Effects of growth conditions on biofilm formation by *Actinobacillus pleuropneumoniae*

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Abstract – Biofilm formation is an important virulence trait of many bacterial pathogens. It has been reported in the literature that only two of the reference strains of the swine pathogen *Actinobacillus pleuropneumoniae*, representing serotypes 5b and 11, were able to form biofilm in vitro. In this study, we compared biofilm formation by the serotype 1 reference strain S4074 of *A. pleuropneumoniae* grown in five different culture media. We observed that strain S4074 of *A. pleuropneumoniae* is able to form biofilms after growth in one of the culture conditions tested brain heart infusion (BHI medium, supplier B). Confocal laser scanning microscopy using a fluorescent probe specific to the poly-N-acetylglucosamine (PGA) polysaccharide further confirmed biofilm formation. In accordance, biofilm formation was susceptible to dispersin B, a PGA hydrolase. Transcriptional profiles of *A. pleuropneumoniae* S4074 following growth in BHI-B, which allowed a robust biofilm formation, and in BHI-A, in which only a slight biofilm formation was observed, were compared. Genes such as *tadC*, *tadD*, genes with homology to autotransporter adhesins as well as genes pgaABC involved in PGA biosynthesis and genes involved in zinc transport were up-regulated after growth in BHI-B. Interestingly, biofilm formation was inhibited by zinc, which was found to be more present in BHI-A (no or slight biofilm) than in BHI-B. We also observed biofilm formation in reference strains representing serotypes 3, 4, 5a, 12 and 14 as well as in 20 of the 37 fresh field isolates tested. Our data indicate that *A. pleuropneumoniae* has the ability to form biofilms under appropriate growth conditions and transition from a biofilm-positive to a biofilm-negative phenotype was reversible.

*Actinobacillus pleuropneumoniae* / biofilm / growth condition / transcriptomic

1. INTRODUCTION

*Actinobacillus pleuropneumoniae*, a member of the *Pasteurellaceae*, is an important swine pathogen responsible for economic losses in the swine industry. To date, 15 serotypes of *A. pleuropneumoniae* have been described based on capsular antigens [3, 10]. The virulence of the bacteria is mediated by the coordinated action of several virulence factors, namely the capsule, lipopolysaccharides (LPS), Apx toxins and outer membrane proteins involved in iron uptake [4, 11, 14, 18, 19, 28, 29].

It is widely accepted that the majority of bacteria in virtually all ecosystems (natural,
engineered and pathogenic ecosystems) grow in matrix-enclosed biofilms [7]. The matrix provides biofilm cells with a protected microenvironment containing nutrients, secreted enzymes and DNA. The matrix also contributes to the increased resistance to antibiotics and host defenses exhibited by biofilm cells [15]. All members of the Pasteurellaceae are inhabitants of mucosal surfaces of mammals and therefore formation of a biofilm may be crucial to their persistence in vivo. However, biofilms have only been investigated in a few species of the Pasteurellaceae family [16]. In A. pleuropneumoniae, the formation of biofilms on polystyrene microtiter plate is dependent on the production of poly-N-acetylglucosamine (PGA) a linear polymer of N-acetylglucosamine residues in β(1,6) linkage [17, 20]. The production of PGA is encoded by the genes pgaABCD [20]. A novel insertion element, ISApl1, was recently identified in an A/T rich region of the pgaC gene of the biofilm-negative A. pleuropneumoniae strain HB04 [25]. PGA is a substrate for dispersin B (DspB), a biofilm-releasing glycosyl hydrolase produced by Aggregatibacter (Actinobacillus) actinomycetemcomitans and A. pleuropneumoniae [20, 22]. It has also been reported that only 2 of the 15 A. pleuropneumoniae reference strains, representing serotypes 5b and 11, were able to form a biofilm in vitro and that the transition from a biofilm-positive to biofilm-negative phenotype was irreversible [21]. However, Li et al. [24] recently observed slight biomass of biofilm when the A. pleuropneumoniae serotype 1 reference strain S4074 was grown in serum-free TSB but not in serum-containing TSB. In addition, an enhanced biofilm formation was observed in luxS [24] and hns [8] mutants of A. pleuropneumoniae strain S4074. The aims of the present study were: (i) to re-evaluate biofilm formation by A. pleuropneumoniae reference strain S4074 (serotype 1) under different growth conditions using a standard microtiter plate and crystal violet staining protocol; (ii) to evaluate the ability of 16 reference strains and 37 fresh field isolates to form biofilm in the growth condition shown to allow the best biofilm formation and (iii) to determine the transcriptomic profile of A. pleuropneumoniae strain S4074 when grown in that culture condition.

2. MATERIALS AND METHODS

2.1. Bacterial strains and growth conditions

Bacterial strains used in the present study are listed in Table I. Bacteria were grown on brain heart infusion agar plates (BHI; Difco Laboratories, Detroit, MI, USA) supplemented with 15 μg/mL nicotinamide adenine dinucleotide (NAD). A colony was transferred into 5 mL of Luria-Bertani broth (LB; Difco), tryptic soy broth (TSB; Difco), Mueller Hinton broth (MH; Difco) or BHI (BHI-A; Difco or BHI-B; Oxoid Ltd, Basingstoke, Hampshire, UK) with 5 μg/mL NAD and incubated at 37 °C overnight with agitation. This culture was used for the biofilm assays.

2.2. Biofilm assay in microtiter plates

The microtiter plate biofilm assay is a static assay particularly useful for examining early events in biofilm formation [27]. The wells of a sterile 96-well microtiter plate (Costar® 3599, Corning, NY, USA) were filled in triplicate with a dilution (1/100) of an overnight bacterial culture. Following an incubation of 6 or 24 h at 37 °C, the wells were washed by immersion in water and excess water was removed by inverting plates onto a paper towel. The wells were then filled with 100 μl of Lowry crystal violet (0.1%) and the plate was incubated for 2 min at room temperature. After removal of the crystal violet solution, the plate was washed and dried in a 37 °C incubator for 30 min and 100 μl of ethanol (70%) were added to the wells. Absorbance was measured at 590 nm using a spectrophotometer (Powerwave, BioTek Instruments, Winooski, VT, USA).

2.3. Scanning laser confocal microscopy

The same biofilm assay protocol was used as described previously. After the 6 or 24 h incubation, the wells were filled with 100 μL of Wheat Germ Agglutinin (WGA)-Oregon Green 488 (Molecular Probes, Eugene, OR, USA) diluted 1/100 in PBS and the plate was incubated for 30 min at room temperature in the dark. The plate was then washed with water and filled with PBS. The plate was observed with a confocal microscope (Olympus FV1000

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Table 1. A. pleuropneumoniae strains used in the present study.

| Strains       | Relevant traits | Source                        |
|---------------|-----------------|--------------------------------|
| **Reference strains** |                  |                                |
| S4074         | Serotype 1      | K.R. Mittal<sup>1</sup>        |
| 4226          | Serotype 2      | K.R. Mittal<sup>1</sup>        |
| 1421          | Serotype 3      | K.R. Mittal<sup>1</sup>        |
| 1462          | Serotype 4      | K.R. Mittal<sup>1</sup>        |
| K17           | Serotype 5a     | K.R. Mittal<sup>1</sup>        |
| L20           | Serotype 5b     | K.R. Mittal<sup>1</sup>        |
| FEMO          | Serotype 6      | K.R. Mittal<sup>1</sup>        |
| WF.83         | Serotype 7      | K.R. Mittal<sup>1</sup>        |
| 405           | Serotype 8      | K.R. Mittal<sup>1</sup>        |
| 13261         | Serotype 9      | K.R. Mittal<sup>1</sup>        |
| 13039         | Serotype 10     | K.R. Mittal<sup>1</sup>        |
| 56153         | Serotype 11     | K.R. Mittal<sup>1</sup>        |
| 832985        | Serotype 12     | K.R. Mittal<sup>1</sup>        |
| N273<sup>4</sup> | Serotype 13    | M. Gottschalk<sup>1</sup>      |
| 3906<sup>4</sup> | Serotype 14    | M. Gottschalk<sup>1</sup>      |
| HS143         | Serotype 15     | M. Gottschalk<sup>1</sup>      |

| **Field strains** | Relevant traits | Source                        |
|------------------|-----------------|--------------------------------|
| 05-7430, 05-7431| Serotype 1      | M. Ngeleka<sup>2</sup>        |
| 111A, 719, 2398, 2521 | Serotype 1   | D. Slavic<sup>3</sup>        |
| 05-4817, 05-C996, 06-996 | Serotype 5a  | S. Messier<sup>4</sup>        |
| 04-37943, 04-3128, 05-508 | Serotype 5a | M. Ngeleka<sup>2</sup>        |
| 05-6501, 06-4091 | Serotype 5b    | S. Messier<sup>1</sup>        |
| 03-14796, 03-22382, 03-22383, 05-4832 | Serotype 5b | M. Ngeleka<sup>2</sup>        |
| 366A, 400, 564D, 888 | Serotype 5b  | D. Slavic<sup>3</sup>        |
| 05-3695, 06-3008, 06-3060, 06-4108 | Serotype 7 | S. Messier<sup>4</sup>        |
| 04-37257, 05-14401 | Serotype 7    | M. Ngeleka<sup>2</sup>        |
| 881, 986, 1951, 4648 | Serotype 7  | D. Slavic<sup>3</sup>        |
| 05-13146, 05-14657, 05-20080, 05-20081, 05-2983 | Serotype 15 | M. Ngeleka<sup>2</sup>        |

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4 These strains are NAD-independent and belong to biotype II.

IX81). WGA was excited at 488 nm and detected using 520 nm filters. The images were processed using Fluoview software (Olympus).

2.4. Transcriptomic microarray experiments

2.4.1. RNA extractions

For the microarray experiments, BHI-A or BHI-B broths were inoculated with 500 µL of an overnight culture of A. pleuropneumoniae serotype 1 strain S4074 and grown at 37 °C in an orbital shaker until an optical density of 0.6 was reached. Ice-cold RNA degradation stop solution (95% ethanol, 5% buffer-saturated phenol), shown to effectively prevent RNA degradation and therefore preserve the integrity of the transcriptome [2], was added to the bacterial culture at a ratio of 1:10 (vol/vol). The sample was mixed by inversion, incubated on ice for 5 min, and then spun at 5 000 g for 10 min to pellet the cells. Bacterial RNA isolation was then carried out using the QIAGEN RNeasy MiniKit (QIAGEN, Mississauga, ON, Canada), as prescribed by the manufacturer. During the extraction, samples were subjected to...
an on-column DNase treatment, as suggested by the manufacturer and then treated with Turbo DNase (Ambion, Austin, TX, USA) to ensure that all DNA contaminants were eliminated. The RNA concentration, quality and integrity were assessed spectrophotometrically and on gel.

2.4.2. Microarray construction and design

For the construction of AppChip2, 2033 ORFs from the complete genome sequence of *A. pleuropneumoniae* serotype 5b strain L20, representing more than 95% of all ORFs with a length greater than 160 nt, were amplified and spotted in duplicate on the chip. Spotted sheared genomic DNA from *A. pleuropneumoniae* L20 and porcine DNA are used as controls (GEO: GPL6658). Additional information concerning chip production is described by Gouéré et al. [13].

2.4.3. Microarray hybridizations

cDNA synthesis and microarray hybridizations were performed as described [6]. Briefly, equal amounts (15 μg) of test RNA and control RNA were used to set up a standard reverse transcription reaction using random octamers (BioCorp, Montreal, QC, Canada), SuperScript II (Invitrogen, Carlsbad, CA, USA) and aminoallyl-dUTP (Sigma, St. Louis, MO, USA), and the resulting cDNA was indirectly labelled using a monofunctional NHS-ester Cy3 or Cy5 dye (Amersham, Buckinghamshire, UK). The labelling efficiency was assessed spectrophotometrically. Labelled samples were then combined and added to the AppChip2 for overnight hybridization. Five hybridizations were performed for the serotype 1 strain S4074 BHI-A versus BHI-B experiments. All slides were scanned using a Perkin-Elmer ScanArray Express scanner.

2.4.4. Microarray analysis and bioinformatics

Microarray data analysis was conducted with the TM4 Suite of software from the J. Craig Venture Institute [30] as described by Deslandes et al. [9]. Briefly, raw data was first generated using SpotFinder v.3.1.1. Locally weighted linear regression (lowess) was then performed in the Microarray Data Analysis System (MIDAS) in order to normalize the data. The Significance Analysis of Microarray (SAM) algorithm [33], which is implemented in TIGR Microarray Expression Viewer (TMEV), was used to generate a list of differentially expressed genes. During SAM analysis, a false discovery rate (FDR) of 0% was estimated for the serotype 1 strain S4074 BHI-A versus BHI-B experiments.

2.5. Effects of DspB and zinc on biofilm formation

Biofilms were grown for 6 or 24 h in BHI-B as described above. The wells were washed with water and then filled with 100 μL of PBS containing 0.2, 2.0 or 20 μg/mL of DspB (Kane Biotech Inc, Winnipeg, MB, Canada) as described by Izano et al. [17]. After incubation at 37 °C for 5 min, the wells were rinsed with water and stained with crystal violet. To monitor the effect of zinc on biofilm formation, bacteria were grown for 6 or 24 h in BHI-B supplemented with 50–250 μg/mL of ZnCl2.

2.6. Statistical analysis

The statistical significance (p value) of differences in biofilm phenotypes (mean optical density values) was determined by a paired, one-tailed t-test using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA, USA).

3. RESULTS

3.1. Biofilm formation and growth conditions

The ability of the *A. pleuropneumoniae* serotype 1 reference strain S4074 to form biofilms was evaluated using different growth media (Fig. 1). No biofilm was present in the wells containing bacterial cells grown in LB broth while only a slight biofilm was observed in wells containing cells grown in TSB, MH or BHI-A broths after 24 h of incubation. However a pronounced biofilm (p < 0.01) was formed when strain S4074 was grown in BHI-B broth. This was not due to an increased growth in BHI-B compared to BHI-A as similar growth curves were observed in both media.

We then evaluated biofilm formation by all the reference strains of *A. pleuropneumoniae* after growth for 6 or 24 h in BHI-B. Similarly to what was observed with the serotype 1, we found that growth in BHI-B, but not BHI-A, allows biofilm formation in reference strains representing serotypes 4, 5a and 14. In addition
to the already reported biofilm formation in serotypes 5b and 11, we also observed biofilms for serotype 3 and 12 reference strains. Moreover, biofilm formation (OD\textsubscript{590nm} > 0.1) was observed in 20 (54%) of the 37 fresh field isolates of serotypes 1, 5, 7 and 15 that were tested (Fig. 2). In general, serotypes 5a, 5b and 7 field isolates tend to form more biofilms (mean OD of 1.15, 1.47 and 1.47 after 24 h) than isolates from serotypes 1 and 15 (mean OD of 0.36 and 0.80 after 24 h).

When *A. pleuropneumoniae* strain S4074 grown in BHI-A (no or slight biofilm) was transferred to BHI-B we observed the formation of a pronounced biofilm (*p* < 0.05). When these cells were then transferred back to BHI-A, the phenotype returned to a slight biofilm (*p* < 0.05). This was also observed with field isolates representing different serotypes (data not shown).

### 3.2. Scanning laser confocal microscopy

We observed that for many reference strains, including strain S4074, and field isolates, pronounced biofilms were present after a short incubation period of only 6 h (Fig. 2). The biofilm was visualized by confocal laser scanning microscopy using a fluorescent probe (WGA-Oregon Green) specific to the PGA matrix polysaccharide (Fig. 3). It is evident from these micrographs that *A. pleuropneumoniae* strain S4074 does not form biofilm when grown in BHI-A while a thick PGA matrix is formed by *A. pleuropneumoniae* serotype 5b strain L20 grown in the same condition. However, both strains showed a pronounced biofilm when grown in BHI-B. In the case of strain S4074, the biofilm is even more important after 6 h than 24 h of incubation (Fig. 3). Because scanning laser confocal microscopy allows optical sectioning of the biofilm either in the horizontal or the vertical dimension it is possible to evaluate the thickness of the biofilm. We evaluated the thickness of *A. pleuropneumoniae* strain S4074 biofilm to be of ~ 25 μm after growth in BHI-B for 6 h (Fig. 3C) and even greater (~ 65 μm) for *A. pleuropneumoniae* strain L20.

### 3.3. Transcriptomic profiling under different growth conditions

To assess the transcriptional response of *A. pleuropneumoniae* S4074 after growth in BHI-B compared to BHI-A, transcript profiling experiments using DNA microarrays were performed. Overall, 232 genes were significantly differentially expressed during growth in BHI-B; 152 being up-regulated and 80 being down-regulated (Tab. II). The genes that showed the highest level of up-regulation after growth in BHI-B belonged to the “amino acid biosynthesis”, “energy metabolism”, “transport and binding proteins”, “cell envelope” and “hypothetical/unknown/unclassified” functional classes (Fig. 4). Genes such as *tadC* and *tadD* (tight adherence proteins C and D), genes with homology to autotransporter adhesins (APL\_0443 and APL\_0104) as well as genes *pgaABC* involved in PGA biosynthesis were up-regulated after growth in BHI-B. A cluster of genes involved in dipeptide transport (*dppABCDF*) and genes involved in the synthesis of an urease (*ureAEFG*) were also up-regulated. Down-regulated genes after growth in BHI-B mostly belonged to the “transport and binding proteins”, “cell envelope”, “protein synthesis” and “hypothetical/
unknown/unclassified” functional classes. Most notably, cys genes involved in sulphate transport systems were down-regulated, as well as a gene (APL_1096) sharing 59% identity with the DspB gene of A. actinomycetemcomitans.

3.4. Effect of DspB on biofilm formation

Enzymatic treatment with DspB of biofilms of A. pleuropneumoniae strains S4074 and L20 grown for 6 or 24 h almost completely dispersed them ($p < 0.05$) confirming the presence of PGA in the biofilm matrix.

3.5. Effect of zinc on biofilm formation

Chemical analysis showed differences in some divalent cations concentration between BHI-A (Fe < 0.10 ppm, Zn 2.03 ppm) and BHI-B (Fe 0.10 ppm, Zn 1.75 ppm) while no differences were observed for others (Ca, Cu, Mg, Mn). We therefore hypothesized that the difference in biofilm formation observed after growth in BHI-B compared to BHI-A might be due to cations concentration. Since the concentration of zinc was found to be higher in BHI-A (no or slight biofilm) we tested a
possible inhibitory effect of this cation on biofilm formation. The addition of ZnCl₂ to BHI-B inhibited, in a dose-dependent manner, the formation of biofilms by *A. pleuropneumoniae* strains S4074 and L20 (Fig. 5). A complete inhibition (*p* < 0.01) was observed when 100 µg/mL of ZnCl₂ was added to BHI-B, a concentration which did not affect growth after 24 h (data not shown). A similar inhibition was also observed with the addition of ZnSO₄, ZnO, and Zn₃(PO₄)₂ but not with MgCl₂ or CaCl₂ thus confirming that the inhibition was due to the addition of zinc. Biofilm formation in *A. actinomycetemcomitans* was also inhibited.

![Image](image-url)
Table II. *A. pleuropneumoniae* strain S4074 genes that are up- or down-regulated after growth in BHI-B compared to growth in BHI-A.

| Locus tag | Gene | Description | Fold change |
|-----------|------|-------------|-------------|
| **Amino acid biosynthesis** | | | |
| APL_0728 | ilvH | Acetolactate synthase small subunit | 5.707 |
| APL_0662 | aspC | Putative aspartate aminotransferase | 5.324 |
| APL_0427 | gdhA | NADP-specific glutamate dehydrogenase | 4.943 |
| APL_0727 | ilvI | Acetolactate synthase large subunit | 4.204 |
| APL_0099 | ilvG | Acetolactate synthase isozone II large subunit (AHAS-II) | 3.915 |
| APL_1499 | thrC | Threonine synthase | 3.198 |
| APL_0097 | ilvD | Dihydroxy-acid dehydratase | 3.142 |
| APL_0393 | leuA | 2-isopropylmalate synthase | 3.000 |
| APL_0098 | ilvM | Acetolactate synthase isozone II small subunit (AHAS-II) | 2.934 |
| APL_2027 | hisF | Imidazole glycerol phosphate synthase subunit hisF | 2.833 |
| APL_0702 | serC | Phosphoserine aminotransferase | 2.788 |
| APL_0432 | leuB | 3-isopropylmalate dehydrogenase | 2.643 |
| APL_0899 | dapA | Dihydrodipicolinate synthase | 2.401 |
| APL_0211 | glyA | Glycine/serine hydroxymethyltransferase | 2.398 |
| APL_0133 | cysB | HTH-type transcriptional regulator CysB | 2.340 |
| APL_1853 | ilvC | Ketal-acid reductoisomerase | 2.313 |
| APL_0072 | ilvE | Branched-chain-amino-acid aminotransferase | 2.001 |
| APL_0859 | trpCF | Tryptophan biosynthesis protein trpCF | 1.883 |
| APL_2025 | hisH | Imidazole glycerol phosphate synthase subunit hisH | 1.777 |
| APL_2026 | hisA | Phosphoribosylformiminino-5-aminoimidazole carboxamide ribotide isomerase | 1.739 |
| APL_1198 | APL_1198 | Putative NAD(P)H nitroreductase | 1.708 |
| APL_0139 | leuC | 3-isopropylmalate dehydratase large subunit 2 | 1.605 |
| APL_1230 | serB | Phosphoserine phosphatase | 1.438 |
| APL_0620 | aroG | Phospho-2-dehydro-3-deoxyheptonate aldolase | 1.428 |
| APL_1873 | dapE | Succinyl-diaminopimelate desuccinylase | 1.380 |
| **Biosynthesis of cofactors, prosthetic groups, and carriers** | | | |
| APL_0207 | Dxs | 1-deoxy-D-xylulose-5-phosphate synthase (DXPS) | −1.555 |
| APL_1461 | menA | 1,4-dihydroxy-2-naphthoateoctaprenyltransferase | −1.631 |
| APL_0382 | ribD | Riboflavin biosynthesis protein | −1.726 |
| APL_1408 | gshA | Glutathione biosynthesis bifunctional protein GshAB | −1.789 |
| **Cell envelope** | | | |
| APL_1494 | ftpA | Fine tangled pili major subunit | 5.705 |
| APL_1921 | pgaA | Biofilm PGA synthesis protein PgaA precursor | 5.308 |
| APL_0460 | pilD | Lipoprotein Plp4 | 3.801 |
| APL_1923 | pgaC | Biofilm PGA synthesis N-glycosyltransferase PgaC | 3.591 |
| APL_1922 | pgaB | Biofilm PGA synthesis lipoprotein PgaB precursor | 3.093 |
| APL_0006 | ompP2A | Outer membrane protein P2 | 2.515 |
| APL_0550 | tadC | Tight adherence protein C | 1.985 |
| APL_0442 | sanA | SanA protein | 1.776 |
| APL_0549 | tadD | Tight adherence protein D | 1.749 |
| APL_0332 | hlpB | Lipoprotein HlpB | 1.627 |
| APL_1364 | ghmA | Putative phosphoheptose isomerase | 1.386 |
| APL_0873 | rlpB | Putative rare lipoprotein B | −1.391 |

Continued on next page
| Locus tag | Gene | Description | Fold change |
|-----------|------|-------------|-------------|
| APL_1028  | APL_1028 | Possible lipooligosaccharide N-acetylglucosamine glycosyltransferase | -1.445 |
| APL_0747  | mepA | Penicillin-insensitive murein endopeptidase precursor | -1.446 |
| APL_0436  | mreC | Rod shape-determining protein MreC | -1.585 |
| APL_1086  | ompW | Outer membrane protein W precursor | -1.606 |
| APL_1029  | APL_1029 | Hypothetical protein | -1.650 |
| APL_1424  | oxaA | Inner membrane protein OxaA | -1.772 |
| APL_0933  | ompP1 | Putative outer membrane protein precursor | -2.808 |

**Cellular processes**

| Locus tag | Gene | Description | Fold change |
|-----------|------|-------------|-------------|
| APL_1489  | Tpx | Putative thiol peroxidase | 2.252 |
| APL_0988  | hkiE | Catalase | -1.461 |
| APL_0669  | APL_0669 | Putative iron dependent peroxidase | -1.483 |
| APL_1442  | apxID | RTX-I toxin secretion component | -1.506 |
| APL_1346  | ftsY | Cell division protein FtsY-like protein | -1.530 |

**Central intermediary metabolism**

| Locus tag | Gene | Description | Fold change |
|-----------|------|-------------|-------------|
| APL_1615  | Gst | Putative glutathione S-transferase | 3.269 |
| APL_1614  | ureE | Urease accessory protein UreE | 2.601 |
| APL_1613  | ureF | Urease accessory protein UreF | 2.478 |
| APL_1612  | ureG | Urease accessory protein UreG | 2.165 |
| APL_1618  | ureA | Urease gamma subunit UreA | 1.653 |

**DNA metabolism**

| Locus tag | Gene | Description | Fold change |
|-----------|------|-------------|-------------|
| APL_1931  | tagI | 3-methyladenine-DNA glycosidase | -1.500 |
| APL_1474  | dnaG | DNA primase | -1.551 |
| APL_1282  | dnaQ | DNA polymerase III subunit | -1.579 |
| APL_1255  | parE | DNA topoisomerase IV subunit | -1.630 |
| APL_1505  | holC | DNA polymerase III subunit | -1.663 |

**Energy metabolism**

| Locus tag | Gene | Description | Fold change |
|-----------|------|-------------|-------------|
| APL_1197  | APL_1197 | 3-hydroxyacid dehydrogenase | 3.100 |
| APL_0841  | pntB | NAD(P) transhydrogenase subunit beta | 2.726 |
| APL_1908  | xylA | Xylose isomerase | 2.243 |
| APL_0894  | fdsH | Formate dehydrogenase, iron-sulfur subunit | 2.161 |
| APL_1425  | napC | Cytochrome c-type protein NapC | 2.159 |
| APL_1799  | torC | Pentahemicytochrome | 2.156 |
| APL_0892  | fdsG | Formate dehydrogenase, nitrate-inducible, major subunit | 2.116 |
| APL_1798  | torA | Trimethylamine-N-oxide reductase precursor | 1.977 |
| APL_0381  | gtlC | Anaerobic glycerol-3-phosphate dehydrogenase subunit C | 1.919 |
| APL_0842  | pntA | NAD(P) transhydrogenase subunit alpha | 1.903 |
| APL_0895  | fdaI | Formate dehydrogenase, cytochrome b556 subunit | 1.816 |
| APL_1208  | adhC | Putative alcohol dehydrogenase class 3 | 1.801 |
| APL_0971  | APL_0971 | Putative acyl CoA thioester hydrolase | 1.796 |
| APL_0652  | manB | Phosphomannomutase | 1.677 |
| APL_0483  | APL_0483 | Predicted nitroreductase | 1.668 |
| APL_0142  | glxK | Glycerate kinase | 1.564 |
| APL_0452  | sucC | Succinyl-CoA synthetase beta chain | 1.515 |
| APL_0461  | APL_0461 | Predicted hydrolases of the HAD superfamily | 1.456 |

*Continued on next page*
### Table II. Continued.

| Locus tag | Gene | Description | Fold change |
|-----------|------|-------------|-------------|
| APL_0687  | Dld  | D-lactate dehydrogenase | 1.439       |
| APL_1510  | gpsA | Glycerol-3-phosphate dehydrogenase (NAD(P)+) | 1.414       |
| APL_1427  | napH | Ferredoxin-type protein NapH-like protein | 1.360       |
| APL_0789  | APL_0789 | Dioxygenase | 1.253       |
| APL_0983  | tktA | Transketolase 2 | 1.233       |
| APL_1036  | pflB | Formate acetyltransferase | -1.653     |
| APL_1498  | mgsA | Methylglyoxal synthase | -1.790     |
| APL_1840  | ubiC | 4-hydroxybenzoate synthetase (chorismate lyase) | -1.952     |
| APL_0857  | sdaA | L-serine dehydratase | -3.016     |

**Fatty acid and phospholipid metabolism**

| Locus tag | Gene | Description | Fold change |
|-----------|------|-------------|-------------|
| APL_1407  | Psd  | Phosphatidylycerine decarboxylase | -1.419       |
| APL_1384  | fabH | 3-oxoacetyl-[acyl-carrier-protein] synthase 3 | -1.826       |
| APL_1385  | plsX | Fatty acid/phospholipid synthesis protein PlsX | -2.706       |

**Mobile and extrachromosomal element functions**

| Locus tag | Gene | Description | Fold change |
|-----------|------|-------------|-------------|
| APL_1056  | APL_1056 | Transposase | 1.560       |
| APL_0985  | APL_0985 | Transposase | 1.271       |

**Protein fate**

| Locus tag | Gene | Description | Fold change |
|-----------|------|-------------|-------------|
| APL_0871  | pepE | Peptidase E | 2.551       |
| APL_1101  | pepA | Putative cytosol aminopeptidase | 1.913       |
| APL_0254  | pepD | Aminoacyl-histidine dipeptidase | 1.903       |
| APL_1883  | ptrA | Protease 3 precursor | 1.680       |
| APL_0928  | hscB | Co-chaperone protein HscB-like protein | 1.377       |
| APL_1068  | secF | Protein-export membrane protein SecF | -1.496     |
| APL_0321  | dsbB | Disulfide bond formation protein B | -1.557     |
| APL_1035  | pflA | Pyruvate formate-lyase 1-activating enzyme | -1.774     |

**Protein synthesis**

| Locus tag | Gene | Description | Fold change |
|-----------|------|-------------|-------------|
| APL_1821  | rpmE | 50S ribosomal protein L31 | 2.211       |
| APL_0484  | rimK | Ribosomal protein S6 modification protein | 1.533       |
| APL_1781  | rpsM | 30S ribosomal protein S13 | -1.401     |
| APL_0205  | APL_0205 | Predicted RNA methyltransferase | -1.538     |
| APL_0399  | ksgA | Dimethyladenosine transferase | -1.578     |
| APL_0679  | glnS | Glutamyl-tRNA synthetase | -1.584     |
| APL_0641  | traB | tRNA pseudouridine synthase B | -1.742     |
| APL_1383  | trnB | tRNA (guanine-N(7)-)-methyltransferase | -1.756     |
| APL_0574  | APL_0574 | tRNA-specific adenosine deaminase | -1.778     |
| APL_0723  | Tgt  | Queuine tRNA-ribosyltransferase | -1.937     |

**Purines, pyrimidines, nucleosides, and nucleotides**

| Locus tag | Gene | Description | Fold change |
|-----------|------|-------------|-------------|
| APL_0958  | purH | Bifunctional purine biosynthesis protein PurH | 1.856       |
| APL_0593  | guaB | Inosine-5'-monophosphate dehydrogenase | 1.485       |
| APL_1343  | Cdd | Cytidine deaminase | 1.278       |
| APL_1014  | deoD | Purine nucleoside phosphorylase DeoD-like protein | -1.430     |
| APL_0351  | Ndk | Nucleoside diphosphate kinase | -1.531     |
| APL_1839  | Udp | Uridine phosphorylase | -1.617     |
| APL_1075  | purA | Adenylosuccinate synthetase | -1.762     |

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Continued on next page
| Locus tag   | Gene | Description                                           | Fold change |
|-------------|------|-------------------------------------------------------|-------------|
| **Regulatory functions**                                      |                      |                         |             |
| APL_0059    | narP | Nitrate/nitrite response regulator protein            | 2.552       |
| APL_0823    | glpR | Glycerol-3-phosphate regulon repressor                | 1.908       |
| APL_1295    | argR | Arginine repressor                                    | 1.896       |
| APL_0126    | APL_0126 |                                               | 1.580       |
| APL_0395    | rseA | Putative sigma-E factor negative regulatory protein    | 1.524       |
| APL_1668    | rbsR | Ribose operon repressor                                | 1.302       |
| APL_1270    | sprT | Putative SprT-like protein                            | −1.483      |
| APL_1233    | malT | HTH-type transcriptional regulator MalT               | −1.484      |
| APL_1540    | tldD | TldD-like protein                                     | −1.578      |
| **Transcription**                                           |                      |                         |             |
| APL_0560    | rhlB | ATP-dependent RNA helicase RhlB                       | 1.409       |
| APL_0423    | rnhA | Ribonuclease HI                                       | 1.345       |
| APL_0201    | nusB | Transcription antitermination protein NusB            | −1.457      |
| **Transport and binding proteins**                          |                      |                         |             |
| APL_0967    | gltS | Sodium/glutamate symport carrier protein               | 4.155       |
| APL_0377    | glpT | Glycerol-3-phosphate transporter                       | 3.247       |
| APL_0664    | dppA | Periplasmic dipeptide transport protein                | 3.168       |
| APL_0869    | abgB | Aminobenzoyl-glutamate utilization-like protein        | 3.004       |
| APL_1857    | merP | Copper chaperone MerP                                 | 2.911       |
| APL_0068    | dppF | Dipeptide transport ATP-binding protein DppF          | 2.860       |
| APL_1665    | gntP_1 | Glucanate permease                                     | 2.723       |
| APL_0066    | dppC | Dipeptide transport system permease protein DppC      | 2.640       |
| APL_1440    | znuA | High-affinity zinc uptake system protein ZnuA precursor | 2.600       |
| APL_0065    | dppB | Dipeptide transport system permease protein DppB      | 2.229       |
| APL_0067    | dppD | Dipeptide transport ATP-binding protein DppD          | 2.036       |
| APL_1448    | afuC | Ferric ABC transporter ATP-binding protein             | 1.855       |
| APL_1319    | ptsB | PTS system sucrose-specific EIIBC component           | 1.744       |
| APL_1320    | thiQ | Thiamine transport ATP-binding protein ThiQ            | 1.569       |
| APL_1622    | cbIM | Predicted ABC transport permease protein CbiM         | 1.433       |
| APL_1620    | cbIO | Predicted ABC transport ATP-binding protein CbiO       | 1.417       |
| APL_1173    | pmuC | Nicotinamide mononucleotide transporter                | 1.408       |
| APL_0749    | APL_0749 |                                               | −1.436      |
| APL_1212    | tehA | Tellurite resistance protein TehA                     | −1.543      |
| APL_0716    | APL_0716 |                                               | −1.547      |
| APL_1253    | APL_1253 |                                               | −1.598      |
| APL_1846    | cysT | Sulfate transport system permease protein cysT         | −1.684      |
| APL_0191    | APL_0191 |                                               | −1.751      |
| APL_1083    | arcD | Putative arginine/ornithine antiporter                | −1.786      |
| APL_2016    | fhuA | Ferrichrome-iron receptor FhuA                        | −2.031      |
| APL_1847    | cysW | Sulfate transport system permease protein cysW        | −2.195      |
| APL_1844    | cysN | Sulphate adenylyl transferase subunit 1               | −2.375      |
| APL_1848    | cysA | Sulfate/thiosulfate import ATP-binding protein cysA   | −2.401      |
| APL_1843    | cysJ | Sulfite reductase [NADPH] flavoprotein alpha-component | −2.757      |
| APL_1127    | APL_1127 |                                               | −3.402      |

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| Locus tag | Gene     | Description                                      | Fold change |
|-----------|----------|--------------------------------------------------|-------------|
| APL_1100  | APL_1100 | Hypothetical protein                              | 3.395       |
| APL_0920  | APL_0920 | Hypothetical protein                              | 2.835       |
| APL_1882  | APL_1882 | Hypothetical protein                              | 2.776       |
| APL_1856  | APL_1856 | Hypothetical protein                              | 2.775       |
| APL_1855  | APL_1855 | Hypothetical protein                              | 2.763       |
| APL_0443  | APL_0443 | Autotransporter adhesin                           | 2.762       |
| APL_1252  | APL_1252 | Hypothetical protein                              | 2.739       |
| APL_0134  | APL_0134 | Hypothetical protein                              | 2.681       |
| APL_0836  | APL_0836 | Putative transcriptional regulator                | 2.661       |
| APL_1588  | APL_1588 | Predicted TRAP transporter solute receptor        | 2.464       |
| APL_1491  | APL_1491 | Hypothetical protein                              | 2.282       |
| APL_0104  | APL_0104 | Autotransporter adhesin                           | 2.231       |
| APL_1069  | fnA      | Ferritin-like protein 1                           | 2.194       |
| APL_1059  | APL_1059 | Hypothetical transposase-like protein             | 2.172       |
| APL_1690  | APL_1690 | Inner membrane protein                            | 2.168       |
| APL_0245  | APL_0245 | Transferrin binding protein-like solute binding   | 2.097       |
| APL_1191  | namA     | NADPH dehydrogenase                               | 2.078       |
| APL_1948  | APL_1948 | Hypothetical protein                              | 2.061       |
| APL_0870  | APL_0870 | Putative C4-dicarboxylate transporter             | 2.034       |
| APL_0643  | APL_0643 | Hypothetical protein                              | 2.029       |
| APL_1743  | APL_1743 | Ser/Thr protein phosphatase family protein        | 1.999       |
| APL_0426  | APL_0426 | Hypothetical protein                              | 1.994       |
| APL_1791  | APL_1791 | Putative periplasmic iron/siderophore binding     | 1.944       |
| APL_0970  | APL_0970 | Hypothetical protein                              | 1.908       |
| APL_1070  | fnB      | Ferritin-like protein 2                           | 1.907       |
| APL_1894  | APL_1894 | Hypothetical protein                              | 1.907       |
| APL_1374  | APL_1374 | Hypothetical protein                              | 1.803       |
| APL_1206  | APL_1206 | Plasmid stability-like protein                    | 1.794       |
| APL_1881  | APL_1881 | Hypothetical protein                              | 1.792       |
| APL_0038  | APL_0038 | Hypothetical protein                              | 1.730       |
| APL_1355  | APL_1355 | Hypothetical protein                              | 1.716       |
| APL_0471  | APL_0471 | Hypothetical protein                              | 1.707       |
| APL_1438  | APL_1438 | Hypothetical protein                              | 1.689       |
| APL_1437  | APL_1437 | Hypothetical protein                              | 1.643       |
| APL_1423  | APL_1423 | Hypothetical protein                              | 1.612       |
| APL_0125  | APL_0125 | Hypothetical protein                              | 1.608       |
| APL_0096  | APL_0096 | Zinc transporter family protein ZIP                | 1.592       |
| APL_0220  | APL_0220 | Putative lipoprotein                              | 1.583       |
| APL_1934  | APL_1934 | Hypothetical protein                              | 1.570       |
| APL_1574  | APL_1574 | Hypothetical protein                              | 1.543       |
| APL_0036  | APL_0036 | Hypothetical protein                              | 1.533       |
| APL_0222  | APL_0222 | Putative lipoprotein                              | 1.518       |
| APL_1088  | APL_1088 | Hypothetical protein                              | 1.512       |
| APL_1207  | APL_1207 | Hypothetical protein                              | 1.510       |
| APL_0463  | APL_0463 | Predicted sortase and related acyltransferases    | 1.448       |
| APL_1859  | APL_1859 | Probable NADH-dependent butanol dehydrogenase 1   | 1.448       |
| APL_1828  | APL_1828 | PiiT protein-like protein                         | 1.447       |

Continued on next page
by zinc (data not shown). Interestingly, genes potentially involved in zinc transport (znuA and APL_0096) were up-regulated after growth in BHI-B (Tab. II).

### 4. DISCUSSION

Biofilm formation is an important virulence trait of many bacterial pathogens including *A. pleuropneumoniae*. It has been previously reported that only 2 of the 15 *A. pleuropneumoniae* reference strains, representing serotypes 5b and 11, were able to form a biofilm in vitro [21]. We observed however an increased stickiness of colonies when strain *A. pleuropneumoniae* S4074 was grown on plates made of BHI from one of two different suppliers. In addition, Li et al. [24] recently observed slight biomass of biofilm when the *A. pleuropneumoniae* serotype 1 reference strain S4074 was grown in serum-free TSB and that an enhanced biofilm formation was observed in *luxS* [24] and *hns* [8] mutants of *A. pleuropneumoniae* S4074. These observations brought us to re-evaluate biofilm formation by strain *A. pleuropneumoniae* S4074 under different growth conditions using a standard microtiter plate and crystal violet staining protocol. Our data indicate that strain S4074 has the ability to form a pronounced biofilm when grown in the appropriate conditions, and that the biofilm was sensitive to DspB treatment and can be inhibited by zinc. Transition from a biofilm-positive to a biofilm-negative phenotype is not irreversible in contrast to what was reported by Kaplan and Mulks [21] under different conditions.

Transcript profiling experiments using DNA microarrays indicated that overall, 232 genes were significantly differentially expressed during growth in BHI-B. Genes such as *tadC*, *tadD*, genes with homology to autotransporter adhesins as well as genes *pgaABC* involved in PGA biosynthesis were up-regulated after

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### Table II. Continued.

| Locus tag | Gene | Description | Fold change |
|-----------|------|-------------|-------------|
| APL_0433  | *msrB* | Methionine sulfoxide reductase B | 1.415 |
| APL_1189  | *APL_1189* | Hypothetical protein | 1.393 |
| APL_0090  | *APL_0090* | Hypothetical protein | 1.360 |
| APL_1709  | *APL_1709* | Hypothetical protein | −1.307 |
| APL_0357  | *APL_0357* | Hypothetical protein | −1.328 |
| APL_1380  | *APL_1380* | Hypothetical protein | −1.394 |
| APL_1729  | *APL_1729* | Hypothetical protein | −1.401 |
| APL_1062  | *APL_1062* | Hypothetical protein | −1.468 |
| APL_0179  | *APL_0179* | Hypothetical protein | −1.481 |
| APL_0940  | *APL_0940* | Hypothetical protein | −1.482 |
| APL_1273  | *APL_1273* | Putative fimbrial biogenesis and twitching motility protein PilF-like protein | −1.488 |
| APL_1131  | *APL_1131* | Hypothetical protein | −1.540 |
| APL_0583  | *APL_0583* | Hypothetical protein | −1.585 |
| APL_1096  | *APL_1096* | Hypothetical protein (59% ID dispersine B) | −1.594 |
| APL_0936  | *APL_0936* | Hypothetical protein | −1.616 |
| APL_1115  | *APL_1115* | Hypothetical protein | −1.639 |
| APL_0811  | *APL_0811* | Hypothetical protein | −1.682 |
| APL_1898  | *ap2029* | Hypothetical protein | −1.798 |
| APL_1654  | *gidB* | Methyltransferase GidB | −1.816 |
| APL_0340  | *APL_0340* | Hypothetical protein | −1.893 |
| APL_1381  | *APL_1381* | Hypothetical protein | −1.926 |
| APL_0053  | *typA* | GTP-binding protein | −2.043 |
| APL_1681  | *APL_1681* | Hypothetical protein | −2.233 |
growth in BHI-B. While we can hypothesize that these genes might be important for the formation of the biofilm itself, it is also interesting to note that many of the same genes (\textit{tadB}, \textit{rcpA}, gene APL_0443 with high homology to the Hsf autotransporter adhesin of \textit{Haemophilus influenzae} as well as genes \textit{pgaBC} involved in biofilm biosynthesis) were up-regulated, when the transcriptomic profile of \textit{A. pleuropneumoniae} was determined after contact with porcine lung epithelial cells [1], thus emphasizing the possible importance of biofilm formation for the establishment of the infection.

Initial steps in biofilm development require the transcription, early on, of genes involved in reversible attachment and motility, before a subsequent switch towards the transcription of genes involved in the irreversible attachment of bacteria [35]. This second irreversible attachment might require the synthesis of adhesive organelles, such as the curli fibers (\textit{csg} genes). Interestingly, gene APL_0220 is a putative lipoprotein of the CsgG family, responsible for the transport and assembly of curli fibers. The up-regulation of other genes possibly involved in adhesion processes (\textit{tadC}, \textit{tadD}, Hsf homolog APL_0443) might indicate that bacterial cells were entering or in the middle of this irreversible attachment phase. In \textit{A. actinomyce- tenucomitans}, the Tad locus is essential for biofilm formation [32]. The fact that the transcription of a zinc-specific transporter (\textit{znuA}) was increased, combined with the decrease in transcription of an hypothetical Zn-dependant
protease (APL_1898) and lower concentration of this metal in BHI-B lead us to believe that Zn restriction might be a signal leading to increase biofilm formation.

It is tempting to speculate that growth in BHI-B affected the expression of regulators which in turn affected PGA expression and biofilm formation. Indeed, it has been recently shown that an enhanced biofilm formation was observed in a hns mutant of *A. pleuropneumoniae* strain S4074 [8] and that over-expression of RpoE in a rseA mutant is sufficient to alleviate repression of biofilm formation by H-NS\(^1\). However, other genes have been shown to affect biofilm formation in *A. pleuropneumoniae*. An enhanced biofilm formation was observed in a quorum sensing (*luxS*) mutant [24] while a mutant in the ArcAB two-component system facilitating metabolic adaptation to anaerobicity (*arcA*) [5] and an autotransporter serine protease (AasP) mutant were deficient in biofilm formation [31]. It is interesting to note that many genes involved in branched-chain amino acid biosynthesis (*ilv* genes) were up-regulated after growth in BHI-B. Limitation of branched-chain amino acids was shown to be a cue for expression of a subset of in vivo induced genes in *A. pleuropneumoniae*, including not only genes involved in the biosynthesis of branched-chain amino acids, but also other genes that are induced during infection of the natural host [34].

Our data indicate that many strains of *A. pleuropneumoniae* have the ability to form biofilms under appropriate growth conditions. This is an important observation considering that *A. pleuropneumoniae* biofilm cells exhibit increased resistance to antibiotics compared to planktonic cells [17] and may also exhibit increased resistance to biocides [12]. Biofilms are often associated with chronic infections but the fact that *A. pleuropneumoniae* can form an important biofilm after only 6 h of incubation suggests that biofilm formation might also play a role in acute infections.

We have undertaken the screen of a large library of mini-Tn10 isogenic mutants of *A. pleuropneumoniae* S4074 in order to identify other genes that are involved in biofilm

\(^1\) Bosse J.T., Sinha S., O’Dwyer C.A., Rycroft A.N., Kroll J.S., Langford P.R., H-NS is a specific regulator of biofilm formation in *Actinobacillus pleuropneumoniae*, Proceedings of the International Pasteurellaceae Society meeting, Sorrento, Italy, 2008, p. 110.
formation and/or regulation. A better understanding of biofilm formation in *A. pleuropneumoniae* might lead to the development of molecules or strategies to interfere with biofilm formation and prevent infection in pigs. In that respect, we made an important, and unexpected, observation that zinc could completely inhibit biofilm formation in *A. pleuropneumoniae* and *A. actinomycetemcomitans*, which also synthesizes PGA [20]. We do not know at this time how zinc interferes with PGA biosynthesis and biofilm formation but some glycosyltransferases have been shown to be inhibited by zinc [23]. Hypozincemia which occurs during infection and inflammation [26] might therefore favour biofilm formation by *A. pleuropneumoniae*. Knowing that PGA functions as a biofilm matrix polysaccharide in phylogenetically diverse bacterial species such as *Staphylococcus aureus*, *S. epidermidis*, and *Escherichia coli* [20], it would be worth investigating whether zinc can also interfere with PGA biosynthesis in these other bacterial pathogens.

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