growth rate for wild-type was further reduced in CDM-ILV, there was no detectable growth of the \textit{lrp} mutant in this medium. The ability to grow in CDM-ILV was restored in the complemented mutant. In addition to an inability to grow in CDM-ILV, the \textit{lrp} mutant displayed a slightly longer lag time in all growth media as compared to wild-type \textit{A. pleuropneumoniae} (data not shown).

**Luciferase expression from \textit{iviI} and \textit{iviG} in wild-type, \textit{lrp} mutant, and complemented \textit{lrp} mutant.** To examine the effect of the loss of Lrp on expression from putative Lrp-regulated \textit{ivi} promoters, luciferase activity expressed from the promoters in plviI and plviG, which contain promoter::\textit{luxAB} fusions, in each strain was compared in CDM+ILV and CDM–ILV broth media (Fig. 7A&B). The luciferase activity expressed
from the *ivi*I promoter showed a basal level of expression of ~500 RLU/OD in CDM+ILV media in either a wild-type background (AP225/pGZRS39/pIviI), *lrp* mutant background (AP359/pGZRS39/pIviI), or in the complemented mutant (AP359/pTW415/pIviI), with no significant increase over the course of the experiment. However, luciferase activity in the wild-type background increased on average over seven fold in CDM-ILV as compared to CDM+ILV in one hour, and over ten fold in two hours. In sharp contrast, when the *ivi*I promoter was placed in an *lrp* mutant background, no increase in luciferase activity was observed in CDM-ILV. In the complemented mutant, induction of luciferase expression from the *ivi*I promoter in CDM–ILV was restored. The apparent decrease in relative RLU/OD in CDM-ILV as compared to CDM+ILV in the *lrp* mutant is due to the lack of growth of the mutant in CDM-ILV medium (see Table 3). Expression from the *ivi*I promoter was strongly up-regulated in CDM-ILV in the wild-type and complemented mutant strains containing Lrp and unresponsive in the Lrp mutant. These data suggest that Lrp is directly involved in the regulation of the *A. pleuropneumoniae ilvIH* promoter within pIviI in response to BCAA limitation.

The luciferase activity of strains containing pIviG was also examined. The luciferase activity expressed from the *ivi*G promoter in wild-type, the *lrp* mutant, and the complemented mutant showed an initial level of expression of ~100 RLU/OD in CDM+ILV media that increased 2-3 fold within 3 hours in all three backgrounds. In the wild-type background, expression from the *ivi*G promoter was further increased two-fold in CDM-ILV as compared to CDM+ILV (Fig. 7B). This additional increase in expression from *ivi*G was not seen in the *lrp* mutant, and was partially restored in the
complemented mutant. Again, the apparent decrease in relative RLU/OD in CDM-ILV as compared to CDM+ILV in the \textit{lrp} mutant is due to the lack of growth of the mutant in CDM-ILV medium (see Table 3). These data suggest that Lrp is also directly involved in the regulation of the \textit{iviG} promoter in response to BCAA limitation, although the increase in expression for \textit{iviG} is distinctly lower and slower than the rapid and robust response seen with \textit{iviI}.

\textbf{Discussion}

In this study, we report the identification of the \textit{A. pleuropneumoniae} \textit{lrp} gene and its cloning, sequencing, protein purification, and mutation by deletion disruption using a chloramphenicol selection/sucrose counter-selection procedure. The \textit{A. pleuropneumoniae} Lrp is similar in amino acid sequence and function to the extensively studied Lrp from \textit{E. coli}. The functional domains for DNA binding, transcriptional activation, and leucine response identified in \textit{E. coli} Lrp (43) are also highly conserved in \textit{A. pleuropneumoniae} and suggest the functions of \textit{A. pleuropneumoniae} Lrp may be similar. This is further supported by the \textit{in vitro} binding of purified \textit{A. pleuropneumoniae} His$_6$-Lrp binding \textit{in vitro} to the \textit{E. coli ilvI} promoter in an EMSA experiment (data not shown). These results demonstrate that \textit{A. pleuropneumoniae} does have an \textit{lrp} gene and its protein function is similar to that of \textit{E. coli} Lrp.

However, differences may exist between these similar proteins. A study by Platko and Calvo (43) show multiple mutations in Lrp can affect DNA-binding, activation, and the leucine response of Lrp. While \textit{A. pleuropneumoniae} has the same
amino acids at 21 of the 22 sites identified as critical for these functions, *E. coli* has a serine at position 125 of *E. coli* Lrp whereas *A. pleuropneumoniae* has an alanine at the homologous position (Fig. 1). The serine was shown to be important in the activation of transcription by Lrp in *E. coli*. The unconserved alanine in *A. pleuropneumoniae* Lrp suggests a difference in regulation could exist between *E. coli* and *A. pleuropneumoniae* Lrp. In *Haemophilus influenzae*, a bacterium closely related to *A. pleuropneumoniae*, Lrp was shown to affect the expression of fewer proteins (18) than in *E. coli* (15), which suggests that a difference between the roles of Lrp in *E. coli* and *A. pleuropneumoniae* could be expected.

Our main hypothesis guiding this study was that a subset of *ivi* genes responding similarly to BCAA limitation are also regulated by a similar mechanism. Since Lrp had been identified as a regulator of *ilvI* in *E. coli* (44, 46), we speculated that an *A. pleuropneumoniae* Lrp would not only regulate the *A. pleuropneumoniae* *ivi* gene, *ilvI* (19), but also other *ivi* genes we previously identified as being up-regulated by BCAA limitation (59).

Since it had been previously shown that the *E. coli* His<sub>6</sub>-Lrp behaves as the native protein does (36), we began to address our hypothesis by using purified *A. pleuropneumoniae* His<sub>6</sub>-Lrp to determine if *A. pleuropneumoniae* Lrp has a role in the regulation of *ivi* genes shown to induce under BCAA limitation. The observation that two of the eight identified clone inserts bound *A. pleuropneumoniae* His<sub>6</sub>-Lrp supports that a subset of clones shown to induce under BCAA limiting conditions are regulated similarly. The remaining six *ivi* clone inserts that did not bind *A. pleuropneumoniae*
HisE-Lrp could be regulated by countless other mechanisms such as other protein regulators, amino acid attenuation, or could be regulated indirectly by Lrp.

The Lrp regulon in *E. coli* is extensive, including ~10% of all *E. coli* genes (54). We identified *A. pleuropneumoniae* homologues of several genes known to be regulated by Lrp in *E. coli*, including *ilvG* (45), *serA* (67), and *lrp* itself (61), and tested these by EMSA. Lrp also regulates a variety of virulence-associated genes including fimbriae in *E. coli* (5) and haemolysin in *Xenorhabdus nematophila* (10, 26). Therefore, we also tested binding of Lrp to the putative *A. pleuropneumoniae* promoters of Apx toxin genes, two fimbrial operons, and the *nqr* gene. *A. pleuropneumoniae* Lrp did bind to its own promoter, suggesting that regulation of the *lrp* gene may be similar in *A. pleuropneumoniae* and *E. coli*. However, Lrp failed to bind to the *ilvG* or *serA* promoters, which suggests that regulation of BCAA biosynthesis by Lrp in *A. pleuropneumoniae* is different, or at least not as complex, than in *E. coli* (18). *A. pleuropneumoniae* Lrp did not bind to the *apxI, apxII, apxIV, apf*, or *flp-rcp-tad* promoters under the assay conditions used, suggesting that either Lrp does not regulate these genes in *A. pleuropneumoniae*, the effect of Lrp is indirect, or the assay conditions established for binding to the *ilvIH* promoter are not optimal for all DNA fragments. Lrp did bind to the DNA fragment containing the *nqr* operon promoter. However, it should be noted that this fragment potentially also contains the promoter region for a divergently transcribed upstream open reading frame. *A. pleuropneumoniae nqrA* has been shown to be strongly expressed and antigenic *in vivo* (11) and has been shown to be essential for survival during infection (52). In *Vibrio cholerae*, mutants in Nqr affect virulence gene expression (25). However, there is no *nqr* operon in *E. coli*. This is the first report of
potential regulation of nqr by Lrp. These results indicate that the A. pleuropneumoniae Lrp regulon, while not as extensive as that characterized for E. coli, is not limited to genes involved in BCAA biosynthesis and does include both in vivo induced and virulence-associated genes.

While these experiments were not designed to allow accurate calculation of binding affinities of LRP, we were able to estimate the binding constant for the ilvIH (iviI) promoter to be 125 nM. This estimate is greater than the calculated Kd for E. coli LRP binding to the E. coli ilvIH gene (8 nM) and to the lrp gene (35 nM) (61) but still within a reasonable range for a DNA-binding protein.

The ability of A. pleuropneumoniae His6-Lrp to bind to the DNA inserts of ivi clones that did not respond to the limitation of BCAAs was not analyzed. Given that certain promoters can be regulated by E. coli Lrp in the presence of leucine and other promoters in the absence of leucine (15, 34, 40), it is distinctly possible Lrp from A. pleuropneumoniae may bind to additional ivi clone DNA inserts from clones that failed to induce under BCAA limitation. If this is the case, there may be additional ivi genes that are regulated by Lrp that have not been identified within the scope of this study.

While the demonstrated binding of recombinant A. pleuropneumoniae His6-Lrp to DNA fragments in vitro suggests regulation by Lrp, it is not proof that expression of these genes is controlled by Lrp. To complement this data, we analyzed expression from the iviI and iviG promoters in wild-type A. pleuropneumoniae, an lrp mutant, and a complemented mutant. In the presence of leucine, isoleucine, and valine, there was minimal expression from the iviI promoter in all three backgrounds regardless of the presence of Lrp. In the absence of leucine, isoleucine, and valine, expression from the
iviI promoter was strongly and rapidly up-regulated in the wild-type and complemented mutant strains containing Lrp but not in the Lrp mutant. These results suggest that the iviI promoter shows Lrp-dependent activation that is antagonized by leucine, or by a combination of leucine, isoleucine, and/or valine, with a low basal level of expression in the absence of Lrp, and that Lrp is critical for the regulation of the ilvIH (iviI) in A. pleuropneumoniae.

The role of A. pleuropneumoniae Lrp in the expression of the iviG promoter is less clear. In CDM+ILV, there is an increase in basal activity from this promoter with increased growth (or possibly growth rate) in all three backgrounds, which was not seen with the iviI promoter. In the absence of leucine, isoleucine, and valine, expression from the iviG promoter was up-regulated in the wild-type and complemented mutant strains containing Lrp but not in the Lrp mutant. This pattern of up-regulation in response to BCAA limitation is similar to, but dramatically smaller and less rapid, than the response seen with the iviI promoter. These results suggest that the iviG promoter also shows Lrp-dependent activation that is antagonized by leucine, or by a combination of leucine, isoleucine, and/or valine, but in addition shows expression independent of Lrp. How the Lrp-independent expression of iviG is regulated has not been determined.

Mutation of the lrp gene does not appear to affect the growth rate of A. pleuropneumoniae in complex media or in CDM+ILV, except for a slightly longer lag time in broth cultures. However, the lrp mutant fails to grow in chemically defined medium in the absence of BCAAs. Growth in CDM-ILV is restored in the complemented mutant, indicating that the lack of growth is due to the mutation in lrp. Whether this lack of growth CDM-ILV is solely due to the lack of induction of the ilvIH
operon in the absence of LRP, or to lack of regulation of additional genes involved in
BCAA biosynthesis, will require more extensive analysis of the Lrp regulon in A.
*pleuropneumoniae*. It should be noted that the lack of growth of the *lrp* mutant in CDM–
ILV could influence the expression assays. The rapid and robust response to BCAA
limitation seen with the *iviI* promoter strongly suggests that the lack of expression in the
*lrp* mutant strain is due to the *lrp* mutation and not to lack of growth. The lower and
slower response seen with the *iviG* promoter makes it more difficult to draw this
conclusion with complete confidence. However, since *A. pleuropneumoniae* His<sub>6</sub>-Lrp
binds to the *iviG* fragment *in vitro*, the regulation of this promoter by Lrp remains likely.

While the discovery of *A. pleuropneumoniae* Lrp binding to and regulating the
expression of the *ilvI* promoter in *A. pleuropneumoniae* is novel because few regulators
have been identified and examined in this organism, we were not surprised since Lrp had
been shown to regulate *ilvI* in *E. coli*. In contrast, the binding of *A. pleuropneumoniae*
Lrp to the *iviG* promoter is quite surprising. The p*iviG* insert contains the terminal 3’ end
of the *cps1A* gene and the terminal 5’ end of the *cps1B* gene of *A. pleuropneumoniae*
serotype 1, but the promoter in pIviG is in an antisense orientation to both *cps1A* and
*cps1B*. *Cps1A* and *cps1B* encode putative glycosyl transferases involved in the synthesis
of *A. pleuropneumoniae* capsular polysaccharide. Capsule is required for virulence in
this respiratory pathogen. An antisense transcript expressed from the *iviG* promoter
could affect the expression of *cps1B* alone, both *cps1AB*, or the entire capsule
biosynthetic operon. While the role of each gene in the biosynthesis of capsule has not
been established, it is known that serotype 1 capsule is composed of a repeating N-acetyl-
2-dioxy-β-D-glucopyranosyl and α-D-galactopyranosyl disaccharide that is partially O-
acetylated (2). Two possible roles of a transcript antisense to the capsule biosynthesis operon could be to reduce the total amount of capsule or to alter the antigenic structure by reducing the O-acetylation. Reducing the amount of capsule could serve to expose surface adhesins necessary for attachment to respiratory epithelial cells. The fact that *A. pleuropneumoniae* Lrp binds to this region *in vitro* and possibly regulates the expression of the *iviG* promoter raises the possibility that Lrp may play a role in regulation of capsule biosynthesis of *A. pleuropneumoniae*. Future experiments comparing the amount or type of capsule produced in wild-type and the *lrp* mutant are needed.

In *E. coli*, Lrp has been implicated in the regulation of genes involved in virulence such as fimbriae (5), but to our knowledge, this study is the first time that Lrp has been implicated in the regulation of genes specifically induced during infection of the host. Furthermore, it is interesting to speculate on the affect of an *lrp* mutant on the virulence of *A. pleuropneumoniae* and compare it to what is known about the global regulator, Fur. Like Lrp, Fur has been shown to be both a positive and negative regulator (23) but can be modulated by iron rather than leucine. A Fur mutant in *A. pleuropneumoniae* has recently been shown to have reduced virulence (29). An *A. pleuropneumoniae lrp* mutant may also be attenuated if Lrp is necessary for the correct regulation of genes important in causing disease. Infection trials with the *lrp* mutant are needed to address this subject.

This is the first report to identify an *A. pleuropneumoniae* Lrp homolog. While the role of Lrp in the regulation of *ilvI* in *E. coli* has been extensively studied, this work addresses the role of *A. pleuropneumoniae* Lrp in the regulation of *A. pleuropneumoniae* virulence-associated genes, *in vivo* induced genes, and BCAA biosynthetic genes. *A.
pleuropneumoniae Lrp was shown to bind to the promoter of A. pleuropneumoniae ilvI and regulate the expression under BCAA limitation. Furthermore, Lrp was shown to bind to the putative nqr promoter and the A. pleuropneumoniae serotype 1 capsule biosynthesis operon, suggesting for the first time that Lrp is involved in the regulation of A. pleuropneumoniae serotype 1 capsule biosynthesis and nqr expression. Our results suggest that the Lrp regulon in A. pleuropneumoniae differs from that found in E. coli, and is potentially more extensive than the limited regulon found in H. influenzae.

In summary, our previous IVET studies with A. pleuropneumoniae led to the hypothesis that limitation of branched chain amino acids is an important environmental cue for respiratory pathogens of mammals, which need to survive and multiply in an anatomical location where these amino acids are in short supply. Analysis of the in vivo induced gene promoters identified in that work demonstrated that 25% of those ivi promoters were upregulated on chemically defined medium lacking branched chain amino acids as compared to medium containing BCAAs (59). In this study, we have shown that two of these ivi promoters, as well as two additional genes, are regulated by the global regulatory protein Lrp. These results suggest that the ability to synthesize BCAAs, and the ability to produce a functional Lrp protein, may be required for respiratory pathogens of mammals, which further suggests that inhibition of BCAA synthesis or Lrp function might be a fruitful avenue for the development of new classes of antibiotics that would target respiratory pathogens.
Acknowledgements

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<tr>
<th>Strain or plasmid</th>
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<td><strong>Strains</strong></td>
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<td>AP100</td>
<td><em>A. pleuropneumoniae</em> ATCC 27088, serotype 1A, passaged through pigs</td>
<td>ATCC*</td>
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<tr>
<td>AP225</td>
<td>A spontaneous nalidixic acid-resistant mutant of AP100</td>
<td>(20)</td>
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<tr>
<td>APTW405</td>
<td>An <em>lrp</em> single-crossover mutant of AP225</td>
<td>This work</td>
</tr>
<tr>
<td>AP359</td>
<td>An <em>lrp</em> double-crossover mutant of AP225</td>
<td>This work</td>
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<tr>
<td><em>E. coli</em> CV975</td>
<td>*F. ara thi Δ(lac-pro) ilvIH:*Mu dI1734</td>
<td>(44)</td>
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<td><em>E. coli</em> XL1-Blue</td>
<td>Δ(mcrA)Δ83 Δ(mcrCB-hsdSMR-mrr)Δ73</td>
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<td>mRF'</td>
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<td>λpir* recA thi pro hsd (rK⁻ mK⁺) RP4-2-Tc::Mu&lt;br&gt;Km::Tn7 Tmp' Sm'</td>
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<td>pUC19</td>
<td>Ap&lt;sup&gt;f&lt;/sup&gt;; high-copy-number cloning vector</td>
<td>(58)</td>
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<tr>
<td>pTF86</td>
<td>Ap&lt;sup&gt;f&lt;/sup&gt;; <em>A. pleuropneumoniae</em> IVET vector containing promoterless <em>luxAB</em> and <em>ribBAH</em> genes downstream of a unique <em>BamHI</em> cloning</td>
<td>(19)</td>
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pQE30  Ap<sup>+</sup>;  protein expression vector  (QIAGEN)

pCV294  Ap<sup>+</sup>;  *E. coli* (His<sub>6</sub>-Lrp) protein expression vector

pER187  Ap<sup>+</sup>  Cm<sup>+</sup>;  chloramphenicol acetyl-transferase cassette (CAT) containing vector

pUM24Cm  Cm<sup>+</sup>  Kan<sup>+</sup>;  *sacR-sacB-nptI* cassette containing vector

pER187  Ap<sup>+</sup>  Cm<sup>+</sup>;  chloramphenicol acetyl-transferase cassette (CAT) containing vector

pGZRS39  Kan<sup>+</sup>;  *A. pleuropneumoniae* shuttle vector

pKB11  Ap<sup>+</sup>;  *A. pleuropneumoniae* nqr promoter region cloned into pKB10, a derivative of pTF86 with the ribBAH genes deleted

pTW296  Ap<sup>+</sup>;  *E. coli* ilvIH promoter (amplified by PCR and cloned into BamHI site of pUC19)

pTW313  Ap<sup>+</sup>;  *A. pleuropneumoniae* lrp (amplified by PCR and cloned into pQE30)

pTW328  Ap<sup>+</sup>;  *E. coli* ilvIH promoter (constructed by cloning the ~300-bp BamHI fragment from pTW296 into the BamHI site of pTF86)

pTW338  Ap<sup>+</sup>;  *rnd*-lrp-ftsK<sup>+</sup> (amplified by PCR and cloned into SphI/SalI sites of pUC19)

pTW355  Ap<sup>+</sup>;  *rnd*-Δlrp-ftsK<sup>+</sup> (constructed by inverse
PCR with pTW338 as template)

pTW401  Ap<sup>i</sup> Cm<sup>i</sup>;  *rnd<sup>·</sup>*-Δ*lrp*::CAT-ftsK<sup>*</sup>  (constructed by cloning a PCR generated CAT cassette into the *Pst*I site of pTW355)

pTW402  Ap<sup>i</sup> Cm<sup>i</sup> Kan<sup>i</sup>;  *rnd<sup>·</sup>*-Δ*lrp*::CAT-ftsK<sup>·</sup>-*sacR*-sacB-nptI  (constructed by cloning the 3.4-kb *BamHI* sacR-sacB-nptI fragment into a partially *BamHI* digested pTW401)

pTW404  Ap<sup>i</sup> Cm<sup>i</sup> Kan<sup>i</sup>;  *rnd<sup>·</sup>*-Δ*lrp*::CAT-ftsK<sup>·</sup>-*sacR*-sacB-nptI  (constructed by cloning the *SphI*/SacI insert of pTW402 into pGP704)

pTW415  Kan<sup>i</sup>;  *rnd<sup>·</sup>*-lrp-ftsK<sup>·</sup>  (constructed by cloning the *SphI*/SacI insertion of pTW338 into pGZRS39)

*plviA<sup>b</sup>  Ap<sup>i</sup>;  pTF86 containing a 333-bp insert that is homologous to the *mrp* gene

*plviG<sup>b</sup>  Ap<sup>i</sup>;  pTF86 containing a 211-bp insert that is homologous to, but in an antisense orientation to, the *cps1AB* genes involved in capsule biosynthesis

*plviI<sup>b</sup>  Ap<sup>i</sup>;  pTF86 containing a 623-bp insert that is homologous to the *ilvi* gene required for branched chain amino acid biosynthesis
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<th>Ap&lt;sup&gt;c&lt;/sup&gt;; pTF86 containing a 175-bp insert that is homologous to the comJ promoter (30)</th>
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<td>plviX&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Ap&lt;sup&gt;c&lt;/sup&gt;; pTF86 containing a 490-bp insert that is homologous to the vapBvapC promoter (30)</td>
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<td>Ap&lt;sup&gt;c&lt;/sup&gt;; pTF86 containing a 782-bp insert that is homologous to ftsY, which encodes the signal recognition particle receptor (30)</td>
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<tr>
<td>plvi17g&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Ap&lt;sup&gt;c&lt;/sup&gt;; pTF86 containing a 290-bp insert that is homologous to, but in an antisense orientation to, the coaE gene (30)</td>
</tr>
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<sup>a</sup> ATCC, American Type Culture Collection

<sup>b</sup> plvi plasmids contain in vivo-induced promoter fragments fused to promoterless luxAB genes of pTF86.
Table 2. Primers used for this study

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<th>Primer</th>
<th>Sequence of primer$^a$ (5’ to 3’)</th>
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<td>GGCCATCAGTGGATCCAAGAGC</td>
<td><em>E. coli ilvIH</em> promoter forward primer</td>
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<td>MM363-BamHI</td>
<td>CTCGGATCCAGACAAACATCTCC</td>
<td><em>E. coli ilvIH</em> promoter reverse primer</td>
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<td>MM379-SalI</td>
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<td><em>lrp</em> downstream primer</td>
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<tr>
<td>MM430-BamHI</td>
<td>CTGGGAAGACGGATCCCATGGAACATAAAAAAC</td>
<td><em>lrp</em> upstream primer</td>
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<td>MM437-SphI</td>
<td>CCCATCGGATCCGTTACATAAGCATGCTCGGC</td>
<td><em>nqr</em> promoter forward primer</td>
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<td>MM438-BamHI</td>
<td>GTGTACCTGCGATAGGTTGATCCAAGCC</td>
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<td>MM450-SphI</td>
<td>GCAGCGGGCATGCGTTGTTGGGATTTTGGTA</td>
<td><em>rnd</em> internal forward primer</td>
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<tr>
<td>MM451-SalI</td>
<td>AAGCGGTCGATTTTCGTCATTTTTGTTGC</td>
<td><em>fisK</em> internal reverse primer</td>
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<tr>
<td>MM459-PstI</td>
<td>AACTGCAGGAATGTCATTTGTTGGGATTTTC</td>
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<tr>
<td>MM460-PstI</td>
<td>ATCTGCAGGTTCAAGCAATTCCGGATCTAATG</td>
<td><em>lrp</em> inverse PCR reverse primer</td>
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<td>MM478-lux</td>
<td>GCTGCCTCCATCCATGGGGTTTCC</td>
<td>pTF86 <em>lux</em> primer</td>
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<td>MM480</td>
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<td><em>lrp</em> forward PCR screen primer</td>
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<tr>
<td>MM481</td>
<td>CGGGTAATCGCAGTAAAGTGC</td>
<td><em>lrp</em> reverse PCR screen primer</td>
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MM489-PstI  ATGACCCTGCAAGACGAAATTGAGCTCGG  pER187::CAT forward primer
MM508       CTAATGAAGAAAGCAGACAAAGTA  CAT forward PCR screen primer
MM509       GGGGCAGGTTAGTGACATT  CAT reverse PCR screen primer
MM511-PstI  GTGCAGCTCTAGACTGCAAGCCTCGTC  pER187::CAT reverse primer
MM525-BamHI AAAGGGATCCGCCAGACGAAT  ilvG promoter forward primer
MM526-XbaI  ACCGACATCTTAGACCGACATAAAGTA  ilvG promoter reverse primer
MM527-BamHI GAAATGGATCCGCCTGATTTAGCAC  lrp promoter forward primer
MM528-XbaI  CGTTCAATTCTAGATTAACTCCTTATTITA  lrp promoter reverse primer
MM529-BamHI GGAACGGATCGCTCAATAACATCGCC  serA promoter forward primer
MM530-XbaI  CGCATATTAGAAATTTTGAGTGGAGGC  serA promoter reverse primer
MM531       GTTTTCACGACGTAGACGTGT  pUC19 forward primer
MM532       CACAGGAAACAGCTATGACCATG  pUC19 reverse primer
MM533-T4    CTGGGGGATGAGTGCGACACC  pTF86 T4 primer
MM534-BamHI GCAATATCGGATCCGCTCAATGTAATC  flp-rcp-tad promoter forward primer
MM535-XbaI  TGGCAATGAGTCTAGAAATACGAGTGTC  flp-rcp-tad promoter reverse primer
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<td>MM537-XbaI</td>
<td>CGATGCGCGCTCTAGATCCGAAAGC</td>
<td>apf promoter reverse primer</td>
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<tr>
<td>MM538-BamHI</td>
<td>CCGTAATTGGATCCAAAATACCGTAAGCAG</td>
<td>apxI promoter forward primer</td>
</tr>
<tr>
<td>MM539-XbaI</td>
<td>AAGTCTAGACATCCGCAAATAGCGAGGCAAC</td>
<td>apxI promoter reverse primer</td>
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<tr>
<td>MM540-BamHI</td>
<td>TACGGATCTTTGGTACAAAAAATTTTACAG</td>
<td>apxII promoter forward primer</td>
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<tr>
<td>MM544-BamHI</td>
<td>AACCAGGATCCCAACAGAACACAAAGC</td>
<td>apxIV promoter forward primer</td>
</tr>
<tr>
<td>MM545-XbaI</td>
<td>CACAAAACGTCTAGACCCACCATAAAT</td>
<td>apxIV promoter reverse primer</td>
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</table>

* Restriction sites are underlined.
Table 3. Specific growth rates of wild-type, *lrp* mutant, and complemented mutant in various growth media.

<table>
<thead>
<tr>
<th>Medium</th>
<th>AP225 (wild type)</th>
<th>AP359 (<em>lrp</em> mutant)</th>
<th>AP359/pTW415 (complemented mutant)</th>
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<tbody>
<tr>
<td>BHIV</td>
<td>0.83 ± 0.05</td>
<td>0.81 ± 0.08</td>
<td>ND</td>
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<tr>
<td>HIV</td>
<td>0.83 ± 0.02</td>
<td>0.90 ± 0.05</td>
<td>ND</td>
</tr>
<tr>
<td>CDM+ILV</td>
<td>0.36 ± 0.05</td>
<td>0.39 ± 0.09</td>
<td>0.39 ± 0.12</td>
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<tr>
<td>CDM-ILV</td>
<td>0.26 ± 0.03</td>
<td>No growth</td>
<td>0.26 ± 0.02</td>
</tr>
</tbody>
</table>

*a* Data presented are the average of at least three separate growth curves. $\mu$ was calculated as $\ln 2 / T_d$ where $T_d$ is the doubling time during exponential growth.

*b* ND = Not done.
FIGURE LEGENDS

Fig. 1. Lrp domain and amino acid alignment. Lrp amino acid sequence from 9 bacterial species aligned with ClustalX (55) and shaded with Boxshade v3.31. Residues that are identical in the majority of species are shown on a black background while residues that are functionally conserved are shown on a gray background. The location of the Lrp functional domains for DNA binding (dashed line), transcriptional activation (solid line), and leucine response (dotted line) are indicated above the sequence (32, 43). The position of the amino acid important in the activation of transcription by Lrp in *E. coli* but unconserved in *A. pleuropneumoniae* is indicated by the symbol *. The following species abbreviations and GenBank numbers were used: Ec, *Escherichia coli* (BAA35614); Ka, *Klebsiella aerogenes* (AAD12584); Aa, *Actinobacillus actinomycetemcomitans*; Pm, *Pasteurella multocida* (AAK02338); Hi, *Haemophilus influenzae* (AAC23241); Hd, *Haemophilus ducreyi* (AAP96279); Ap, *Actinobacillus pleuropneumoniae* serotype 1; Pa, *Pseudomonas aeruginosa* (AAG08693); Lp, *Legionella pneumophila* (AAU27568). The *A. actinomycetemcomitans* sequence was identified by similarity in the University of Oklahoma unfinished *A. actinomycetemcomitans* genome and translated.

Fig. 2. Induction and purification of *Actinobacillus pleuropneumoniae* serotype 1 His$_6$-Lrp. (Panel A) SDS-PAGE of *A. pleuropneumoniae* serotype 1 His$_6$-Lrp protein induction samples. Lanes in panel A: M, 10 µl of LOW SDS Page standard; 1, 0.4 ml culture-equivalent of cell extract before induction; 2, 0.1 ml culture-equivalent cell
extract after induction; 3, 0.05 ml culture-equivalent induced soluble fraction; 4, 0.05 ml culture-equivalent induced insoluble fraction. Lysozyme, used in the lysis procedure, appears at the 14 kDa size in lanes 3 and 4. (Panel B) SDS-PAGE of A. pleuropneumoniae serotype 1 His$_6$-Lrp purification samples. Lanes in panel B: 1, 35 µl culture-equivalent of cell extract; 2, 35 µl culture-equivalent of cell extract flow through; 3, 17.5 µl culture-equivalent of first wash flow through; 4, 35 µl culture-equivalent of second wash flow through; 5, 35 µl culture-equivalent of protein elution; 6, 140 µl culture-equivalent of concentrated His$_6$-Lrp; 7, 280 µl culture-equivalent of concentrated His$_6$-Lrp; M, 10 µl of LOW SDS Page standard.

Fig. 3. Analysis of A. pleuropneumoniae His$_6$-Lrp binding to ivi clone DNA inserts using electrophoretic mobility shift assays. The iviI, iviG, iviA, lrp, and nqr labeled DNA fragments were mixed with 0, 5, 30, or 60 ng of His$_6$-Lrp in a binding reaction. Gels from at least two replicate experiments were scanned using a Storm PhosphorImager and the quantitative data used to calculate the percent of labeled DNA bound to protein (% bound).

Fig. 4. Alignment of the promoter regions from A. pleuropneumoniae iviI, iviG, lrp, and nqr. The transcriptional start points identified by primer extension are shown in bold and underlined. The putative -10 and -35 regions are shown in bold and shaded, and the consensus sequences are shown at the bottom of the figure. Dashes indicate gaps to align the promoter regions. Sequences with at least 80% homology to the consensus binding site for E. coli LRP are boxed with a solid line; sequences with 73-80%
homology are boxed with a dashed line. Where overlapping potential binding sites were identified, the site with the best homology is marked.

Fig. 5. Construction of the knock-out construct, pTW404. An inverse PCR technique was performed to amplify around the lrp containing plasmid, pTW338, in opposite directions and generate a ~100-bp deletion from the center of lrp to make pTW355. A chloramphenicol acetyl transferase resistance (CAT) cassette DNA fragment was generated by PCR and cloned into the PstI site of pTW355 to form pTW401. A BamHI fragment, containing the sacR, sacB, and nptI, was excised from pUM24Cm and ligated into a BamHI site in pTW401 to generate pTW402. The insert from pTW402 was cloned into the conjugative suicide vector pGP704 to form pTW404.

Fig. 6. Confirmation of the A. pleuropneumoniae lrp mutant. (A) Genetic maps of the lrp locus in wild-type, pTW404, and predicted double-crossover mutant strains. The predicted genomic DNA EcoRI fragment sizes of wild-type and double-crossover mutant strains are shown along with predicted EcoRI fragments for the knock-out construct, pTW404. Abbreviations used: E, EcoRI; X_A, site of genetic recombination site A; X_B, site of genetic recombination site B; X_AX_B, resulting double-crossover event at sites X_A and X_B. (B) A 2% agarose gel with PCR reactions using A. pleuropneumoniae lrp specific primers, MM480 and MM481. PCR reactions using the following DNA templates were loaded into each lane: AP359 lrp mutant genomic DNA (lane 1), AP100 wild-type genomic DNA (lane 2), knock-out construct pTW404 (lane 3), no DNA (lane labeled “-“), Invitrogen 1-kb DNA ladder (lane M). Southern blots probed with lrp (C),
CAT cassette (D), and pGP704 BglII fragment (E) are shown. Lanes in Southern blot panels: 1, AP100 wild-type genomic DNA; 2, AP359 lrp mutant genomic DNA; 3, knock-out construct pTW404.

Fig. 7. Relative expression from iviI and iviG promoters in wild-type and lrp mutant backgrounds in CDM-ILV and CDM+ILV media. Bacterial strains were inoculated into CDM+ILV and CDM-ILV broth to an optical density of ~0.1 at 520 nm (OD\textsubscript{520}) and luciferase activity expressed from the promoter:luxAB fusions was measured over time. Luciferase activity was first normalized to RLU per OD\textsubscript{520}, and relative expression for each culture at each time point was calculated as RLU/OD in CDM-ILV divided by RLU/OD in CDM+ILV. Panel A shows the expression from the iviI promoter in wild-type (AP225/pGZRS39/iviI, white bar), lrp mutant (AP359/pGZRS39/iviI, dark gray bar), and complemented lrp mutant (AP359/pTW415/iviI, light gray bar). Panel B shows the expression from the iviG promoter in wild-type (AP225/pGZRS39/iviG, white bar), lrp mutant (AP359/pGZRS39/iviG, dark gray bar), and complemented lrp mutant (AP359/pTW415/iviG, light gray bar). Data are presented as the means ± the standard deviation of three experiments in panel A and of six experiments in panel B.
| Ec  | 1   | MVDSKRPGKDLDRIDNILELDKGRISNVELSKRVLGSLTPCLERVK |
| Ka  | 1   | MVDSKRPGKDLDRIDNILELDKGRISNVELSKRVLGSLTPCLERVK |
| Aa  | 1   | MEKLPKALDAIDIKILNELQRGKISNDLSKVLGSPTCLERVK     |
| Pm  | 1   | MEKKLMKALDSIDIKILNELQRGKISNDLSKVLGSPTCLERVK     |
| Hi  | 1   | MSKEIKKMEKKNAIKILNELQRGKISNDLSKVLGSPTCLERVK     |
| Hd  | 1   | MEYKKSALDKQIDLNILELDQRGKISNDLSKVLGSPTCLERVK     |
| Ap  | 1   | MEHKLPKALDAIDIKILNELQRGKISNDLSKVLGSPTCLERVK     |
| Pa  | 1   | MRTQHQQSKRELDKDRNILRILQEEGRISITEFELGERVLGSTTPCTERVK |
| Lp  | 1   | MKNPTKKNYPNEKNTEYPDIEILDKIDRKLILNIEKNQQTINQALADIVGISAPPCFRVK |

| Ec  | 51  | RLREQQGFIOTGYTALLNPHYLDASLLVFEITEIINRGGAPDVFEQFNTAVOKLLEEIQECHLVS |
| Ka  | 51  | RLREQQGFIOTGYTALLNPHYLDASLLVFEITEIINRGGAPDVFEQFNTAVOKLLEEIQECHLVS |
| Aa  | 48  | RLEKQGVMGYRALNPEALSPLLVEITLVRGKPDVFEEFNAAVQOQLEIQQECHLVS |
| Pm  | 48  | RLEKQGVMGYRALNPEALSPLLVEITLVRGKPDVFEEFNAAVQOQLEIQQECHLVS |
| Hi  | 55  | RLEKQGVMGYRALNPEALSPLLVEITLVRGKPDVFEEFNAAVQOQLEIQQECHLVS |
| Hd  | 49  | RLEKQNVQIMGYRALNPEALSPLLVEITLVRGKPDVFDFNYAIKOLDEIQQECHLVS |
| Ap  | 49  | RLEKQNVQIMGYRALNPEALSPLLVEITLVRGKPDVFDFNYAIKOLDEIQQECHLVS |
| Pa  | 50  | RLREQGLIMYHARLNPQHKLKASLLVFEISLDYKSGDTFEFERRAVLKLPHVECHLVS |
| Lp  | 61  | RLREEEKITVNDVALVPDPSKVRPLIVFVNITLEKQREDLLAHFERRKMQQEPEVMQQYFVS |

| Ec  | 111 | GDFDYLLKTRVPMAYRKLLEGLTTLLPGVNDTRTYVMEEEVKQSNRLVIKTR |
| Ka  | 111 | GDFDYLLKTRVPMAYRKLLEGLTTLLPGVNDTRTYVMEEEVKQSNRLVIKTR |
| Aa  | 108 | GDFDYLLKTRVADMAAYRKLLEGLTTLLPGVNDTRTYVMEEEVKQTNFLQLK-- |
| Pm  | 108 | GDFDYLLKTRVADMAAYRKLLEGLTTLLPGVNDTRTYVMEEEVKQTNFLQLK-- |
| Hi  | 115 | GDFDYLLKTRVADMAEYRKLLEGLTTLLPGVNDTRTYVMEEEVKQTNFLQLK-- |
| Hd  | 109 | GDFDYLLKTRVADMAEYRKLLEGLTTLLPGVNDTRTYVMEEEVKQTNFLQLK-- |
| Ap  | 109 | GDFDYLLKTRVADMAEYRKLLEGLTTLLPGVNDTRTYVMEEEVKQTNFLQLK-- |
| Pa  | 110 | GDFDYLLKTRVADMAEYRKLLEGLTTLLPGVNDTRTYVMEEEVKQSNFLQLK-- |
| Lp  | 121 | GDTDYLIIIHVKDMNHYNEFARRVFANENIKFRSSFCNKTQKFQVLDE- |
### A

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