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Proteomic Characterization of Host Response to *Yersinia pestis* and Near Neighbors

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Abstract

Host-pathogen interactions result in protein expression changes within both the host and the pathogen. Here, results from proteomic characterization of host response following exposure to *Yersinia pestis*, the causative agent of plague, and to two near neighbors, *Y. pseudotuberculosis* and *Y. enterocolitica*, are reported. Human monocyte-like cells were chosen as a model for macrophage immune response to pathogen exposure. Two-dimensional electrophoresis followed by mass spectrometry was used to identify host proteins with differential expression following exposure to these three closely related *Yersinia* species. This comparative proteomic characterization of host response clearly shows that host protein expression patterns are distinct for the different pathogen exposures, and contributes to further understanding of *Y. pestis* virulence and host defense mechanisms. This work also lays the foundation for future studies aimed at defining biomarkers for presymptomatic detection of plague.

Key Words: Yersinia, host-pathogen interactions, 2-dimensional electrophoresis, presymptomatic detection, biomarkers, proteomics

Introduction

Yersinia pestis, the etiological agent of plague, is a gram-negative, highly communicable, enteric bacterium [1, 2]. There have been three recorded pandemics of plague as well as recent smaller outbreaks, substantiating the deadly effects of *Y. pestis* virulence and the ability of this human pathogen to overcome host defenses. The genus *Yersinia* also contains two other human pathogens, *Y. enterocolitica* and *Y. pseudotuberculosis*, and it is believed that *Y. pestis* evolved from *Y. pseudotuberculosis* within the past 10,000 years [3].

While *Y. enterocolitica* and *Y. pseudotuberculosis* cause severe intestinal distress, only *Y. pestis* frequently results in highly contagious person-to-person disease transmission and death. All three *Yersinia* species function via a Type III secretion mechanism common to several human, animal and plant pathogens [4, 5]. In Type III secretion, a series of pathogen-specific proteins form a syringe-like structure capable of injecting virulence factors into the host, subsequently affecting a variety of host pathways. For example, virulence factors such as YopE and YopT of *Y. pestis* are known to act on the cytoskeleton, while YopP induces apoptosis [6]. Therefore, cytoskeletal proteins and programmed cell death pathways are expected to be affected by exposure to *Y. pestis*.

Although *Y. pestis* can be phagocytosed by the host, it can also evade the immune response by manipulating the host defense mechanisms [4, 6-8]. Once infection is established, *Y. pestis* multiplies rapidly leading to necrosis of lymph nodes, which can result in death if untreated [2]. This condition is known as bubonic plague, and in some patients the infection can spread through the blood stream resulting in systemic plague, or to the lungs resulting in the

highly contagious and deadly form of the disease known as pneumonic plague. In the current age of heightened awareness toward bioterrorism, it is critical to be able to quickly and accurately detect exposure to pathogens such as *Y. pestis* in order to administer appropriate medical treatment and carry out triage procedures as necessary.

Previously, changes in gene expression in response to pathogen exposures have been reported [9-11]. However, the critical proteins and pathways involved, as well as the underlying mechanisms of virulence and host response are still poorly understood. Here, 2-dimensional electrophoresis (2-DE) has been utilized to characterize the proteomic host response of monocyte-like U937 cells after exposure to three *Yersinia* species. U937 cells were previously used to characterize protein changes from HIV infection [12]. Proteomic results reported here for U937 cells exposed to three distinct *Yersinia* pathogens demonstrate some shared host expression alterations indicating a common immune response to *Yersinia*; however, distinct pathogen-specific alterations were also observed in these host cells. The discovery of pathogen-specific host expression profiles supports efforts to identify biomarkers for early detection of plague based upon host response to *Y. pestis, Y. enterocolitica* and *Y. pseudotuberculosis*.

Materials and Methods

Cell culture. The human monocyte-like cell line, U937 (ATTC: CRL-1593-2), was maintained in RPMI media w / L-glutamine, 10 mM HEPES, Sodium Bicarbonate (ATCC), and 10% FCS (Invitrogen) in 5% CO_2 at 37 °C. Cells were resuspended to contain 1 x 10^7 cells / 10 cm plate prior to *Yersinia* exposure.

Pathogen Exposure. *Yersinia pestis* (KIM D27), *Y. enterocolitica* (WA, serovar 0:8) and *Y. pseudotuberculosis* (serotype 1, PB1 [13]) from glycerol stocks were grown on tryptose blood agar plates at 26 °C for 2 days. Single colonies were then selected and grown on tryptose blood agar slants at 26 °C for 2 days. Bacteria were washed off of slants using 2 mL of 0.033 M potassium phosphate, pH 7.0. Cells were lightly vortexed and left at room temperature for 30 min. 100 µL of cell suspension was diluted ten-fold into potassium phosphate buffer, and the OD_{620} was measured (1 OD_{620} = 1.2 x 10^9 colony forming units / ml). Bacteria cells were then added to tissue culture wells for a multiplicity of infection (MOI) ratio of 5:1. U937 cells were incubated with bacteria or 0.033 M phosphate buffer (negative, unexposed control) for 4 hours at 37 °C in 5% CO_2.

Protein Extraction. Following incubation, cells were washed with PBS and 500 µL of lysis buffer (2 M thiourea, 5 M urea, 0.25% CHAPS, 0.25% Tween 20, 0.25% SB 3-10, 100 mM dithiothreitol (DTT), 0.25% carrier ampholytes pH 3-10, 10% isopropanol, 12.5% water-saturated isobutanol, 5% glycerol, 1 mM sodium vanadate, and protease inhibitor (adapted from [14]) was added to the plate to solublize the proteins. Cells were collected with a rubber policeman into 1.5 mL centrifuge tubes and mixed by nutation at room temperature for 30 min and were stored at –80 °C overnight. Protein concentration (ADV01, Cytoskeleton) was determined prior to and after use of a 2D protein cleanup kit (Amersham).

2-Dimension Electrophoresis. Protein lysates (250 µg) were mixed with rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS) in a total volume of 450 µL. Equal amounts (250 µg) of protein were isoelectrically focused using 24 cm pH 3-10 NL and pH 4.5-5.5 strips (Amersham)

followed by 26 x 20 cm 12.5% SDS-PAGE using the Ettan Dalt system (Amersham). Gels were stained with SYPRO ruby (Bio-Rad) and visualized using a CCD camera (AlphaInnotech). Protein expression levels from protein spots on gels were compared between the different pathogen exposure samples. Gel analysis was performed using the ProPic imaging software (HT Analyzer, Genomics Solutions) to determine differential expression. Protein spots that were at least two fold increased or decreased after one of the *Yersinia* pathogen exposures are reported.

Protein Identification. Differentially expressed proteins spots were excised and placed in microcentrifuge tubes containing 100 μL of 10% methanol. Following reduction with DTT and alkylation with iodoacetamide, proteins were digested with trypsin by robotic sample handling (ProGest)(Proteomic Research Services, Ann Arbor, MI). For matrix assisted laser desorption ionization-mass spectrometry (MALDI-MS) analysis, protein spotting onto MALDI plates was performed robotically (ProMS, Genomic Solutions) using ZipTips. Peptides were eluted from the C18 material with matrix (α-cyano 4-hydroxy cinnamic acid in 60% acetonitrile, 0.2% TFA). MALDI-MS data was acquired on an Applied Biosystems Voyager DE-STR mass spectrometer and the observed peptide masses were submitted to ProFound using the NCBI database. Those samples that proved inconclusive following MALDI-MS were analyzed by LC/MS/MS on a Micromass Q-Tof2 using a 75 μm C18 column at a flow-rate of 200 nL / min. The MS/MS data were used for database search using MASCOT.

Results and Discussion

A human monocyte-like cell line, U937, was chosen for proteomic characterization of the host response to *Y. pestis, Y. enterocolitica* and *Y. pseudotuberculosis* exposure. While cell culture is not likely to reproduce the true response of a human to a pathogen, these cells were chosen to represent the human monocyte-derived immune response, thereby mimicking a blood-borne exposure to *Yersinia* [15, 16]. Following 4 hour exposure to the pathogens, host cells were lysed and the resulting soluble protein fractions were analyzed by 2-DE. Fig. 1 highlights a region of 2-DE gels showing both upregulated and downregulated differential expression following exposure to *Yersinia*. Fig. 1A shows the unexposed sample, and Fig. 1B-D show the *Y. pestis, Y. enterocolitica* and *Y. pseudotuberculosis* exposed samples respectively. From the protein spot patterns highlighted in Fig. 1, it is clear that the protein expression patterns are distinct for the three pathogens indicating that, even without identification of the protein spots, it is possible to crudely distinguish these three different pathogen exposures. However, a full proteomic characterization of host response to *Yersinia* and other pathogens is needed to identify specific biomarkers for the detection of infectious diseases, as well as to provide a better understanding of the mechanisms of virulence and pathogenesis.

Highlighting the complexity of proteomic characterization of host response to pathogen exposure, Fig. 2A shows a 2-DE gel of protein samples from U937 cells separated first on a non-linear pH 3-10 IPG gradient followed by molecular weight separation on a 12.5% acrylamide gel. Some regions are clearly overcrowded, making it difficult to detect all the individual protein spots. In order to further separate the most crowded acidic proteomic region, the protein samples

were next separated using pH 4.5 to 5.5 IPG strips (Fig. 2B), and the narrow pH first dimension strip was shown to be effective at separating the acidic proteome. Therefore, use of several range pH strips rather than solely a broad range pH 3-10 strip can result in a more thorough 2-DE separation with an increased number of protein spots detected.

Here, to screen host response at the proteomic level, both pH 3-10 and pH 4.5-5.5 IPG strips were used. Fig. 3 shows a region of a broad pH range 2-DE gel before (A) and after (B) *Y. pestis* exposure (*Y. pseudotuberculosis* and *Y. enterocolitica* exposures not shown), and the locations of six differentially expressed proteins identified by mass spectrometry. Fig. 4 shows a region of a narrow acidic pH range 2-DE gel before (A) and after (B) *Y. pestis* exposure, and the locations of four differentially expressed proteins identified by mass spectrometry. Proteins that showed at least a two fold change (log2 = 1) following *Yersinia* exposure were isolated from gels and identified by peptide mapping and mass spectrometry. The identities of the differentially expressed proteins are listed in Table 1.

From this initial proteomic view of host response to *Yersinia* pathogens, three proteins were identified with notable differences in expression after exposure to *Y. pestis* compared to the two other pathogens. Peroxiredoxin III (Fig. 4, protein spot **1**), which was upregulated after exposure to *Y. pestis*, is a known part of the mitochondrial antioxidant system and is induced by oxidative stress on the cell [17]. Human monocyte/neutrophil elastase inhibitor (M/NEI) (Fig. 3, protein spot **2**), which was also upregulated after exposure to *Y. pestis*, is a fast-acting stoichiometric inhibitor of neutrophil serine proteases [18, 19]. Finally, heterogenous nuclear ribonucleoprotein complex K protein (HnRNP K) (not shown in figures, protein spot **3** in Table

1), which is involved in apoptosis, showed no change in expression following *Y. pestis* exposure, but was significantly downregulated after *Y. pseudotuberculosis* and *Y. enterocolitica* exposures. HnRNP K is one of a large family of nucleic acid binding proteins, of which many members have been implicated to have roles in telomere and telomerase regulation [20]. The differential expression of these proteins is of particular interest from a detection point of view because of the ability to differentiate between exposures to the different *Yersinia* pathogens. Further work is required in order to understand the mechanistic interpretation of these results and to verify that these proteins could be used as biomarkers for detection in animal studies, or in humans in the event of a plague outbreak.

Several of the differentially expressed proteins identified in this study showed changes in expression common to all three pathogen exposures. For example, heat shock protein 27 (Hsp 27, Fig. 3, protein spot **4**), which is involved in apoptosis, was upregulated after exposure to all three *Yersinia* pathogens. Heat shock proteins are synthesized in response to a wide variety of stressful stimuli including viral and microbial infections with the biological purpose of rendering the host cells resistant to further and more severe stress [21-23]. Another protein, Cathepsin D (Fig. 3, protein spot **5**), was also upregulated after exposure to all three pathogens. Cathepsin D is an aspartic protease usually found in the lysosome where it functions in protein catabolism [24]. Cathepsin D functions in a wide variety of tissues during cytoskeletal remodeling and in apoptosis, both processes of which are common after bacterial infection and are known to be caused by *Yersinia* virulence factors [6]. RACK1, or receptor for activated C-kinase 1, (Fig. 3, protein spot **6**) was also upregulated after exposure to all three *Yersinia* pathogens. RACK1 is an intracellular receptor proteins that binds activated protein kinase C (PKC), and this binding is

thought to play a role in the activation-induced translocation of PKC from the cytosol to the cytoskeletal membrane [25, 26]. In addition, 14-3-3 protein tau (Fig. 4, protein spot **7**) was upregulated after exposure to all three pathogens. 14-3-3 proteins are a family of highly conserved signal transduction proteins that play a role in a wide variety of cellular functions including the regulation of signal transduction, apoptosis, cell cycle control, and nutrient-sensing pathways [27]. Macrophage capping protein (MCP, Fig. 3, protein spot **8**) was upregulated after exposure to all three pathogens, and is a calcium-activated barbed end actin capping protein that also functions in cytoskeletal remodeling [28]. Finally, RCL (Fig. 4, protein spot **9**) was upregulated after exposure to all three pathogens. While the function of RCL is unknown, it appears to be a growth-related c-Myc responsive element [29, 30].

Two other proteins of interest, tyrosine 3-monooxygenase theta (not shown in figures, protein spot **10** in Table 1) and WD40-repeat containing protein Ciao 1 (Fig. 4, protein spot **11**), showed downregulation after exposure to all three *Yersinia* pathogens. Tyrosine monooxygenases catalyze the first step in the biosynthesis of catacholamines and have been reported to require binding of 14-3-3 tau for optimal activity [31]. WD40 Ciao 1, which is involved in apoptosis, has been shown to specifically modulate the transactivation activity of the Wilms tumor suppressor protein (WT1). WT1 is a transcription factor capable of activating or repressing transcription of various cellular genes that has also been reported to play a role in controlling apoptosis [32]. Finally, protein disulfide isomerase (PDI, Fig. 4, protein spot **12**) was upregulated following *Y. pseudotuberculosis* exposure but this protein spot was undetectable after the exposure to the other two pathogens representing a probable downregulation. PDI is a member of the thioredoxin family of proteins that assists in the folding of proteins containing disulfide bonds and is abundant in the lumen of the endoplasmic reticulum [33].

While mechanistic interpretation of some of the host protein expression alterations detected here supports some of the known effects of *Yersinia* virulence factors, it is apparent from results within that *Y. pestis*, *Y. enterocolitica* and *Y. pseudotuberculosis* affect different host pathways and that the host response is distinct for each pathogen. In some cases, the host response to *Y. pestis* was more similar to *Y. pseudotuberculosis*, and in some cases more similar to *Y. enterocolitica*. However, from these results, it appears that exposure to *Y. pestis* results in the most divergent host response, and this supports the more severe clinical manifestation of *Y. pestis* compared to *Y. pseudotuberculosis* and *Y. enterocolitica*. More detailed analyses of host response are needed to further understand the different virulence mechanisms and the evolution of these three *Yersinia* species that are pathogenic to humans.

In conclusion, several of the differentially expressed host proteins identified here are involved in protein synthesis, cytoskeletal interactions, immune responses and apoptosis. These results are in support of the known functions of *Yersinia* virulence factors in cytoskeletal modulation and apoptosis[6]. Future experiments including the use of more relevant host models such as animals, and more sensitive and quantitation proteomic technologies such as 2-D differential in-gel electrophoresis (DIGE) [34, 35] are necessary to identify and validate biomarkers for *Yersinia* exposure. From the work reported here, shared expression changes were detected that were common to all three pathogens representing a common host response; however, pathogen-specific expression alterations were clearly detected in host cells suggesting that host response could be used, in the absence of other information, to detect exposure to a pathogen and to identify the pathogen in question.

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Figure Legends

FIG 1. 2-DE gels showing differential protein expression after pathogen exposures. Panel A shows the control, unexposed sample. Panel B shows the *Y. pestis* exposed sample. Panels C and D show *Y. pseudotuberculosis* and *Y. enterocolitica* exposed samples respectively. Both increased and decreased protein expression patterns are highlighted in the four samples.

FIG 2. 2-DE separation of U937 cells. Panel A shows a 2-DE gel using a broad range pH 3-10 NL IPG strip. Panel B shows a 2-DE gel using a narrow pH 4.5-5.5 IPG strip.

FIG 3. Differentially expressed proteins after *Y. pestis* exposure using broad range pH 3-10 strips. Panel A shows a region of a 2-DE gel of unexposed U937 cells. Panel B shows a region of a 2-DE gel of *Y. pestis* exposed cells. Differentially expressed proteins, after exposure to *Y. pestis*, are noted with circles and numbers. Protein identities are listed shown in Table 1.

FIG 4. Differentially expressed proteins after *Y. pestis* exposure using pH 4.5-5.5 strips. Panel A shows a region of a 2-DE gel of unexposed U937 cells. Panel B shows a region of a 2-DE gel of *Y. pestis* exposed cells. Differentially expressed proteins, after exposure to *Y. pestis*, are noted with circles and numbers. Protein identities are listed shown in Table 1.

Table 1. Differentially expressed host proteins identified from U937 cells following exposure to *Yersinia*.

Protein #	Protein Name	Accession number	log2 Expression Change after Pathogen Exposure		
			Y. pestis	***Y. pseudotuberculosis***	***Y. enterocolitica***
1	Peroxiredoxin 3	**IPI00374151**	1.7	-0.1	0.5
2	M/NEI	**ILEU_HUMAN**	2.4	1.2	ND
3	HnRNP K	**ROK_HUMAN**	0.0	-1.1	-0.6
4	Heat shock protein 27	**HSB1_HUMAN**	1.9	0.8	1.6
5	Cathepsin D, chain B	**1LYWB**	1.6	1.1	0.2
6	RACK1	**IPI00013896**	1.9	1.2	1.5
7	14-3-3 protein tau	**IPI00018146**	0.7	1.0	1.7
8	Macrophage capping protein	**IPI00027341**	4.3	2.9	3.1
9	RCL	**IPI00007926**	1.0	0.4	0.4
10	Tyrosine 3-monooxygenase theta	**143Z_HUMAN**	0.1	-0.9	-1.4
11	WD40-repeat containing protein Ciao 1	**IPI00008791**	-1.1	-0.5	-0.7
12	Protein disulfide isomerase A4 precursor	**IPI00009904**	ND	1.7	ND

ND: Protein spot was not detected on the gel following pathogen exposure representing probable downregulation, and reflecting the limits of detection by 2-DE.