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Changes in Gene Expression of *Actinobacillus pleuropneumoniae* in Response to Anaerobic Stress Reveal Induction of Central Metabolism and Biofilm Formation[§]

Lu Li[1,3], Jiawen Zhu[1,2], Kui Yang[4], Zhuofei Xu[1,2], Ziduo Liu[1,3], and Rui Zhou[1,2]*

[1]State Key Laboratory of Agricultural Microbiology, [2]College of Veterinary Medicine, [3]College of Life Science and Technology, [4]Modern Education Technology Center, Huazhong Agricultural University, Wuhan 430070, P. R. China

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***Actinobacillus pleuropneumoniae* is an important porcine respiratory pathogen causing great economic losses in the pig industry worldwide. Oxygen deprivation is a stress that *A. pleuropneumoniae* will encounter during both early infection and the later, persistent stage. To understand modulation of *A. pleuropneumoniae* gene expression in response to the stress caused by anaerobic conditions, gene expression profiles under anaerobic and aerobic conditions were compared in this study. The microarray results showed that 631 genes (27.7% of the total ORFs) were differentially expressed in anaerobic conditions. Many genes encoding proteins involved in glycolysis, carbon source uptake systems, pyruvate metabolism, fermentation and the electron respiration transport chain were up-regulated. These changes led to an increased amount of pyruvate, lactate, ethanol and acetate in the bacterial cells as confirmed by metabolite detection. Genes encoding proteins involved in cell surface structures, especially biofilm formation, peptidoglycan biosynthesis and lipopolysaccharide biosynthesis were up-regulated as well. Biofilm formation was significantly enhanced under anaerobic conditions. These results indicate that induction of central metabolism is important for basic survival of *A. pleuropneumoniae* after a shift to an anaerobic environment. Enhanced biofilm formation may contribute to the persistence of this pathogen in the damaged anaerobic host tissue and also in the early colonization stage. These discoveries give new insights into adaptation mechanisms of *A. pleuropneumoniae* in response to environmental stress.**

***Keywords*:** *Actinobacillus pleuropneumoniae*, anaerobic, gene expression, central metabolism, biofilm

*For correspondence. E-mail: rzhou@mail.hzau.edu.cn; Tel.: +86-27-8728-1878; Fax: +86-27-8728-2608
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Introduction

Actinobacillus pleuropneumoniae, a member of the family *Pasteurellaceae*, is the etiologic agent of porcine contagious pleuropneumonia, which causes large economic losses in the pig industry globally (Bosse *et al.*, 2002). *A. pleuropneumoniae* infection is characterized by fibrinohemorrhagic necrotizing pneumonia and fibrinous pleuritis, often leading to a fatal outcome. The disease course can be peracute, acute and chronic. The chronically infected swine can carry the pathogen for a long time and become the major disease reservoir for *A. pleuropneumoniae* (Rycroft and Garside, 2000). The infection and persistence of this pathogen is mediated by multiple known and putative infection-associated factors (Bosse *et al.*, 2002; Chiers *et al.*, 2010). The confirmed *A. pleuropneumoniae* virulence factors include some anaerobic respiration enzymes (Jacobsen *et al.*, 2005; Buettner *et al.*, 2008a), indicating that central metabolism is likely to be essential for full virulence of this pathogen. Additionally, maltose transporters, D-ribose binding and transport proteins, and the glycerol uptake facilitator and transporter have been found to play roles in infection of *A. pleuropneumoniae* (Lone *et al.*, 2009a, 2009b). A recent study that monitored the transcriptional profile of *A. pleuropneumoniae* during the acute infection phase in the pig also demonstrated the importance of metabolic adjustments in virulence (Klitgaard *et al.*, 2012). Hence, the regulation of basic metabolism of *A. pleuropneumoniae* is not only essential for fitness under various conditions but also presumably related to virulence.

In recent years, it has also been suggested that biofilm formation plays important roles in *A. pleuropneumoniae* infection. Poly-*N*-acetylglucosamine (PGA) mediates *A. pleuropneumoniae* biofilm formation and PGA synthesis genes are located in the *pga* operon (Kaplan *et al.*, 2004). Biofilm formation is prevalent in field isolates of *A. pleuropneumoni*ae (Kaplan and Mulks, 2005). Under proper growth conditions, more strains can form biofilms (Labrie *et al.*, 2010). The *A. pleuropneumoniae* biofilm mediates increased resistance to antibiotics (Izano *et al.*, 2007). In addition, biofilm formation is a stress response in *A. pleuropneumoniae* (Bosse *et al.*, 2010) and it has been suggested to contribute to colonization and persistence of this pathogen *in vivo* (Auger *et al.*, 2009).

Gene regulation in *A. pleuropneumoniae* grown under conditions that mimic conditions in the host has been examined (Deslandes *et al.*, 2007; Auger *et al.*, 2009; Lone *et al.*, 2009b; Li *et al.*, 2012). The results of these studies indicated that

this bacterium can use complicated mechanisms for adaptation to the host environment. Among the stresses that *A. pleuropneumoniae* encounters in the host, one critical stress derives from rapid oxygen consumption during the early infection stage and nearly complete oxygen exhaustion during the persistent stage in the damaged and necrotic tissues (Buettner *et al.*, 2009). The sequenced genome of *A. pleuropneumoniae* reveals the genes encoding DMSO reductase, fumarate reductase, trimethylamine oxide reductase and nitrite/nitrate reductase and enzymes with roles in fermentation (Xu *et al.*, 2008). Previous research has reported that genes encoding the anaerobic enzymes (DmsA, FrdABCD, and AspA) and global anaerobic regulators (ArcA and HlyX) are essential for *A. pleuropneumoniae* infection (Baltes *et al.*, 2003, 2005; Jacobsen *et al.*, 2005; Buettner *et al.*, 2008a, 2008b, 2009). These investigations indicate that anaerobic survival and persistence is important for *A. pleuropneumoniae* infection of its host. Studies on changes in gene expression after the shift to anaerobic conditions in *A. pleuropneumoniae* would contribute to the understanding of the pathogenesis of this bacterium. Therefore, in this study, transcriptional profiles were compared using microarrays between *A. pleuropneumoniae* cultures under aerobic and anaerobic conditions. The expression of genes involved in central metabolism and the infection process were analyzed and further confirmed by phenotypic characterizations.

Materials and Methods

Bacterial strains and growth conditions

A. pleuropneumoniae 4074 (serovar 1 reference strain) was cultured in Tryptic Soy Broth (TSB) (Difco Laboratories, USA) or on Tryptic Soy Agar (TSA) (Difco Laboratories) supplemented with 10 μg/ml of nicotinamide adenine dinucleotide (NAD) and 10% (v/v) filtered cattle serum at 37°C. For aerobic conditions, *A. pleuropneumoniae* was cultured in an aerobic incubator at 200 rpm for RNA collection and detection of metabolites, and without shaking for biofilm detection. For anaerobic conditions, *A. pleuropneumoniae* was cultured in an Anaerobic Environment Chamber (Bactron, USA) with 5% CO_2, 10% H_2 and 85% N_2 (Buettner *et al.*, 2008a) according to the manufacturer's instructions.

Microarray construction

Microarrays used in this study have been described in previous studies (Li *et al.*, 2011, 2012). Briefly, the microarray consists of 15744, 60-mer, oligonucleotide probes *in situ* synthesized by Agilent Technologies. The probes were designed based on the whole genome sequences of *A. pleuropneumoniae* 4074 (serovar 1), JL03 (serovar 3), and L20 (serovar 5) (GenBank accession nos.: AACK00000000, CP000687, and CP000569) including 2132 ORFs. Each probe was spotted twice on the array.

Sample preparation and microarray experiment

A. pleuropneumoniae was cultured aerobically overnight and sub-cultured into fresh medium using an inoculation dose of 10^6 CFU/ml. The bacteria were cultured under aerobic conditions to mid-log phase (3 h) and then divided into two separate groups. One group was continued under aerobic conditions for 1 h (OD_{600nm} = 0.417 ± 0.008), while the other was cultured under anaerobic conditions for 1 h (OD_{600nm} = 0.333 ± 0.015). Three independent experiments were performed. Total RNA was extracted and treated with DNase I using RNA-Solv Reagent (Promega, USA) according to the manufacturer's instructions. Three genes of *A. pleuropneumoniae*, *apxIIA*, *luxS*, and *rbsB*, were targets for amplification from the resultant RNA as template to rule out DNA contamination. The concentration and quality of the RNA samples were analyzed using a Nanodrop ND-1000 (Thermo Fisher Scientific, USA) and an Agilent BioAnalyzer 2100 (Agilent, USA). The RNA samples with A260/A280 ratios of approximately 2.0 and 2100 RIN ≥7.0 were considered to be of good quality. The samples were further purified using a QIAGEN RNeasy Mini Kit (QIAGEN, USA). After that, 2 μg RNA were reverse-transcribed into cDNA and then transcribed into cRNA. After purification, 4 μg cRNA from control samples (aerobically cultured) and test samples (anaerobically cultured) were labeled with Cy5 and Cy3 NHS ester (GE Healthcare), respectively, and purified. Hybridization and scanning were conducted according to the Agilent microarray experimental protocols (Agilent).

Data analysis

The signal intensities were normalized using Feature Extraction Software (Agilent) and transformed into $\log_2$ values. The genes with positive signals (flags = P or M) in all hybridizations were selected for further analysis. The genes with P value < 0.05 (one class t-test) were selected as differentially expressed genes using the software TM4. The cut-off of false discovery rate was ≤5%. Gene annotations and functions were analyzed according to a previous study (Li *et al.*, 2012). All the data are MIAME compliant and the raw data have been deposited in the NCBI GEO database under the number GSE39801.

Real-time quantitative RT-PCR

RNA was extracted as described above and reverse-transcribed into cDNA using Superscript II reverse transcriptase (Invitrogen, USA). Real time quantitative RT-PCR (qRT-PCR) was performed using ABI Power SYBR Green PCR Master Mix (ABI, USA) and the 7900 HT Sequence Detection System (ABI) with 50°C, 5 min; 95°C, 10 min; 40 cycles of 95°C, 15 sec and 60°C, 1 min. The primers used for qRT-PCR are listed in Supplementary data (Table S1). The relative transcription level of each gene was determined by normalization to that of the *dfp* and *comEA* genes, respectively, which displayed no change in the present microarray analysis using the $2^{-\Delta Ct\Delta Ct}$ method (Li *et al.*, 2012).

Determination of the bacterial metabolites

A. pleuropneumoniae was cultured aerobically overnight and sub-cultured into fresh medium at an inoculation dose of 10^6 CFU/ml. The bacteria were cultured under aerobic conditions to mid-log phase (3 h) and then divided into two separate groups. One group was continued under aerobic conditions for 30, 60, 90, and 120 min, and the other group

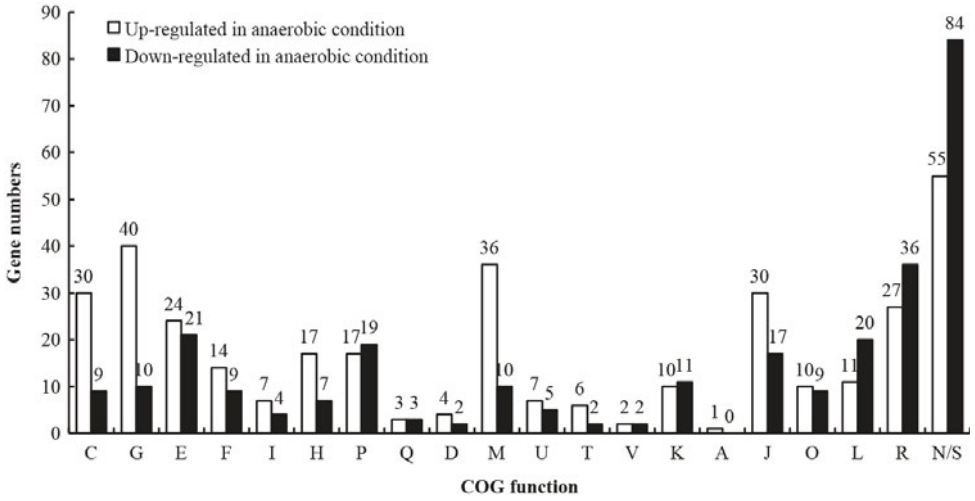

Fig. 1. Functional classification of differentially expressed genes. Gene functions are sorted according to COG categories: C, Energy production and conversion; G, Carbohydrate transport and metabolism; E, Amino acid transport and metabolism; F, Nucleotide transport and metabolism; I, Lipid transport and metabolism; H, Coenzyme transport and metabolism; P, Inorganic ion transport and metabolism; Q, Secondary metabolite biosynthesis, transport and catabolism; D, Cell cycle control, cell division; M, Cell wall/membrane/envelope biogenesis; U, Intracellular trafficking, secretion, and vesicular transport; T, Signal transduction mechanisms; V, Defense mechanisms; K, Transcription; A, RNA processing and modification; J, Translation, ribosomal structure and biogenesis; O, Posttranslational modification, protein turnover, chaperones; L, Replication, recombination and repair; R, General function prediction only; N/S, Function unknown or not in COG classes.

was cultured under anaerobic conditions for 30, 60, 90, and 120 min. Then, the bacterial cells were separated from the supernatant by centrifugation. The concentrations of pyruvate, lactate, ethanol, acetate and glucose in the bacterial supernatant were determined using the appropriate quantitative detection kits for bacteria or dairy products (Genmed Scientifics, Inc, USA) according to the manufacturer's instructions. The medium without bacteria was used as negative control. The metabolite concentrations in the bacterial supernatants were normalized to that in the culture medium control.

Biofilm formation detection

The detection of biofilm formation was performed using the 96-well microplate assay as previously reported (Kaplan and Mulks, 2005). Plates with the bacterial inocula (10^6 CFU/ml) were incubated from 6 to 72 h at 37°C, without shaking, in an aerobic incubator or an anaerobic chamber. Crystal violet was used to detect the quantity of biofilm. The relative amount of biofilm was determined as the ratio of OD_{600nm} values of biofilm/bacterial CFU.

Table 1. Validation of microarray results by real-time quantitative RT-PCR (qRT-PCR)

Gene locus_tag	Gene name	Fold change of microarray	Fold change of qRT-PCR	
			Ref gene *dfp*	Ref gene *comEA*
APJL_0642	*manB*	1.54	4.30±1.09	4.05±1.11
APJL_1143	*pfkA*	1.73	16.43±1.20	6.50±1.32
APJL_1132	*eno*	1.77	2.06±1.01	3.38±1.23
APJL_1891	*lldD*	1.81	3.06±1.06	3.41±1.11
APJL_1861	*spoT*	1.26	8.24±1.06	7.76±1.17
APJL_2061	*aldA*	2.28	6.42±1.08	8.57±1.28
APJL_0945	*arcB*	-1.13	-2.50±1.10	-2.66±1.23
APJL_0049[a]	*arcA*[a]	-1.90[a]	-3.59±1.07[a]	-3.82±1.19[a]
APJL_2012	-	-1.57	-2.81±1.01	-3.88±1.18

[a] Fold change was not significant in microarray analysis but significant by qRT-PCR confirmation (P<0.005).

Statistical methods

Microarray data were analyzed using the one class *t*-test in SAM (significance analysis of microarray). A two-tailed paired Student's *t* test was used for qRT-PCR, metabolite determination and biofilm assay.

Results and Discussion

Overview of changes in gene expression of *A. pleuropneumoniae* in response to anaerobic stress

To investigate the gene expression profile of *A. pleuropneumoniae* in response to the stress caused by anaerobic conditions, a transcriptomic study was conducted to compare the gene expression under aerobic and anaerobic conditions using microarrays of the whole genome of *A. pleuropneumoniae*. The results showed that 631 genes were differentially expressed, including 351 up-regulated and 280 down-regulated genes (P<0.05) with a false discovery rate of 4.2% under anaerobic conditions (Supplementary data Table S2). These genes accounted for 27.7% of the total ORFs of the *A. pleuropneumoniae* genome. According to the COG database, the functions of these genes could be assigned to 20 categories (Fig. 1). A large proportion of the differentially expressed genes (37.1%) encoded proteins associated with metabolism, indicating extensive changes in metabolic status of *A. pleuropneumoniae*. At the same time, 15.1% of the regulated genes were involved in cellular processes and signaling. Genes involved in transcription, translation, rep-

lication and recombination made up 15.9% of the differentially expressed genes. Additionally, 32% of the differentially expressed genes had only predicted functions or unknown functions. Genes involved in central metabolism and infection-associated characters were selected for further analyses and are described below.

To validate the microarray results, the expression changes of nine genes were tested by qRT-PCR. The differential expression trends revealed by qRT-PCR were the same as those from the microarray analysis (Table 1). The *arcA* gene did not show significant changes in microarrays (P=0.08) but showed significant down-regulation from the results of qRT-PCR (P<0.005). Thus, the *arcA* gene was designated as a differentially expressed gene.

Expression profile of genes involved in central metabolism after the shift to anaerobic growth

Thirty-five out of the sixty-five genes involved in central metabolism were differentially expressed, indicating that the *A. pleuropneumoniae* central metabolism was extensively changed when shifting to anaerobic conditions. Gene expression changes in glycolysis, carbon source uptake systems, pyruvate metabolism, citric acid cycle (TCA cycle) and pentose phosphate pathway are shown in Fig. 2A. The *manB* gene encoding phosphomannomutase, *pfkA* encoding phosphofructokinase, *pgk* encoding phosphoglycerate kinase and *eno* encoding an enolase involved in glycolysis were up-regulated. At the same time, the *ptsB* gene encoding a sucrose-specific phosphotransferase (PTS) system enzyme, the *malKEFG* genes encoding ABC-type maltose transport system subunits, the APJL_1411 and APJL_1410 genes encoding mannose-specific PTS system enzymes and the *glpF* gene encoding glycerol uptake permease were all up-regulated. The carbon sources transported by these enzymes can enter the glycolysis pathway and participate in energy production. Up-regulation of these genes indicates that *A. pleuropneumoniae* enhances carbon source uptake and glycolysis under anaerobic conditions to make sufficient energy for survival. Another mannose-specific PTS system encoded by APJL_1693, APJL_1694 and APJL_1695 was down-regu-

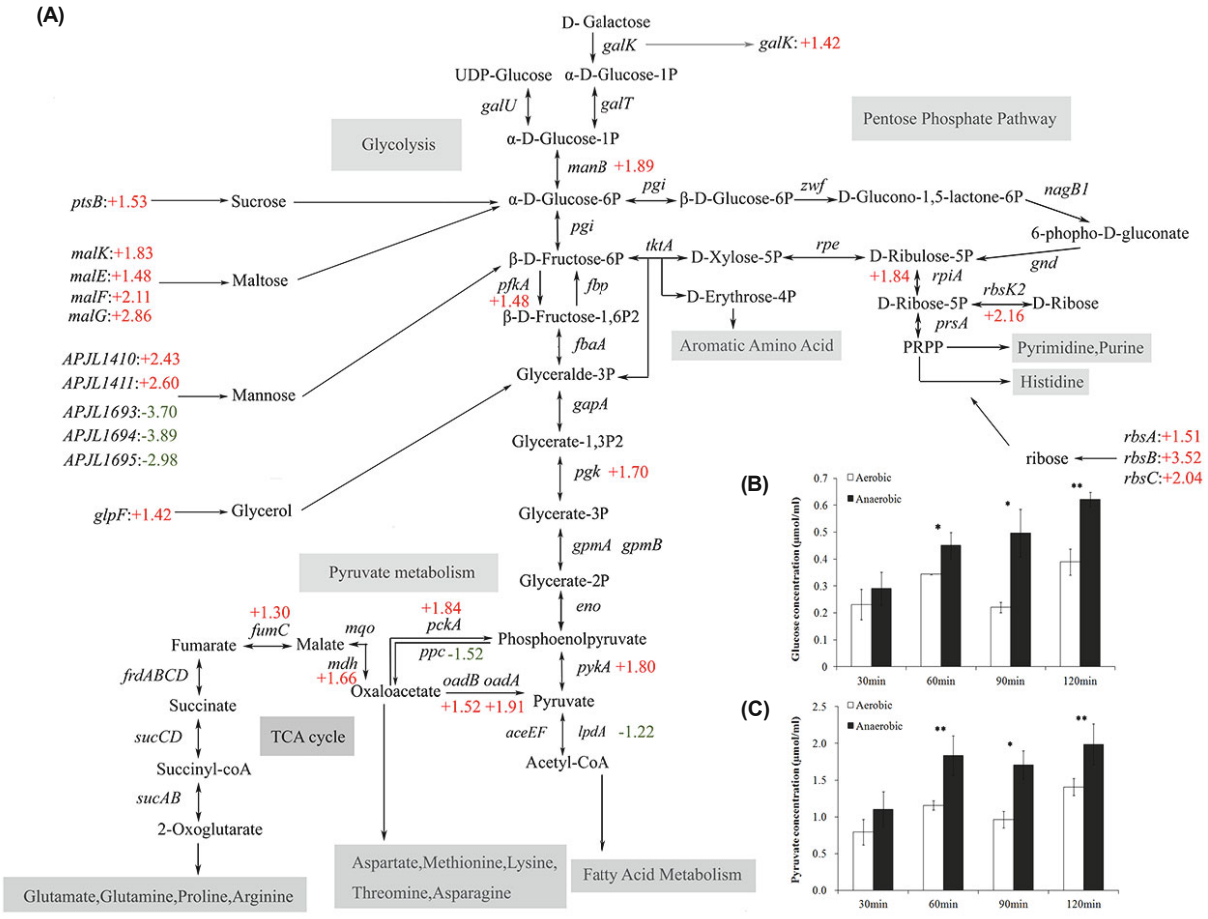

Fig. 2. Gene expression changes in central metabolism under anaerobic conditions. (A) Differentially expressed genes encoding proteins involved in glycolysis, carbon source uptake systems, pyruvate metabolism, citric acid cycle (TCA cycle) and pentose phosphate pathway. (B) and (C) The concentrations of glucose and pyruvate in the *A. pleuropneumoniae* culture supernatant under aerobic and anaerobic conditions. The bacteria were cultured under aerobic conditions to mid-log phase (3 h) and then divided into two separate groups. Each group was cultured under aerobic and anaerobic conditions, respectively, for 30, 60, 90, and 120 min. Then, the concentrations of glucose and pyruvate in the bacterial supernatant were detected. Data are shown as means ± SD from four independent replications. The asterisk shows significant differences (one asterisk P<0.05 and two asterisks P<0.01).

lated for unknown reasons. In pyruvate metabolism, the *pykA* gene encoding pyruvate kinase, *pckA* encoding phosphoenolpyruvate carboxykinase and *oadAB* encoding oxaloacetate decarboxylase were up-regulated, while genes *ppc* encoding phosphoenolpyruvate carboxylase and *lpdA* encoding dihydrolipoamide dehydrogenase were down-regulated. Three genes involved in the TCA cycle, the *frdD* encoding fumarate reductase, *fumC* encoding fumarate hydratase and *mdh* encoding malate dehydrogenase, were up-regulated. The TCA cycle is normally repressed under low oxygen conditions in other bacteria, for example, in *Staphylococcus aureus* (Fuchs *et al.*, 2007). Induction of these three genes in *A. pleuropneumoniae* may be due to the incomplete TCA cycle in *A. pleuropneumoniae* (Xu *et al.*, 2008). Therefore, the regulation of the TCA cycle is possibly insignificant for *A. pleuropneumoniae*, while the up-regulation of *frdD*, *fumC*, and *mdh* may be associated with induction of pyruvate metabolism and anaerobic respiration using fumarate as electron acceptor. In the pentose phosphate pathway, *rpiA* encoding ribose 5-phosphate isomerase, *rbsK2* encoding ribokinase, and *rbsABC* encoding a ribose specific ABC transporter were up-regulated.

Bacteria can shift to fermentation in a low oxygen environment for energy metabolism. In *A. pleuropneumoniae*, the TCA cycle is incomplete but the genes involved in fermentation are present according to the sequenced genome and previous studies (Buettner *et al.*, 2008a; Xu *et al.*, 2008). In this study, six genes involved in fermentation were regulated (Fig. 3A). Gene *lldD* encoding L-lactate dehydrogenase, *aldA* encoding aldehyde dehydrogenase and *adh3* encoding alcohol dehydrogenase were induced. Another gene encoding alcohol dehydrogenase (*adh1*) was slightly down-regulated.

In the electron respiration transport chain (Table 2), genes *rnfBCDGE* and *ndh* encoding NADH dehydrogenase, *dmsBC* encoding DMSO reductase and *frdD* encoding fumarate reductase were up-regulated, whereas *torD* encoding trimethylamine-n-oxide reductase was down-regulated. These regulations suggest that *A. pleuropneumoniae* preferentially uses DMSO and fumarate as electron acceptors to perform anaerobic respiration, which is consistent with previous studies that show both DMSO reductase and fumarate reductase are essential virulence factors involved in *A. pleuropneumoniae* infection (Baltes *et al.*, 2003; Jacobsen *et al.*, 2005; Buettner *et al.*, 2008a). Additionally, *yrhG* encoding a putative formate-nitrite transporter and *sufI2* encoding a copper-containing nitrite reductase were up-regulated, im-

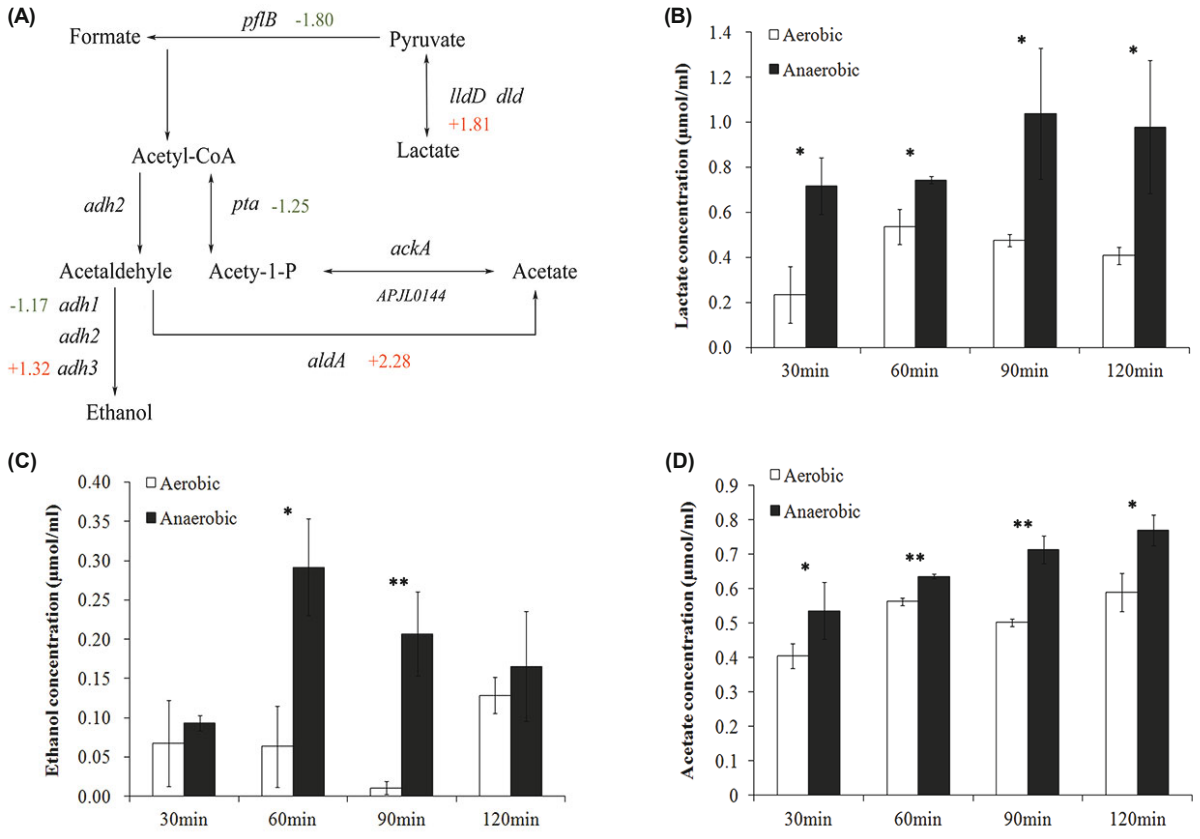

Fig. 3. Gene expression changes in fermentation pathway under anaerobic conditions. (A) Differentially expressed genes encoding proteins involved in fermentation. (B), (C), (D) The concentrations of lactate, ethanol and acetate in the *A. pleuropneumoniae* culture supernatant under aerobic and anaerobic conditions. The bacteria were cultured under aerobic conditions to mid-log phase (3 h) and then divided into two separate groups. Each group was cultured under aerobic and anaerobic conditions, respectively, for 30, 60, 90, and 120 min. Then, the concentrations of lactate, ethanol and acetate in the bacterial supernatant were measured. Data are shown as means ± SD from four independent replications. The asterisk shows significant differences (one asterisk $P<0.05$ and two asterisks $P<0.01$).

Table 2. **Differentially expressed genes encoding proteins involved in electron respiration transport chain**

Gene locus_tag	Gene name	Description	Fold change	*P*-value
APJL_0167	*rnfB*	putative ferredoxin II, iron sulfur protein	1.19	0.024
APJL_0168	*rnfC*	putative iron-sulfur binding NADH dehydrogenase	1.43	0.044
APJL_0170	*rnfD*	putative Na-translocating NADH-quinone reductase	1.55	0.024
APJL_0171	*rnfG*	electron transport complex protein	1.52	0.037
APJL_0172	*rnfE*	Na-translocating NADH-quinone reductase	1.27	0.041
APJL_1282	*ndh*	NADH dehydrogenase	1.52	0.033
APJL_1553	*frdD*	fumarate reductase, 13 kDa hydrophobic protein	1.31	0.044
APJL_1706	*dmsB*	anaerobic dimethyl sulfoxide reductase chain B	1.43	0.047
APJL_1707	*dmsC*	anaerobic dimethyl sulfoxide reductase chain C/DMSO reductase anchor subunit	1.70	0.006
APJL_1833	*torD*	trimethylamine-n-oxide oxidoreductase	-1.46	0.018

plying that nitrite could also be used as anaerobic terminal electron acceptor in *A. pleuropneumoniae*.

Metabolites produced in central metabolism increased after the shift to anaerobic growth

To further verify the gene expression profile of central metabolism, the concentrations of pyruvate, lactate, ethanol, acetate and glucose in the *A. pleuropneumoniae* culture supernatant under aerobic and anaerobic conditions were determined (Figs. 2B and 2C and Figs. 3B, 3C, and 3D). The concentration of pyruvate was significantly increased ($P<0.05$) at 60, 90, and 120 min after initiating anaerobic culture. The lactate and acetate concentrations increased from 30 min to 120 min, and the ethanol concentration increased at 60 and 90 min ($P<0.05$) after the shift to anaerobic culture. The increases were not from the result of increased bacterial numbers as *A. pleuropneumoniae* grew slower under anaerobic conditions (Supplementary data Fig. S1). The glucose concentration under anaerobic conditions was higher ($P<0.05$). This fact may be due to the increased utilization of other sources of carbon according to up-regulation of sucrose, maltose, mannose, glycerol and ribose uptake systems under anaerobic conditions. Another possible reason is that *A. pleuropneumoniae* may secrete glucose into the growth medium during anaerobic growth.

The results of metabolite detection confirmed the gene expression changes in central metabolism, indicating that *A. pleuropneumoniae* enhances glycolysis, pyruvate metabolism and fermentation to fulfill energy production needs under anaerobic conditions. Bacterial basic metabolism is not only essential for growth but also contributes to pathogenesis. For example, in *E. coli*, the level of the metabolite fructose-6-phosphate in central metabolism directly controls the expression of a universal stress protein, which is involved in stress resistance, cell motility, adhesion and many other infection-associated behaviors (Persson *et al.*, 2007). In *Staphylococcus aureus*, it has been accepted that basic cell physiology determines not only survival but also virulence (Fuchs *et al.*, 2007). In *A. pleuropneumoniae*, many genes involved

Table 3. **Differentially expressed genes encoding proteins involved in cell surface and cell wall structures**

Gene locus_tag	Gene name	Description	Fold change	*P*-value
Pilus and biofilm formation				
APJL_1519	*ftpA*	fine tangled pili major subunit	1.67	0.012
APJL_1968	*pgaA*	biofilm PGA synthesis protein PgaA precursor	1.56	0.001
Peptidoglycan biosynthesis				
APJL_1299	*murA*	UDP-*N*-acetyl-glucosamine-1-carboxyvinyltransferase	1.53	0.020
APJL_0020	*murC*	UDP-*N*-acetylmuramate-L-alanine ligase	2.14	0.025
APJL_0014	*murE*	UDP-*N*-acetylmuramyl-tripeptide synthetase	1.38	0.001
APJL_0015	*murF*	UDP-*N*-acetylglucosamine-*N*-acetylmuramyl-pentapeptide synthetase	1.35	0.005
APJL_0016	*mraY*	phospho-*N*-acetylmuramoyl-pentapeptide-transferase E	1.22	0.013
APJL_0019	*murG*	UDP-*N*-acetylglucosamine-*N*-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol *N*- acetylglucosamine transferase	1.61	0.017
APJL_0505	-	Monofunctional biosynthetic peptidoglycan transglycosylase	1.32	0.007
APJL_0196	*mrcA*	penicillin-binding protein 1A	-1.36	0.029
APJL_1632	*mrdA*	penicillin binding protein 2	1.22	0.037
APJL_0013	*ftsI*	penicillin-binding protein 3	1.68	0.049
APJL_0955	*dacB*	penicillin-binding protein 4, D-alanyl-D-alanine carboxypeptidase	-1.85	0.013
Lipopolysaccharide biosynthesis				
APJL_0912	*lpxL*	lipid A acyltransferase	1.21	0.027
APJL_1427	*rfaC*	lipopolysaccharide heptosyltransferase-1	1.93	0.013
APJL_1151	*waaA*	3-deoxy-D-manno-octulosonic-acid transferase	1.64	0.035
APJL_1578	*rffC*	Lipopolysaccharide biosynthesis protein RffC	1.92	0.011

in metabolism have been identified as being essential in the infection process (Chiers *et al.*, 2010). *A. pleuropneumoniae* metabolic modulation in response to anaerobic stress discovered in this study can be important both in the early infection stage when oxygen is consumed rapidly, and also in later phases when bacteria persist in purulent abscesses.

Genes encoding cell surface structures were up-regulated after the shift to anaerobic growth

It was evident that a great number of genes involved in cell surface and cell wall structures were up-regulated in cells in the anaerobic environment (Table 3). The *pgaA* gene encoding biofilm PGA synthesis protein PgaA (Kaplan *et al.*, 2004) was up-regulated. In addition, nine genes involved in peptidoglycan biosynthesis and four genes involved in LPS biosynthesis were up-regulated. Meanwhile, the *ftpA* gene encoding the fine tangled pili major subunit was also up-regulated. These genes have been suggested to play roles in adhesion of *A. pleuropneumoniae*, which is crucial in the early infection phase (Chiers *et al.*, 2010). The LPS also contributes to nutrient acquisition and the host's defense mechanism inside pigs (Chiers *et al.*, 2010). Regulation of these genes implies that *A. pleuropneumoniae* may use the rapid decrease of oxygen level as a stress signal during early infection. Biofilm formation may also play important roles in the persistent stage and will be further discussed.

Biofilm formation was enhanced during anaerobic growth

Since the *A. pleuropneumoniae* biofilm formation gene *pgaA* was up-regulated, biofilm formation under aerobic and anaerobic conditions was quantitatively measured using the 96-well microplate method. *A. pleuropneumoniae* 4074 did not form biofilms under aerobic conditions in TSB medium, which is in agreement with previous studies (Kaplan and Mulks, 2005; Li *et al.*, 2008). But 4074 formed obvious biofilms under anaerobic conditions (Fig. 4A). The relative biofilm formation (biofilm/CFU) was significantly enhanced (P<0.01) from 36 h to 60 h in anaerobic culture (Fig. 4B).

In *Aggregatibacter actinomycetemcomitans*, genes encoding fimbriae assembly and LPS synthesis proteins, the known biofilm determinants of this bacterium, have been shown to be up-regulated in the anaerobic environment, contributing to biofilm formation (Hall-Stoodley *et al.*, 2004). In this study, genes encoding LPS biosynthesis and the pilus subunit were also up-regulated when *A. pleuropneumoniae* was shifted to anaerobic conditions. In another study, the *ftpA* gene from *A. pleuropneumoniae* encoding the fine tangled pili major subunit was up-regulated in the BHI-B medium, in which *A. pleuropneumoniae* 4074 can form a robust biofilm structure (Labrie *et al.*, 2010). Based on these observations, induction of LPS and pilus biosynthesis genes probably also contribute to biofilm formation in *A. pleuropneumoniae* under anaerobic conditions.

Biofilm formation plays an important role in chronic infection by many pathogens (Hall-Stoodley *et al.*, 2004). The biofilm structure can protect the bacteria in a microenvironment containing sufficient nutrients. This structure also contributes to resistance against antibiotics and the host defense system (Fux *et al.*, 2005). Biofilm formation is prevalent in field isolates of *A. pleuropneumoniae* (Kaplan and Mulks, 2005) and mediates antibiotic resistance (Izano *et al.*, 2007). The genes in the *pga* operon can be up-regulated by the alternative sigma factor σ^E, suggesting that biofilm formation is a stress response to host damage and contributes to the persistence of *A. pleuropneumoniae* (Bosse *et al.*, 2010). Therefore, biofilm induction in the anaerobic environment may protect *A. pleuropneumoniae* from host damage and clearance to achieve long-lasting survival inside the tonsils and lung lesions. *A. pleuropneumoniae* biofilm may also play roles in early infection (Kaplan and Mulks, 2005; Labrie *et al.*, 2010). At this stage, *A. pleuropneumoniae* may use the anaerobic environment as a stress signal to induce biofilm formation, contributing to colonization.

Other differentially expressed genes after the shift to anaerobic growth

Iron acquisition proteins and proteases are also involved in the infection process of *A. pleuropneumoniae* (Negrete-Abascal *et al.*, 1994, 1998; Bosse *et al.*, 2002). In this study,

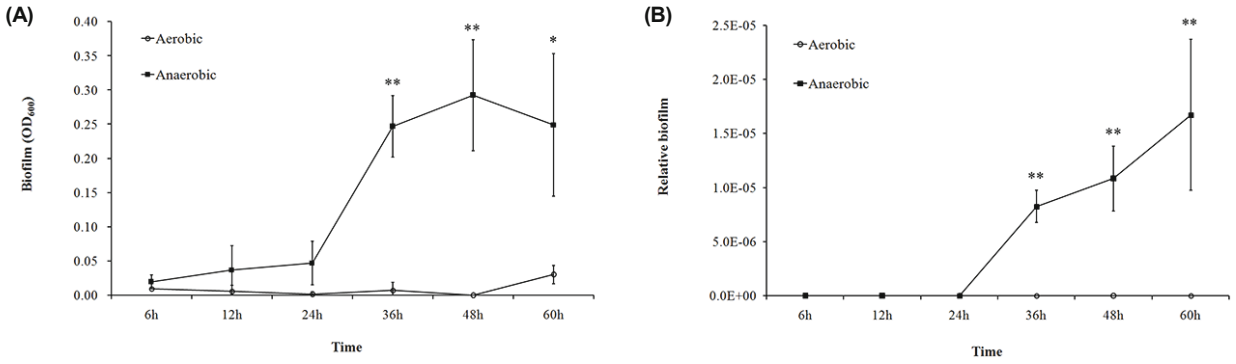

Fig. 4. *A. pleuropneumoniae* biofilm formation under aerobic and anaerobic conditions. Biofilm formation was detected using the 96-well microplate assay. *A. pleuropneumoniae* was cultured under aerobic and anaerobic conditions respectively for 72 h without shaking. Crystal violet was used to detect the quantity of biofilm. Biofilm formations are represented by OD_{600} values of biofilms normalized to that of the TSB medium control (A) and relative biofilm determined as the ratio of normalized OD_{600} values of biofilm / bacterial CFU (B). Data are shown as means ± SD from six independent replications. The asterisk shows significant differences (one asterisk P<0.05 and two asterisks P<0.01).

eighteen genes involved in iron metabolism were differentially expressed under anaerobic conditions (Supplementary data Table S3). The iron acquisition proteins encoded by these genes can utilize many forms of iron, including heme, ferrichrome/ferric hydroxamate, transferrin, and enterochelin, which may contribute to metabolic enzyme activity. Five genes encoding proteases without reported function in *A. pleuropneumoniae* were differentially expressed (Supplementary data Table S3). These proteases might have roles in nutrient acquisition for basic metabolic adjustment and/or defend the bacteria from the host's defensive mechanisms under anaerobic conditions.

Additionally, twenty-six differentially expressed genes were annotated as regulators (Supplementary data Table S4). Among these regulators, the two-component signal transduction system (TCSTS) encoded by *arcB* and *arcA* has been reported to be an important anaerobic regulatory system (Salmon *et al.*, 2005; Wong *et al.*, 2007). In *A. pleuropneumoniae*, ArcA acts primarily as a repressor, down-regulating many genes involved in carbohydrate, energy and inorganic ion metabolism (Buettner *et al.*, 2008a). Another gene belonging to the TCSTS, *narQ* encoding the nitrate/nitrite sensor protein, was up-regulated.

Two stringent response genes, *spoT* and *dksA*, were up-regulated. Gene *spoT* encodes the (p)ppGpp hydrolase that balances the (p)ppGpp in response to various stresses (Magnusson *et al.*, 2005). The *dksA* gene encodes the dosage-dependent DnaK suppressor protein that is also an important stringent response regulator (Paul *et al.*, 2004). The APJL_2012 gene encoding cAMP receptor protein (CRP), a catabolite gene activator regulating carbon utilization systems and virulence genes in many bacteria (Gorke and Stulke, 2008), was down-regulated. In addition, the *luxS* gene (encoding autoinducer-2 production protein) functional in *A. pleuropneumoniae* biofilm formation and virulence (Li *et al.*, 2008, 2011), and the *cspC* gene (encoding cold-shock-like protein C, a stress-related regulator), which can be repressed after acute infection of *A. pleuropneumoniae* (Deslandes *et al.*, 2010), were induced.

These signal transduction systems and stress related regulators may play important roles in gene expression modulation during *A. pleuropneumoniae* response to anaerobic stress.

In summary, *A. pleuropneumoniae* gene expression under anaerobic conditions was compared with that under aerobic conditions in this study. We found that 27.7% of the coding genes in the genome of *A. pleuropneumoniae* were differentially expressed after the shift to anaerobic conditions, of which a large proportion was found to be involved in metabolism. The changes in gene expression and metabolite production demonstrated that central metabolism of *A. pleuropneumoniae* underwent several changes in response to anaerobic stress, allowing it to fulfill the energy requirements and basic metabolic needs for *A. pleuropneumoniae* to survive in an anaerobic environment. The genes involved in *A. pleuropneumoniae* cell surface structures, including biofilm formation, were also up-regulated. Biofilm formation was increased under anaerobic conditions. This induction may contribute to colonization in the acute phase and persistence in the damaged host tissue as well. Some global anaerobic regulators and unreported stress-related regulators in *A. pleuropneumoniae* may play roles in these anaerobic regulations.

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