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Effects of bacterial inactivation methods on downstream proteomic analysis

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ABSTRACT

Inactivation of pathogenic microbial samples is often necessary for the protection of researchers and to comply with local and federal regulations. By its nature, biological inactivation causes changes to microbial samples, potentially affecting observed experimental results. While inactivation-induced damage to materials such as DNA has been evaluated, the effect of various inactivation strategies on proteomic data, to our knowledge, has not been discussed. To this end, we inactivated samples of *Yersinia pestis* and *Escherichia coli* by autoclave, ethanol, or irradiation treatment to determine how inactivation changes liquid chromatography-tandem mass spectrometry data quality as well as apparent protein content of cells. Proteomic datasets obtained from aliquots of samples inactivated by different methods were highly similar, with Pearson correlation coefficients ranging from 0.822 to 0.985 and 0.816 to 0.985 for *E. coli* and *Y. pestis*, respectively, suggesting that inactivation had only slight impacts on the set of proteins identified. In addition, spectral quality metrics such as distributions of various database search algorithm scores remained constant across inactivation methods, indicating that inactivation does not appreciably degrade spectral quality. Though overall changes resulting from inactivation were small, there were detectable trends. For example, one-sided Fischer exact tests determined that periplasmic proteins decrease in observed abundance after sample inactivation by autoclaving ($\alpha = 1.71 \times 10^{-2}$ for *E. coli*, $\alpha = 4.97 \times 10^{-4}$ for *Y. pestis*) and irradiation ($\alpha = 9.43 \times 10^{-7}$ for *E. coli*, $\alpha = 1.21 \times 10^{-5}$ for *Y. pestis*) when compared to controls that were not inactivated. Based on our data, if sample inactivation is necessary, we recommend inactivation with ethanol treatment with secondary preference given to irradiation.

1. Introduction

Mass spectrometry-based proteomics has become an increasingly important tool for examining changes in protein content of microorganisms under varied experimental conditions. However, many research groups do not have the necessary equipment and experience to perform proteomic analysis by mass spectrometry; therefore samples generated by one research group have to be shipped to a second group with proteomic capability. Regulations surrounding shipping and handling of microorganisms are dictated by the biological risk associated with each organism. Microorganisms are assigned biosafety levels (BSL) that range from 1–4 (Centers for Disease Control and Prevention, 2009; Zaki, 2010). The BSL assigned to each microorganism specifies the handling precautions and shipping requirements for a particular microorganism. For example, BSL-1 organisms such as non-pathogenic *Escherichia coli* can be handled on an open laboratory bench, as well as transferred to other facilities with minimal preparation. Organisms with a BSL rating of 2 or higher must be inactivated (non-viable and non-infectious state) before they can be removed from the laboratory. Sample inactivation also occurs in scenarios outside of research laboratories. Since the 2001 anthrax letter cases (in which letters containing live *Bacillus anthracis* were sent through the U.S. mail), mail sent to Congress, the White House, and other federal agencies is irradiated to kill potential biological agents (Government Accountability Office, 2008). As a result, samples that have been sent through the U.S. mail system, perhaps recovered in a forensic investigation, will have been inactivated by irradiation.

The effectiveness of irradiation and other procedures for inactivating microorganisms has been widely documented (Coohill and Sagripanti, 2008; Lasch et al., 2008; Rose and O'Connell, 2009; Vatansever et al., 2013; Waje et al., 2009; Whitney et al., 2003). In addition, several studies have assessed the effects of inactivation on microbial identification by ELISA and PCR (Sagripanti et al., 2011; Talbot et al., 2010), and MALDI-TOF MS (Lasch et al., 2008; Talbot et al., 2010). However, the effects of various inactivation methods on liquid chromatography-tandem mass spectrometry (LC-MS/MS) data quality and apparent protein expression profiles of cells have to our knowledge not been investigated. To this end we evaluated the effects of irradiation, ethanol treatment, and autoclaving, on observed expression profiles in *E. coli* and *Yersinia pestis*. Our results demonstrate that the inactivation procedures we

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tested have minimal impact on apparent protein expression and spectral data quality.

2. Methods

2.1. Bacterial strains and culture conditions

Bacterial strains used for this research were *E. coli* ATCC 15597 (obtained from the American Type Culture Collection) and *Y. pestis* KIM D27, an isogenic derivative of KIM 10+ (biovar Mediaevalis) that was passaged until the pigmentation phenotype was lost due to spontaneous deletion of the *pgm* locus (Une and Brubaker, 1984). We obtained *Y. pestis* KIM D27 from Ellen Panisko (Pacific Northwest National Laboratory), who received it from Vladimir Motin at the University of Texas Medical Branch. Both *E. coli* ATCC 15597 and *Y. pestis* pgm-cultivation and manipulation were done at BSL-2 conditions until bacterial inactivation was verified.

All *E. coli* ATCC 15597 cultures were cultured in TSB broth or agar (TSB; BD 286220) at 37 °C. *Y. pestis* KIMD27 was cultured in Brain Heart Infusion broth or agar (BHI; BD 211059) at 30 °C. Starter cultures were established by streaking frozen glycerol stocks onto the appropriate agar plate and were incubated overnight at the appropriate temperature. To achieve biological replication, three replicate broth cultures were inoculated with separate individual colonies in 50 mL of the appropriate broth medium in 250 mL glass flasks. These cultures were incubated for 16 h at the appropriate temperature with shaking at 150 rpm. Bacteria were collected by centrifugation for 10 min at 5500 × *g* in a Thermo-Fisher swinging bucket centrifuge. The supernatant was decanted and the pellet was washed twice in 50 mL of phosphate-buffered saline (PBS; Gibco 20012). The washed pellet was brought to a final volume of 50 mL in PBS. Bacteria were enumerated by plating on the appropriate media prior to inactivation.

2.2. Sample inactivation

In addition to the untreated control, three inactivation treatments were used on 12 mL of each culture: irradiation, ethanol, and autoclaving. The control and irradiated samples were placed into 15 mL conical tubes while the other samples were placed in 50 mL conical tubes. Bacteria were irradiated with a Colbalt-60 source for 24 h at 0.47 kGy/h for a total exposure of 11.3 kGy. Autoclaved samples were exposed for 20 min at 30 psi [206.8 kPa] and 121 °C in a Getinge autoclave. For the ethanol treatment, ethanol was added to the PBS cell suspension for a final concentration of 40% ethanol, and the cells were incubated in this solution for 30 min at room temperature. Following ethanol inactivation treatments, cells were transferred to a clean tube and were collected by centrifugation and resuspended in PBS, transferred to a new tube, collected by centrifugation and brought up to a final volume of 12 mL PBS. Ten percent of each sample was plated on the appropriate bacterial culture media and incubated for 48 h to verify that the cells had been inactivated and were non-viable. All samples were stored at 4 °C prior to proteomic preparation.

2.3. Whole mount transmission electron micrographs

After inactivation, bacteria were fixed in 2.5% glutaraldehyde in PBS (Electron Microscopy Sciences, Hatfield, PA, USA # 16000) for transmission electron micrograph (TEM) imaging. Cells were stained with Nano-W (Nanoprobes, Yaphank, NY, USA #2018) prior to imaging on a Tecnai T-12 transmission electron microscope (FEI Co., Hillsboro, OR) operating at 120 kV with a LaB filament. Images were collected digitally using a 2 × 2K UltraScan 1000 charge-coupled device (Gatan Inc., Pleasanton, CA).

2.4. Digestion of proteins

For peptide preparation, ~4×10^8 CFU *Y. pestis* or of ~2×10^9 CFU *E. coli* were pelleted and resuspended in 8 M urea and 0.5 mM 2-mercaptoethanol in Tris–HCl, pH 8. Samples were incubated for 1 h at 60 °C with shaking at 300 rpm. Insoluble cell material was removed by centrifugation. To the supernatant, 400 μL of 50 mM ammonium bicarbonate was added followed by 1 μg of trypsin (Promega; # V5280). Samples were incubated at 37 °C overnight. Solid phase extraction (SPE) was performed with a vacuum manifold using Strata C-18 T columns following the manufacturer's protocol. The flow rate for vacuuming steps was 0.5 mL/min; the vacuum seal was released after each solution was removed. Briefly, 1 mL of 100% methanol (Chromasolv, Sigma Aldrich, St. Louis, MO) was added to activate the resin, followed by a conditioning rinse of 1 mL of 0.1% trifluoroacetic acid (TFA, Sigma Aldrich, St. Louis, MO) water, followed by the sample. The samples were washed with 5% acetonitrile in 0.1% TFA water, and eluted with 80% acetonitrile in 0.1% TFA water into clean, low protein-binding 1.5 mL microfuge tubes. Samples were dried to near completeness (5–10 μL remaining) with a Thermo-Fisher SpeedVac and resuspended in 25 μL of 0.5% formic acid. The resuspended samples were transferred to high performance liquid chromatography (HPLC) vials with 250 μL inert glass inserts, capped with screw caps, and stored at −20 °C prior to analysis.

2.5. Liquid chromatography tandem mass spectrometry

Reverse-phase liquid chromatography separation of digested peptide samples was performed on an Agilent Infinity 1260 HPLC system, using a column consisting of a fused silica capillary (40 cm long × 150 μm inner diameter) packed in-house with Jupiter C18 resin (5-μm particle size, 300 Å pore size; Phenomenex, Torrance, California). Mobile phases were: A, 5% acetonitrile/0.1% formic acid; and B, 95% acetonitrile/0.1% formic acid. Peptides were eluted during the following 160-min gradient: 100% Solvent A for 10 min; 0% B to 7.5% B over 1 min; 7.5% B to 45% B over 109 min; 45% B to 95% B over 2 min; 95% B for 10 min, 95% B to 0% B over 4 min and 100% A for 23 min. Column washes (5-μL injections of 50% isopropyl alcohol/50% acetone/0.1% formic acid) were run between sample injections. To achieve analytical replication, each sample was injected three times. Run order was randomized to prevent statistical artifacts relating to run order or column age.

The eluent from the liquid chromatography was introduced directly into a Thermo Scientific LTQ Orbitrap XL mass spectrometer via electrospray ionization using an etched silica capillary as the emitter (Kelly et al., 2006). Precursor (survey) mass spectra were acquired using the Orbitrap mass analyzer at a resolution of 30,000, followed by collision-induced dissociation (CID) fragment ion scans of the seven most abundant precursors, collected at low resolution using the ion trap mass analyzer. CID normalized collision energy was 35% and monoisotopic precursor selection was enabled. Dynamic exclusion of precursors already selected for fragmentation was applied (repeat count 2, repeat duration 30 s, exclusion list size 250, exclusion list duration 180 s).

2.6. Data processing and analysis

LC-MS/MS data was analyzed to obtain peptide-spectrum matches (PSMs) using a three step pipeline. DeconMSn (Mayampurath et al., 2008) preprocessed the data by accurately determining the monoisoptic mass and the charge state of parent ions from tandem mass spectrometry. Additional preprocessing was done with DTARefinery (Fenyö and Beavis, 2003; Petyuk et al., 2010), which uses a high-stringency X!Tandem search to recalibrate precursor masses for correction of systematic mass drift. Afterward the post-processed data was searched against a database using MS-GF+ (Kim et al.,