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Branched-Chain Amino Acids Are Required for the Survival and Virulence of *Actinobacillus pleuropneumoniae* in Swine▽

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In *Actinobacillus pleuropneumoniae*, which causes porcine pleuropneumonia, *ilvI* was identified as an in vivo-induced (*ivi*) gene and encodes the enzyme acetohydroxyacid synthase (AHAS) required for branched-chain amino acid (BCAA) biosynthesis. *ilvI* and 7 of 32 additional *ivi* promoters were upregulated in vitro when grown in chemically defined medium (CDM) lacking BCAA. Based on these observations, we hypothesized that BCAA would be found at limiting concentrations in pulmonary secretions and that *A. pleuropneumoniae* mutants unable to synthesize BCAA would be attenuated in a porcine infection model. Quantitation of free amino acids in porcine pulmonary epithelial lining fluid showed concentrations of BCAA ranging from 8 to 30 μmol/liter, which is 10 to 17% of the concentration in plasma. The expression of both *ilvI* and *lrp*, a global regulator that is required for *ilvI* expression, was strongly upregulated in CDM containing concentrations of BCAA similar to those found in pulmonary secretions. Deletion-disruption mutants of *ilvI* and *lrp* were both auxotrophic for BCAA in CDM and attenuated compared to wild-type *A. pleuropneumoniae* in competitive index experiments in a pig infection model. Wild-type *A. pleuropneumoniae* grew in CDM+BCAA but not in CDM−BCAA in the presence of sulfonylurea AHAS inhibitors. These results clearly demonstrate that BCAA availability is limited in the lungs and support the hypothesis that *A. pleuropneumoniae*, and potentially other pulmonary pathogens, uses limitation of BCAA as a cue to regulate the expression of genes required for survival and virulence. These results further suggest a potential role for AHAS inhibitors as antimicrobial agents against pulmonary pathogens.

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia, a disease of significant economic importance throughout the swine-raising areas of the world (6, 48). This pathogen possesses several well-studied virulence factors, including Apx toxins (20), capsular polysaccharides (57, 58), lipopolysaccharide (1, 17, 41), fimbriae (63), and iron-scavenging proteins (13, 50), which aid in the pathogenesis of acute pleuropneumonia marked by edema, hemorrhage, and necrosis (6, 26). In a search for additional virulence factors of this pathogen, we developed an in vivo expression technology (IVET) system and used this genetic tool to identify *A. pleuropneumoniae* gene promoters that are upregulated in vivo in the swine lung during infection compared to growth on laboratory media (22, 55).

One of the *A. pleuropneumoniae* in vivo-induced (*ivi*) promoters that we identified drives the *ilvIH* operon, which encodes both large and small subunits of acetohydroxy acid synthase isozyme III (AHAS) (55). AHAS enzymes catalyze pivotal steps in the biosynthesis of the branched-chain amino acids (BCAA) isoleucine, leucine, and valine (31). In a survey of IVET, signature-tagged mutagenesis, and microarray studies of other pathogens, we observed that genes involved in BCAA biosynthesis were frequently identified in studies of pathogens that cause pneumonia, meningitis, or septicemia but not in pathogens of the gastrointestinal tract (55). This observation suggests that the ability to synthesize BCAA is critical for pathogens of the respiratory tract but not for gastrointestinal pathogens. BCAA are essential amino acids that must be acquired from ingested food for most mammals, including humans and pigs, and it is possible that fluids in "clean" body sites such as the lungs have only limited supplies of BCAA compared to the digestive tract.

To test whether limitation of BCAA affects the expression of *A. pleuropneumoniae* genes that are induced in vivo, we compared expression from the *A. pleuropneumoniae ivi* promoters in a chemically defined medium (CDM) containing or lacking BCAA (55). We found that 25% (8 of 32) of the *ivi* promoters were upregulated during growth in CDM lacking BCAA compared to complete CDM. These included the *ilvI* promoter, as well as promoters for other genes potentially involved in survival within the host and virulence, such as *hfq*, a global regulator that binds sRNAs and mRNA and affects expression of virulence-associated genes in many pathogens (9, 49). These results strongly suggest that the environmental conditions encountered by *A. pleuropneumoniae* during infection of the swine lung include limitation of BCAA.

The goals of the present study were to quantify free BCAA in porcine pulmonary secretions, to evaluate the effect of these concentrations of BCAA on expression of genes required for BCAA biosynthesis, and to test whether *A. pleuropneumoniae*

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics[a]	Source or reference
Strains		
A. pleuropneumoniae		
AP100	ATCC 27088, serotype 1A, passaged through pigs	ATCC[b]
AP225	*A. pleuropneumoniae* ATCC 27088, serotype 1A, nalidixic acid resistant, passaged through pigs	23
AP359	*lrp* double-crossover mutant of AP225	54
AP364	*ilvI* single-crossover mutant of AP225	This study
AP365	*ilvI* double-crossover mutant of AP225	This study
E. coli		
XL1-Blue mRF′	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *proAB lacI*^{q}*Z*ΔM15 Tn*10* (Tet^r)]	Stratagene
S17-1(λ*pir*)	Δ*pir recA thi pro hsd* (r_K^- m_K^+) RP4-2-Tc::Mu Km::Tn*7*; Tmp^r Sm^r	43
Plasmids		
pUC18	Ap^r; high-copy-number cloning vector	62
pGZRS18/19	Ap^r; *A. pleuropneumoniae-E. coli* shuttle vectors	59
pGZRS39	Kan^r; *A. pleuropneumoniae-E. coli* shuttle vector	59
pER187	Ap^r Cm^r; CAT cassette-containing vector	42
pUC4K	Ap^r Kan^r; Kan cassette-containing vector; source of the Kan promoter	53
pUM24Cm	Cm^r Kan^r; *sacR-sacB-nptI* cassette-containing vector	40
pGP704	Ap^r; broad-host-range suicide vector	34
pGP704SacKan	Cm^r Kan^r; *sacR-sacB-nptI* from pUM24Cm cloned into pGP704	This study
pTW415	Kan^r; *rnd′-lrp-ftsK′* from AP100 cloned into pGZRS39	54
pTW429	Ap^r; 653 bp of the 5′ end of *ilvI* and 789 bp of the 3′ end of *ilvI* amplified by PCR from AP100 and cloned into SphI/SalI-digested pUC18	This study
pRL100	Ap^r Cm^r; 300 bp containing Kan promoter from pUC4K cloned upstream of the CAT cassette of pER187	This study
pRL101	Ap^r Cm^r; KanP-CAT cassette from pRL100 inserted into NsiI site in center of *ilvI* in pTW429	This study
pRL102	Ap^r Cm^r Kan^r; 2.5-kb fragment from pRL101 containing Δ*ilvI*::KanP-CAT cloned into pGP704SacKan	This study
pRL103	Ap^r; 2.8-kb fragment containing full *ilvIH* genes amplified from AP100 genomic DNA cloned into SalI/XbaI-digested pGZRS19	This study
pIviI	Ap^r; pTF86 *A. pleuropneumoniae* IVET vector containing a 623-bp insert with the *ilvI* promoter upstream of promoterless *luxAB* and *ribBAH* genes	22

[a] Cm^r, chloramphenicol resistance; Tet^r, tetracycline resistance; Tmp^r, trimethoprim resistance; Ap^r, ampicillin resistance; Sm^r, streptomycin resistance; Kan^r, kanamycin resistance.
[b] ATCC, American Type Culture Collection.

mutants that cannot synthesize BCAA were attenuated. *A. pleuropneumoniae* deletion-disruption mutants of the *ilvI* biosynthetic gene and the *lrp* gene, which encodes a global regulator required for expression of several genes involved in BCAA biosynthesis, were constructed and shown to be attenuated in a porcine infection model. The low levels of available BCAA in pulmonary secretions and the attenuation of these mutants led us to examine the effect of small molecule inhibitors of AHAS on growth of *A. pleuropneumoniae* in vitro. Several AHAS inhibitors were shown to prevent growth in CDM lacking BCAA but not complete CDM. These results demonstrate that *A. pleuropneumoniae*, and likely other bacterial pathogens of the respiratory tracts of other mammals, encounter conditions where BCAA are available only in limited supply during infection, that these low levels of BCAA can affect bacterial gene expression, and that these pathogens must be able to synthesize BCAA to survive and cause disease in the lung.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, and growth conditions. The bacterial strains and plasmids used in the present study are listed in Table 1. *A. pleuropneumoniae* strains were routinely grown on Bacto brain heart infusion (BHI) (Becton Dickinson, Sparks, MD) or CDM (55) supplemented with 10 μg of β-NAD (V factor; Sigma Chemical, St. Louis, MO)/ml and incubated either at 35°C with 5% CO_2 for agar media or at 35°C shaking at 160 rpm for broth media. To make CDM containing various concentrations of BCAA, a BCAA stock was added separately to CDM lacking BCAA to final concentrations equivalent to 10, 20, 50, or 100% of the BCAA concentration in complete CDM. For growth rate, in vitro competitive index, and experimental infection experiments, Bacto heart infusion broth (Becton Dickinson) supplemented with 10 μg of β-NAD/ml was also used. For plasmid selection in *A. pleuropneumoniae*, ampicillin and kanamycin were added at 50 μg/ml. For mating experiments, nalidixic acid was added at 50 μg/ml, and chloramphenicol was added at 2 μg/ml.

Escherichia coli XL1-Blue (Stratagene, La Jolla, CA) and *E. coli* S17-1 (λ*pir*), used for cloning and mating, respectively, were grown on Luria-Bertani (LB) medium at 37°C on agar medium and at 37°C with rapid shaking in broth medium. For plasmid selection in *E. coli*, ampicillin was added at 100 μg/ml, kanamycin was added at 100 μg/ml, and chloramphenicol was added at 10 μg/ml.

Primers used for construction of plasmids or for quantitative reverse transcription-PCR (RT-PCR) are listed in Table 2.

Collection and analysis of BALF. Bronchoalveolar lavage fluid (BALF) was collected from five healthy 12- to 14-week-old Yorkshire-Landrace pigs by using standard veterinary procedures (52). All animal use protocols were approved by the Michigan State University Institutional Animal Care and Use Committee. Pigs were kept off feed for 4 h prior to the collection of BALF. Pigs were anesthetized, placed in ventral recumbency, and intubated to ensure respiratory function, and a flexible catheter was inserted through the tracheal tube. A total of 25 ml of phosphate-buffered saline (PBS) containing 0.0005% methylene blue, kept at body temperature, was slowly introduced into the lungs. After 1 min, the fluid was aspirated with a syringe. This procedure was repeated with a second 25 ml of lavage fluid. The two samples were combined, and the total volume of the aspirated BALF was measured. BALF was centrifuged at 1,300 × *g* for 20 min to remove cells and filtered through a Millipore Centrifree cartridge (Millipore, Billerica, MA).

The volume of pulmonary epithelial lining fluid (ELF) in the recovered BALF

TABLE 2. Primers used in this study

Primer	Sequence (5′–3′)[a]	Description
MM727	CGTTGGAGGATTGCATGCAAAAACTTTCCG	Forward primer for construction of pTW429 (first *ilvI* fragment)
MM512	GAGAATGCATCTCCACCAATGTATAAAACCG	Reverse primer for construction of pTW429 (first *ilvI* fragment)
MM513	AACCGTTATGCATTATATTCCGATTGTGGG	Forward primer for construction of pTW429 (second *ilvI* fragment)
MM734	CTGATGTCGACCTACGCATCTGTTCTC	Reverse primer for construction of pTW429 (second *ilvI* fragment)
MM668	GTTGTGTGGAATTCTGAGCGGATAAC	Forward primer for construction of pRL100 (Kan promoter)
MM710	CCCATTGGTACCCATATAAATCAGCATC	Reverse primer for construction of pRL100 (Kan promoter)
MM744	CAATAAACCCGTCGACCAATCAAATCC	Forward primer for construction of pRL103 (full *ilvIH* operon plus promoter)
MM745	CGTCATCTAGAGTTATAAAGCAATTAGGGT	Reverse primer for construction of pRL103 (full *ilvIH* operon plus promoter)
MM588	CCATGCCGCGTGAATGA	16S forward Q-PCR primer
MM589	TTCCTCGCTACCGAAAGAACTT	16S reverse Q-PCR primer
MM590	TGTCGGTCAGCACCAAATGT	*ilvI* forward Q-PCR primer
MM591	GCGACGAGGTTTTTCAAACG	*ilvI* reverse Q-PCR primer
MM592	AATTGCTTGAAGCACCGCTATT	*lrp* forward Q-PCR primer
MM593	CGTCCGGCTTACCTCTGACT	*lrp* reverse Q-PCR primer

[a] Restriction sites inserted by PCR are underlined.

was calculated both by measurement of the concentration of methylene blue in the BALF (3, 52) and by using urea as a marker of dilution (39). Concentration of free amino acids and of urea in the BALF were measured by physiological amino acid analysis on a Hitachi I-8800 amino acid analyzer (Hitachi High Technologies America, Pleasanton, CA) (45) at the Michigan State University Macromolecular Structure Facility and compared to the concentration of urea in a plasma sample collected immediately prior to the lavage procedure. Using urea as a marker for dilution, which was found to be more reproducible than the methylene blue method, the volume of ELF in each sample was calculated as follows: (the concentration of urea in the BALF × the volume of BALF)/the concentration of urea in plasma (39). The dilution factor was calculated as the volume of BALF divided by the volume of ELF. The concentration of each amino acid in ELF was calculated as the concentration in BALF times the dilution factor.

Luciferase reporter assays. Expression from the *ilvI* promoter was quantified by using a luciferase expression plasmid (pIviI) in which the *ilvI* promoter drives the expression of promoterless *luxAB* genes (54). Wild-type *A. pleuropneumoniae* strain AP225 containing pIviI grown overnight on BHI agar supplemented with 10 μg of V factor/ml (BHIV) containing 50 μg of ampicillin/ml was suspended in CDM−BCAA and then diluted to an optical density at 520 nm (OD_{520}) of ~0.2 in 30 ml of prewarmed CDM containing 50 μg of ampicillin/ml and supplemented with 0, 10, 20, 50, or 100% of the concentration of BCAA found in complete CDM. Growth, measured as OD_{520}, and luciferase activity were determined at 0, 1, 2, and 3 h time points. Luciferase activity was measured as relative light units (RLU) using *N*-decyl aldehyde substrate (Sigma) and a Turner model 20e luminometer (Turner Designs, Sunnyvale, CA) as previously described (22, 55). Each sample was measured in triplicate, and the average RLU were normalized to the optical density of the culture. Three biological replicates of the complete experiment were performed.

Quantitative RT-PCR analysis of gene expression. Wild-type *A. pleuropneumoniae* AP100 was grown to mid-exponential phase (OD_{520} = 0.5 to 0.6) in complete CDM+BCAA and cells pelleted by centrifugation and resuspended to an OD_{520} of ~0.2 in 30 ml of prewarmed CDM supplemented with 0, 10, 20, 50, or 100% of the concentration of BCAA found in complete CDM. One hour after the shift to fresh medium, 30 ml of ice-cold methanol was added to each culture to stop growth, the resulting samples were chilled on ice for at least 5 min, and the cells were pelleted by centrifugation. RNA was isolated by using an RNeasy Midi kit protocol (Qiagen, Valencia, CA), with the modification that 5 mg of lysozyme/ml was used instead of the recommended 400 μg/ml. Residual genomic DNA was removed with Turbo DNase (Ambion, Austin, TX), and the RNA was concentrated. The RNA concentration was measured by using a spectrophotometer (Nanodrop, Wilmington, DE), and the RNA quality was verified by gel electrophoresis. The RNA was used as a template for RT, using random hexamers and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Quantitative PCR (Q-PCR) was performed using gene-specific primers (Table 2) and SYBR green PCR Core reagents (Applied Biosystems, Foster City, CA), according to the manufacturer's protocol. The results were analyzed by using the SDS 2.1 software (Applied Biosystems) and the relative standard curve method, with normalization to 16S rRNA. Each sample was measured in triplicate, and three biological replicates of the complete experiment were performed.

Construction and verification of an *A. pleuropneumoniae ilvI* mutant. To construct an *A. pleuropneumoniae ilvI* mutant, we used a similar technique to that previously reported by our laboratory for the construction of an *lrp* mutant (54). The 5′ and 3′ ends of the *ilvI* gene from *A. pleuropneumoniae* AP100 were amplified from genomic DNA by PCR and cloned into pUC18. Primers MM727 and MM512 (Table 2) were used to amplify a 653-bp fragment from the 5′ end of the *ilvI* gene, which was digested with SphI and NsiI. Primers MM513 and MM734 were used to amplify a 789-bp fragment from the 3′ end of the gene, which was digested with NsiI and SalI. These amplicons were ligated into pUC18 which had been digested with SphI and SalI, resulting in pTW429, which contains the *ilvI* gene with 259 bp deleted from the center of the gene. Next, the Kan promoter-chloramphenicol resistance (Cm^r) cassette was digested from pRL100 with PstI and ligated into the newly generated NsiI site of pTW429 to generate pRL101. The 2.5-kb insert containing *ilvI-5′–KanP-cat–ilvI-3′* was digested from pRL101 with SphI and SacI and ligated into SphI/SacI-digested pGP704SacKan to generate pRL102. This conjugal suicide plasmid was electroporated into *E. coli* S17-1(λ*pir*) and conjugated into nalidixic acid-resistant *A. pleuropneumoniae* AP225 as previously described (35). Transconjugants were isolated on BHIV agar supplemented with 2 μg of chloramphenicol/ml and 50 μg of nalidixic acid/ml. Screening for single- or double-crossover mutants was performed by PCR analysis of the *ilvI* locus. A single-crossover transconjugant (AP364) was exposed to chloramphenicol selection and sucrose counterselection, as previously described (35), to generate a double-crossover *ilvI* mutant. This mutant, designated AP365, was confirmed by PCR and Southern blot to contain the deleted-disrupted *ilvI* gene and the Cm^r cassette in the appropriate location in the AP225 chromosome but not the pGP704 vector, the kanamycin resistance gene, or the *sacR-sacB* cassette.

Preparation of challenge inocula. Bacterial cultures were grown at 35°C, shaking at 160 rpm, in heart infusion broth containing 10 μg of V factor/ml and 5 mM $CaCl_2$ to an OD_{520} of 0.8. Cells were harvested by centrifugation for 10 min at 5,000 × *g*, washed once with sterile PBS, diluted in PBS to the appropriate cell density, and administered within 60 min of preparation. The actual CFU/ml in the inocula were calculated by viable cell count on BHIV agar.

Experimental infection of pigs. Three separate experimental infection experiments were performed. In the first experiment, 15 10-week-old specific-pathogen-free (SPF) Yorkshire-Landrace crossbred pigs (Whiteshire Hamroc, Albion, IN) were divided into five groups of three pigs by a random-stratified sampling procedure, balancing each group for body weight. Group 1 was challenged by percutaneous intratracheal inoculation with 4×10^6 CFU of wild-type *A. pleuropneumoniae* AP225 in 10 ml of PBS, as previously described (23, 28). Group 2 received 4×10^6 CFU of AP359, an *lrp* mutant of AP225 (54). Group 3 received 2×10^7 CFU of AP359. Group 4 received 4×10^6 CFU of AP359 complemented with plasmid pTW415, which contains the *lrp* gene in a pGZRS18 vector (54). Group 5 received 10 ml of PBS.

In the second experiment, eight 10-week-old SPF pigs were divided into four groups of two pigs. Group 1 was challenged by intratracheal inoculation with 10^6

CFU of wild-type *A. pleuropneumoniae* AP225 in 10 ml of PBS. Group 2 received 10^6 CFU of AP359, the *lrp* mutant, by intratracheal inoculation. Group 3 received a mixture of 5×10^5 CFU each of the wild type and the mutant intratracheally. Group 4 received with 2.5×10^6 each of the wild type and the mutant in 2 ml of PBS, inoculated intranasally.

In the third experiment, 10 10-week-old SPF pigs were divided into four groups. Group 1 (three pigs) received 10^6 CFU of AP225. Group 2 (three pigs) received 5×10^5 CFU each of the wild type and the *lrp* mutant. Group 3 (two pigs) received 5×10^5 CFU of the wild type and the *ilvI* mutant. Group 4 (two pigs) received PBS only. All pigs in this experiment were inoculated intratracheally.

After experimental infection, pigs were monitored for the development of clinical signs of pleuropneumonia, including elevated rectal temperature, increased respiratory rate, dyspnea, decreased appetite, and decreased activity (depression), as previously described (23, 28). Pigs were euthanized by lethal injection either when mild-to-moderate clinical signs, particularly dyspnea and/or depression, were seen or at the end of the experiment. All animals were necropsied, and lungs were examined macroscopically for pleuropneumonia lesions. The percentage of lung tissue and pleural surface area affected was estimated for each of the seven lung lobes, and the total percent pneumonia and pleuritis was calculated by using a formula that weights the contribution of each lung lobe to the total lung volume (28). BALF and lung tissue samples from six areas of the lungs were collected and processed for culture and histopathology in all experiments and for quantitative plate counts in experiments 2 and 3. All animal use protocols were approved by the Michigan State University Institutional Animal Care and Use Committee.

Determination of competitive indices. For in vivo competitive index determination, serial 10-fold dilutions of BALF samples and of lung samples homogenized in a Stomacher-80 (Seward Laboratory Systems, Bohemia, NY) were plated on BHIV agar containing 25 μg of nalidixic acid/ml for a total viable count of wild-type and mutant bacteria and on BHIV containing 1 μg of chloramphenicol/ml to select for the mutants. The numbers of wild-type bacteria were calculated as the CFU on BHIV containing nalidixic acid minus the CFU on BHIV containing chloramphenicol. The competitive index for each sample was calculated by using the following formula: the competitive index at time X = the ratio of mutant to the wild type at time X/the ratio of the mutant to the wild type at time zero. The average competitive index for each animal was calculated as the average of all seven specimens (BALF plus lung), with the exception that data for any specimen with no growth were excluded. Similar methods were used to calculate competitive indices in broth media.

Determination of MICs of AHAS inhibitors. Four sulfonylurea herbicide compounds—metsulfuron methyl, primisulfuron methyl, and chlorsulfuron (all purchased from Riedel-de-Haen Laborchemikalien, Seelze, Germany) and chlorimuron ethyl (Chem Service, West Chester, PA)—and the two imidazolinone herbicide compounds imazapyr and imazaquin (Riedel-de-Haen) were tested for their ability to inhibit the growth of *A. pleuropneumoniae* in CDM+BCAA and CDM−BCAA. Serial twofold dilutions of each inhibitor were made in CDM+BCAA and CDM−BCAA in either test tubes or microtiter plates. *A. pleuropneumoniae* AP100 was grown overnight on BHIV agar and suspended in either CDM+BCAA or CDM−BCAA, and this suspension was used to inoculate both tubes and microtiter plate wells at a concentration of $\sim 5 \times 10^7$ CFU/ml. Tubes were incubated overnight at 35°C shaking at 160 rpm; microtiter plates were incubated overnight at 35°C under 5% CO_2. MICs were determined as the lowest concentration of chemical compound that inhibited growth. The full experiment was repeated four times.

RESULTS

Analysis of free amino acid concentration in porcine pulmonary ELF. To determine whether BCAA are present in only limited amounts in the porcine lung, we measured the concentrations of free amino acids in porcine plasma and BALF from healthy Yorkshire-Landrace feeder pigs and calculated the levels of free amino acids in pulmonary ELF using urea as a marker of dilution. Free amino acid concentrations in porcine plasma and ELF are shown in Table 3. The amino acid concentrations found in plasma were similar to those previously reported for porcine plasma (11). Many amino acids, including the essential amino acids lysine and threonine, were present in ELF at ~50% of the concentration in plasma, and aspartic acid and glutamic acid were present in higher concentrations in ELF than in plasma. In contrast, for the BCAA leucine, isoleucine, and valine, the available levels in pulmonary ELF ranged from 8.4 to 30 μmol/liter, which was 9.8 to 16.8% of the levels found in porcine plasma. The concentrations of BCAA in porcine ELF were 10 to 20% of the amounts of these amino acids in the CDM used for *A. pleuropneumoniae* (55).

TABLE 3. Concentration of free amino acids in porcine plasma and pulmonary ELF

Amino acid[a]	Amino acid concn in μmol/liter (avg ± SD)		Avg ELF/plasma ratio (%)[b]	Amino acid concn in CDM in μmol/liter
	Plasma	ELF		
Alanine	509 ± 204	92.9 ± 45.2	20.5	370
Arginine	29.6 ± 39.1	2.6 ± 5.7	24.4	240
Aspartic acid	5.9 ± 3.9	47.9 ± 25.8	640	1,250
Cysteine	71.7 ± 15.8	8.7 ± 10.9	10.5	700
Glutamic acid	132 ± 41.7	172 ± 80.4	114	2,950
Glutamine	926 ± 612	76.3 ± 61.4	12.8	110
Glycine	816 ± 166	291 ± 177	38.7	110
Histidine	15.7 ± 20.5	0.1 ± 0.1	59.9	43
Isoleucine*	80.6 ± 9.8	8.4 ± 9.5	9.8	80
Leucine*	146 ± 25.7	25.5 ± 21.8	16.6	230
Lysine*	57.1 ± 82.3	11.9 ± 26.7	54.2	90
Methionine*	27.7 ± 7.2	1.6 ± 1.5	5.2	30
Phenylalanine*	68.3 ± 18.1	18.1 ± 11.8	26.1	50
Proline	820 ± 206	345 ± 202	46.2	140
Serine	125 ± 26.4	58.2 ± 39.2	42.9	160
Threonine*	63.6 ± 11.9	28.2 ± 15.9	46.8	140
Tyrosine	62.2 ± 13.1	5.5 ± 4.1	9.2	130
Valine*	171 ± 30	30.4 ± 23.4	16.8	170

[a] Values for asparagine, cystine, and tryptophan were below detectable limits on most or all samples. *, Essential amino acid.
[b] ELF/plasma amino acid concentration ratios were calculated for each pig, and the averages of these ratios are presented.

Expression of genes involved in BCAA biosynthesis in CDM. We have previously shown that *A. pleuropneumoniae ilvI*, which is required for BCAA biosynthesis, is upregulated both in vivo during infection (22) and in CDM lacking BCAA (CDM−BCAA) compared to complete CDM (CDM+BCAA) (55) and that leucine-responsive regulatory protein, encoded by the gene *lrp*, is required for the response of *ilvI* to BCAA limitation (54). To determine whether expression of *ilvI* and *lrp* correlates with the concentrations of BCAA available in the porcine lung, we used luciferase reporter assays and quantitative RT-PCR to measure the expression of these genes in CDM samples containing various concentrations of BCAA.

Expression of *ilvI*, as measured using a luciferase reporter assay (Fig. 1), increased rapidly in CDM−BCAA (0% BCAA), with a 6-fold increase at 1 h and a 26-fold increase at 3 h, compared to the 0-h time point. *ilvI* expression was significantly higher ($P \leq 0.02$) in CDM−BCAA at 1, 2, and 3 h than in all other concentrations of BCAA tested. Expression of *ilvI* also increased significantly, albeit more slowly, in CDM plus 10% BCAA, reaching 14-fold times the baseline level by 3 h, which was significantly increased compared to expression in 20, 50, and 100% BCAA ($P \leq 0.01$).

We also measured changes in expression of both *ilvI* and *lrp* in CDM containing different concentrations of BCAA by quantitative RT-PCR (Table 4). At 1 h after a shift to fresh medium, the expression of *ilvI* was strongly upregulated in

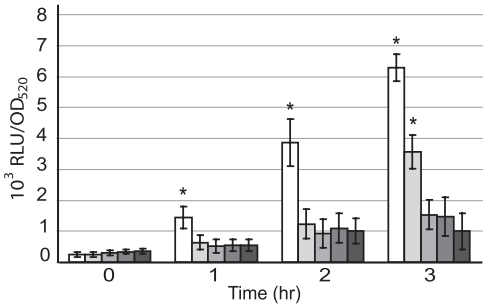

FIG. 1. Expression from the *ilvI* promoter in CDM containing various concentrations of BCAA. *A. pleuropneumoniae* AP225/pIviI was grown in CDM containing 0, 10, 20, 50, and 100% of the amount of BCAA in complete CDM+BCAA. Growth was measured as the OD_{520}, and the luciferase activity expressed from the *ilvI* promoter-*luxAB* fusions was measured as RLU. Luciferase activity was normalized to RLU per OD_{520} for each sample. The data are presented as the means ± the standard deviations from three separate experiments. Asterisks indicate values that are significantly different ($P \leq 0.02$) from all other values at the same time point, as determined by using a Student *t* test.

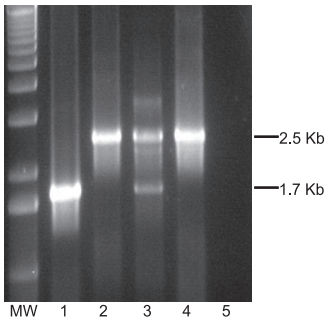

FIG. 2. Confirmation of the *ilvI* mutant. PCR was performed using *A. pleuropneumoniae ilvI*-specific primers MM727 and MM734 and the following templates: AP225 wild-type genomic DNA (lane 1), AP365 double-crossover *ilvI* mutant (lane 2), AP364 single-crossover *ilvI* mutant (lane 3), knockout plasmid pRL102 (lane 4), and no DNA (lane 5).

CDM−BCAA compared to CDM plus 100% BCAA and moderately upregulated in CDM plus 10% and plus 20% BCAA, which paralleled the results seen with the reporter assays. Expression of *lrp* was also upregulated in CDM−BCAA and CDM plus 10% BCAA, although to a much smaller degree than *ilvI*. The results using reporter assays and Q-PCR indicated that expression of *ilvI* and *lrp* was highest in CDM containing no BCAA but also increased in CDM containing the low concentrations of BCAA found in porcine ELF.

Growth of wild-type, *ilvI* mutant, and *lrp* mutant *A. pleuropneumoniae* in CDM. To investigate whether the levels of BCAA in porcine ELF are sufficient for the growth and virulence of *A. pleuropneumoniae* that is unable to synthesize these amino acids, we constructed *A. pleuropneumoniae* serotype 1 strains with mutations in the *ilvI* gene (AP365) (see Materials and Methods) and in the *lrp* gene (AP359) (54). The construction of the *ilvI* mutant was confirmed by both PCR and Southern blot analyses. PCR analysis showed the predicted 2.5-kb product with *ilvI*-specific primers in AP365, the double-crossover mutant, compared to a 1.7-kb product in wild-type AP225; both products were seen with AP364, a single-crossover mutant (Fig. 2). In addition, AP365 was shown to contain the Kan-promoter-Cm^r cassette in a chromosomal location immediately upstream of the *ilvH* gene and to lack the pGP704 vector (data not shown).

Growth of wild-type *A. pleuropneumoniae* AP100, the *ilvI* mutant, a complemented *ilvI* mutant containing plasmid pRL103, the *lrp* mutant, and a complemented *lrp* mutant containing plasmid pTW415 were compared on CDM agar plates containing various concentrations of BCAA. Both mutants grew well on 100 and 50% BCAA but showed reduced growth on 20% BCAA, a faint haze of growth on 10% BCAA, and no growth on 0% BCAA (data not shown). In contrast, wild-type *A. pleuropneumoniae* and both complemented mutants grew well on all concentrations of BCAA.

Exponential growth rates in CDM broth containing various concentrations of BCAA were determined for wild-type *A. pleuropneumoniae* and both *ilvI* and *lrp* mutants (Table 5). Specific growth rates were highest for all three strains in CDM plus 100% BCAA and dropped for all three strains as the concentration of BCAA in the medium dropped. The growth rates for the wild type were higher than for the *lrp* mutant, although both strains grew in all of the media, despite very low growth rates in CDM−BCAA. The growth rates were lowest in all media for the *ilvI* mutant, which failed to grow at all in CDM−BCAA and grew very poorly in CDM plus 10% BCAA.

Evaluation of virulence of *A. pleuropneumoniae ilvI* and *lrp* mutants in pigs. To measure the relative virulence of an *A. pleuropneumoniae lrp* mutant, we first compared wild-type *A. pleuropneumoniae* AP225, the *lrp* mutant (AP359), and a complemented mutant (AP359/pTW415) for virulence in pigs at a

TABLE 4. Q-PCR analysis of *ilvI* and *lrp* expression

Medium	Avg ± SD[a]	
	ilvI	*lrp*
CDM + 0% BCAA	27.1 ± 0.32	2.82 ± 0.48
CDM + 10% BCAA	6.93 ± 1.32	2.35 ± 0.34
CDM + 20% BCAA	6.21 ± 1.99	1.89 ± 0.72
CDM + 50% BCAA	3.91 ± 1.91	1.70 ± 0.93

[a] Data are presented as the ratio of the concentration of specific RNA from cultures grown in the medium indicated divided by the concentration of RNA from cultures grown in CDM + 100% BCAA. Transcript levels for each gene were normalized to the level of 16S rRNA. The data represent the average of triplicate samples from two replicate experiments.

TABLE 5. Specific growth rates of the wild type, the *ilvI* mutant, and the *lrp* mutant in CDM containing various concentrations of BCAA

Medium	Avg specific growth rate μ (h^{-1}) ± SD[a]		
	AP100 (wild type)	AP365 (*ilvI* mutant)	AP359 (*lrp* mutant)
CDM + 100% BCAA	0.91 ± 0.08	0.67 ± 0.08	0.75 ± 0.09
CDM + 20% BCAA	0.60 ± 0.09	0.47 ± 0.06	0.53 ± 0.05
CDM + 10% BCAA	0.41 ± 0.01	0.34 ± 0.04	0.42 ± 0.04
CDM + 0% BCAA	0.33 ± 0.03	NG	0.26 ± 0.04

[a] Data are presented as the averages from at least three separate growth curves. The specific growth rate μ was calculated as $\ln 2/T_d$, where T_d is the doubling time during exponential growth. NG, no growth.

relatively high infective dose of 4×10^6 CFU/pig. At this dose, all pigs except the uninfected control group developed clinical signs of pneumonia, including elevated rectal temperature, dyspnea, depression, and loss of appetite, within 4 to 8 h postinfection, and most of the infected animals were euthanized due to moderate-to-severe dyspnea and/or depression within 16 to 20 h postinfection. At this dose, there were no significant differences between the groups receiving wild-type AP225, the *lrp* mutant, and the complemented mutant in either percent pneumonia (average = 8.1, 17.3, and 14.3%, respectively), maximum temperature (average = 106.1, 106.2, and 104.0°F, respectively), maximum respiratory rate (85, 85, and 86 breaths/min, respectively), or other parameters measured. The group of pigs that received the *lrp* mutant at a higher dose of 2×10^7 CFU developed disease more rapidly, with all three animals showing clinical signs at 4 h postinfection and requiring euthanasia between 10 and 14 h postinfection. This group had a higher average percent pneumonia of 36.6%. *A. pleuropneumoniae* was cultured from all infected animals but not from uninfected controls. Gross pathology showed typical lesions of severe pleuropneumonia in all infected animals, including hemorrhage, regions of necrosis with fibrin deposits, congestion, edema, and consolidation. Histopathology showed large areas of severe hemorrhage and necrosis surrounded by streaming neutrophils, with bacteria visible within these lesions. There was accumulation of fibrin, blood, and necrotic debris in the affected areas. The alveolar septae were necrotic, with loss of nuclear detail and loss of delineation between air spaces, and vascular walls were inflamed. Edema and hemorrhage were evident, resulting in fluid-filled alveoli and bronchi. The animals receiving the higher dose of the *lrp* mutant were most severely affected, but there was no visible difference between the wild type, the *lrp* mutant, and the complemented mutant receiving the same dose. PBS-challenged control animals did not develop any clinical signs of pneumonia and showed no lung lesions at necropsy. In this experiment, where all animals were infected with a high infective dose and rapidly developed disease, the *A. pleuropneumoniae lrp* mutant showed no loss in virulence compared to wild-type *A. pleuropneumoniae*.

Although the methods used in this first experiment were similar to those we have previously used successfully to measure the attenuation of a riboflavin-requiring mutant of *A. pleuropneumoniae* (23), we were concerned that the high infective dose and rapid severe hemorrhagic lung damage might have masked small differences in virulence between the *lrp* mutant and wild-type *A. pleuropneumoniae*. Therefore, we used a lower infective dose and a more sensitive technique, competitive index analysis, to measure the relative virulence of both *lrp* and *ilvI* mutants in pigs in two sets of experiments. In these experiments, most pigs were infected intratracheally with 10^6 CFU of bacteria, either of the wild-type or mutant alone or as a mixture of 5×10^5 CFU of the wild type and 5×10^5 CFU of the mutant. To test whether *lrp* might be critical for initial adherence and survival in the upper respiratory tract rather than at entry into the lung, two animals were infected with a combination of the wild type and the *lrp* mutant by the intranasal route rather than the intratracheal route. Animals were monitored for clinical signs of disease as in the previous experiment. At this lower infective dose, all animals developed elevated rectal temperatures but otherwise showed only mild clinical signs of respiratory disease, with the exception of pigs 2199 and 2202, which developed moderate-to-severe disease. The two pigs infected with the *lrp* mutant alone showed less severe clinical signs, lower percent pneumonia, and less severe pathology than that seen in pigs infected with either the wild-type alone or with a mixture of wild-type and *lrp* mutant (data not shown).

TABLE 6. Competitive index analysis of virulence of *A. pleuropneumoniae* mutants[a]

Mutant	Pig	Dose (CFU)	Route	Time postinfection (h)	Avg CI
ilvI mutant	33	1×10^6	Intratracheal	42	0.22
	38	1×10^6	Intratracheal	66	0.05
lrp mutant	35	1×10^6	Intratracheal	18	0.24
	2199	1×10^6	Intratracheal	32	0.14
	29	1×10^6	Intratracheal	42	0.06
	41	1×10^6	Intratracheal	66	<0.06
	2203	1×10^6	Intratracheal	66	0.04
	2202	5×10^6	Intranasal	18	0.44
	2205	5×10^6	Intranasal	42	0.19

[a] The table includes data on all pigs from experiments 2 and 3 that received both mutant and wild-type strains. Pigs 2199, 2202, 2203, and 2205 were from experiment 2. Pigs 29, 33, 35, 38, and 41 were from experiment 3. CI, competitive index.

Pigs were euthanized either as disease signs became moderate to severe or at 18, 42, and 66 h postinfection, and BALF and lung samples from six different lung locations were collected. The CFU/ml of BALF or per g of lung tissue were measured for both the wild type and the mutant, and competitive indices were calculated as a measure of how well the mutant competed with virulent wild-type *A. pleuropneumoniae* (Table 6).

In these competitive index experiments, we found that neither the *lrp* mutant nor the *ilvI* mutant competed well with wild-type *A. pleuropneumoniae*. This was true for the *lrp* mutant in infections by both the intratracheal and the intranasal routes. For both mutants, the competitive index tended to drop over time, with average CIs of 0.2 to 0.4 for animals euthanized 18 h postinfection, 0.06 to 0.22 at 32 to 42 h, and 0.04 to 0.06 at 66 h. The competitive index was always lower than 1 and declined as the disease progressed. These results indicate that both the *ilvI* mutant and the *lrp* mutant are attenuated in our swine experimental infection model.

Effect of AHAS inhibitors on growth of *A. pleuropneumoniae*. Six compounds that are inhibitors of plant AHAS enzymes routinely used in herbicides, namely, the sulfonylureas metsulfuron methyl, primisulfuron methyl, chlorimuron ethyl, and chlorsulfuron and the imidazolinones imazapyr and imazaquin, were tested for their ability to inhibit the growth of wild-type *A. pleuropneumoniae*. All four of the sulfonylureas that were tested inhibited the growth of *A. pleuropneumoniae* in CDM−BCAA but not CDM+BCAA (Table 7). Metsulfuron methyl was the most effective, with an MIC of 0.5 nmol/ml (0.2 μg/ml), whereas primisulfuron methyl was the least effective, with an MIC of 16 nmol/ml (7.5 μg/ml). In contrast, neither of the imidazolinones tested inhibited growth of *A. pleuropneumoniae* in either CDM+BCAA or CDM−BCAA.

TABLE 7. MICs of AHAS inhibitors against *A. pleuropneumoniae* AP100 in CDM+BCAA and CDM−BCAA

AHAS inhibitor	Avg MIC (nmol/ml)[a]	
	CDM+BCAA	CDM−BCAA
Sulfonylureas		
Chlorimuron ethyl	>2,000	1
Chlorsulfuron	>2,000	2
Metsulfuron methyl	2,000	0.5
Primisulfuron	>2,000	16
Imidazolinones		
Imazapyr	>2,000	≥1,000
Imazaquin	>2,000	≥2,000

[a] Average of at least four independent replicate experiments.

DISCUSSION

Our IVET studies on *A. pleuropneumoniae* (22, 55) and a review of other similar studies (7, 8, 21, 33, 46, 56) identified genes involved in BCAA biosynthesis as in vivo induced or required for survival and virulence in pathogens of relatively clean body sites such as the lungs, cerebrospinal fluid, and bloodstream, but not in pathogens of the gastrointestinal tract. Further, we found that 25% of the *A. pleuropneumoniae* promoters that we had identified as specifically induced in vivo were upregulated in vitro in CDM lacking BCAA (55). These studies suggested that a previously unrecognized environmental condition—limitation of the BCAA leucine, isoleucine, and valine—exists in the healthy mammalian lung, that bacteria can sense this environmental condition and respond to it, and that pulmonary pathogens unable to synthesize BCAA will be attenuated. Since no data were available on the actual concentrations of BCAA in mammalian lung fluids, we first measured the free amino concentrations in porcine pulmonary ELF and serum. We found that most amino acids, with the exception of aspartic acid and glutamic acid, were present in lower concentrations in ELF than in serum, although many were present at roughly 40 to 50% of the serum level, including the essential amino acids lysine and threonine. In contrast, the essential BCAA were present in pulmonary ELF at ~10 to 17% of the concentration in serum. This is the first report of actual free amino acid concentrations in porcine ELF. When tested in vitro in a CDM, the low concentrations of BCAA found in pulmonary ELF led to reduced growth rates of wild-type *A. pleuropneumoniae*, as well as to increased expression of genes required for BCAA biosynthesis. During infection, even a slight reduction in growth rate of a pathogen can allow the host natural defenses to clear the pathogen before disease develops.

To test whether the ability to synthesize BCAA is critical for survival and virulence of *A. pleuropneumoniae* in its natural swine host, we constructed two mutants that require exogenous BCAA for growth. The first mutant contained a mutation in the *ilvI* gene, which encodes the large subunit of acetohydroxy acid synthase, an enzyme required for biosynthesis of all three BCAA. The *A. pleuropneumoniae* genome contains two sets of genes that encode putative AHAS enzymes, *ilvIH* and *ilvGM* (18, 61), and there was concern that the *ilvGM*-encoded enzyme might substitute for that encoded by *ilvIH*. However, the *ilvI* mutant failed to grow in vitro in the absence of exogenous BCAA, indicating that the *ilvGM*-encoded AHAS enzyme did not substitute for the *ilvIH*-encoded AHAS under these conditions.

The second mutant was constructed to knock out *lrp*, which encodes a global regulator of BCAA synthesis and degradation in *E. coli* (10, 12, 38). We have previously shown that an *lrp* homologue is required for *ilvI* expression in *A. pleuropneumoniae* (54). Lrp is frequently a pleiotropic regulator of a wide variety of genes in addition to those involved in amino acid biosynthesis and catabolism, although the Lrp regulon varies between even closely related species (14, 30). Lrp has also been shown to regulate a variety of virulence-associated genes in many other pathogens (2, 19, 24, 29, 32, 60). Although Lrp is essential for virulence in *Xenorhabdus nematophila* (15, 29), it appears to act as a virulence repressor in *Salmonella enterica* subtype Typhimurium (2).

In initial attenuation experiments comparing wild-type and Lrp$^-$ *A. pleuropneumoniae* in experimental infections in pigs using the high inoculating dose of 4×10^6 CFU, the pigs rapidly developed severe hemorrhagic pneumonia, and there was no obvious difference in severity or pathology between the wild-type and mutant infections. However, when a lower inoculating dose of 10^6 was used, the pigs infected with the *lrp* mutant showed less severe clinical signs and less severe pathology than those infected with the wild type. We chose to use a competitive index design for further studies. Competitive index experiments can be a more sensitive measure of virulence attenuation than standard infection experiments to calculate differential LD_{50} values for wild-type and mutant strains (4). In competitive index experiments using a combined dose of 1×10^6 CFU for intratracheal inoculation and 5×10^6 CFU for intranasal inoculation, we found that neither the *lrp* mutant nor the *ilvI* mutant competed well with wild-type *A. pleuropneumoniae* and that the *lrp* mutant was attenuated in animals infected by both methods.

The observation that the *lrp* mutant is virulent at high doses but attenuated at lower doses might be explained by the disease process. At high infecting doses, lung damage occurs very rapidly in this disease. This is most likely due to the extracellular Apx toxins, since growth of the infecting inoculum under conditions that enhance Apx toxin production leads to increased virulence and increased rapidity of disease progression. The Apx toxins, which can be hemolytic or cytotoxic or both, are the key virulence factors leading to severe lung damage (47). These toxins kill neutrophils that are attracted to the site of infection, releasing toxic neutrophil contents into tissues, which causes tissue damage; the Apx toxins are also cytotoxic for porcine alveolar epithelial cells (27, 51). The severe damage to cells and tissues that results can lead to release of cytoplasmic contents such as hemoglobin and free amino acids from host cells. In addition, *A. pleuropneumoniae* produces extracellular proteases that may degrade proteins within the lung tissues (36, 37). Together, Apx toxins plus secreted proteases could release sufficient BCAA to allow the *lrp* mutant to survive and grow. In contrast, at lower infecting doses, there is generally a lag of several hours before the development of clinical signs, and the reduction in tissue damage at the early stages of infection may result in decreased release of free amino acids from host cells and therefore insufficient levels of BCAA to allow multiplication of the infecting *lrp* or *ilvI* mutants. It is likely that BCAA are present in limiting amounts at the initial stages of

A. pleuropneumoniae infection and that the damage caused by the disease leads to increased availability of BCAA.

We observed that limitation of BCAA stimulated the expression of both *ilvI* and *lrp* in the present study, with levels of gene expression increased with decreasing concentrations of BCAA. Expression measured both by Q-PCR to quantitate RNA transcripts for *ilvI* and *lrp* and by luciferase reporter assay for *ilvI* showed elevated RNA and reporter enzyme at 1 h after a shift to conditions of BCAA limitation. We had previously reported that BCAA limitation induced expression in vitro of 25% of the *A. pleuropneumoniae* gene promoters identified as specifically expressed in vivo in our IVET studies (55). These results suggest that low levels of BCAA may be an important environmental cue regulating gene expression in *A. pleuropneumoniae* and other pulmonary pathogens. Recent studies in our laboratory to identify the *A. pleuropneumoniae* transcriptome in response to limitation of BCAA indicate that expression of many genes is modulated by BCAA limitation, including increased expression of several adhesins (M. H. Mulks, unpublished data). These results suggest a model in which BCAA limitation acts as an early signal regulating expression of genes required for survival and virulence, such as adhesins, whose expression may later be altered by increased levels of BCAA.

The results reported here demonstrate that the ability to synthesize BCAA is critical for the survival and virulence of *A. pleuropneumoniae* in the swine lung. These results suggested that BCAA biosynthesis is a potential target for the development of antimicrobials against *A. pleuropneumoniae* and similar pathogens. Many AHAS inhibitors have been developed as potent herbicides that show little toxicity for mammals (16). We tested two classes of inhibitors of AHAS enzymes, sulfonylureas and imidazolinones, for their ability to inhibit growth of *A. pleuropneumoniae*. We predicted that these inhibitors would have minimal effect on growth in CDM+BCAA, where the ability to synthesize BCAA is not required because there are sufficient levels of exogenous BCAA, and would have significant effect on growth in CDM−BCAA, where the bacteria must be able to synthesize BCAA. Our results show that all four sulfonylureas that were tested inhibited growth of wild-type *A. pleuropneumoniae* in CDM−BCAA but not in CDM+BCAA. However, neither imidazolinone had any effect on growth in either medium. Since both classes of compounds inhibit AHAS enzymes, the difference is likely to be in uptake of the compound into bacterial cells. Sulfonylureas have also been shown to inhibit growth of *Mycobacterium tuberculosis* both in vitro and in a mouse model (25, 44) and of *Brucella suis* in macrophages (5). These results indicate that AHAS inhibitors have excellent potential for development as antimicrobials against infections of the respiratory tract or other "clean" body sites, although their utility against fulminant *A. pleuropneumoniae* infection may be limited in vivo due to the effect of the Apx toxins. However, only a few pulmonary pathogens, such as *A. pleuropneumoniae* and *Mannheimia haemolytica*, produce large amounts of repeat-in-toxin toxins, and AHAS inhibitors may function well in vivo against pathogens that cause less destruction of tissues and blood cells, or as prophylactic measures against spread of diseases such as porcine pleuropneumonia within a herd.

In summary, our IVET studies on *A. pleuropneumoniae* led us to hypothesize that BCAA limitation is an environmental condition encountered by this pathogen in the healthy pig lung and that the ability to synthesize BCAA would be critical for full virulence of this pathogen. We have shown that BCAA are indeed present in only limited amounts in porcine pulmonary ELF, that the growth of wild-type *A. pleuropneumoniae* was reduced in vitro when such low levels of BCAA were available and was inhibited by sulfonylureas, and that two mutants unable to synthesize BCAA were attenuated in an experimental infection model in swine. We have also shown that the concentration of available BCAA affects expression of *ilvI* and *lrp*, with increased expression correlating with decreased BCAA concentration. Further studies to identify the full *A. pleuropneumoniae* transcriptome that responds to BCAA limitation and regulatory molecules that control this response are in progress. These results demonstrate how data from IVET and signature-tagged mutagenesis studies can be used to define the environment encountered by pathogens in vivo and to suggest promising avenues for the development of new tools to control infectious diseases.

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