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Human fluids alter DNA-acquisition in *Acinetobacter baumannii*

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ABSTRACT

Transformation is one of the mechanisms of acquisition of foreign genetic material leading to the emergence of multidrug resistant (MDR) bacteria. Recently, human serum albumin (HSA) was shown to specifically increase transformation frequency in the nosocomial pathogen *Acinetobacter baumannii*. To further assess the relevance of HSA as a possible modulator of *A. baumannii* transformation in host-pathogen interactions, in this work we examined the effect of different human fluids. We observed a significant increase in transformation frequencies in the presence of pleural fluid, whole blood cells and liquid ascites, and to a lesser extent with urine. The observed effects correlate with both HSA and bacterial content found in the assayed patient fluids. Taken together, these results are in agreement with our previous findings that highlight HSA as a possible host signal with the ability to trigger natural transformation in *A. baumannii*.

1. Introduction

Acinetobacter baumannii is a pathogen associated with severe multi-drug-resistant (MDR) infections with mortality levels as high as 60%. Last year, *A. baumannii* topped The World Health Organization (WHO) high priority list of resistant pathogens for antibiotic research and development [1, 2].

Among the genus *Acinetobacter*, natural transformation has been mainly studied in *Acinetobacter baylyi*, a non-pathogenic *A. baumannii* relative [3]. Subsequently, our group has studied this mechanism in the naturally competent clinical strain *A. baumannii* A118 [4, 5], showing that a) this clinical isolate can acquire different DNA sources [4, 6, 7],; b) albumin is a specific inducer of natural competence*;* and c) sub-inhibitory concentrations of meropenem, one of the last resources of antibiotics, increases transformation frequency in all strains tested [7-9].

Recently, several *in-vitro* studies have shown that *A. baumannii* can respond to extracellular stimuli such as bile salts, mucin, light, antibiotics and human serum, among others, modifying the expression of genes involved in biofilm formation, degradation of phenylacetic acid, metabolic pathways, and genes coding for the Type VI secretion system (T6SS) [10, 11]. In addition, *in-vivo* studies performed with a life-threatening bacteremia animal model using *A. baumannii* ATCC 17878-infected mice showed up-regulation of genes associated with three iron uptake systems, whereas genes related to metabolism, quorum sensing, and biofilm formation were down-regulated, highlighting the ability of *A. baumannii* to adapt to fluctuating environments [12].

Previous findings from our group identified both bovine and human serum albumin as inducers of natural transformation in *A. baumannii,* evidence supported by the induction of two competence genes*, comEA* (a small DNA-binding periplasmic protein important for DNA uptake) and *pilQ* (the outer secretin protein found in type IV pili that allows double-stranded DNA to enter into the periplasm) [7, 9]. Notably, casein, extracellular matrix/basal membrane components, as well as norepinephrine and mucin did not significantly enhance the transformation rate of this bacterium, showing that albumin effect is specific

[9]. These observations support the idea that HSA is a host component that enhances acquisition of foreign genetic material, thus increasing the ability of the bacterium to adapt to environmental conditions. To further assess this notion, in the present work we analyzed the effect of different HSA-containing human fluids on transformation frequency and competence-related gene expression.

2. Materials and methods

2.1. Bacterial strains

Acinetobacter baumannii clinical strains A118 and A42 were used for transformation assays. A118 and A42 were recovered from a blood sample and an endotracheal aspirate, respectively [4]. Both strains are kanamycin-susceptible [4, 5]. Total genomic DNA from *A. baumannii* Ab144 and plasmid DNA from *Escherichia coli* TOP10 cells harboring pDsRedAK were obtained using Wizard® Genomic DNA Purification Kit and QIAprep Spin Miniprep Kit following manufacturer instructions (Promega, Madison, WI and Qiagen Germantown, MD, USA*),* respectively.

2. 2. Natural transformation assays

Natural transformation assays were done as previously described [4]. Briefly, 50 µL of late stationary-death phase cultures of *A. baumannii* cells were transferred to 50 µL of sterile Luria-Bertani (LB) with 100 ng of the pDsRed plasmid DNA and/or gDNA. These cultures were incubated for 1 hour at 37°C and then plated on LB agar with 10 µg/mL kanamycin. Transformation events were scored by counting Kan^R colonies, while total CFUs was assessed by plating serial dilutions on LB agar. Negative controls with no DNA addition were included in every tested condition. Different host fluids were assayed including pleural fluid (PF) 4%, human whole blood (HWB) 0.2%, ascites fluid (AF) 4%, urine (U) 4%, nasal fluid (NA) 0.2%, all from Innovative research (MI, CA, USA). All experiments were performed in triplicate and statistical analysis was performed. Transformation events were scored as mentioned above.

2.3. Real-time RT-qPCR.

Previously extracted, and DNase treated RNA from *A. baumannii* strains A118 was synthesized to cDNA using the manufacturer protocol provided within the iScript™ Reverse Transcription Supermix for RT-qPCR (BioRad, Hercules, CA, USA). The cDNA concentrations were measured with a DeNovix DS-11+ Spectrophotometer; each sample was then diluted to a concentration of 50ng/uL. Real-time quantitative PCR (RT-qPCR) was conducted using the iQ™SYBR® Green Supermix through manufacturer's instructions. At least 3 biological replicates of cDNA were used and were run in quadruplet. All samples were then run on the CFX96 Touch™ Real-Time PCR Detection System (BioRad, Hercules, CA, USA).

The *comEA* and *pilQ* -two selected competence genes- transcript levels of each sample were normalized to the *recA* rRNA transcript levels for each cDNA sample. The relative quantification of gene expression was performed using the comparative threshold method $2^{-\Delta\Delta Ct}$. The ratios obtained after normalization were expressed as folds of change compared with cDNA samples isolated from bacteria cultures on LB. Statistical analysis (Mann-Whitney test) was performed using GraphPad Prism (GraphPad software, San Diego, CA, USA). A *P*-value < 0.05 was considered significant.

2.4. Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical analysis (Mann-Whitney test) was performed using GraphPad Prism (GraphPad software, San Diego, CA, USA). A *P*-value < 0.05 was considered significant.

3. Results and discussion

In order to assess the possible effect of HSA-containing host fluids on competence during *A. baumannii* colonization/infection, here, we examined various human fluids where *A. baumannii* can be recovered. Accordingly, we first analyzed data stating the frequency of *A. baumannii* collected between January 2017 to June 2018 in a teaching hospital that allocates 400 beds. From a total of

5,700 positive samples comprising urine (3,075), human blood (1,652), sputum (251), bronchoalveolar lavage (297), tracheal aspirate (307), abdominal fluid (100), and pleural fluid (18), the largest *A. baumannii* contents were found in the bronchoalveolar lavage (17.17%), tracheal aspirate (19.22%) and pleural fluid (PF) (16.67%). On the other hand, samples acquired from blood, sputum, abdominal liquid, and urine had percentages of 1.94, 7.57, 5 and 1.27%, respectively. Based on these observations, human pleural fluid, whole blood cells, ascites fluid, urine, and nasal fluid were chosen to test the effect of human host fluids on transformation frequency of *A. baumannii* strains A118 and A42.

3.1. Exposure to pleural fluid enhances transformation frequencies of A. baumannii and competence-associated gene expression

A. baumannii strains A118 and A42 were treated with PF using the largest fluid concentration (4%) permissive for the growth of *A. baumannii*. As shown in Fig. 1A, a statistically significant effect on transformation frequencies was observed in the presence of PF 4% in the two different strains when transformed with both DNA sources. Strain A118 showed increases in transformation frequency by 47.95- and 9.29-fold with plasmid and genomic DNA, respectively (*P*-value <0.05), whereas in strain A42 transformation was increased by 11.83- and 15.08-fold with the same DNA sources (Fig. 1A).

Our previous reports demonstrated that either bovine or human serum albumin have the ability to induce the expression of *comEA* and *pilQ*, two competence-related genes highly conserved within the *A. baumannii* group [7]. Considering the high HSA amounts present in PF (Supplementary Fig S1), we investigated whether the latter fluid could induce expression of the aforementioned competence-related genes. Accordingly, retrotranscription coupled to quantitative polymerase chain reaction (RT-qPCR) was performed in the presence or absence of PF 4%. As shown in Fig. 1B, we observed that the levels of *comEA* and *pilQ* transcripts were increased by 8.306- and 43.975-fold, respectively, upon exposition to this host fluid.

Pleural fluid is an essential lubricant that allows pleurae to function during respiratory movements. When excess fluid accumulates in the pleural cavity, an effusion occurs. Pleural effusions related to *Acinetobacter* pneumonia are common, and secondary parapneumonic effusion complications have also been reported [13]. In 2014, a multistate survey effort by the Centers for Diseases Control and Prevention (CDC) reported pneumonia to have the highest incidence of health care associated infections, with *A. baumannii* being the causative pathogen of 3.6% of cases [1]. Colonization of pleural fluid had been associated with considerable morbidity and, due to the increased risk of MDR infection, antibiotic treatment before microbial testing is extremely discouraged in patients with pleural effusion [14]. This is further supported by our results, as exposing *A. baumannii* to human pleural fluid, which normally contains between 50-70% albumin [7], significantly increased both transformation frequencies and expression of competence-related genes, thus increasing the probability of acquisition of foreign genetic material and contributing to its success as a pathogen in antibiotic-rich environments.

3.2. Human whole blood induces natural transformation in A. baumannii

Human whole blood was used at a concentration of 0.2% in order to avoid potential complement activation and opsonophagocytosis that could lead to bacterial death [15] (Supplementary Fig S1). A statistically significant increase in transformation frequencies of 4.9- and 3.45-fold was observed in strain A118 when transformed with plasmid and genomic DNA, respectively (*P*-value <0.05) (Fig. 2A). Although no statistical significance resulted from strain A42, it is noteworthy that a trend of induction of transformation was observed in this human fluid with both plasmid and genomic DNA, with increments by 1.74- and 2.18-fold transformation frequencies (Fig. 2B).

3.3. Ascites fluid increases natural transformation frequencies

To keep consistency with the assessment of PF performed in this study, AF was also tested at a percentage of 4%. As shown in Fig. 2A, we observed a

statistically significant increase in transformation frequencies of strain A118 with plasmid and genomic DNA by 5.25- and 9.45-fold, respectively. As observed with HWB, although no statistically significant, strain A42 showed a similar tendency with an increase in transformation frequencies by about 1.64- and 7.55-fold with the same DNA sources (Fig. 2B).

3.4. Human urine has a strain-specific effect on transformability of A. baumannii

Human urine was used at a concentration of 4%. As shown in Fig. 2A, an increase of transformation frequency by 1.52- and 1.63-fold was observed when A118 was transformed with both plasmid and genomic DNA, respectively. In A42, instead, a decrease in transformation frequency by 3.91- and 8.52-fold was observed, although showing no statistical significance.

In A118, the increase in transformation frequency could be explained in part by the presence of Ca^{2+} in urine (10-24 nmoles/l), since we previously demonstrated that $CaCl_2$ has the ability to induce both transformation frequencies and competence-related gene expression in the same strain [7]. It is noteworthy that such effects were previously observed with 1 mM ($1x10^6$ nmoles/l) Ca^{2+}, a concentration significantly higher than what is present in UR 4% (0.6 -14.4 nmoles/L), and, therefore, the extent of the induction observed in this work is proportional to the lower cation content in this human fluid, highlighting the strength of Ca^{2+} as a transformation inducer for *A. baumannii* A118. On the other hand, in strain A42, the observed opposite effect characterized by slight decrease in transformation frequencies in the presence of UR 4% suggests that the mechanism of Ca^{2+}-mediated modulation of transformation in *A. baumannii* may be strain-specific, as observed for T6SS and two-component regulatory system functionality, host colonization, multidrug efflux and biofilm formation [16-18].

In addition to the low Ca^{2+} concentrations, no traces of albumin were observed in UR 4% and, consistently with our previous data, the condition tested here could not be enough to trigger significant transformation events [7]. It is worth pointing out that serum albumin transports calcium, thereby exposing *A. baumannii* to

both elements in the host [19]. In this way, HSA may act as a signal triggering transcriptional responses, whereas calcium could positively impact on natural competence by binding to the foreign DNA and reducing electrostatic repulsion with the bacterial cell surface [20].

3.5 Nasal fluid did not have a significant effect on natural transformation

NF was used to test a human fluid that contains both albumin and also mucin (Supplementary Fig S1). As shown in Fig. 2A-B, 0.2% NF produced a slight, non-significant decrease of transformation frequency in both strains A118 and A42 when transformed with either plasmid or genomic DNA.

Similarly to mucin, nasal fluid is known to have a protein content in the range of 414-895 mg/100mL, from which lysozymes represent 10-30% of the total amount. Lysozymes are capable of breaking ß-1,4 linkages of the *A. baumannii* cell wall, thus degrading the pathogenic cell and completely prohibiting the chance for transformation. Mucin and nasal fluid also utilize the mucous layer developed to act as a barrier that prevents any undesired interactions with pathogenic bacteria. Accordingly, any interaction between *A. baumannii* and free roaming DNA from its environment would be disrupted as well [19]. Therefore, both the previous results and those obtained here with mucin, where we observed a decrease in transformation frequency, are in correlation with each other [9].

4. Conclusions

Pleural fluid, nasal fluid, human whole blood, human urine and ascites fluid were selected for this study due to their role and impact during bacterial infection in a human host. Fluids with a significant albumin composition such as pleural fluid, ascites fluid and whole blood notably increased transformation frequencies within two representative strains of *A. baumannii,* A118 and A42. However, human fluids that had little to no traces of albumin, such as urine and nasal fluid, had a lesser impact on natural transformation within the species. This further confirms the inductive role of albumin in horizontal gene transfer of *A. baumannii*, and

even suggests the likelihood of gene acquisition of *A. baumannii in vivo* during respiratory and blood infections by the naturally competent pathogen.

So far, the mechanisms by which albumin aids in *A. baumannii* natural transformation remain unknown. We hypothesize that either albumin or albumin-derived peptides can induce the expression of competence-associated genes leading to an increase in natural transformation by a not yet described intracellular-signaling pathway (Fig. 3). This works contributes to a further understanding on albumins effect on *A. baumannii* competence associated genes. PF 4% has shown to have a positive regulatory effect on on *A. baumannii pilQ* and *comEA* gene expression. *pilQ* allows for the entry of genetic material into the bacterial cell and contributes to the expression of twitching motility in *A. baumannii*, whereas *comEA* further aids in guiding genetic material towards the periplasmic region of the cell, thus allowing for greater success in the entry and integration of genetic material into the *A. baumannii* genome.

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Figure 1. Effects of pleural fluid on *A. baumannii* natural transformation and differential expression of competence genes *PilQ* and *comEA.* A) Transformation assays were performed with LB and LB plus PF 4%. Cultures were transformed with either plasmid (black) or genomic (grey) DNA and plated onto LB agar plates supplemented with 10ug/ml of kanamycin. Experimental controls are presented as solely LB broth. Data is presented as means and error bars represent standard deviation. Asterisks represent statistical significance with a p- value <0.05 (Mann Whitney t test (n=3 to 13). B) Real-time Quantitative PCR was performed to identify the variance in expression levels of *A. baumannii* strains A118 and A42 competence genes *PilQ* and *comEA* in exposure to PF 4%. Fold changes were calculated using the double $^{\triangle}$Ct analysis. Data are presented as the mean and the errors bars represent standard deviation. At least three independent samples were used, and four technical replicates were performed from each sample. Asterisks represent statistical significance (*P*-value <0.05) as determined by the Mann Whitney t-test (n= 3 to 4).

Figure 2. Natural transformation frequencies with host human fluids.
Transformation assays of *A. baumannii* strain A) A118 and B) A42 were performed with LB broth, LB plus HWB 0.2%, LB plus AF 4%, LB plus UR 4%, and LB plus NF 0.2%. Experimental controls were transformed solely with LB broth. Cultures were transformed with plasmid DNA (black) or genomic DNA (grey) and plated on LB agar supplemented with 10ug/mL of kanamycin. CFUs were plated on LB agar. The data is presented as the mean and error bars represent standard deviation. At least three independent replicates were conducted and asterisks above represent statistical significance with a *P*-value <0.05 (Mann Whitney t test (n=3 to 13).

Figure 3. Schematic representation of albumin effect on natural transformation.

Supplementary Material

Figure S1. SDS-PAGE of human fluids. Human fluids; human serum albumin (HSA), pleural fluid (PF), ascites fluid (AF), urine (UR), human whole blood (HWB), and nasal fluid (NF) were diluted and resuspended in 1X PBS. Fluids were visualized on SDS-PAGE, from left to right: Ladder (Ladd), 0.2% HSA, 0.2% PF 4% PF, 0.2% AF, 4% AF, 4% UR, 0.2% HWB, and 0.2% NF.

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Highlights

- A significant increase in transformation frequencies in *A. baumannii* strains in the presence of pleural fluid, whole blood cells, and liquid ascites was observed.
- human serum albumin, present in different human fluids, can trigger natural transformation in *A. baumannii*.
- Plueral fluid has shown to have an upregulating effect on *A. baumannii* genes, *pilQ* and *comEA*.

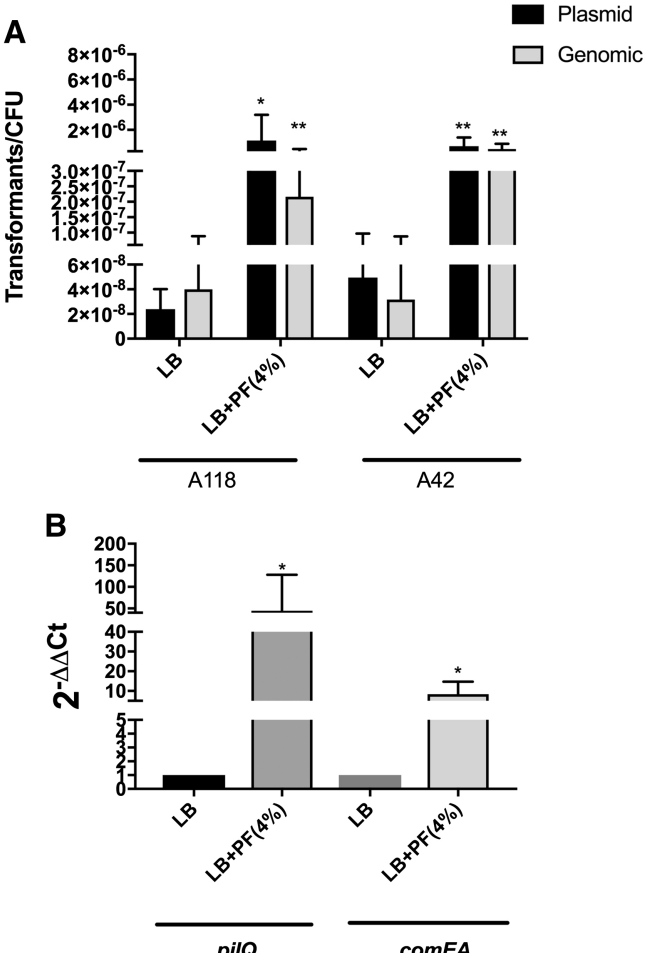
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Figure 1

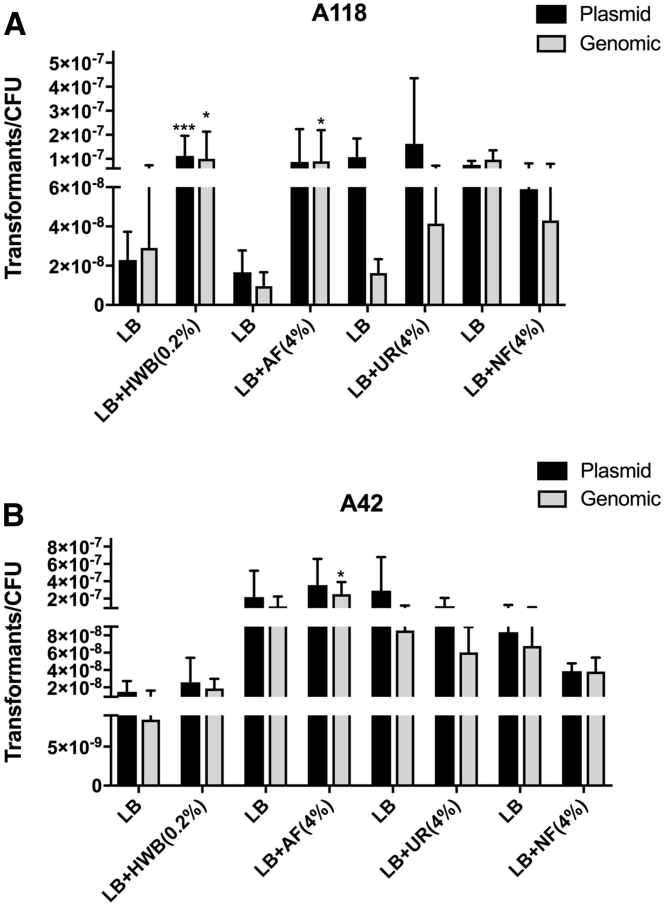
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Figure 2

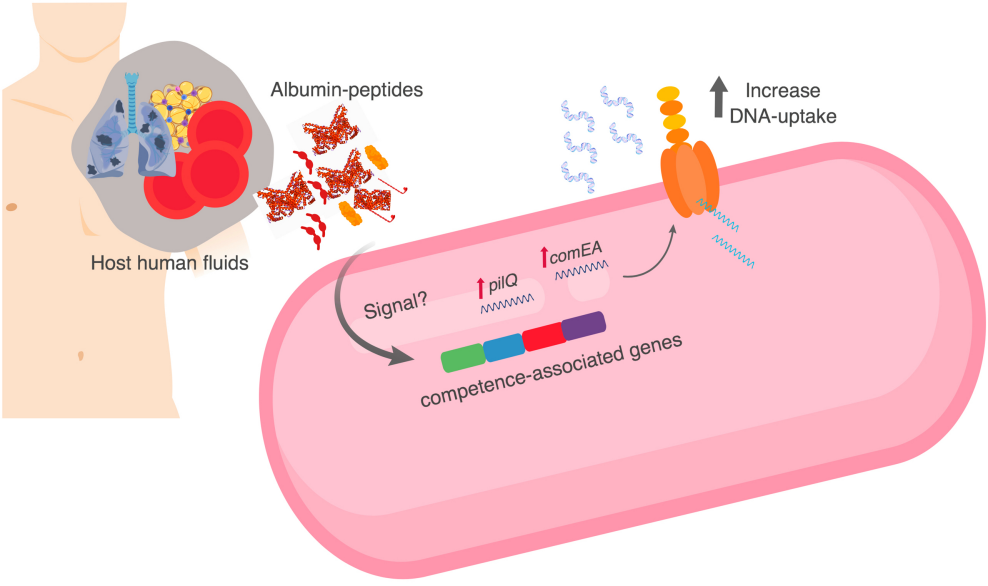
Albumin-peptides
Host human fluids
Increase
DNA-uptake
Signal?
pilQ
comEA
competence-associated genes

Figure 3

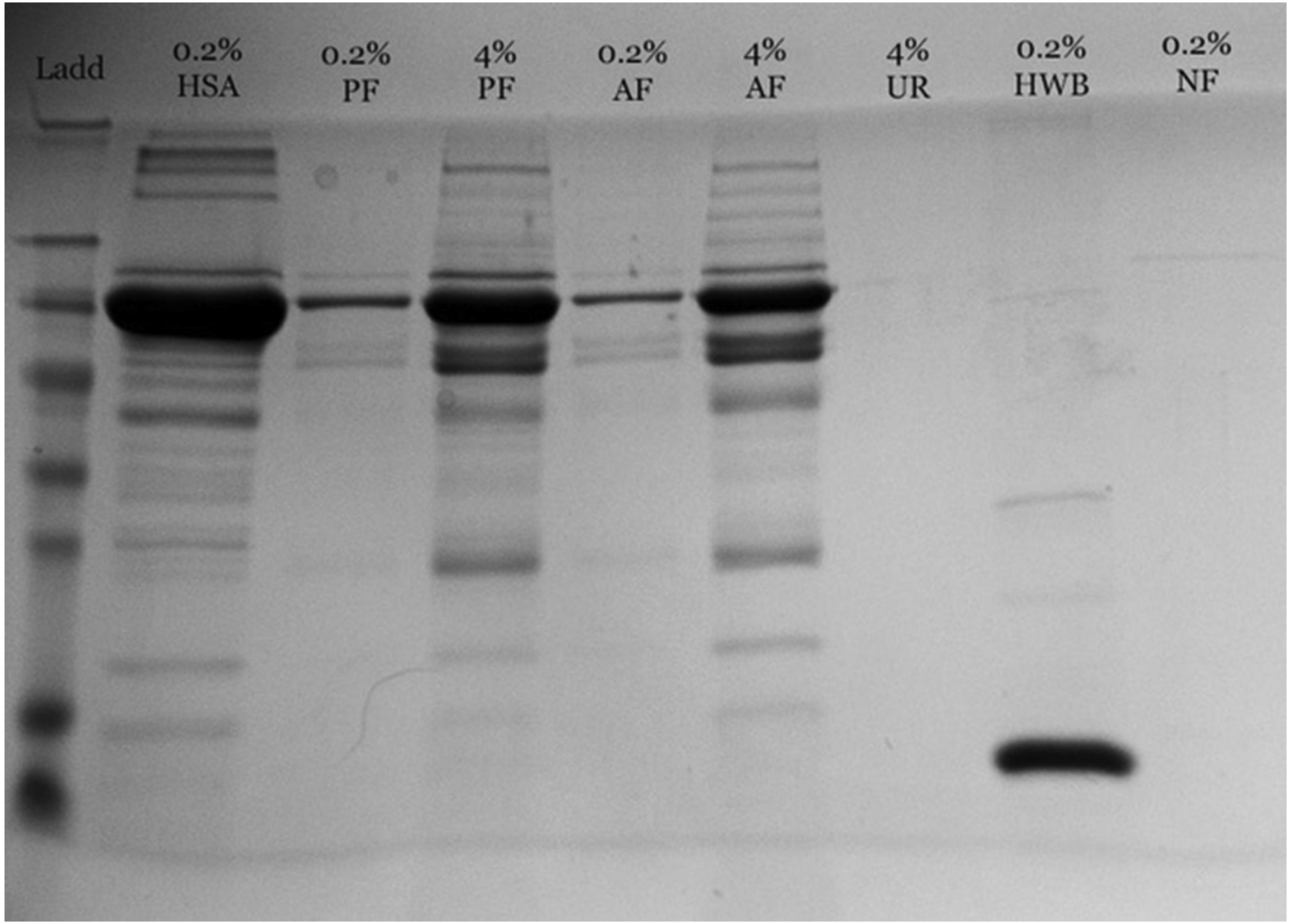

Figure 4