Novel Bacterial Protein Extraction Platform for Proteomic Analysis

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ABSTRACT:

S. pneumoniae is a Gram positive coccus, one of the leading causes of bacterial pneumonia, otitis media and meningitis. Bacterial resistance to antibiotics is of major concern as multidrug resistance has increased substantially in recent years. A more detailed understanding of how an antibiotic affects the cellular proteins could provide insights into ways to circumvent the development of resistance. In order to detect changes in the bacterial proteome resulting from antibiotic treatment, the ability to obtain micrograms of protein is critical. We have developed a novel methodology of combining extraction reagents, detergents and pressure cycling to reproducibly extract soluble cytoplasmic proteins away from the bacterial cell wall. The extracted proteins were then analyzed with 2D gel electrophoresis (2DE) and MS identification of differentially expressed proteins across samples treated with multiple antibiotic delivery regimens. A bolus dose of 40 ug/mL (~5X the MIC of 6.7 ug/mL for S. pneumoniae I6891) was given in the 1X dosing bolus with three evenly spaced (2 hour intervals) doses of 13.3 ug/mL given in the 3P (pulsatile) dosing scheme. This validated extraction methodology will allow highly purified bacterial proteins for more detailed proteomic studies in many important clinical conditions.

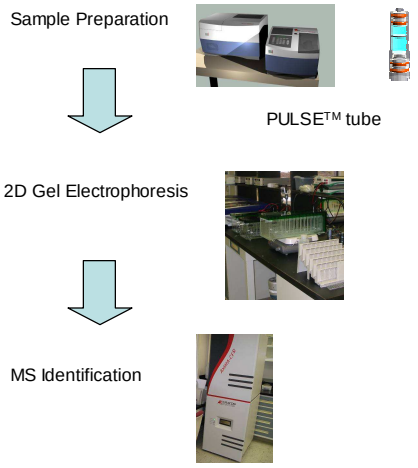


Diagram 1. Experimental Work Flow

METHODS:

Bacterial cell pellets before and after treatment with amoxicillin (~10^8 cfu) were suspended in 500 ul of 10 mM Tris, 1mM EDTA containing 0.5 mg/ml lysozyme (Sigma L-6876, St. Louis, MO), 15 uM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) (Sigma, St. Louis, MO) and 50 U/ml benzonase (Qiagen, Valencia, CA) and mixed at 4°C for 1 hour. Extraction of proteins was performed by adding 1 ml of (1% w/v C7 (Sigma C-0856), 7M urea, 2M thiourea and 40mM Tris base) solution to 500 ul of treated sample from above and placing this mixture in PULSE™ tubes. Each sample in a PULSE™ tube was subjected to ten pressure cycles, each cycle consisting of 20 seconds at 35 kpsi followed by 20 seconds at ambient pressure. The lysates were then sonicated at 70% maximum power on ice 4 times, 10 seconds each and placed in 2.0 mL Eppendorf tubes for centrifugation at 2,500 g for 5 minutes, 20°C to remove foam from the sonication process. A subsequent 21,000 g spin for 10 minutes, 20°C was performed to remove the cellular debris and aggregates. The solubilized proteins were reduced and alkylated with 5 mM TBP and 10 mM acrylamide at room temperature for 90 minutes. The proteins were precipitated in 9 volumes of acetone at room temperature for 1 hour. Protein pellets were spun down at 3,000 g for 20 minutes, 20°C and resuspended in 150 ul of solution containing 2% w/v CHAPS (Sigma C-9426), 7M urea, 2M thiourea and 40mM Tris base. For Iso-electric Focusing (IEF) and 2DE, the protein conductivity was confirmed as less than 100 uS/cm and protein concentrations were determined by the Bradford Reagent (Sigma B-6916, St. Louis, MO.).

2DE was run to examine the quality of the extracted protein, using samples normalized to 0.35 mg/mL total protein by dilution into ion-exchanged 7M urea, 2M thiourea, 65 mM CHAPS (conductivity ≤ 100 uS/cm). Immobilized pH gradient (IPG) pH 3-10 and 4-7 were rehydrated overnight with 200 µL of the sample. IEF was performed for 95 kVh in a Bio-Rad IEF device (Bio-Rad Protean IEF Cell). The IPGs were equilibrated twice for 10 minutes each in SDS equilibration buffer (6 M urea, 2% SDS, 50 mM Tris-acetate buffer (pH 7.0), 0.01% bromophenol blue), prior to second dimension PAGE in 8-16% polyacrylamide gel. Following electrophoresis, the gels were fixed for two hours in 7% acetic acid / 10% methanol and stained with Sypro Ruby (Molecular Probes, Invitrogen Inc.) overnight; followed by destaining with 7% acetic acid / 10% methanol and imaged.(Bio-Rad Gel Doc EQ System).

RESULTS:

Table 1: Protein extraction from ~10^8 cfu bacteria, bolus and pulsed amoxicillin treatment

Protein concentration (mg/mL)	Treatment
1.8	control
1.8	control
1.9	amoxicillin treated 1X bolus dosing, 15 minutes
2.2	amoxicillin treated 1X bolus dosing, 2 hours 15 minutes
2.1	amoxicillin treated 1X bolus dosing, 4 hours 15 minutes
1.9	amoxicillin treated 3 P dosing, 15 minutes
1.9	amoxicillin treated 3 P dosing, 2 hours 15 minutes
1.6	amoxicillin treated 3 P dosing, 4 hours 15 minute

Figure 1: 2DE of bacterial proteins at pI 3-10

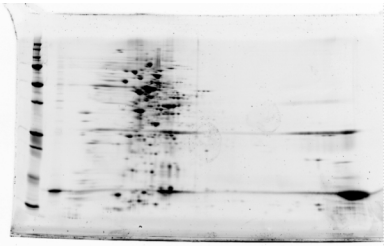


Figure 2: 2DE of bacterial proteins at pI 4-7

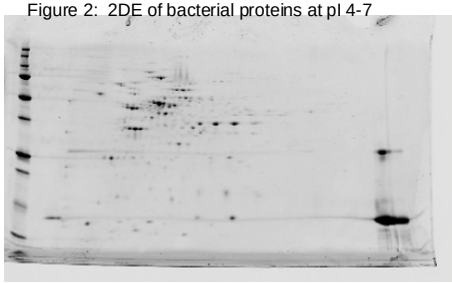
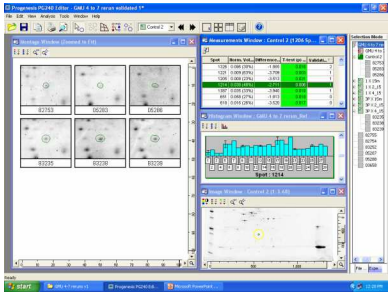


Figure 3: Progenesis software analysis of differentially expression proteins



CONCLUSION:

- The protocol can successfully extract reproducible amount of bacterial proteins across different treatment groups.
- The bacterial proteins, after careful sample preparation, can generate well resolved protein spots on 2D gels.
- Clear well resolved protein spots from 2DE allow the detection of differentially expressed proteins across treatment groups.