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Analysis of the *Actinobacillus pleuropneumoniae* ArcA Regulon Identifies Fumarate Reductase as a Determinant of Virulence▽†

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The ability of the bacterial pathogen *Actinobacillus pleuropneumoniae* to grow anaerobically allows the bacterium to persist in the lung. The ArcAB two-component system is crucial for metabolic adaptation in response to anaerobic conditions, and we recently showed that an *A. pleuropneumoniae arcA* mutant had reduced virulence compared to the wild type (F. F. Buettner, A. Maas, and G.-F. Gerlach, Vet. Microbiol. 127:106–115, 2008). In order to understand the attenuated phenotype, we investigated the ArcA regulon of *A. pleuropneumoniae* by using a combination of transcriptome (microarray) and proteome (two-dimensional difference gel electrophoresis and subsequent mass spectrometry) analyses. We show that ArcA negatively regulates the expression of many genes, including those encoding enzymes which consume intermediates during fumarate synthesis. Simultaneously, the expression of glycerol-3-phosphate dehydrogenase, a component of the respiratory chain serving as a direct reduction equivalent for fumarate reductase, was upregulated. This result, together with the in silico analysis finding that *A. pleuropneumoniae* has no oxidative branch of the citric acid cycle, led to the hypothesis that fumarate reductase might be crucial for virulence by providing (i) energy via fumarate respiration and (ii) succinate and other essential metabolic intermediates via the reductive branch of the citric acid cycle. To test this hypothesis, an isogenic *A. pleuropneumoniae* fumarate reductase deletion mutant was constructed and studied by using a pig aerosol infection model. The mutant was shown to be significantly attenuated, thereby strongly supporting a crucial role for fumarate reductase in the pathogenesis of *A. pleuropneumoniae* infection.

Actinobacillus pleuropneumoniae is the causative agent of a porcine pleuropneumonia that results in high economic losses worldwide (16). After *A. pleuropneumoniae*-containing aerosols are inhaled, the pathogen colonizes the respiratory epithelium of its host and persists on tonsils, on healthy lung epithelium, and in sequestered lesions (14). The persistence of bacterial pathogens in the respiratory tracts of clinically healthy carriers is of major importance in the spread of infection in both animals and humans (6, 10, 29).

Previously, we have shown that the ability of *A. pleuropneumoniae* to adapt to low redox conditions is essential for its long-term persistence on intact and diseased respiratory tract epithelia (4, 26). In particular, the *arcA* deletion mutant of *A. pleuropneumoniae* was severely attenuated in this respect (8). A role in virulence for ArcA has also been implicated for intracellular bacterial pathogens such as *Mycobacterium tuberculosis* (42, 45), invasive pathogens such as *Haemophilus influenzae* (13, 59), and the enteric pathogens *Vibrio cholerae* (50) and *Shigella flexneri* (7). However, the molecular mechanisms responsible for this attenuation are only partially resolved.

In *Escherichia coli*, the transcriptional regulator ArcA functions primarily as a major metabolic modulator, downregulating many genes involved in respiratory metabolism (24) and upregulating genes necessary for fermentative metabolic activities under anaerobic conditions (48). The importance of basic metabolic pathways for virulence is increasingly being recognized. For example, it was recently shown that deletion of the genes encoding some enzymes of the tricarboxylic acid (TCA) cycle resulted in attenuation in *Salmonella enterica* serovar Typhimurium (54). The glyoxylate shunt is required for persistence of *M. tuberculosis* (34) and fungal virulence (32), and genes involved in energy metabolism are differentially expressed in active versus persistent infections with *Chlamydia trachomatis* (19). Based on these considerations, we set out to investigate whether ArcA-mediated regulation of metabolic functions could be partially responsible for the attenuation and reduced persistence of the *A. pleuropneumoniae arcA* mutant. Thus, the ArcA regulon of *A. pleuropneumoniae* was analyzed by whole-genome microarray and two-dimensional difference gel electrophoresis (2D DIGE) analyses. The results suggested that attenuation of the *A. pleuropneumoniae arcA* mutant was due to its inability to anaerobically adapt its metabolism in order to use fumarate as a terminal electron acceptor and to provide succinate and other essential metabolic intermediates via the reductive branch of the citric acid cycle. This hypothesis was supported by the attenuation of a fumarate reductase (*frd*) deletion mutant in vivo.

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MATERIALS AND METHODS

Bacterial strains, plasmids, primers, and growth conditions. The bacterial strains, plasmids, and primers used in this study are listed in Table 1. *A. pleuropneumoniae* wild-type (wt) and mutant strains were cultured at 37°C and 5% CO_2 in PPLO medium or on PPLO agar (Difco GmbH, Augsburg, Germany), both supplemented with NAD (10 μg/ml; Merck AG, Darmstadt, Germany), L-cysteine hydrochloride (260 μg/ml; Sigma Chemical Company, Deisenhofen, Germany), L-cystine dihydrochloride (10 μg/ml; Sigma), and dextrose (1 mg/ml). For cultivation of the complemented mutants, kanamycin (25 μg/ml) was added. For anaerobic growth, supplemented medium (PPLO medium) was preincubated 48 h prior to inoculation in an anaerobic chamber (Don Whitley Scientific, Shipley, England) in an atmosphere containing 5% CO_2, 10% H_2, and 85% N_2 at 37°C. Anaerobicity of the medium was confirmed using a dissolved oxygen sensor (CellOx 325; WTW, Weilheim, Germany) linked to an inoLab instrument (WTW, Weilheim, Germany). For RNA and protein preparations, this medium was inoculated with 1% of an aerobically grown log-phase culture in supplemented PPLO medium with an optical density at 600 nm (OD_{600}) of 0.3, and bacteria were grown anaerobically for 6 h, until they reached late exponential growth phase, and were then harvested by centrifugation. Due to severe autoaggregation under anaerobic conditions, the growth phase was assessed by determination of the total protein content (8).

Microarray analysis. RNA isolation was carried out using a FastRNA Pro Blue kit (Qbiogen, Heidelberg, Germany), and further purification was done using an RNeasy Mini-Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA contamination was removed using a Turbo DNA-free kit (Ambion, Austin, TX). RNA concentration and quality were determined spectrophotometrically and by agarose gel electrophoresis, respectively.

For cDNA synthesis, a 10-μl volume containing 2 to 5 μg of total RNA and 2 μg of random hexamers (Gibco-BRL) was heated at 70°C for 5 min and quick-cooled on ice. cDNA synthesis was performed at 42°C for 1.5 h in a 25-μl reaction mixture containing the RNA, the primer mixture, 1× first-strand buffer (Invitrogen), 500 U Superscript II (Invitrogen), 10 mM dithiothreitol (Invitrogen), and 1.7 nmol of either Cy3-dCTP or Cy5-dCTP (Amersham) at 37°C for 90 min. Unincorporated nucleotides were removed using a MinElute kit (Qiagen), according to the manufacturer's protocol. Labeled samples were combined and hybridized to the *A. pleuropneumoniae* serotype 5b strain L20 microarray (12). This microarray contains PCR products representing each of the 2,025 open reading frames of the genome of *A. pleuropneumoniae* serotype 5b strain L20. Details on the construction and content of the microarray (AppChip1) are available on the Institute for Biological Sciences website (http://ibs-isb.nrc-cnrc.gc.ca/glycobiology/appchips_e.html). Array hybridization and washing were performed as described previously (12). In total, six hybridizations were performed for six independent biological repeats of the *A. pleuropneumoniae* wt and the *A. pleuropneumoniae* Δ*arcA* mutant. The scanned images were analyzed with GenePix Pro 6.0 to generate a data format compatible for further statistical analysis using Genespring GX7.3 (Agilent). The spot intensities of mutant and wt samples were defined as signal and control channels, respectively. The signal channel was normalized to the control channel using LOWESS (intensity dependent), and the percentile was calculated by using all genes not marked absent by GenePix Pro analysis. The final ratio of expression (mutant/wt) was generated using the means for all biologicals and dye swaps. Differentially expressed genes were selected using "filtering on confidence" (*t* test *P* value) plus multiple testing (Benjamini and Hochberg false discovery rate) at a cutoff of ≤0.05 in order to generate a comprehensive list. For biological relevance, a >2-fold change in expression was considered significant; accordingly, discussion was focused on these genes.

2D DIGE. Anaerobic cultures were cooled on ice, and bacteria were harvested by centrifugation (13,000 × *g*, 10 min, 4°C) and washed once with a buffer containing 10 mM Tris-HCl (pH 8.0) and 5 mM magnesium acetate. Pellets were resuspended in 50 mM Tris-HCl (pH 7.3) and stored at −70°C overnight. Cells were thawed and then were ruptured using a FastPrep Instrument (Qbiogene, Heidelberg, Germany) three times for 40 s each time at an intensity setting of 5.0, and cell debris was removed by centrifugation (15,000 × *g*, 30 min). Protein concentration was determined by a MicroBC assay (Uptima Interchim, Montluçon Cedex, France) and confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Proteins were precipitated with trichloroacetic acid, washed with acetone, and solubilized in lysis buffer composed of 30 mM Tris-HCl (pH 8.0), 7 M urea (Roth, Karlsruhe, Germany), 2 M thiourea (Sigma), and 4% (wt/vol) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS; Roth) to a protein concentration of 5 mg/ml. Labeling with CyDyes and 2D gel electrophoresis was performed according to the manufacturer's instructions (Amersham Biosciences AB, Uppsala, Sweden), and four independent biological repeats of the *A. pleuropneumoniae* wt and its *arcA* mutant were used. Gels were scanned with a Typhoon Trio scanner (Amersham), and the intensity of pixel values was adjusted such that the maximum volume of each image was between 50,000 and 90,000. Quantitative evaluation and statistical analyses of the 2D DIGE results was performed using DeCyder 6.5 software (GE Healthcare) according to the manufacturer's instructions. All spots calculated as having an expression difference of statistical significance (unpaired *t* test, $P \leq 0.05$) were highlighted. If, for a certain protein, a horizontal string of spots occurred, these spots were numbered with the lowest number being given to the spot showing the highest calculated difference between the two strains (also see Table S2 in the supplemental material).

Protein identification by Q-TOF MS/MS or MALDI-TOF MS. Protein spots identified as differentially expressed by 2D DIGE were excised, trypsinized, and removed from the gel according to the method of Wilm and Mann (51). For electrospray injection quadrupole time-of-flight tandem mass spectrometry (ESI Q-TOF MS/MS), 3 μl of the peptide solution was loaded into a sample tip (Nanoflow Probe Tip, long; Waters, Milford, MA). Peptide sequences were determined from MS/MS fragmentation data recorded with an ESI Q-TOF MS (Q-TOF Ultima, Waters) in positive reflection mode. Proteins were identified using the program ProteinLynx Global Server (version 2.1; Waters) by searching against the *A. pleuropneumoniae* serotype 5b strain L20 genome database (ftp://ftp.ncbi.nlm.nih.gov./genomes/Bacteria/Actinobacillus_pleuropneumoniae_L20/NC_009053.faa), as downloaded on 10 April 2007. Alternatively, matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) MS was carried out by using a VoyagerDE Pro (Applied Biosystems, Foster City, CA); 1 μl of the peptide solution was mixed with 1 μl of matrix (α-cyano-4-hydroxycinnamic acid [Bruker Daltonics, Billerica, MA], 5 mg/ml, in 50% acetonitrile with 0.1% trifluoroacetic acid) and then spotted on the target plate. Peptide spectra were acquired in positive reflection mode, averaging about 1,000 laser shots per MALDI-TOF spectrum. Mass spectra were calibrated using calibration mixtures CalMix1 and CalMix2 (Applied Biosystems). The peptide mass spectra were analyzed with the peptide mass fingerprint algorithm on the Mascot web site (http://www.matrixscience.com) by searching against the NCBI nonredundant database. The search algorithm was set to allow carbamidomethylation on cysteine residues, oxidation on methionine residues, and a maximum of one missed trypsin cleavage. The peptide mass tolerance was 0.2 Da. Proteins were considered to be positively identified when the probability-based score was above the significance threshold ($P \leq 0.05$). Identification was confirmed by comparing the calculated molecular masses and isoelectric point values from the identified proteins with the observed values for the 2D gel.

Construction and in vitro testing of an isogenic *A. pleuropneumoniae* Δ*frd* in-frame deletion mutant. DNA fragments of approximately 1,300 bp located upstream and downstream of the intended deletion were amplified from genomic *A. pleuropneumoniae* AP76 DNA using primers ofrd_1 and ofrd_2 and primers ofrd_3 and ofrd_4, respectively (Table 1). The downstream fragment was cloned into the vector pCR2.1-TOPO, resulting in pFRD811. Both pFRD811 and the upstream fragment were restricted with BsmBI and PspOMI and ligated, resulting in pFRD820 harboring the truncated *frdA* gene. Plasmid pFRD820 was restricted with NotI and PspOMI, and the fragment containing the truncated *frdA* gene was ligated into pEMOC2, resulting in pFRD710 (Table 1). The *A. pleuropneumoniae* Δ*frd* mutant was constructed by conjugation with *E. coli* β2155 harboring the plasmid pFRD710 (Table 1) as described previously (41). Colonies with the correct PCR profile were confirmed by Southern blotting (47), and the recombination site was confirmed by nucleotide sequencing analysis (Seqlab, Göttingen, Germany). The absence of genomic rearrangements was confirmed by pulsed-field gel electrophoresis (41). The specificity of the mutation with respect to the fumarate reductase-negative phenotype was confirmed by complementation in *trans* with plasmid pFRD1300 (Table 1) and subsequent assaying of fumarate reductase activity (25). Briefly, absorbance at 340 nm was measured for a solution containing 8.4 mM sodium fumarate (Sigma), 116.3 μM NADH (Roth), and 1.25 mM Tris (pH 7.3). The reaction was started by adding an equal volume of bacterial whole-cell lysates. Bacterial cultures and preparation of whole-cell lysates were performed as described for 2D DIGE.

Virulence studies. Comparisons of the virulence of the *A. pleuropneumoniae* wt and the *frd* deletion mutant were assessed by using an aerosol infection model after determining clinical, lung, and reisolation scores as previously described (8). Briefly, *A. pleuropneumoniae*-free pigs (German Landrace, male, 8 to 9 weeks of age) were randomly assigned to two groups (wt group, 7 pigs; Δ*frd* group, 8 pigs) and cared for in accordance with the principles outlined by the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (European Treaty Series, nos. 123 [http://conventions.coe.int

TABLE 1. Bacterial strains and primers used in this study

Strain, plasmid, or primer	Characteristic(s)[a]	Source and/or reference
Strains		
E. coli DH5α F′	F′ *endA1 hsdR17*(r_κ^- m_κ^-) *supE44 thi-1 recA1 gyrA*(Nal^r) *relA1* Δ(*lacZYA-argF*)*U169 deoR* [ϕ80d*lac*Δ(*lacZ*)M15]	44
E. coli β2155	*thrB1004 pro thi strA hsdS lacZ*ΔM15 (F′ *lacZ*ΔM15 *laqI*^{q} *traD36 proA*$^+$ *proB*$^+$)Δ*dap*::*erm*(Erm^r) *recA*::RPA-2-*tet*(Tc^r)::Mu-Km (Km^r) λ *pir*	11
E. coli TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 deoR araD139* Δ(*ara leu*)*7697 galU galK rpsL*(Str^r) *endA1 nupG*	TOPO TA cloning
A. pleuropneumoniae wt	*A. pleuropneumoniae* serotype 7 isolate 76	1
A. pleuropneumoniae Δ*arcA*	Unmarked *arcA* deletion mutant of *A. pleuropneumoniae* wt	8
A. pleuropneumoniae Δ*frd*	Unmarked *frd* deletion mutant of *A. pleuropneumoniae* wt	This work
Plasmids		
pCR2.1-TOPO	*E. coli* cloning vector for fast and efficient cloning of *Taq* polymerase-amplified PCR products	TOPO TA cloning; Invitrogen, Groningen, The Netherlands (52)
pFRD811	pCR2.1-TOPO-based plasmid containing a 1,300-bp PCR product obtained with primers ofrd_3 and ofrd_4, starting at position 1534 downstream of the *frdA* start codon and ending 251 bp downstream of the *frdC* start codon	This work
pFRD820	PCR product obtained with primers ofrd_1 and ofrd_2, digested with BsmBI and PspOMI and ligation of the 1,231-bp fragment into pFRD811 digested with PspOMI and BsmBI, resulting in pFRD820	This work
pEMOC2	Transconjugation vector based on pBluescript SK with *mobRP4*, a polycloning site, Cm^r, and transcriptional fusion of the *omlA* promoter with the *sacB* gene	Accession no. AJ868288 (3)
pFRD710	pEMOC2-based plasmid carrying the truncated *frdABC* fragment excised from pFRD820 using NotI and PspOMI and ligated into pEMOC2 restricted with NotI and PspOMI	This work
pLS88	Broad host range shuttle vector from *Haemophilus ducreyi*; Str^r Sm^r Km^r	58
pFRD1300	pLS88-based complementation plasmid carrying the PCR product of primers ofrd_5 and ofrd_6 encoding the entire *frdABCD* operon and 477-bp upstream sequence cloned into the EcoRI restriction site	This work
Primers		
ofrd_1	5′-ATGCGGGCCCCGGGGTTTCCACTTCTAATA-3′; forward primer containing an internal PspOMI site (underlined) comprising positions 477–458 upstream of the *frdA* gene start codon	This work
ofrd_2	5′-ATGCCGTCTCATTCACCGCGACAACCTTCAG-3′; reverse primer containing an internal BsmBI site (underlined) comprising positions 734–753 of the *frdA* gene	This work
ofrd_3	5′-ATGCCGTCTCATGAATTCATTTTAGATGTGGCGCA-3′; forward primer containing an internal BsmBI site (underlined) comprising positions 1534–1553 of the *frdA* gene	This work
ofrd_4	5′-ATGCGCGGCCGCGCATGGTAAAGTAATGCGGC-3′; reverse primer containing an internal NotI site (underlined) comprising positions 232–251 of the *frdC* gene	This work
ofrd_5	5′-ACGTACCAATTGGATTACAGTGCGATTACTGC-3′; forward primer containing an internal MfeI site (underlined) comprising positions 477–458 upstream of the *frdA* gene start codon	This work
ofrd_6	5′-ACGTACCAATTGCGGGGTTTCCACTTCTAATA-3′; reverse primer containing an internal MfeI site (underlined) comprising position 18 upstream to position 2 downstream of the *frdD* stop codon	This work
ofrd_7	5′-CAATGCGAATGCGGTGGTTA-3′; forward primer comprising positions 522–541 of the *frdA* gene	This work
ofrd_8	5′-CGTTTCGCCGGTTGTGATTT-3′; reverse primer comprising positions 1714–1733 of the *frdA* gene	This work

[a] Nal^r, nalidixic acid resistance; Erm^r, erythromycin resistance; Tc^r, tetracycline resistance; Km^r, kanamycin resistance; Str^r, streptomycin resistance.

/Treaty/EN/Treaties/Html/123.htm] and 170 [http://conventions.coe.int/Treaty/EN/Treaties/Html/170.htm]).

Based on the specifications for the validation of *A. pleuropneumoniae* vaccines given in the European Pharmacopoeia (http://www.pheur.org), the occurrence of coughing, dyspnea, and vomiting was assessed daily within the first 7 days postinfection. In order to quantify the results, a score of 1 was given for each of the symptoms, resulting in a maximum score of 3 per pig and day.

The lung lesion score was determined according to the scoring system adopted by the European Pharmacopoeia. Briefly, upon necropsy, each of the seven porcine lung lobes was scored separately with a score of 0 (healthy) to 5 (completely altered), resulting in a maximum score of 35 per pig.

In addition, a histopathological analysis of lung lesions was performed. Briefly, pigs were necropsied on day 7 or day 21 postinfection, and samples were fixed in 10% formaldehyde, embedded in paraffin wax, sectioned at 5 μm, stained with hematoxylin and eosin, and analyzed by light microscopy.

The reisolation score was determined as described previously (33). Briefly, defined locations of each of the seven lung lobes were plated on selective meat blood agar (27). A score for reisolation of 0 was given if no growth of *A.*

pleuropneumoniae occurred or if growth occurred only in the area of the plate inoculated by direct contact with the tissue sample; by using a sterile loop, each sample was fractionated twice, and a score of 1 was given if colonies were also present in the fractionated streaks. To confirm the identity of the challenge strain, one colony for each location or at least three individual colonies were analyzed by PCR using the primers ofrd_7 and ofrd_8. The reisolation score was determined by adding the individual scores for each pig, and the arithmetic means and standard deviations were determined.

Immune response. The humoral immune response of pigs was determined by two different enzyme-linked immunosorbent assays (ELISAs). Antibody levels directed against the *A. pleuropneumoniae* ApxII toxin were analyzed using recombinant ApxII protein as the coating antigen (30), and the response is quantified in ELISA units (EU) based on an external standard. Activities of ≤10 EU in the sera are considered negative, those of 11 to 25 EU are intermediate, and those of >25 EU are positive. Antibodies directed against outer membrane-associated proteins were determined by an ELISA using a detergent extract of *A. pleuropneumoniae* grown under iron restriction as the coating antigen (20). Quantification of the immune response was done by determining the antibody titer in comparison to that of an internal negative control consisting of an equal mixture of all serum samples taken at the arrival of the pigs. The titer was defined as the highest serum dilution resulting in an OD twice as high as that of the negative control serum at a dilution of 1:100.

RESULTS

Identification and functional classification of the *A. pleuropneumoniae* ArcA regulon. In the presence of ArcA (as in *A. pleuropneumoniae* wt), among the 2,025 predicted protein-encoding genes on the microarray chip, the expression levels of 16 and 77 genes were upregulated >2-fold and between 1.5- and 2-fold, respectively. The expression levels of 42 and 64 genes were downregulated >2-fold and between 1.5- and 2-fold, respectively (see Table S1 in the supplemental material). An analysis of the ArcA regulon based on the database of Clusters of Orthologous Groups of proteins (COGs) (53) revealed that ArcA controls the expression of genes in almost all prokaryotic COGs, with large differences in the numbers of up- and downregulated genes in some functional categories. Thus, in the category "carbohydrate transport and metabolism," seven genes were downregulated in the presence of ArcA and none was upregulated. In the "energy production and conversion" category, 21 genes were downregulated and 6 genes were upregulated in the presence of ArcA. In the category "inorganic ion transport and metabolism," 12 genes were downregulated in the presence of ArcA compared to 4 that were found to be upregulated. In contrast, in the categories "replication, recombination and repair," "transcription," and "cell cycle control, cell division, and chromosome partitioning," considerably more genes were upregulated than downregulated in the presence of ArcA (Fig. 1).

Transcription data were supplemented by 2D DIGE (Fig. 2), which revealed a total of 156 protein spots that were regulated statistically significantly ($P \leq 0.05$) in the presence of ArcA in *A. pleuropneumoniae*. Definitive protein identification was possible for 52 of these spots, representing 34 different proteins with 16 proteins that were upregulated and 18 that were downregulated (see Table S2 in the supplemental material).

The microarray data were in accordance with the 2D DIGE data for the proteins downregulated significantly in the presence of ArcA. Thus, for 16 of the 18 proteins identified, the encoding genes were also identified by microarray analysis as being downregulated significantly. For the proteins upregulated significantly in the presence of ArcA, the data were partly confirmed by microarray data. Thus, of the 16 proteins identified, 7 were found by microarray analysis, and 6 of these were significantly upregulated in the presence of ArcA. For the remaining nine proteins, the encoding genes were not identified as being significantly regulated due to variations in signal ratios for the biological repeats. For the gene-protein pairs identified by both microarray and 2D DIGE, the results of both methods correlated with the line of best fit, which showed a slope of 1.9 and an R^2 value of 0.65 (see Fig. S1 and Table S3 in the supplemental material).

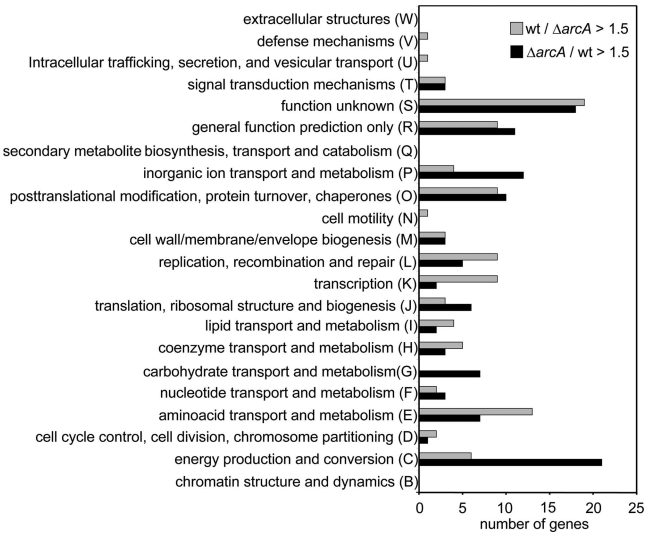
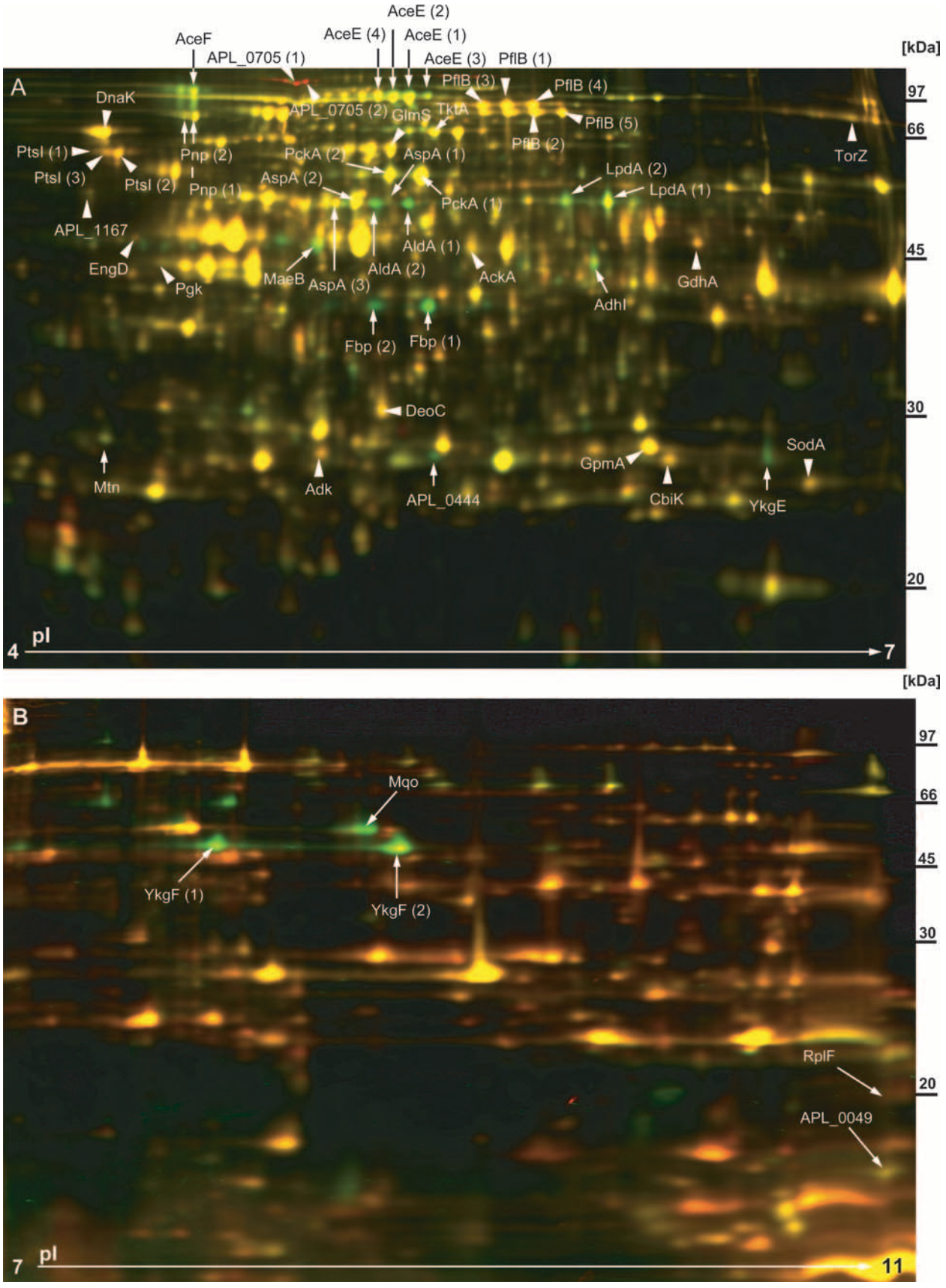


FIG. 1. Functional classification of ArcA-regulated genes according to the COGs database. The classification was adopted from the genome annotation for *A. pleuropneumoniae* serotype 5b L20 from the NCBI database (GenBank accession no. CP000569).

Predicted impact of ArcA on *A. pleuropneumoniae* metabolism. In silico analysis of the ArcA regulon led to the hypothesis that (i) ArcA of *A. pleuropneumoniae* modulates its metabolism toward the synthesis of fumarate as the terminal electron acceptor and (ii) glycerol-3-phosphate is used as the reduction equivalent for the reduction of fumarate and that, after oxidation to dihydroxyacetone phosphate, it serves as a precursor for fumarate synthesis (Fig. 3A). Transcripts and proteins of the three subunits of the pyruvate dehydrogenase complex, AceE, AceF and LpdA, were downregulated approximately threefold in the presence of ArcA in the *A. pleuropneumoniae* wt. This result suggests that consumption of pyruvate, by oxidative decarboxylation into acetyl-coenzyme A (acetyl-CoA) for the introduction of reduced carbon atoms into the citric acid cycle, is downregulated. In addition, a BLASTP homology search against *E. coli* citric acid cycle enzymes revealed that the citric acid cycle of *A. pleuropneumoniae* is incomplete; it lacks a homologue for citrate synthase (GltA). Therefore, potentially, acetyl-CoA cannot react with oxaloacetate to form citrate. Furthermore, homologues of aconitase and isocitrate dehydrogenase (AcnA, AcnB, Icd), catalyzing the transformation of citrate into α-ketoglutarate, are also missing from the *A. pleuropneumoniae* genome. These results led us to hypothesize that phosphoenolpyruvate, not being fed into the oxidative branch of the citric acid cycle, is carboxylated to oxaloacetate by phosphoenolpyruvate carboxylase, as can occur with *E. coli* (2). The absence of a citrate synthase in *A.*


A
AceE (2)
AceF
AceE (4)
AceE (1)
APL_0705 (1)
AceE (3)
PflB (1)
[kDa]
PflB (3)
PflB (4)
DnaK
APL_0705 (2)
GlmS
TktA
PflB (5)
PflB (2)
PtsI (1)
Pnp (2)
PckA (2)
AspA (1)
TorZ
PtsI (3)
PtsI (2)
Pnp (1)
AspA (2)
LpdA (2)
LpdA (1)
PckA (1)
APL_1167
AldA (1)
EngD
MaeB
AspA (3)
AldA (2)
AckA
GdhA
Pgk
AdhI
Fbp (2)
Fbp (1)
DeoC
SodA
GpmA
Mtn
Adk
APL_0444
CbiK
YkgE
97
66
45
30
20
pI
4
7
[kDa]
B
Mqo
YkgF (1)
YkgF (2)
RplF
APL_0049
97
66
45
30
20
pI
7
11

pleuropneumoniae may prevent oxaloacetate consumption by the generation of citrate. Additionally, oxaloacetate is unlikely to be used for gluconeogenesis, as the latter is strongly repressed in the presence of ArcA. Two key enzymes of gluconeogenesis, phosphoenolpyruvate carboxykinase (PckA) and fructose-1,6-bisphosphatase (Fbp), were downregulated in the *A. pleuropneumoniae* wt compared to the *arcA* deletion mutant. Therefore, oxaloacetate is most likely converted to L-malate by malate dehydrogenase (Mdh), an enzyme favoring the NADH-consuming reduction of oxaloacetate to malate (39). L-Malate could then be converted into fumarate by FumC, the only fumarase found in *A. pleuropneumoniae*. An alternative possibility, the irreversible oxidation of malate to oxalacetate by the membrane-bound malate quinone oxidoreductase (Mqo; 28), is unlikely since transcription of *mqo* is downregulated 20-fold in the presence of ArcA. Also, malic enzyme (MaeB), which catalyzes the oxidative decarboxylation of malate into pyruvate, is downregulated 4.6-fold in the presence of ArcA. The second decarboxylating malate dehydrogenase, *E. coli* homologue (MaeA), is missing in *A. pleuropneumoniae*. However, fumarate could serve as the terminal electron acceptor in an electron transfer reaction of the respiratory chain catalyzed by the fumarate reductase enzyme complex (FrdABCD; 5). Fumarate reductase is typically linked with glycerol-3-phosphate dehydrogenase through a simple electron transport chain (49), transferring reduction equivalents directly from glycerol-3-phosphate to fumarate and thereby generating a proton gradient (23). Transcription of the three genes encoding the anaerobic glycerol-3-phosphate dehydrogenase (*glpABC*) was upregulated >2-fold in the presence of ArcA. This enzyme complex catalyzes the oxidation of glycerol-3-phosphate to dihydroxyacetone phosphate which, in turn, is a glycolysis intermediate and can be converted into fumarate (Fig. 3A).

The hypothesized use of glycerol-3-phosphate as a reduction equivalent and fumarate as an alternative electron acceptor was supported by the simultaneous downregulation of other enzymes commonly involved in anaerobic metabolism, including (i) enzymes involved in fermentation, such as an alcohol dehydrogenase (AdhI) and an aldehyde dehydrogenase (AldA), which were repressed 5- and 16-fold, respectively (Fig. 3C); (ii) dehydrogenases of the respiratory chain, which require L-lactate (LldD), D-lactate (Dld), proline (PutA), or formate (FdhD, FdxG [APL_0892], FdxG [APL_0893], FdxH, FdnI, FdnE) as reduction equivalents; and (iii) terminal reductases using nitrite (NrfA, NrfB, NrfC, NrfD, NrfE), nitrate (NapA, NapF), or oxygen (CydA, CydB) as oxidation equivalents in the respiratory chain (Fig. 3B).

Impact of the *frdABCD* genes, encoding fumarate reductase, on virulence. To test whether fumarate reductase may be partly responsible for the attenuation of the *A. pleuropneumoniae* Δ*arcA* mutant, fumarate reductase was deleted by genomic exchange against an *frdABCD* operon harboring an in-frame-deleted version of the *frdA* sequence. The deletion mutant was confirmed by PCR, Southern blot analysis, sequencing, and pulsed-field gel electrophoresis (data not shown). By use of an enzymatic assay, it was shown that NADH consumption in the presence of fumarate was considerably lowered in the mutant and could be restored by complementation in *trans* (data not shown). Pigs infected with the *A. pleuropneumoniae* Δ*frd* mutant had clearly but not significantly reduced clinical and reisolation scores. The lung lesion score was reduced by an amount that was statistically significant ($P \leq 0.05$) (Table 2), and the quality of lung lesions was completely different than those seen with the wt. Thus, pigs infected with the *A. pleuropneumoniae* Δ*frd* mutant had a mild bronchiolo-interstitial pneumonia, whereas animals infected with the *A. pleuropneumoniae* wt showed a severe fibrinous pleuropneumonia and a moderate focal purulent pneumonia with abscess formation. Similar observations were made on day 7 postinfection and day 21 postinfection (Fig. 4). Histopathology of lung tissue obtained from an uninfected control pig revealed a diffuse interstitial pneumonia comparable to that in animals infected with the *A. pleuropneumoniae* Δ*frd* mutant (data not shown).

DISCUSSION

The role of central metabolic enzymes in bacterial virulence and persistence is an area of burgeoning research (19, 32, 34, 54). In particular, in view of gene conservation in many bacterial species, it may be a fruitful research field for the identification of potential targets for new therapeutic drugs. A suitable approach to defining metabolic pathways involved in virulence is the analysis of isogenic mutants in global regulators that are attenuated for virulence, such as ArcA. ArcA is an established global regulator that affects metabolic genes in *Enterobacteriaceae* (31, 46). The inactivation of *arcA* has been shown to cause attenuation in a number of pathogens, such as *M. tuberculosis* (45), *V. cholerae* (50), *S. flexneri* (7), *H. influenzae* (13, 59), and *A. pleuropneumoniae* (8). However, the consequences of *arcA*-induced changes of metabolic enzymes for virulence have not been investigated to date.

The ArcA regulon of *A. pleuropneumoniae* is considerably smaller than that of *E. coli* (31, 46). Possible reasons include the twofold-smaller genome size of *A. pleuropneumoniae* and the more-restricted environmental niche it inhabits, the por-

FIG. 2. 2D DIGE analysis. The *A. pleuropneumoniae* wt (shown here labeled with Cy5 [red]) and *A. pleuropneumoniae* Δ*arcA* (shown here labeled with Cy3 [green]) proteins were precipitated directly from whole-cell lysates and separated by using Immobiline DryStrips with pI gradients of 4 to 7 (A) or 7 to 11 (B). The *A. pleuropneumoniae* wt and *A. pleuropneumoniae* Δ*arcA* mutant were grown anaerobically in four independent cultures (each). By using three fluorescent dyes, Cy2 (blue [internal standard]), Cy3 (green [either wt or Δ*arcA*]) and Cy5 (red [either wt or Δ*arcA*]), on each gel, an internal standard and a protein preparation of the *A. pleuropneumoniae* wt and *A. pleuropneumoniae* Δ*arcA* mutant were separated simultaneously. Fluorescence was detected using a Typhoon Trio fluorescence scanner. Quantitative and statistical analyses of differences in protein expression levels were performed with DeCyder 2D 6.5 software. Each gel shown here represents one out of four gels that were run and analyzed. Arrowheads highlight protein spots that were significantly ($P \leq 0.05$) upregulated in the presence of ArcA. Arrows indicate protein spots that were significantly ($P \leq 0.05$) downregulated in the presence of ArcA. Protein spots of interest were excised from preparative gels, trypsinized and, after recovery of peptides from the gel, analyzed by MS.

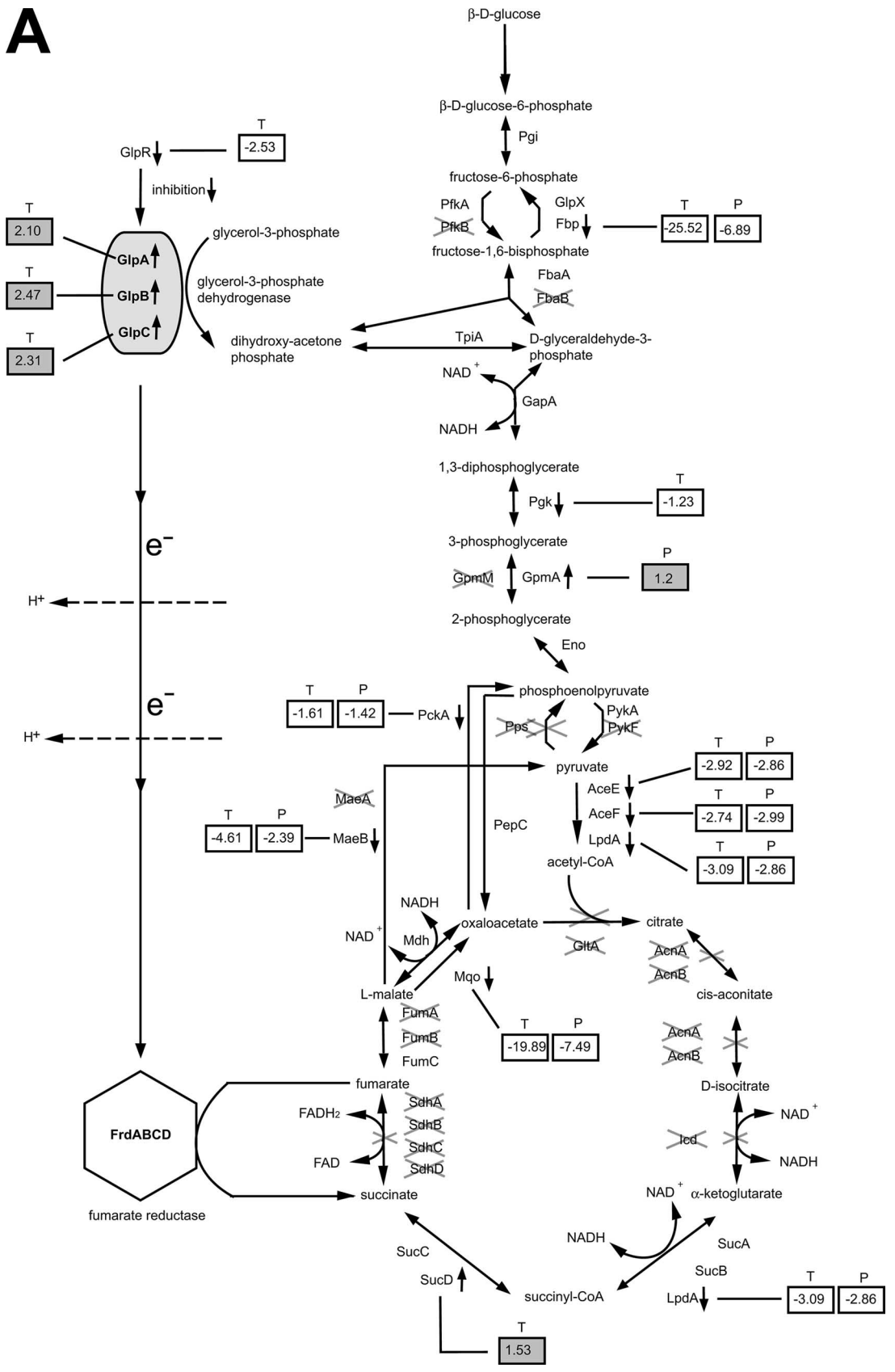

A
β-D-glucose
β-D-glucose-6-phosphate
Pgi
fructose-6-phosphate
PfkA
PfkB
GlpX
Fbp
T -25.52
P -6.89
fructose-1,6-bisphosphate
FbaA
FbaB
GlpR
T -2.53
inhibition
T 2.10
T 2.47
T 2.31
GlpA
GlpB
GlpC
glycerol-3-phosphate
glycerol-3-phosphate dehydrogenase
dihydroxy-acetone phosphate
TpiA
D-glyceraldehyde-3-phosphate
NAD+
GapA
NADH
1,3-diphosphoglycerate
Pgk
T -1.23
3-phosphoglycerate
GpmM
GpmA
P 1.2
2-phosphoglycerate
Eno
phosphoenolpyruvate
T -1.61
P -1.42
PckA
Pps
PykA
PykF
pyruvate
T -2.92
P -2.86
AceE
AceF
T -2.74
P -2.99
LpdA
T -3.09
P -2.86
acetyl-CoA
MaeA
T -4.61
P -2.39
MaeB
PepC
NADH
NAD+
Mdh
oxaloacetate
GltA
citrate
AcnA
AcnB
cis-aconitate
Mqo
T -19.89
P -7.49
L-malate
FumA
FumB
FumC
fumarate
AcnA
AcnB
D-isocitrate
FADH2
FAD
SdhA
SdhB
SdhC
SdhD
Icd
NAD+
NADH
FrdABCD
fumarate reductase
succinate
NAD+
α-ketoglutarate
NADH
SucA
SucC
SucD
SucB
LpdA
T -3.09
P -2.86
succinyl-CoA
T 1.53
e−
H+
e−
H+

FIG. 3

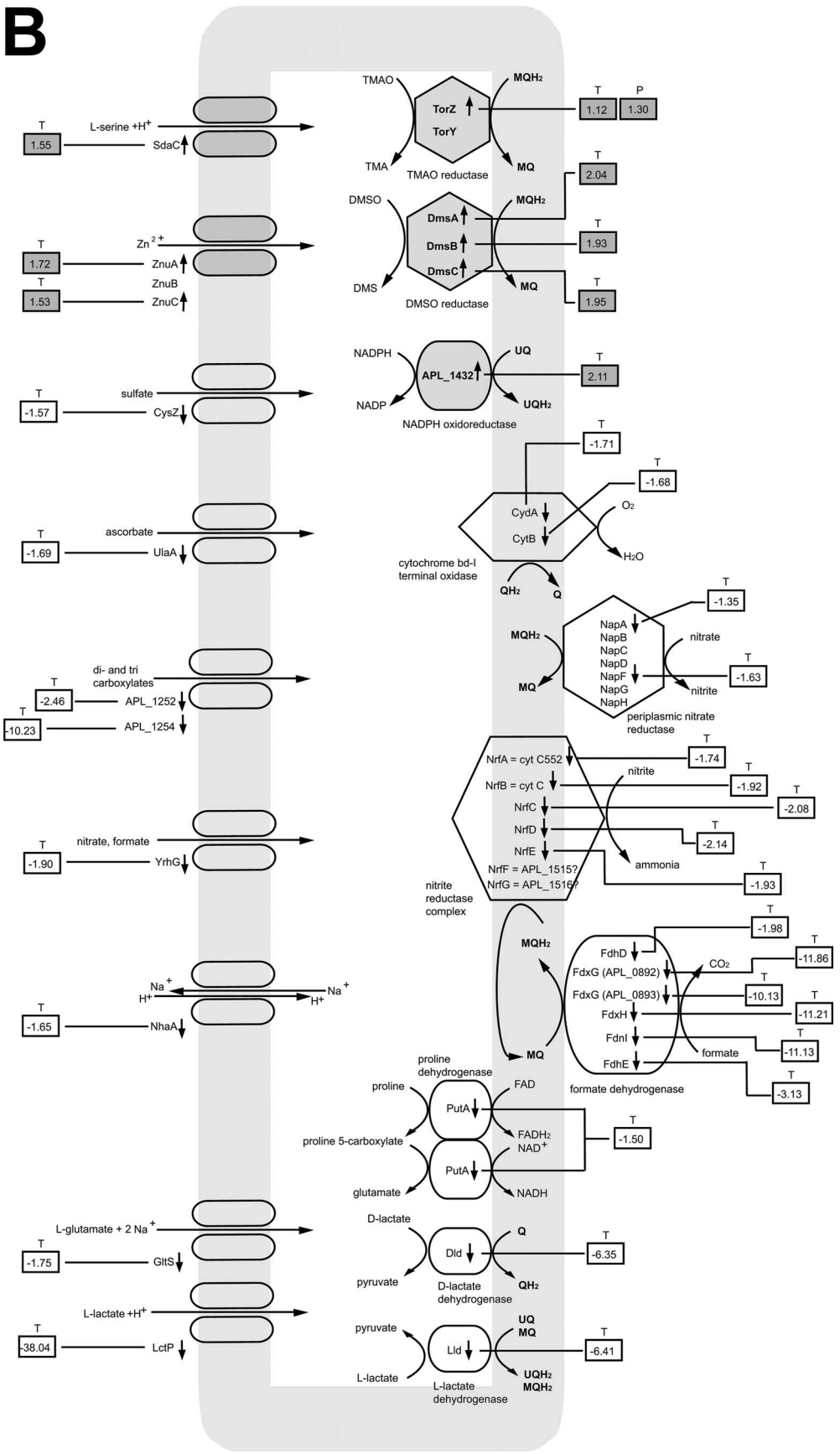


FIG. 3—*Continued.*

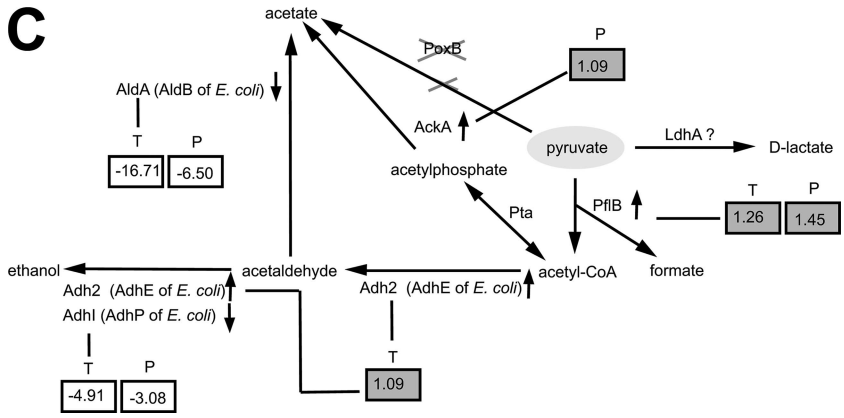


FIG. 3. Impact of ArcA-regulated genes on metabolic pathways. (A) Glycolysis and citric acid cycle. (B) Dehydrogenases, terminal reductases, and membrane transporter. (C) Fermentation. (A to C) T, transcript; P, protein. The degree of ArcA-dependent regulation is given within the boxes when results obtained by microarray analysis (transcript) or by 2D DIGE (protein) were statistically significant ($P \leq 0.05$; gray-highlighted box, upregulated in the presence of ArcA; unhighlighted box, downregulated in the presence of ArcA). Dehydrogenases are indicated by a rounded rectangle, terminal reductases are indicated by hexagons, and membrane transporters are indicated by rounded rectangle pairs. Biochemical pathways were adopted from those for *E. coli* K-12 (BioCyc, http://www.biocyc.org). The sequence and annotation of the *A. pleuropneumoniae* serotype 5b strain L20 genome have recently been published (GenBank accession no. CP000569). If *A. pleuropneumoniae* carries no homologue to the respective *E. coli* protein, its name is crossed out.

cine respiratory tract. In accordance with the ArcA regulon of its close relative *H. influenzae* (59), ArcA of *A. pleuropneumoniae* appears to act primarily as a repressor of transcription. In *A. pleuropneumoniae*, as well as in *H. influenzae* (59), the gene most strongly downregulated by the presence of ArcA was the L-lactate permease gene. This downregulation under anaerobic conditions is energetically efficient, since the transport is at the expense of the proton gradient and L-lactate seems not to be a favored substrate. Genes like the L-lactate dehydrogenase gene *lldD* or the formate dehydrogenase genes *fdnI*, *fdxH*, and *fdhE* were also downregulated in *A. pleuropneumoniae* as well as in *H. influenzae* by the presence of ArcA. However, overall, the ArcA regulon of *A. pleuropneumoniae* is larger than that described for *H. influenzae* (59), and there was no correlation between the genes upregulated by the presence of ArcA in both species. The three genes most strongly upregulated in *A. pleuropneumoniae* exhibit homology to a methylation subunit of a type III restriction modification system, a restriction enzyme, and a hypothetical DNA/RNA helicase. These genes are located next to each other in the genome of *A. pleuropneumoniae* and, to our knowledge, the homologues in other species have not previously been described as being regulated by ArcA. As in *Shewanella oneidensis* (21), genes encoding dimethyl sulfoxide reductase (*dmsABC*) and cytochrome *d* oxidase (*cydAB*) were up- and downregulated in the presence of ArcA, respectively. This contrasts with *E. coli*, where ArcA-dependent *dms* gene regulation is unknown and *cydAB* is upregulated by ArcA.

We analyzed the ArcA regulon of *A. pleuropneumoniae* with respect to its possible effect on virulence and persistence in the

TABLE 2. Virulence of *A. pleuropneumoniae* parent and *frd* mutant strain following aerosol challenge

Challenge strain	No. of animals	Challenge dose (OD_{600} [aerosolized] per 4 pigs)[a]	Day of necropsy	Serological response with[b]:		Arithmetic mean (± SD) for[c]:		
				Detergent wash	ApxII	Clinical score	Lung lesion score	Reisolation score
AP76 wt	1	0.5	2[d]	ND	ND	21	15.4	7
	3	0.5	7	ND	ND	6 ± 3	21.21 ± 11.07	5 ± 2.65
	3	0.5	21	21,333 ± 6,034	64 ± 9	3.33 ± 2.31	14.92 ± 14.18	1.67 ± 2.89
AP76 Δ*frd*	4	0.45	7	ND	ND	1.5 ± 1.29	6 ± 4.77	1.5 ± 1.73
	4	0.45	21	22,400 ± 5,543	66 ± 19	2.75 ± 0.03	0.73 ± 0.57	0.25 ± 0.5
P value[e]						0.093	0.046	0.106

[a] Bacteria were grown aerobically in the presence of Tween 80 (0.1%) to the respective OD_{600} and diluted 1:30,000 with sterile saline; 13 ml of this dilution was aerosolized in the aerosol chamber.

[b] Detergent wash, arithmetic mean of the highest serum dilution resulting in an OD twice as high as that of the negative control serum at a dilution of 1:100 ± standard deviation. ApxII, arithmetic mean (in EU) of the serum activity ± standard deviation. ND, not determined.

[c] The clinical (33) and lung (22) scores were determined as described previously.

[d] Animal died on day 2; these results are not included in the statistical comparison of scores between wt- and Δ*frd*-infected pigs.

[e] The statistical analysis comparing *A. pleuropneumoniae* wt- and *A. pleuropneumoniae* Δ*frd* mutant-infected animals was performed using the Wilcoxon signed-rank test, and results were considered significant when the *P* value was ≤0.05. Animals infected with the *A. pleuropneumoniae* wt and the *A. pleuropneumoniae* Δ*frd* mutant, respectively, were considered one group, independent of the day of necropsy, as half of the animals was sacrificed on day 7 and on day 21 in either group. The *A. pleuropneumoniae* wt-infected animal, which died on day 2 postinfection, was not included in order to not overassess the scores for the *A. pleuropneumoniae* wt group.

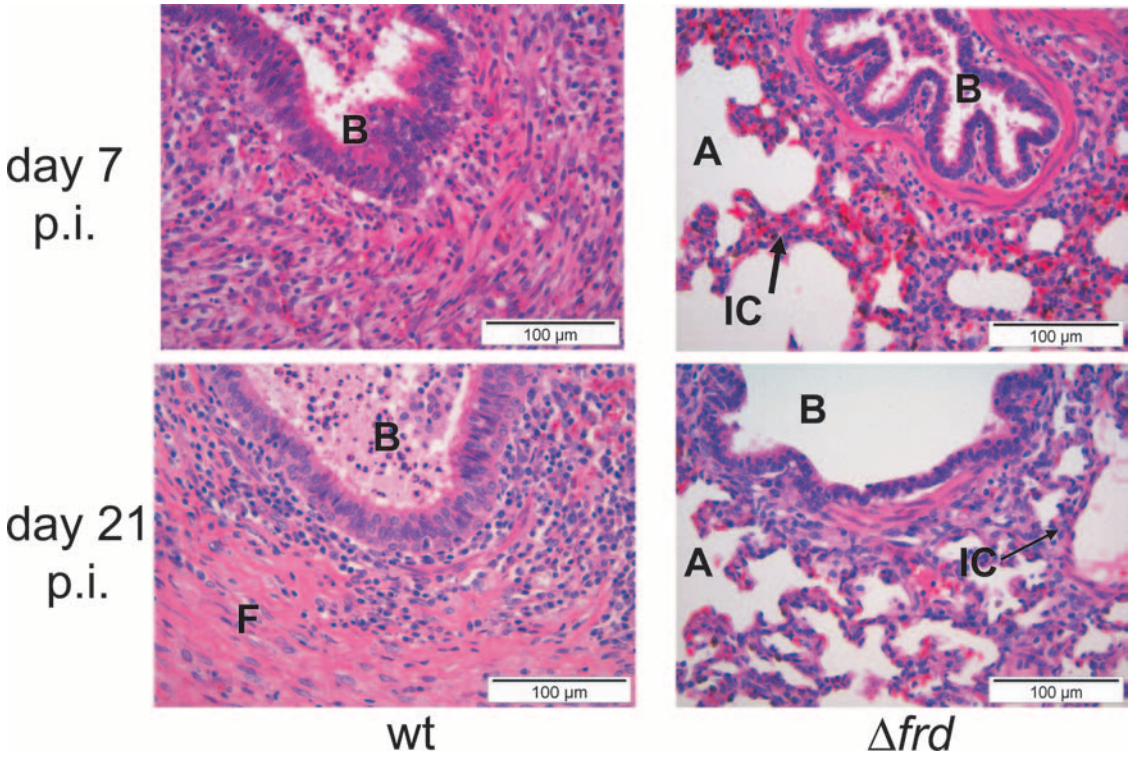


FIG. 4. Histopathological examination of lung lesions. Sections were obtained from pigs necropsied on day 7 postinfection or on day 21 postinfection. Animals were infected with either the *A. pleuropneumoniae* wt or the *A. pleuropneumoniae* Δ*frd* mutant. A, alveole; B, bronchus; F, fibrin; IC, interstitial consolidation.

porcine respiratory tract. The results suggested an ArcA-dependent coordinated regulation of genes involved in the pathway leading to fumarate synthesis. This implied that fumarate is used as the terminal electron acceptor, with glycerol-3-phosphate being the most likely reduction equivalent (49). We assume that fumarate is synthesized from dihydroxyacetone phosphate, the oxidation product of glycerol-3-phosphate, which is processed during glycolysis, and that phosphoenolpyruvate is then introduced into the reductive branch of the citric acid cycle to generate fumarate. In support of this hypothesis, the transcription of glycerol-3-phosphate dehydrogenase was upregulated, whereas several competing steps (the pyruvate dehydrogenase, malate:quinone reductase, and malic enzyme steps) were strongly downregulated. These findings emphasize the necessity of a thorough pathway analysis, as genes encoding enzymes with crucial functions (phosphoenolpyruvate carboxylase, fumarate reductase) may be constitutively expressed.

The midpoint potentials for the glycerol-3-phosphate/dihydroxyacetone phosphate and the fumarate/succinate redox pairs are −0.19 V and 0 V, respectively. The coupling of these two systems has been calculated for *E. coli* to yield a $-\Delta G^\circ$ of about 8.8 kcal (37). This energy facilitates the export of approximately two protons from the cytosol into the periplasm, thereby generating a proton gradient (38). If a stoichiometry of three to four protons is transported per ATP synthesized (56), the oxidation of two molecules of glycerol-3-phosphate coupled to the reduction of two molecules of fumarate leads to synthesis of at least one ATP by oxidative phosphorylation plus two ATPs by substrate chain phosphorylation. Importantly, the NAD^+ balance is neutral, facilitating ongoing glycolysis.

A. pleuropneumoniae, *H. pylori* (43), and *H. influenzae* (NC_000907) lack citric acid cycle enzymes. In these organisms, the citric acid cycle functions as a noncyclic branched pathway, as has been reported for several anaerobic organisms (57). Homology analyses revealed that *A. pleuropneumoniae* lacks the initial steps of the oxidative branch of the TCA cycle but can synthesize TCA cycle intermediates through to α-ketoglutarate via the reductive branch. Therefore, in *A. pleuropneumoniae*, the TCA cycle appears to be directed toward (i) synthesis of the metabolic intermediates succinate, succinyl-CoA, and α-ketoglutarate, which cannot be produced by the TCA cycle upon deletion of *frd*; (ii) energy production by reduction of fumarate with glycerol-3-phosphate as the reduction equivalent (49), as proposed for *H. pylori* (35); and (iii) fermentation to achieve a neutral NAD^+ balance (9).

In order to support a predicted role for fumarate reductase in virulence, *A. pleuropneumoniae* fumarate reductase was inactivated by deletion. The deletion mutant was attenuated for infection so that it caused less-severe lung lesions than the parent strain. An essential role for fumarate reductase in bacterial virulence has previously been described for *H. pylori*, where it was hypothesized that inactivation would abolish a crucial energy-producing source required to penetrate the mucus layer of the gastric epithelia (18). For *A. pleuropneumoniae*, the reduction of fumarate might likewise be an efficient energy source in its environment on the intact respiratory epithelium. In both environments, the gastric mucus and the epithelial

lining fluid, phospholipids are abundant and phospholipases might provide a rich source of glycerol-3-phosphate (15), which is predicted to serve directly as the reduction equivalent.

The fumarate reductase of *H. pylori* is a potential therapeutic target (17, 36), but none of the three known inhibitory compounds available were suitable for therapeutic use (36). However, more recently, nafuredin, a novel noncommercial anthelminthic substance inhibiting fumarate reductase (55), has been used successfully to treat experimental *Haemonchus contortus* infections in sheep (40). Since higher eukaryotes do not carry homologues to the bacterial or helminthal fumarate reductase enzyme complex, nafuredin could have potential as a novel therapeutic drug to treat not only helminthal but also at least some bacterial infections.

Taken together, these data lead us to propose that fumarate reductase activity may be crucial not only for virulence of *A. pleuropneumoniae* but also for a number of other bacterial pathogens that effectively colonize mucous membranes in the respiratory and gastrointestinal tracts, by providing either energy by anaerobic respiration or important metabolic intermediates or both of the above. Therefore, it might be worthwhile to investigate the use of nafuredin or other fumarate reductase inhibitors as novel therapeutics for the treatment of bacterial infections of mucosal surfaces.

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