OPEN ACCESS Freely available online

PLoS one

Modulation of Gene Expression in *Actinobacillus pleuropneumoniae* Exposed to Bronchoalveolar Fluid

Abdul G. Lone[1], Vincent Deslandes[2,3], John H. E. Nash[4¤], Mario Jacques[2,3], Janet I. MacInnes[1]*

1 Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada, 2 Groupe de Recherche sur les Maladies Infectieuses du Porc, Université de Montréal, St-Hyacinthe, Québec, Canada, 3 Centre de Recherche en Infectiologie Porcine, Université de Montréal, St-Hyacinthe, Québec, Canada, 4 Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada

Abstract

Background: *Actinobacillus pleuropneumoniae*, the causative agent of porcine contagious pleuropneumonia, is an important pathogen of swine throughout the world. It must rapidly overcome the innate pulmonary immune defenses of the pig to cause disease. To better understand this process, the objective of this study was to identify genes that are differentially expressed in a medium that mimics the lung environment early in the infection process.

Methods and Principal Findings: Since bronchoalveolar lavage fluid (BALF) contains innate immune and other components found in the lungs, we examined gene expression of a virulent serovar 1 strain of *A. pleuropneumoniae* after a 30 min exposure to BALF, using DNA microarrays and real-time PCR. The functional classes of genes found to be up-regulated most often in BALF were those encoding proteins involved in energy metabolism, especially anaerobic metabolism, and in cell envelope, DNA, and protein biosynthesis. Transcription of a number of known virulence genes including *apxIVA* and the *g*ene for SapF, a protein which is involved in resistance to antimicrobial peptides, was also up-regulated in BALF. Seventy-nine percent of the genes that were up-regulated in BALF encoded a known protein product, and of these, 44% had been reported to be either expressed *in vivo* and/or involved in virulence.

Conclusions: The results of this study suggest that in early stages of infection, *A. pleuropneumoniae* may modulate expression of genes involved in anaerobic energy generation and in the synthesis of proteins involved in cell wall biogenesis, as well as established virulence factors. Given that many of these genes are thought to be expressed *in vivo* or involved in virulence, incubation in BALF appears, at least partially, to simulate *in vivo* conditions and may provide a useful medium for the discovery of novel vaccine or therapeutic targets.

Citation: Lone AG, Deslandes V, Nash JHE, Jacques M, MacInnes JI (2009) Modulation of Gene Expression in *Actinobacillus pleuropneumoniae* Exposed to Bronchoalveolar Fluid. PLoS ONE 4(7): e6139. doi:10.1371/journal.pone.0006139

Editor: Rosemary Jeanne Redfield, University of British Columbia, Canada

Received February 2, 2009; **Accepted** June 8, 2009; **Published** July 3, 2009

Copyright: © 2009 Lone et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by Discovery grants from the Natural Sciences and Engineering Research Council of Canada to JIM and to MJ and the Ontario Ministry of Agriculture Food and Rural Affairs (JIM). VD is a recipient of a FQRNT (Fonds québécois de la recherche sur la nature et les technologies) scholarship. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: macinnes@uoguelph.ca

¤ Current address: Office of Biotechnology, Genomics and Population Health, Public Health Agency of Canada, Ottawa, Ontario, Canada

Introduction

Actinobacillus pleuropneumoniae is a species-specific swine pathogen that causes a necrotizing, fibrinohaemorrhagic pneumonia with pleurisy [1]. Depending upon the immune status of the animal, disease can range in severity from peracute to chronic [2,3]. Although a protective immune response is usually acquired through the adaptive immune system following acute infection, vaccines offer only partial protection against this organism.

The lungs, which are the primary site of infection by *A. pleuropneumoniae*, have a large surface area that is directly in contact with the external environment. There are no published data for swine, but in the human lung, there is an average of 480 million alveoli [4] with an area of 120 to 140 m^2 [5]. The lungs are protected by both innate and adaptive immune systems. Two major components are involved in the innate immune system: a cellular component comprised of leukocytes as well as airway and alveolar epithelial cells, and a humoral component which includes surfactant lipids and proteins, collectins, defensins, cathelicidins, lysozyme, and lactoferrin [6]. Most of these innate immune components reside in a thin layer of fluid lining the lung epithelial cell layer and some have been shown to directly kill bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* [7–9]. Inhaled particles, including pathogens, first encounter the resident immune system in this fluid layer, which forms the first line of defense [10,11]. Little work has been done to examine bacterial gene expression in BALF, but in a recent study Schwab et al. [12] found that *Myobacterium tuberculosis* genes encoding proteins which enabled the organism to use surfactant lipids as a substrate and those for synthesis of phthiolocerol dimycocerosate (PDIM), a protective cell wall component, were up-regulated in the presence of a whole lung surfactant preparation.

A. pleuropneumoniae is capable of overcoming innate pulmonary immune mechanisms of the pig. It can rapidly multiply and spread in naive herds, with some pigs dying within 24 h of infection without showing any clinical signs. Several virulence factors have

been described in *A. pleuropneumoniae* to explain its pathogenesis; factors for colonization, nutrient acquisition, immune evasion and tissue destruction have all been implicated in the disease process [13,14]. Although some aspects of pathogenesis can be explained by the production of tissue-damaging RTX toxins and the ability of the pathogen to acquire nutrients such as iron in the host, factors involved in bacterial survival and rapid multiplication in the host are largely unknown.

To identify genes that may be involved in survival and pathogenesis of *A. pleuropneumoniae* in the host we used porcine bronchoalveolar fluid as a medium to simulate, in part, the lung environment. By analogy with other species, BALF collected from swine likely contains plasma proteins and proteins with unknown functions [15] as well as proteins with diverse functions including anti-oxidation, lipid-metabolism, and tissue repair and proliferation in addition to innate immune components and dissolved minerals. Because BALF contains components that perform diverse functions in the lungs, *A. pleuropneumoniae* gene expression in this fluid could mimic gene expression in the host. Therefore, the objective of this study was to identify *A. pleuropneumoniae* genes that are differentially expressed in BALF to better understand survival and pathogenesis of this important swine pathogen early in the disease process.

Results and Discussion

Differential gene expression in BALF

The survival of *A. pleuropneumoniae* CM5 was assessed in BALF before carrying out experiments to identify differentially expressed genes since this fluid contains many antibacterial substances [6,16]. No significant decrease was observed in *A. pleuropneumoniae* CM5 cell numbers following incubation for 30 min in BALF, while 70% of the *E. coli* DH5α cells were killed at this time.

Genes that were differentially expressed by *A. pleuropneumoniae* after 30 min of incubation in BALF were identified with DNA microarrays by hybridization of Cy3-labeled cDNA from the BALF-incubated bacteria (target sample) and Cy5-labelled cDNA from the BHI-incubated bacteria (reference sample). One hundred and fifty-six genes were differentially expressed in BALF at a false discovery rate (FDR) of 1.07%; 52 genes were down-regulated while 104 were up-regulated. Forty-one (26%) of these genes encode hypothetical proteins (Table 1).

Differential expression of selected genes representing various biological functional classes of interest was confirmed by real-time PCR analysis. Although fold change in gene expression measured by real-time PCR was generally higher, there was a good correlation between the two data sets, and no genes that were deemed up-regulated with the microarrays were demonstrated to be down-regulated by qRT-PCR, and vice-versa (Table 2). The reason why the three *nqr* genes tested appeared to be overestimated in the microarray analysis is not clear, but these slightly divergent results were not likely due to dynamic range or % G+C considerations.

The genes found to be most frequently up-regulated in BALF were those encoding proteins involved in energy metabolism and in cell envelope, DNA, and protein biosynthesis (Table S1). Genes encoding proteins for co-factor biosynthesis, toxin production and secretion and trafficking of ions and biomolecules were also up-regulated while genes encoding proteins involved in protein folding and stabilization, nucleotide biosynthesis, and mobile elements were down-regulated. Representative genes belonging to these functional classes are described below.

Modulation of gene expression for enhanced protein synthesis and energy generation in BALF

Incubation of *A. pleuropneumoniae* CM5 in BALF for 30 min resulted in increased expression of genes encoding 30S and 50S ribosomal subunit proteins and tRNA modification enzymes (Table S1). Such up-regulation of ribosomal genes could play a role in synthesis of the proteins described below.

Genes encoding proteins involved in energy metabolism were also up-regulated in BALF, with some showing an increase of more than 6-fold. Most of these genes encoded enzymes involved in anaerobic respiration, including those that were part of the dimethyl sulfoxide reductase (*dms*) operon), periplasmic nitrate reductase (*nap*) operon, nitrite reductase (*nrf*) operon and a primary dehydrogenase, hydrogenase 2 (*hya*) operon (Table S1). Dimethyl

Table 1. Functional classes of differentially expressed genes.

Functional class	No. up-regulated	No. down-regulated	Total differentially expressed genes
Protein biosynthesis	16	1	17
Amino acid biosynthesis	0	9	9
Cofactor biosynthesis (heme and vitamins)	6	0	6
Nucleotide biosynthesis	0	3	3
Lipid biosynthesis	1	0	1
Cell envelope biosynthesis	11	0	11
Detoxification and toxin production	5	1	6
DNA metabolism	8	0	
Energy metabolism	22	4	26
Protein folding and stabilization	0	4	4
Transcriptional regulators	0	3	3
Secretion and trafficking	13	5	18
Transposon functions	0	3	3
Unclassified and unknowns	22	19	41
Total	104	52	157

doi:10.1371/journal.pone.0006139.t001

Table 2. Verification of microarray data by real-time PCR.

Gene	Gene name	Fold change by real-time PCR	Fold change by microarray
dmsA	Anaerobic dimethyl sulfoxide reductase chain A precursor	17.90±6.52	5.74
dmsB	Anaerobic dimethyl sulfoxide reductase chain B	10.12±3.34	2.78
nqrB	Na^+-translocating NADH quinone reductase subunit B	4.62±1.63	7.65
nqrC	Na^+-translocating NADH quinone reductase subunit C	4.57±1.3	6.35
nqrE	Na^+-translocating NADH quinone reductase subunit E	4.84±1.59	6.36
napB	Nitrate reductase cytochrome ctype subunit	11.61±3.94	4.69
napF	Ferredoxin-type protein NapF	15.94±5.35	6.42
napD	Putative NapD protein	18.59±7.25	3.93
apxIVA	RTX toxin protein	4.07±2.02	1.93
dapA	Dihydrodipicolinate synthase	0.09±0.02	0.20
leuC	3-isopropylmalate dehydratase large subunit 2	0.15±0.14	0.28
ilvH	Acetolactate synthase small subunit	0.13±0.17	0.27

doi:10.1371/journal.pone.0006139.t002

sulfoxide (DMSO) reductase catalyzes the transfer of electrons to dimethyl sulfoxide and other substrates; the periplasmic-nitrate and nitrite reductases are involved in transfer of electrons to nitrate and nitrite respectively [17]. Hydrogenase 2, a primary dehydrogenase, uses the hydrogen produced by formate hydrogen lyase from formate as a substrate [18] for energy production [19,20]. A putative formate transporter, *focA*, was also up-regulated in BALF.

Previous studies have shown that *A. pleuropneumoniae* up-regulates transcription of genes encoding enzymes involved in anaerobic metabolism in porcine lungs and lung washings [21–23]. *A. pleuropneumoniae* recovered from BALF following infection have increased expression of hydrogenase 2 [21], aspartate ammonia lyase (Asp) [24] and DMSO reductase [25], with DMSO reductase levels being elevated in cells recovered from both acute and chronic infections [22,23].

The components present in BALF that could lead to up-regulation of anaerobic energy-metabolism genes in *A. pleuropneumoniae* are largely unknown; however, glutathione in the airway epithelium might be an activator of HlyX, which is the *A. pleuropneumoniae* equivalent of FNR in *E. coli* [26]. For example, it has been reported HlyX up-regulates DMSO reductase (*dms*) and aspartate ammonia lyase (*asp*), which breaks down aspartate to fumarate and ammonia. Fumarate is used as an electron acceptor under anaerobic conditions in *A. pleuropneumoniae* [27–30]. The fact that a significant change of expression of *hlyX* was not observed may be because differences in the level of expression of this gene tend to be small. Moreover, like *fnr*, regulation of the *hlyX* gene product is likely affected by a multitude of factors including protein stability, growth phase and nutrient availability [31,32].

Up-regulation of the genes encoding the periplasmic nitrate (Nap) reductase in BALF suggests a role for nitrate metabolism in *A. pleuropneumoniae* energy production in the host. Nap uses nitrate as an electron acceptor. As nitrate has a higher redox potential than most other electron acceptors under anaerobic conditions [17,33] it is a preferred electron acceptor. Nitrate is formed from nitric oxide in the animal and is present in various body fluids [34–37] where it can serve as a cue for the up-regulation of nitrate-responsive genes in *A. pleuropneumoniae*. Nap has a higher affinity for nitrate than membrane-bound nitrate reductase (Nar) [38], and it can be used for nitrate utilization in body fluids with low nitrate concentrations such as are found in the respiratory tract.

Nitrite reductase (Nrf) is another nitrate metabolism-related enzyme whose genes were up-regulated in BALF. This enzyme converts nitrite, a potential bacterial cell-damaging substance produced by nitrate reductase, to ammonia. Nrf can also convert nitric oxide to ammonium [39], thus inactivating a key defense molecule of the host.

Given that *A. pleuropneumoniae* is a host-associated pathogen which resides in oxygen-deprived environments in both the acute and carrier states of the disease, the major production of energy is likely through anaerobic metabolism. The absence of three main TCA-cycle enzymes (citrate synthase, aconitase and isocitrate dehydrogenase) in the genomes of serotype 3 and serotype 5 *A. pleuropneumoniae* again points to the importance of anaerobic metabolism in the survival of this organism [40]. In addition, many upper respiratory tract pathogens including *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Pasteurella multocida*, *Neisseria meningitidis*, carry genes for anaerobic energy generation, consistent with the notion that anaerobic metabolism might have an important role in the survival and virulence of bacterial pathogens in the respiratory tract.

Some of the genes encoding enzymes involved in anaerobic energy production in *A. pleuropneumoniae* have been shown to be essential for virulence. For example, knockout mutants of *hlyX* are unable to survive in lung epithelium, sequestered lungs or tonsils [29]; *dmsA* mutants are attenuated in acute disease [25]; and *asp* mutants cause less severe lung lesions than the wild type organism [24]. Similarly, in *Bordetella pertussis*, another respiratory tract pathogen, the FNR homolog, Btr [41] is essential for survival of this pathogen in mice [42].

The role of the nitrate-inducible energy metabolism genes, *nap* and *nrf*, is unknown in *A. pleuropneumoniae*. Nitrate metabolism has, however, been shown to be essential for the entry and replication of *Salmonella* Typhi in epithelial cells [43] and for the survival and virulence of *Mycobacterium bovis* in mice [44,45].

In addition to the genes encoding enzymes of energy metabolism discussed above, the transcription of Na^+ -translocating NADH-quinone reductase (NQR) was also enhanced in BALF (Table 3 and S1). NQR is a primary Na^+ pump that translocates Na^+ ions outside the cytoplasmic membrane to generate a sodium motive force, instead of a proton motive force, for energy production [46,47]. The NQR enzyme is a complex of six subunits encoded by the *nqrABCDEF* operon [48,49]; all six genes

Table 3. BALF up-regulated virulence-associated genes reported in other studies.

Genes	Type of study	Reference no.
bioD1, nhaB, apxIVA, rps, dmsA, hya	SCOTS (acute infection; 7 days PI))	[22]
nqrB, dnaG, rpsT, rplL, rho, secA, truD, nusA, atpD, sap, rps, rpl	SCOTS (chronic infection; 21 days PI)	[23]
dmsA	Knockout mutation	[25]
nqr, hemA, napB, atp, ccm, recR, tonB, galU, cpxC, gloB	Signature tagged mutagenesis (24 h PI)	[51]
sec, nusG	*In vivo* expression technology (12 and 16 h PI)	[84]
exbB2, atp, dnaK	Signature tagged mutagenesis (20 h PI)	[85]
apxIVA, malF, malG, APL_0668 (predicted periplasmic lipoprotein involved in iron transport)	*In vivo* transcript profiling of *A. pleuropneumoniae* by microarray	Deslandes (personal communication)

Complete gene names are given in Table S1.
doi:10.1371/journal.pone.0006139.t003

are present in all of the genomes of *A. pleuropneumoniae* reported to date. Another Na^+-cycling gene, *nhaB* (an Na^+/H^+ antiporter), which, like NQR, could be involved in energy generation or in sodium homeostasis [46], was also up-regulated in BALF.

In previous studies, the *nqrB* [23] and *nhaB* [22] genes and the NqrA (AopA) protein [50], which are all involved in Na^+-cycling, have been reported to be up-regulated in *A. pleuropneumoniae* when it is grown *in vivo*. The importance of NQR, the major Na^+-cycling enzyme, in survival and pathogenesis of *A. pleuropneumoniae* is unknown. However, BHI containing 2-*n*-nonyl-4-hydroxyquinoline *N*-oxide (HQNO), an inhibitor of NQR, does not allow growth of *A. pleuropneumoniae* CM5, while *E. coli* DH5α grows well in media containing HQNO (unpublished data). Further, in signature tagged mutagenesis studies, the *nqrB* gene was found to be essential for persistence *A. pleuropneumoniae* in the host [51].

Although genome sequencing has revealed that many bacterial pathogens possess homologues of *nqr* and other primary and secondary sodium pumps [46], the role of Na^+ -cycling in pathogenesis is largely unknown, except in *Vibrio cholerae*. In *V. cholerae*, mutation of *nqr* results in increased expression of *toxT*, a positive regulator of virulence factors including cholera toxin and toxin co-regulated pilus [52,53]. NQR is best known for its involvement in energy transduction, cytoplasmic pH regulation and ion homeostasis in marine and halophilic bacteria [54,55].

Other BALF-up-regulated *A. pleuropneumoniae* genes encoding enzymes of energy metabolism included the heme exporter gene (*ccmC*), ATP synthase epsilon chain *(atpC)*, deoxyribosephosphate aldolase (*deoC)* and 1-phosphofructokinase (*fruK*). The *ccmC* gene is a part of the *ccmABCDEFGH* operon which encodes proteins required for maturation of cytochrome C [56], an essential component of the electron-transfer chain [57]; whereas AtpC is a part of the F1 complex of ATP synthase [58]; DeoC cleaves deoxyribose 5-phosphate to acetaldehyde and glyceraldehyde 3-phosphate for central carbon metabolism [59]; and FruK regulates the flow of glucose through glycolysis [60]. Thus in BALF, *A. pleuropneumoniae* enhances transcription of the genes encoding both the central carbon metabolism and the energy transduction proteins.

The down-regulation of genes encoding TCA cycle related enzymes, phosphoenolpyruvate carboxylase (*pepC* and succinyl-CoA ligase (ADP forming) subunit alpha (*sucD*) genes (Table S1) again points to the importance of anaerobic metabolism in *A. pleuropneumoniae*. Phophoenolpyruvate carboxylase catalyses carboxylation of pyruvate to oxaloacetate and succinyCOA ligase catalyzes the nucleotide-dependent conversion of succinyl-CoA to succinate [61,62]. The genes encoding putative haloacid dehalogenase like hydrolase *(pfhB) and* xylose isomerase (*xylA*) were also down-regulated, which could be because of the absence of the substrates for these enzymes in BALF or because alternate pathways are preferable in that environment. Haloacid dehalogenase catalyzes dehalogenation of L-2-haloalkanoic acids to form D-2-hydroxyalkanoic acids [63] and xylose isomerase converts xylose to xylulose [64]. The xylose transport system permease gene (*xylH*) was also down-regulated in BALF as were the mannitol (PTS system mannitol-specific EIICBA component, *mtlA*) and ribose (D-ribose binding periplasmic protein precursor, *rbsB*) transport systems (Table S1) consistent with the absence of manitol and ribose in BALF or the presence of a preferred substrate. The ferritin-like protein 2 encoding-gene, *ftnB*, which is involved in protection against oxidative damage to iron-sulfur-containing enzymes such as the tricarboxylic acid (TCA) enzyme aconitase [65] was also down-regulated in BALF. Since *A. pleuropneumoniae* lacks aconitase, the target of FtnB is not obvious. This result nevertheless suggests that *A. pleuropneumoniae* is not under oxidative stress in BALF.

Modulation of gene expression for survival and virulence in BALF

Following incubation in BALF, *A. pleuropneumoniae* CM5 up-regulates genes required for cell wall synthesis, repair and recombination of DNA, and secretion and trafficking of ions and biomolecules (Table S1).

Several genes encoding cell wall biosynthesis proteins were up-regulated in BALF, including those required for synthesis of peptidoglycan, LPS and integral membrane proteins (Table S1). Up-regulated genes for peptidoglycan biosynthesis enzymes included phosphoglucosamine mutase (*mrsA*), alanine racemase (*alr*), and D-alanyl D-alanine carboxypeptidase fraction A (*dacA*). MrsA converts glucosamine-6-phosphate to glucosamine-1-phophate which finally yields UDP-N-acetyl glucosamine for both peptidoglycan and LPS biosynthesis [66–68] while Alr catalyses the isomerization of L-alanine into D-alanine which is essential in bacteria for peptidoglycan biosynthesis [66,69], and DacA catalyzes transpeptidation between neighboring peptide chains of N-acetylmuramyl-N-acetylglucosyl polysaccharides to produce cross-links in the cell wall. DacA can also act as a carboxypeptidase to control the amount of cross-linking in peptidoglycan [70,71].

A semi-rough LPS is present in *A. pleuropneumoniae* serotype 1 [72], and the BALF-up-regulated genes encoding LPS biosynthesis proteins included the tetraacyldisaccharide 4′kinase (LpxK) required for lipid-A biosynthesis, and a bifunctional protein (HldE) and UTPglucose-1-phosphate uridylyltransferase (GalU), required for LPS core biosynthesis. Genes encoding capsular

export proteins, CpxA (ATP binding protein) and CpxC (capsule polysaccharide export inner membrane protein) were also up-regulated in BALF. While the genes encoding peptidoglycan and LPS biosynthesis proteins described above are assumed to be essential for the survival of *A. pleuropneumoniae*, a clear role for capsular polysaccharides in the virulence of the bacterium has been demonstrated [73,74]. In addition to cell surface polysaccharides synthesis genes, the genes encoding the outer membrane protein OmpW (outer membrane protein W precursor) and a lipoprotein (outer membrane antigenic lipoprotein B precursor) were also up-regulated in BALF. The up-regulation of genes encoding proteins of cell wall biosynthesis may help the organism to overcome cell surface-damaging components present in BALF.

Transcription of genes encoding proteins involved in replication, and recombination and repair was enhanced in BALF. Genes encoding subunits of DNA polymerase III, various recombination proteins of the RecF machine, and an exonuclease (*uvrA of uvrABC*) were all up-regulated in BALF. Replication and recombination are two intertwined processes [75]; enhancement of transcription of genes involved in these two processes is consistent with active replication of *A. pleuropneumoniae* in BALF. On the other hand, *rec2*, encoding recombination protein2 and involved in transport of DNA across the cell envelope in competent bacteria [76], was down-regulated in BALF as were 3 genes predicted to have transposon functions. The fact that the expression of transposases is reported to be associated with starvation and other stressful conditions is again consistent with BALF being a favorable environment for *A. pleuropneumoniae* [77].

For survival in the host, bacteria require nutrients for biosynthesis of various biomolecules. In BALF, *A. pleuropneumoniae* increased transcription of genes encoding proteins required for transport of various nutrients. For example, complex-carbohydrate transport genes *malF* and *malG*, involved in maltose and maltodextrin transport, were up-regulated in BALF. Similarly, Group A *Streptococcus* enhances transcription of genes encoding proteins required for maltodextrin uptake in saliva and Δ*malE:-malT* strains are attenuated in their growth and in their ability to catabolize α-glucans [78]. Genes for amino acid (9) and nucleotide (3) biosynthesis were down-regulated suggesting that some or all amino acids and nucleotides were either directly or indirectly, available in BALF. Consistent with this finding, amino acid transporters such as BrnQ (for branched chain amino acid) and SdaC (for serine transport) were up-regulated. In contrast, the product of the glycerol uptake facilitator gene, *glpF*, which allows transport of glycerol, erythritol, pentitols, and hexitol,was down-regulated, however, this gene is known to be down-regulated presence of glucose [79].

In BALF, *A. pleuropneumoniae* also increased transcription of genes encoding proteins required for transport of iron and potassium. Genes encoding the cell membrane transport proteins, ExbD and ExbD2, and FbpB (iron (III) ABC transporter, ATP-binding protein), which are involved in energy-coupled transport of the iron-containing compound, transferrin were up-regulated in BALF, as was *fieF*. The cation efflux pump, FieF, probably protects the bacterium from ferrous iron toxicity [80]. The gene encoding PtsN (PTS system, nitrogen regulatory IIA like protein) was also up-regulated in BALF. PtsN has recently been shown to regulate transport of K^+ through its interaction with a K^+ transporter in *E. coli* and could be involved in ion homeostasis needed for optimal survival of *A. pleuropneumoniae* in BALF [81].

Incubation in BALF also led to increased expression of *A. pleuropneumoniae* CM5 genes encoding toxin synthesis and antimicrobial-resistance compounds. The ApxIV RTX toxin is reported to be expressed only *in vivo* [82,83]. Following exposure to BALF we have shown in vitro expression of *apxIVA* for the first time. ApxIVA is a homolog of FrpC in *Neisseria meningitidis*. FrpC is involved in tissue invasion of *N. meningitidis* [84]. The role, if any, of ApxIV in the pathogenesis of *A. pleuropneumoniae*, however, remains to be demonstrated.

The *sapF* gene is a part of the *sapABCDF* operon was up-regulated in BALF. It is involved in resistance to antimicrobial peptides in *Vibrio fischeri* [85], and in non-typable *Haemophilus influenzae* [86]. Also, *sapD* mutants of non-typable *H. influenzae* have been shown to be attenuated in a chinchilla model of otitis media [87]. *A. pleuropneumoniae* possesses a complete *sap* operon, which could have significant role in the survival of the pathogen in the host. Another detoxification molecule, glyoxylase II (*gloB*) is an enzyme involved in conversion of dicarbonyl compounds to less reactive hydroxy acids [88] was also up-regulated. It has been shown to be essential for survival of *A. pleuropneumoniae in vivo* [51] likely by protecting the organism against harmful dicarbonyl compounds present in the host. Expression of the *ostA* gene was also enhanced in *A. pleuropneumoniae* CM5 after incubation in BALF. The role for OstA in *A. pleuropneumoniae* is not known at this time, but in *Helicobacter pylori*, OstA confers protection against organic solvents and antibiotics [89], and in *E. coli*, it is essential for survival and has a direct role in membrane biogenesis and effects the lipid:protein ratio of the cell membrane [90]. In *N. meningitidis*, OstA is required for LPS biosynthesis [90].

Transcription of *secB*, which is a part of the Sec machinery, was also enhanced in BALF. The Sec machinery plays a key role in the translocation of proteins across, and integration of some proteins into, the cytoplasmic membrane of bacteria [91]. In *A. pleuropneumoniae*, *secA* and *secB*, another protein of the Sec machinery, have been shown to be expressed *in vivo* during both acute and chronic infection [23, 92, and 93].

Several genes encoding transcriptional regulators, protein stabilization and folding and transposon functions were down-regulated in BALF. The precise role of these down-regulated transcriptional regulators is unknown in *A. pleuropneumoniae*, but FadR (a member of the GntR family of regulators) is an activator of unsaturated fatty acid acid synthesis in *E. coli*, although the authors do note that the FadR regulon in other gammaproto-bacteria such as *Haemophilus influenzae* is much smaller [94]. Nevertheless, it is reasonable to assume that fatty acids would be freely available in BALF and their synthesis would not be required [95]. MerR transcriptional regulators have similar N-terminal helix-turn-helix DNA binding regions and C-terminal effector binding regions specific to the effector. Most of these regulators respond to oxidative stress and the presence of heavy metals and antibiotics in the medium [96]. The down-regulation of a MerR transcriptional regulator is consistent with the absence of stressors in the medium.

Similarly, the precise role of protein folding and stabilization proteins in *A. pleuropneumoniae* has not been reported, but in other systems, proteins such as HtpG and HtpX are usually up-regulated during stress such as nutrient deprivation and their down-regulation is consistent with BALF being a comparatively non-stressful environment [97,98].

In the current study, genes encoding 22 "unclassified or unknown" proteins were up-regulated while genes encoding a further 19 "unclassified or unknown" proteins were down-regulated. This large set of gene encoding unknown proteins could have a significant role in the survival and pathogenesis of *A. pleuropneumoniae*. In the future, it may be possible to predict functions of unknown genes by using bioinformatic approaches such have been developed for analysis of human microarray data [99].

In summary, incubation in BALF appears to simulate *in vivo* conditions and may provide a useful medium for the discovery of novel vaccine or therapeutic targets. In this environment, *A. pleuropneumoniae* is actively involved in protein and cell envelope biosynthesis and in general, BALF appears to provide a comparatively favorable and nutrient replete environment. Although more than 40% of the genes that were up-regulated following a 30 min exposure to BALF had been reported in earlier in vivo studies (Table 3), we have described an additional 70 genes whose precise role in survival and virulence of *A. pleuropneumoniae* is unknown and merit further study.

Materials and Methods

Collectin and concentration of bronchoalveolar fluid (BALF)

BALF was obtained from ten specific pathogen free pigs, each weighing about 15 kg. The pigs were euthanized, and the lungs were lavaged in situ using a catheter passed through a bronchoscope to instill 100 ml of sterile PBS into the trachea. After ~1 min, lung washings were collected and centrifuged to remove cell debris. The cell-free lavage was concentrated with a 5 kDa molecular weight cut off ultrafiltration device, Vivacell 70 (Vivascience Ltd., Stonehouse, Gloucestershire,UK). A total of about 100 ml of BALF was collected from each pig and concentrated to a final volume of 5 ml. Concentrated BALF from each pig was pooled and sterilized by passage through a 0.22 μm membrane filter. From collection to concentration, BALF was kept at 4°C; the concentrated BALF was stored at −80°C for long-term storage. Molecules less than 5 kDa in molecular weight were not concentrated by this method; nevertheless, the fluid still contained these substances in concentrations found before ultrafiltration and the concentrated BALF represents alveolar epithelial fluid better than unprocessed BALF. The procedure used for BALF collection received approval from the Animal Care Committee of the University of Guelph and was consistent with the Guidelines of the Canadian Council on Animal Care.

Assessment of bacterial survival in BALF

Exponential growth phase cultures of *A. pleuropneumoniae* CM5 and *Escherichia coli* DH5α were incubated in 2 ml of concentrated BALF at 37°C. As a control, bacteria were also incubated in phosphate-buffered saline (PBS). A 50-μl aliquot was taken from each of the cultures after 15 and 30 min of incubation in BALF and PBS and plated onto brain heart infusion (BHI; Becton, Dickinson and Company, Sparks, MD, USA) agar supplemented with 0.01% (wt/vol) β-nicotinamide adenine dinucleotide (NAD). The number of colony-forming units (CFU) was counted after incubation overnight at 37°C. The number of bacteria surviving in BALF at each time point was expressed as the percent of number of bacteria surviving in PBS.

The data were analyzed using one-way analysis of variance (ANOVA); the means were compared using Tukey's method.

Culture conditions for identification of differentially expressed genes in BALF

The virulent *A. pleuropneumoniae* serotype 1 strain CM5 was grown in BHI (Becton, Dickinson and Company) broth supplemented with 0.01% (wt/vol) NAD, at 37°C to an OD_{600} of 0.7 (approximately 10^7 CFU/ml). The cell suspension was split into two equal parts and centrifuged at 10,000 x*g* for 1 min to pellet the cells. One pellet was suspended in pre-warmed concentrated BALF and the other in fresh pre-warmed BHI broth supplemented with NAD. The volume of BALF and BHI broth used to suspend the cell pellets was equal to that of the culture from which the pellets were obtained, so that the resulting cell suspension contained approximately 10^7 CFU/ml. The cell suspensions were incubated with constant agitation at 37°C for 30 min and harvested by centrifugation for RNA extraction.

RNA extraction

RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the instructions of the manufacturer. RNA quantity and quality was determined using an RNA 6000 Nano LabChip read in a Bioanalyzer 2100 instrument (Agilent Technologies, Santa Clara, CA, USA). RNA was treated with Turbo DNA-free (Ambion, Austin, TX, USA) to remove traces of contaminating DNA. For hybridization in microarray experiments, RNA was extracted from 3 independent biological replicates.

Labeling of cDNA and microarray hybridizations

cDNA synthesis was carried out as described previously [100]. Briefly, RNA (15 μg per reaction) from target (BALF-incubated bacteria) and reference (BHI-incubated bacteria) samples was used to synthesize cDNA in the presence of amino-allyl-dUTP (Sigma-Aldrich, St. Louis MO, US), random octamer primers (Biocorps, Montreal, QC, Canada), and SuperScript II transcriptase (Invitrogen, Carlsbad, CA, US). cDNAs were labeled indirectly with mono-functional NHS-ester Cy3 or Cy5 dye (GE Healthcare, Buckinghamshire, UK) and the efficiency of the labeling reactions was determined spectrophotometrically. RNA from three independent biological replicates was used in the labeling reaction. Four hybridizations, including the dye-swap experiment, were carried out between the target and the reference samples. The microarray data from this study were submitted to the Gene Expression Omnibus repository at NCBI and assigned accession number GEO: GSE13006.

Microarray chip design

The AppChip2 microarray chip used in this study is an evolved version of the AppChip1 chip, and like its predecessor, was a part of the *A. pleuropneumoniae* 5b L20 genome sequencing project [101]. For construction of AppChip2, open-reading-frame (ORF) PCR fragments of 160-nucleotide length and above were spotted in duplicate on microarray slides. The spots represent 2033 ORFs, covering 95% of the total ORFs, from the complete genome sequence of the organism. Spotted sheared genomic DNA from *A. pleuropneumoniae* L20 and porcine DNA are used as controls (http://ibs-isb.nrc-cnrc.gc.ca/glycobiology/appchips_e.html). Other details concerning chip production are described elsewhere [102].

Microarray data analysis

Microarray image and data analysis were carried out using the TM4 Suite [103] of software. Briefly, images were analyzed with Spotfinder v3.1.1. Background intensity was subtracted from the integrated intensity of each spot, and the spots that were less than one standard deviation above background intensity were eliminated, as were ones with total intensity less than 10,000. Replicate spots were analyzed subsequent to LOWESS (locally weighted linear regression) normalization of the data. Genes that were represented by good quality spots on a minimum of three replicate slides were considered for downstream analysis using SAM (significance analysis of microarray) to identify differentially expressed genes. A median false discovery rate (FDR = expected rate of falsely identified up- or down-regulated genes [104]) of 1.07% was used to generate a list of differentially expressed genes,

which were classified into various functional classes using the JCVI Comprehensive Microbial Resource [105] tool.

Quantitative real-time PCR

RNA capacity (the maximum RNA concentration that can be used without affecting efficiency of reverse transcription), optimum primer concentration (list of primers is given in Table S2), and gene dynamic ranges were determined before carrying out real-time PCR for relative quantification of target genes. Synthesis of template cDNA was carried out in a 20-µl reaction mixture containing 500 ng RNA, using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Streetsville, ON, Canada). SYBR-Green-dye-based real-time PCR methodology was carried out using MicroAmp Optical 96- well plates (Applied Biosystems) in a StepOnePlus thermocycler (Applied Biosystems) for relative quantification of target genes. The 20-µl PCR reaction mixtures contained 10 µl of 2× Power SYBR Green PCR Master Mix (Applied Biosystems), 100 nM of forward and reverse primer, and 5 µl of template cDNA. The real-time PCR thermal profile included heat-activation of AmpliTaq Gold DNA Polymerase at 95°C for 10 min, and three-step 40-cycle PCR of denaturation at 95°C for 15 sec, primer annealing and extension at 60°C for 1 min.

The Comparative C_T (or $\Delta\Delta$ C_T) method [106] where $\Delta\Delta C_T = (C_{T,\ target} - C_{T,\ syp})_{BALF} - (C_{T,\ target} - C_{T,\ syp})_{BHI}$ was used to determine the relative gene expression of the target genes in BALF. As an endogenous control, the level of prolyl-tRNA-synthetase gene expression was used to normalize target gene expression levels, since this gene exhibited the least variation in expression across various conditions in both the microarray and real-time PCR experiments. Three independent biological replicates were tested in triplicates in the PCR experiments for the relative quantification of target genes.

Supporting Information

Table S1

Found at: doi:10.1371/journal.pone.0006139.s001 (0.26 MB DOC)

Table S2

Found at: doi:10.1371/journal.pone.0006139.s002 (0.04 MB DOC)

Acknowledgments

We thank Drs. Jeff Caswell and Andrew Brooks for providing bronchoalveolar lavage fluid and Jing Zhang and Devon Metcalf for their help with the real-time PCR experiments.

Author Contributions

Conceived and designed the experiments: AGL VD JN MJ JIM. Performed the experiments: AGL VD. Analyzed the data: AGL VD JIM. Contributed reagents/materials/analysis tools: JN. Wrote the paper: AGL VD JN MJ JIM.

References

1. Sebunya TN, Saunders JR (1983) *Haemophilus pleuropneumoniae* infection in swine: A review. J Am Vet Med Assoc 182(12): 1331–1337.
2. Inzana TJ (1991) Virulence properties of *Actinobacillus pleuropneumoniae*. Microb Pathog 11(5): 305–316.
3. Rycroft AN, Garside LH (2000) *Actinobacillus* species and their role in animal disease. Vet J 159(1): 18–36.
4. Ochs M, Nyengaard JR, Jung A, Knudsen L, Voigt M, et al. (2004) The number of alveoli in the human lung. Am J Respir Crit Care Med 169(1): 120–124.
5. Ochs M (2006) A brief update on lung stereology. J Microsc 222(Pt 3): 188–200.
6. Zaas AK, Schwartz DA (2005) Innate immunity and the lung: Defense at the interface between host and environment. Trends Cardiovasc Med 15(6): 195–202.
7. Schnapp D, Harris A (1998) Antibacterial peptides in bronchoalveolar lavage fluid. Am J Respir Cell Mol Biol 19(3): 352–356.
8. Travis SM, Conway BA, Zabner J, Smith JJ, Anderson NN, et al. (1999) Activity of abundant antimicrobials of the human airway. Am J Respir Cell Mol Biol 20(5): 872–879.
9. Hughes J (2007) Review series: Lung function made easy: Assessing gas exchange. Chronic Respiratory Disease 4(4): 205–214.
10. Kendall M, Tetley TD, Wigzell E, Hutton B, Nieuwenhuijsen M, et al. (2002) Lung lining liquid modifies PM(2.5) in favor of particle aggregation: A protective mechanism. Am J Physiol Lung Cell Mol Physiol 282(1): L109–14.
11. Wright JR (2006) The "wisdom" of lung surfactant: Balancing host defense and surface tension-reducing functions. Am J Physiol Lung Cell Mol Physiol 291(5): L847–50.
12. Schwab U, Rohde KH, Wang Z, Chess PR, Notter RH, et al. (2009) Transcriptional responses of mycobacterium tuberculosis to lung surfactant. Microb Pathog 46(4): 185–193.
13. Inzana TJ, Ma J, Workman T, Gogolewski RP, Anderson P (1988) Virulence properties and protective efficacy of the capsular polymer of *Haemophilus* (*Actinobacillus*) *pleuropneumoniae* serotype 5. Infect Immun 56(8): 1880–1889.
14. Bossé JT, Janson H, Sheehan BJ, Beddek AJ, Rycroft AN, et al. (2002) *Actinobacillus pleuropneumoniae*: Pathobiology and pathogenesis of infection. Microbes Infect 4(2): 225–235.
15. Wattiez R, Falmagne P (2005) Proteomics of bronchoalveolar lavage fluid. J Chromatogr B Analyt Technol Biomed Life Sci 815(1–2): 169–178.
16. Schnapp D, Harris A (1998) Antibacterial peptides in bronchoalveolar lavage fluid. Am J Respir Cell Mol Biol 19(3): 352–356.
17. Unden G, Bongaerts J (1997) Alternative respiratory pathways of *Escherichia coli*: Energetics and transcriptional regulation in response to electron acceptors. Biochim Biophys Acta 1320(3): 217–234.
18. Maeda T, Bao B, Sanchez-Torres V, Wood TK (2007) Metabolic engineering to enhance bacterial hydrogen production. Microbial Biotechnol 1: 30–39.
19. Weiner JH, MacIsaac DP, Bishop RE, Bilous PT (1988) Purification and properties of *Escherichia coli* dimethyl sulfoxide reductase, an iron-sulfur molybdoenzyme with broad substrate specificity. J Bacteriol 170(4): 1505–1510.
20. Bilous PT, Weiner JH (1985) Dimethyl sulfoxide reductase activity by anaerobically grown *Escherichia coli* HB101. J Bacteriol 162(3): 1151–1155.
21. Baltes N, Kyaw S, Hennig-Pauka I, Gerlach GF (2004) Lack of influence of the anaerobic [NiFe] hydrogenase and L-1,2 propanediol oxidoreductase on the outcome of *Actinobacillus pleuropneumoniae* serotype 7 infection. Vet Microbiol 102(1–2): 67–72.
22. Baltes N, Gerlach GF (2004) Identification of genes transcribed by *Actinobacillus pleuropneumoniae* in necrotic porcine lung tissue by using selective capture of transcribed sequences. Infect Immun 72(11): 6711–6716.
23. Baltes N, Buettner FF, Gerlach GF (2007) Selective capture of transcribed sequences (SCOTS) of *Actinobacillus pleuropneumoniae* in the chronic stage of disease reveals an HlyX-regulated autotransporter protein. Vet Microbiol 123(1–3): 110–121.
24. Jacobsen I, Hennig-Pauka I, Baltes N, Trost M, Gerlach GF (2005) Enzymes involved in anaerobic respiration appear to play a role in *Actinobacillus pleuropneumoniae* virulence. Infect Immun 73(1): 226–234.
25. Baltes N, Hennig-Pauka I, Jacobsen I, Gruber AD, Gerlach GF (2003) Identification of dimethyl sulfoxide reductase in *Actinobacillus pleuropneumoniae* and its role in infection. Infect Immun 71(12): 6784–6792.
26. Green J, Baldwin ML (1997) HlyX, the FNR homologue of *Actinobacillus pleuropneumoniae*, is a [4Fe-4S]-containing oxygen-responsive transcription regulator that anaerobically activates FNR-dependent class I promoters via an enhanced AR1 contact. Mol Microbiol 24(3): 593–605.
27. Spiro S, Guest JR (1990) FNR and its role in oxygen-regulated gene expression in *Escherichia coli*. FEMS Microbiol Rev 75(4): 399–428.
28. Van Hellemond JJ, Tielens AG (1994) Expression and functional properties of fumarate reductase. Biochem J 304(Pt 2): 321–331.
29. Baltes N, N'diaye M, Jacobsen ID, Maas A, Buettner FF, et al. (2005) Deletion of the anaerobic regulator HlyX causes reduced colonization and persistence of *Actinobacillus pleuropneumoniae* in the porcine respiratory tract. Infect Immun 73(8): 4614–4619.
30. Buettner FF, Bendalla IM, Bosse JT, Meens J, Nash JH, et al. (2009) Analysis of the *Actinobacillus pleuropneumoniae* HlyX (FNR) regulon and identification of iron-regulated protein B as an essential virulence factor. Proteomics 2009 Apr 2. [Epub ahead of print]. PMID. pp 19343711.
31. Soltes GA, MacInnes JI (1994) Regulation of gene expression by the HlyX protein of *Actinobacillus pleuropneumoniae*. Microbiology 140(4): 839–845.
32. Yan A, Kiley PJ (2008) Dissecting the role of the N-terminal region of the *Escherichia coli* global transcription factor FNR. J Bacteriol 190(24): 8230–8233.
33. Gunsalus RP (1992) Control of electron flow in *Escherichia coli*: Coordinated transcription of respiratory pathway genes. J Bacteriol 174(22): 7069–7074.

34. Zeballos GA, Bernstein RD, Thompson CI, Forfia PR, Seyedi N, et al. (1995) Pharmacodynamics of plasma nitrate/nitrite as an indication of nitric oxide formation in conscious dogs. Circulation 91(12): 2982–2988.
35. Ellis G, Adatia I, Yazdanpanah M, Makela SK (1998) Nitrite and nitrate analyses: A clinical biochemistry perspective. Clin Biochem 31(4): 195–220.
36. Guevara I, Iwanejko J, Dembinska-Kiec A, Pankiewicz J, Wanat A, et al. (1998) Determination of nitrite/nitrate in human biological material by the simple griess reaction. Clin Chim Acta 274(2): 177–188.
37. Ekerhovd E, Enskog A, Caidahl K, Klintland N, Nilsson L, et al. (2001) Plasma concentrations of nitrate during the menstrual cycle, ovarian stimulation and ovarian hyperstimulation syndrome. Hum Reprod 16(7): 1334–1339.
38. Potter LC, Millington P, Griffiths L, Thomas GH, Cole JA (1999) Competition between *Escherichia coli* strains expressing either a periplasmic or a membrane-bound nitrate reductase: Does Nap confer a selective advantage during nitrate-limited growth? Biochem J 344 Pt 1: 77–84.
39. Costa C, Macedo A, Moura I, Moura JJ, Le Gall J, et al. (1990) Regulation of the hexaheme nitrite/nitric oxide reductase of *Desulfovibrio desulfuricans*, *Wolinella succinogenes* and *Escherichia coli*. A mass spectrometric study. FEBS Lett 276(1–2): 67–70.
40. Xu Z, Zhou Y, Li L, Zhou R, Xiao S, et al. (2008) Genome biology of *Actinobacillus pleuropneumoniae* JL03, an isolate of serotype 3 prevalent in China. PLoS ONE 3(1): e1450. 10.1371/journal.pone.0001450.
41. Bannan JD, Moran MJ, MacInnes JI, Soltes GA, Friedman RL (1993) Cloning and characterization of *btr*, a *Bordetella pertussis* gene encoding an FNR-like transcriptional regulator. J Bacteriol 175(22): 7228–7235.
42. Wood GE, Khele N, Guiso N, Friedman RL (1998) Identification of *btr*-regulated genes using a titration assay. search for a role for this transcriptional regulator in the growth and virulence of *Bordetella pertussis*. Gene 209(1–2): 51–58.
43. Contreras I, Toro CS, Troncoso G, Mora GC (1997) *Salmonella typhi* mutants defective in anaerobic respiration are impaired in their ability to replicate within epithelial cells. Microbiology 143(Pt 8): 2665–2672.
44. Fritz C, Maass S, Kreft A, Bange FC (2002) Dependence of *Mycobacterium bovis* BCG on anaerobic nitrate reductase for persistence is tissue specific. Infect Immun 70(1): 286–291.
45. Weber I, Fritz C, Ruttkowski S, Kreft A, Bange FC (2000) Anaerobic nitrate reductase (*narGHJI*) activity of *Mycobacterium bovis* BCG *in vitro* and its contribution to virulence in immunodeficient mice. Mol Microbiol 35(5): 1017–1025.
46. Hase CC, Fedorova ND, Galperin MY, Dibrov PA (2001) Sodium ion cycle in bacterial pathogens: Evidence from cross-genome comparisons. Microbiology and Molecular Biology Reviews 65(3): 353.
47. Fadeeva MS, Nunez C, Bertsova YV, Espin G, Bogachev AV (2008) Catalytic properties of Na^+-translocating NADH: Quinone oxidoreductases from *Vibrio harveyi*, *Klebsiella pneumoniae*, and *Azotobacter vinelandii*. FEMS Microbiol Lett 279(1): 116–123.
48. Zhou W, Bertsova YV, Feng B, Tsatsos P, Verkhovskaya ML, et al. (1999) Sequencing and preliminary characterization of the Na^+-translocating NADH:Ubiquinone oxidoreductase from *Vibrio harveyi*. Biochemistry 38(49): 16246–16252.
49. Nakayama Y, Yasui M, Sugahara K, Hayashi M, Unemoto T (2000) Covalently bound flavin in the NqrB and NqrC subunits of Na(+)-translocating NADH-quinone reductase from *Vibrio alginolyticus*. FEBS Lett 474(2–3): 165–168.
50. Cruz WT, Nedialkov YA, Thacker BJ, Mulks MH (1996) Molecular characterization of a common 48-kilodalton outer membrane protein of *Actinobacillus pleuropneumoniae*. Infect Immun 64(1): 83–90.
51. Sheehan BJ, Bosse JT, Beddek AJ, Rycroft AN, Kroll JS, et al. (2003) Identification of *Actinobacillus pleuropneumoniae* genes important for survival during infection in its natural host. Infect Immun 71(7): 3960–3970.
52. Hase CC, Mekalanos JJ (1999) Effects of changes in membrane sodium flux on virulence gene expression in *Vibrio cholerae*. Proc Natl Acad Sci U S A 96(6): 3183–3187.
53. DiRita VJ (2000) Virulence gene regulation inside and outside. Philosophical Transactions of the Royal Society B: Biological Sciences 355(1397): 657–665.
54. Unemoto T, Hayashi M (1993) Na(+)-translocating NADH-quinone reductase of marine and halophilic bacteria. J Bioenerg Biomembr 25(4): 385–391.
55. Kogure K (1998) Bioenergetics of marine bacteria. Curr Opin Biotechnol 9(3): 278–282.
56. Schulz H, Fabianek RA, Pellicioli EC, Hennecke H, Thony-Meyer L (1999) Heme transfer to the heme chaperone CcmE during cytochrome c maturation requires the CcmC protein, which may function independently of the ABC-transporter CcmAB. Proc Natl Acad Sci US A 96(11): 6462–6467.
57. Schultz BE, Chan SI (1998) Thermodynamics of electron transfer in *Escherichia coli* cytochrome bo3. Proc Natl Acad Sci U S A 95(20): 11643–11648.
58. Deckers-Hebestreit G, Altendorf K (1996) The F0F1-type ATP synthases of bacteria: Structure and function of the F0 complex. Annu Rev Microbiol 50: 791–824. 10.1146/annurev.micro.50.1.791.
59. Rashid N, Imanaka H, Fukui T, Atomi H, Imanaka T (2004) Presence of a novel phosphopentomutase and a 2-deoxyribose 5-phosphate aldolase reveals a metabolic link between pentoses and central carbon metabolism in the hyperthermophilic archaeon *Thermococcus kodakaraensis*. J Bacteriol 186(13): 4185–4191. 10.1128/JB.186.13.4185-4191.2004.
60. Mayes PA (2000) Glycolysis and the oxidation of pyruvate. In: Murray KM, Granner DK, Mayes PA, Rodwell VW, eds. Harper's Biochemistry. , United States of America: Appleton and Lange. pp 190.
61. Diesterhaft MD, Freese E (1973) Role of pyruvate carboxylase, phosphoenolpyruvate carboxykinase, and malic enzyme during growth and sporulation of *Bacillus subtilis*. J Biol Chem 248(17): 6062–6070.
62. Yu BJ, Sung BH, Lee JY, Son SH, Kim MS, et al. (2006) *sucAB* and *sucCD* are mutually essential genes in *Escherichia coli*. FEMS Microbiol Lett 254(2): 245–250.
63. Nakamura T, Yamaguchi A, Kondo H, Watanabe H, Kurihara T, et al. (2009) Roles of K151 and D180 in L-2-haloacid dehalogenase from *Pseudomonas* sp. YL: Analysis by molecular dynamics and ab initio fragment molecular orbital calculations. J Comput Chem.
64. Schellenberg GD, Sarthy A, Larson AE, Backer MP, Crabb JW, et al. (1984) Xylose isomerase from *Escherichia coli*. Characterization of the protein and the structural gene. J Biol Chem 259(11): 6826–6832.
65. Velayudhan J, Castor M, Richardson A, Main-Hester KL, Fang FC (2007) The role of ferritins in the physiology of *Salmonella enterica* sv. Typhimurium: A unique role for ferritin B in iron-sulphur cluster repair and virulence. Mol Microbiol 63(5): 1495–1507.
66. Barreteau H, Kovac A, Boniface A, Sova M, Gobec S, et al. (2008) Cytoplasmic steps of peptidoglycan biosynthesis. FEMS Microbiol Rev 32(2): 168–207.
67. Jolly L, Pompeo F, Van Heijenoort J, Fassy F, Mengin-Lecreulx D (2000) Autophosphorylation of phosphoglucosamine mutase from *Escherichia coli*. J Bacteriol 182(5): 1280–1285.
68. Mengin-Lecreulx D, van Heijenoort J (2005) Characterization of the essential gene *glmM* encoding phosphoglucosamine mutase in *Escherichia coli*. J Biol Chem 272(5): 1243–1254.
69. Oikawa T, Tauch A, Schaffer S, Fujioka T (2006) Expression of *alr* gene from *Corynebacterium glutamicum* ATCC 13032 in *Escherichia coli* and molecular characterization of the recombinant alanine racemase. J Biotechnol 125(4): 503–512.
70. Kelly J, Knox J, Moews P, Hite G, Bartolone J, et al. (1985) 2.8-A structure of penicillin-sensitive D-alanyl carboxypeptidase-transpeptidase from *Streptomyces* R61 and complexes with beta-lactams. J Biol Chem 260(10): 6449–6458.
71. Dideberg O, Charlier P, Dive G, Joris B, Frère J, et al. (1982) Structure of a Zn 2-containing D-alanyl-D-alanine-cleaving carboxypeptidase at 2.5 Å resolution. Nature 299: 469–470.
72. Byrd W, Kadis S (1989) Structures and sugar compositions of lipopolysaccharides isolated from seven *Actinobacillus pleuropneumoniae* serotypes. Infect Immun 57(12): 3901–3906.
73. Labrie J, Rioux S, Wade MM, Champlin FR, Holman SC, et al. (2002) Identification of genes involved in biosynthesis of *Actinobacillus pleuropneumoniae* serotype 1 O-antigen and biological properties of rough mutants. J Endotoxin Res 8(1): 27–38.
74. Bandara AB, Lawrence ML, Veit HP, Inzana TJ (2003) Association of *Actinobacillus pleuropneumoniae* capsular polysaccharide with virulence in pigs. Infect Immun 71(6): 3320.
75. Kreuzer KN (2005) Interplay between DNA replication and recombination in prokaryotes. Annu Rev Microbiol 59: 43–67.
76. Clifton SW, McCarthy D, Roe BA (1994) Sequence of the *rec*-2 locus of *Haemophilus influenzae*: Homologies to comE-ORF3 of *Bacillus subtilis* and *msbA* of *Escherichia coli*. Gene 146(1): 95–100.
77. Kharat AS, Coursange E, Noirclerc-Savoye M, Lacoste J, Blot M (2006) IS1 transposition is enhanced by translation errors and by bacterial growth at extreme glucose levels. Acta Biochim Pol 53(4): 729–738.
78. Shelburne SA 3rd, Keith DB, Davenport MT, Horstmann N, Brennan RG, Musser JM (2008) Molecular characterization of group A *Streptococcus* maltodextrin catabolism and its role in pharyngitis. Mol Microbiol 69(2): 436–52.
79. Sweet G, Gandor C, Voegele R, Wittekindt N, Beuerle J, et al. (1990) Glycerol facilitator of *Escherichia coli*: Cloning of *glpF* and identification of the *glpF* product. J Bacteriol 172(1): 424–430.
80. Munkelt D, Grass G, Nies DH (2004) The chromosomally encoded cation diffusion facilitator proteins DmeF and FieF from *Wautersia metallidurans* CH34 are transporters of broad metal specificity. J Bacteriol 186(23): 8036–8043.
81. Lee CR, Cho SH, Yoon MJ, Peterkofsky A, Seok YJ (2007) *Escherichia coli* enzyme IIANtr regulates the K+ transporter TrkA. Proc Natl Acad Sci U S A 104(10): 4124–4129. 10.1073/pnas.0609897104.
82. Cho WS, Chae C (2001) Expression of the *apxIV* gene in pigs naturally infected with *Actinobacillus pleuropneumoniae*. J Comp Pathol 125(1): 34–40. 10.1053/jcpa.2001.0474.
83. Schaller A, Kuhn R, Kuhnert P, Nicolet J, Anderson TJ, et al. (1999) Characterization of *apxIVA*, a new RTX determinant of *Actinobacillus pleuropneumoniae*. Microbiology 145(Pt 8): 2105–2116.
84. Osicka R, Kalmusova J, Krizova P, Sebo P (2001) *Neisseria meningitidis* RTX protein FrpC induces high levels of serum antibodies during invasive disease: Polymorphism of *frpC* alleles and purification of recombinant FrpC. Infect Immun 69(9): 5509–5519.
85. Chen HY, Weng SF, Lin JW (2000) Identification and analysis of the *sap* genes from *Vibrio fischeri* belonging to the ATP-binding cassette gene family required for peptide transport and resistance to antimicrobial peptides. Biochem Biophys Res Commun 269(3): 743–748. 10.1006/bbrc.1999.1506.

86. Mason KM, Bruggeman ME, Munson RS, Bakaletz LO (2006) The non-typeable *Haemophilus influenzae* Sap transporter provides a mechanism of antimicrobial peptide resistance and SapD-dependent potassium acquisition. Mol Microbiol 62(5): 1357–1372.
87. Mason KM, Munson Jr RS, Bakaletz LO (2005) A mutation in the *sap* operon attenuates survival of nontypeable *Haemophilus influenzae* in a chinchilla model of otitis media. Infect Immun 73(1): 599.
88. Kizil G, Wilks K, Wells D, Ala'Aldeen D (2000) Detection and characterisation of the genes encoding glyoxalase I and II from *Neisseria meningitidis*. J Med Microbiol 49(7): 669–673.
89. Chiu HC, Lin TL, Wang JT (2007) Identification and characterization of an organic solvent tolerance gene in *Helicobacter pylori*. Helicobacter 12(1): 74–81.
90. Bos MP, Robert V, Tommassen J (2007) Biogenesis of the gram-negative bacterial outer membrane. Annu Rev Microbiol 61: 191–214.
91. Mori H, Ito K (2001) The Sec protein-translocation pathway. Trends Microbiol 9(10): 494–500.
92. Fuller TE, Shea RJ, Thacker BJ, Mulks MH (1999) Identification of in vivo induced genes in *Actinobacillus pleuropneumoniae*. Microb Pathog 27(5): 311–327. 10.1006/mpat.1999.0309.
93. Fuller TE, Martin S, Teel JF, Alaniz GR, Kennedy MJ, et al. (2000) Identification of *Actinobacillus pleuropneumoniae* virulence genes using signature-tagged mutagenesis in a swine infection model. Microb Pathog 29(1): 39–51.
94. Kazakov AE, Rodionov DA, Alm E, Arkin AP, Dubchak I, et al. (2009) Comparative genomics of regulation of fatty acid and branched-chain amino acid utilization in proteobacteria. J Bacteriol 191(1): 52–64.
95. Meyer KC, Sharma A, Brown R, Weatherly M, Moya FR, et al. (2000) Function and composition of pulmonary surfactant and surfactant-derived fatty acid profiles are altered in young adults with cystic fibrosis. Chest 118(1): 164–174.
96. Brown NL, Stoyanov JV, Kidd SP, Hobman JL (2003) The MerR family of transcriptional regulators. FEMS Microbiol Rev 27(2–3): 145–163.
97. Jenkins DE, Auger EA, Matin A (1991) Role of RpoH, a heat shock regulator protein, in *Escherichia coli* carbon starvation protein synthesis and survival. J Bacteriol 173(6): 1992–1996.
98. Sakoh M, Ito K, Akiyama Y (2005) Proteolytic activity of HtpX, a membrane-bound and stress-controlled protease from *Escherichia coli*. J Biol Chem 280(39): 33305–33310.
99. Wren JD (2009) A global meta-analysis of microarray expression data to predict unknown gene functions and estimate the literature-data divide. Bioinformatics May 15.[Epub ahead of print].
100. Carrillo CD, Taboada E, Nash JH, Lanthier P, Kelly J, et al. (2004) Genome-wide expression analyses of *Campylobacter jejuni* NCTC11168 reveals coordinate regulation of motility and virulence by *flhA*. J Biol Chem 279(19): 20327–203381.
101. Foote SJ, Bossé JT, Bouevitch AB, Langford PR, Young NM, et al. (2008) The complete genome sequence of *Actinobacillus pleuropneumoniae* L20 (serotype 5). J Bacteriol 190(4): 1495–1496.
102. Deslandes V, Nash JH, Harel J, Coulton JW, Jacques M (2007) Transcriptional profiling of *Actinobacillus pleuropneumoniae* under iron-restricted conditions. BMC Genomics 8: 72. doi:10.1186/1471-2164-8-72.
103. Saeed AI, Sharov V, White J, Li J, Liang W, et al. (2003) TM4: A free, open-source system for microarray data management and analysis. Biotechniques 34(2): 374.
104. Pawitan Y, Michiels S, Koscielny S, Gusnanto A, Ploner A (2005 Jul 1) False discovery rate, sensitivity and sample size for microarray studies. Bioinformatics 21(13): 3017–24.
105. Peterson JD, Umayam LA, Dickinson T, Hickey EK, White O (2001) The comprehensive microbial resource. Nucleic Acids Res 29(1): 123.
106. Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3(6): 1101–1108.