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Proteomic Characterization of *Yersinia pestis* Virulence

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ABSTRACT

Yersinia pestis, the etiological agent of plague, functions via the Type III secretion mechanism whereby virulence factors are induced upon interactions with a mammalian host. Here, the *Y. pestis* proteome was studied by two-dimensional differential gel electrophoresis (2-D DIGE) under physiologically relevant growth conditions mimicking the calcium concentrations and temperatures that the pathogen would encounter in the flea vector and upon interaction with the mammalian host. Over 4100 individual protein spots were detected of which hundreds were differentially expressed in the entire comparative experiment. A total of 43 proteins that were differentially expressed between the vector and host growth conditions were identified by mass spectrometry. Expected differences in expression were observed for several known virulence factors including catalase-peroxidase (KatY), murine toxin (Ymt), plasminogen activator (Pla), and F1 capsule antigen (Caf1), as well as putative virulence factors. Chaperone proteins and signaling molecules hypothesized to be involved in virulence due to their role in Type III secretion were also identified. Other differentially expressed proteins not previously reported to contribute to virulence are candidates for more detailed mechanistic studies, representing potential new virulence determinants. For example, several sugar metabolism proteins were differentially regulated in response to lower calcium and higher temperature, suggesting these proteins, while not directly connected to virulence, either represent a metabolic switch for survival in the host environment or may facilitate production of virulence factors. Results presented here contribute to a more thorough understanding of the virulence mechanism of *Y. pestis* through proteomic characterization of the pathogen under induced virulence.

INTRODUCTION

Yersinia pestis, the etiological agent of plague, is a Gram-negative, highly communicable bacterium, known as a natural pathogen and as a biothreat agent. The primary natural route of human infection by *Y. pestis* is via flea-bite from infected rodents (40). Since, the bacteria populate a significant amount of time in the flea vector and mammalian hosts, the physiological conditions present within these organisms can be used to elucidate protein expression relevant to *Y. pestis* virulence.

Early studies of *Yersinia* physiology uncovered a phenomenon known as the low calcium response (LCR), whereby bacterial cultures grown in rich media at elevated temperatures (37°C) would exhibit a growth defect upon chelation of calcium ions. A strong temperature component of the LCR exists, as cells continue to grow logarithmically regardless of calcium at 26°C. The growth effect found at elevated temperature and reduced calcium concentration was proposed to mimic the bacteria entering a mammalian host. Eventually, the growth arrest was shown to be a result of activation of the Type III secretion mechanism, and the growth defect was due to the bacteria dedicating massive resources to the secretion of virulence factors known as Yersinia outer proteins, or Yops (23, 27) (for review, 57). Type III secretion can be activated and virulence factors released into the media (for review, 30) when *Y. pestis* is grown at 37°C in enriched media mimicking mammalian intracellular conditions with respect to Mg^{2+} (20 mM) and Ca^{2+} (0 mM). Once inside the host cell, virulence factors affect a variety of host pathways, with detectable expression changes in the pathogen as well as the host (16, 79). The growth parameters of temperature and calcium have previously been employed to study changes in gene expression (45) and protein expression by chip-based mass spectrometry (70), Fourier transform-ion cyclotron resonance mass spectrometry (47), and accurate mass tags (28).

Many reports have examined bacterial proteomes using two-dimensional electrophoresis (2-DE) (for review, 17), and earlier proteomic analyses of *Y. pestis* have also been completed using 2-DE (68). Specifically, 2-DE has been used to examine different subcellular fractions of *Y. pestis* proteins, leading to numerous new vaccine

targets, including V antigen, W antigen, lcrG, lcrV, and lcrH (68, 52, 56). It was also shown that virulence factors are not expressed at 26°C or at 37°C in the presence of calcium concentrations found in mammalian plasma (2.5 mM) (68). The introduction of 2-dimensional differential gel electrophoresis (2-D DIGE) has improved the quality of gel-based proteomics through multiplex proteomic comparison providing relative quantitation of expression differences and improved gel-to-gel comparisons (78). 2-D DIGE utilizes spectrally resolvable fluorescent dyes to label up to three different protein samples that can be electrophoresed on the same gel (71). Several examples of 2-D DIGE bacterial proteomics have been reported (24, 32), including characterization of Gram-negative bacteria, *Escherichia coli* (1, 72, 78).

Here, we report the characterization of soluble protein expression changes in *Y. pestis* upon transition from the environmental growth conditions of the flea vector to the mammalian host. Both calcium concentration and temperature were adjusted to mimic the flea midgut (26°C, 4 mM calcium), mammalian host plasma (37°C, 4 mM calcium), and host intracellular (37°C, 0 mM calcium) physiological environments. This approach has identified known virulence factors as well as several other proteins that are differentially expressed under host conditions that represent potential novel virulence determinants.

MATERIALS and METHODS

Bacterial cell growth: *Yersinia pestis* (KIM5 D27) bacterial cell colonies were picked from glycerol stocks and grown on tryptose blood agar plates at 26°C for 2 days. Individual colonies were selected and bacteria grown on a tryptose blood agar slant at 26°C for 2 days. The bacteria were washed off of the slants using 2 ml of 0.033 M K-phosphate, pH 7.0. Cells (1.3 ml) were lightly vortexed and diluted 100-fold into K-phosphate buffer. Next, 0.1 ml of the cell suspension was used to inoculate 15 ml of best case scenario (BCS) media in a 125 ml flask. Cells were grown at 26°C at 200 rpm for 8h and an OD_{620} between 0.6 and 0.7 (1 OD_{620} = 1.2 x 10^9 CFU/ml). 4 ml of this culture was then used to inoculate 50 ml BCS media in a 250 ml flask, which was grown at 26°C for an additional 16 h to an OD_{620} between 2.3 and 2.5. Finally, 52 ml of the second culture was used to inoculate 2.175 liters of BCS media, which was divided into eight 125 ml cultures grown in 1 liter flasks at 26°C. After 8 h of growth, four of the flasks were shifted to 37°C, two of which were supplemented with 1.25 ml of 0.4 M $CaCl_2$ solution, while the other two were supplemented with water. Similar additions of $CaCl_2$ or water were repeated for the four flasks growing at 26°C. Cells were grown for an additional 4 h after the temperature and calcium concentration shifts, providing the four different growths for proteomic analysis.

Relevant *Y. pestis* growth conditions. The parameters examined in this study represent the growth conditions that differ within *Y. pestis* transition from the flea vector to a mammalian host. The temperature of the flea midgut is 26°C and contains millimolar concentrations of calcium. The low temperature and high calcium are sufficient to prevent the production of the type III secretion mechanism and secretion of virulence factors, known as Yops. This physiological state is represented by the 26°C with 4 mM calcium sample. The mammalian host plasma has sufficient calcium to prevent the Yop secretion associated with the low calcium response. This physiological state is represented by the 37°C with calcium condition. The 37°C without additional calcium represents the intracellular host cells environment, as the micromolar concentration of calcium within cells initiates the LCR. Therefore, the key comparisons for differential protein expression related to *Y. pestis* virulence are represented by the low temperature

and high calcium of the flea physiological conditions, and the high temperature and low calcium found in a mammalian host cell.

Cell lysis and soluble protein extraction: Cultures were harvested at defined phases by centrifugation and resuspended in 50 mM ammonium bicarbonate pH 7.8. Cells were washed two times in the same buffer and pelleted by centrifugation at 4,000 rpm for 10 minutes. Cell lysis was achieved by bead beating using three 180 sec cycles at 4500 rpm in a Mini-BeadBeater-1 (BioSpec Products, Inc., Bartlesville, OK), with a 5 minute cool down period on ice between cycles. Lysates were immediately placed on ice to inhibit proteolysis and 1X protease inhibitor cocktail (Roche) was added. Protein was quantified using the ADV01 reagent (Cytoskeleton) and analyzed at wavelength of 590. Following protein assay, protein extraction was performed as described in the Ettan 2-D DIGE users guide (Amersham). Briefly, oligonucleotides and other non-protein contaminants were removed by using the 2-D protein cleanup kit (Amersham).

2-D DIGE: Each of the four growth condition samples was aliquoted (50 μg) and labeled with the fluorescent amine-reactive cyanine dyes, Cy3 or Cy5 CyDyes (200 pmol). Equal amounts of all four samples were pooled as a representation of the entire experiment and for comparative analysis. To prepare the internal pooled standard, 50 μg aliquot of the pooled sample were labeled with 200 pmol of Cy2. This pooled standard was used in each gel to normalize protein abundance measurements across each gel facilitating inter-gel spot matching and relative protein quantitation. After labeling protein of each experimental growth sample and the pooled standard with CyDyes for 30 minutes at 4°C, the labeling reaction was quenched with 1 nmol of lysine. Eight gels were used for each experiment and multiplexed analysis was performed according to Table 1. In addition to the fluorescently labeled samples, in two of the gels, additional unlabeled protein sample from each growth condition was added to ensure enough protein was present for subsequent protein identification by mass spectrometry.

Table 1. Experimental design for 2-D DIGE experiment.

Gel Number	Cy3 Labeled Protein Sample	Cy5 Labeled Protein Sample	Cy2 Labeled Pooled Sample	Additional Unlabeled Protein Sample
Gel 1	50 μg 26°	50 μg 37°	50 μg pooled standard	
Gel 2	50 μg 37° w/ Ca	50 μg 26°	50 μg pooled standard	
Gel 3	50 μg 26°	50 μg 26° w/ Ca	50 μg pooled standard	
Gel 4	50 μg 26° w/ Ca	50 μg 37°	50 μg pooled standard	
Gel 5	50 μg 26° w/ Ca	50 μg 37° w/ Ca	50 μg pooled standard	
Gel 6	50 μg 37°	50 μg 37° w/ Ca	50 μg pooled standard	
Gel 7	50 μg 26° w/ Ca	50 μg 37°	50 μg pooled standard	50 μg each growth condition
Gel 8	50 μg 37° w/ Ca	50 μg 26°	50 μg pooled standard	50 μg each growth condition

Multiplexed samples were separated by charge on 24 cm 3-10 NL Immobiline IPG DryStrips, and separated by size on 12.5% polyacrylamide gels. Following electrophoresis, gels were scanned using the Typhoon 9410 imager and protein spots were analyzed using DeCyder™ software.

Decyder Analysis. 2-D DIGE gel images were subjected to DeCyder analysis. CyDye images from individual gels were analyzed by differential in gel analysis, (DIA) and protein spots were detected. Differential protein expression was determined between two samples in the gel based on the ratio of standardized log of abundance of the Cy3 versus Cy5 spot volume over the Cy2 spot volume. The standardized log of abundance is the log abundance of the Cy3- or Cy5-labeled spot over the log abundance of Cy2-labeled pooled standard spot. T-test values were obtained by comparing the differential expression of the Cy3/Cy2 volume as compared to Cy5/Cy2 volume. CyDye images from all the gels within the experiment were analyzed by biological variation analysis, (BVA). Protein spots were normalized to the Cy2 spot volumes. T-test values were obtained by comparing all the replicates from one sample compared to another. Differential spots reported in the text have p values less than or equal to 0.05. DeCyder analysis provided a pick list file containing the pixel location of the differentially expressed protein spots of interest. Protein spots were picked using the ProPic Robotic Workstation (Genomic Solutions) using a custom software and hardware to integrate DeCyder Analysis with the ProPic Workstation (39). Each experiment contained three gel replicates of each of the four growth conditions, and the entire experiment was performed four times.

Protein Digestion and Mass Spectrometry. Protein spot digestion and mass spectrometry characterization were performed by Proteomic Research Services (PRS, Ann Arbor, MI). Differentially expressed protein spots were subjected to robotic in-gel digestion using trypsin (ProGest) following reduction with DTT and alkylation with iodoacetamide. A portion of the resulting digest supernatant was used for matrix assisted laser ionization desorption-mass spectrometry (MALDI-MS) analysis. Spotting was performed robotically (ProMS) with 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 instrument and the observed m/z values were submitted to ProFound for peptide mass fingerprint searching using the NCBInr 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 analyzed using MASCOT.

RESULTS

Differential proteomic characterization. Following logarithmic growth for 48 hours at 26°C, *Y. pestis* was grown at both 26°C and 37°C with and without 4 mM Ca^{2+} for four hours. Bacterial cells were lysed and the soluble protein fraction was isolated. The soluble proteome of *Y. pestis* was examined using the Ettan 2-D DIGE system for comparison of the relevant four different growth conditions. Figure 1 shows 2-D DIGE images of each of the four *Y. pestis* growth conditions: panel A, 26°C with no added calcium; panel B, 26°C with 4 mM calcium (corresponding to flea midgut); panel C, 37°C with no added calcium (corresponding to intracellular host); and panel D, 37°C with 4 mM calcium (corresponding to host plasma). The gel images are representative of the *Y. pestis* proteome of the four growth conditions obtained from four separate experiments.

After 2-DE, gel images were analyzed using DeCyder software to determine the relative expression levels of *Y. pestis* proteins for the relevant growth conditions. DeCyder analysis was used to directly compare the relative protein expression differences in samples from the same gel (differential in gel analysis; DIA) and among all gels in the experiment (biological variation analysis; BVA). Proteomic analysis revealed hundreds of differential protein spots between the four growth conditions. A comparative analysis of all the protein samples on all gels revealed 4151 unique protein spots with 375 protein spots exhibiting differential protein expression within at least one of the four growth conditions. The more relevant protein expression changes that were detected between the flea vector and the physiological host growth conditions were examined more closely using DIA (Table 2).

To illustrate differential protein expression of three selected protein spots, Figure 2 shows DeCyder BVA output images. Protein expression changes for two proteins that were upregulated (Pla and OmpA), and one that was downregulated (fructose-bisphosphate aldolase class II) in host intracellular conditions compared to flea vector conditions are shown. Panel A shows that the Pla protein was upregulated 2.8-fold under host intracellular growth conditions compared to flea midgut conditions. Panel B shows

that the OmpA protein was upregulated 12.3-fold under host intracellular growth conditions compared to flea midgut conditions. Panel C shows that fructose bisphosphate was downregulated 1.5-fold under host intracellular growth conditions compared to flea midgut conditions. The majority of differentially expressed proteins detected in this study were upregulated between the flea midgut and host intracellular conditions, consistent with the report that overall protein production is greatly increased during *Y. pestis* invasion of host immune cells (57).

As an example of comparative proteomics examined under all four experimental growth conditions, Figure 3 shows DeCyder BVA output images for Pla. Differential protein expression levels of Pla are shown for flea midgut compared to host intracellular conditions (panel A); flea midgut compared to host plasma (panel B); 4 mM compared to 0 mM calcium at 37°C (panel C); and 26°C compared to 37°C with 4 mM calcium (panel D). Pla showed an increase of 2.8-fold under host intracellular growth conditions compared to flea midgut conditions. The increase under host plasma conditions compared to flea midgut showed a similar increase of 2.4-fold (panel B). Essentially no change in expression was detected in Pla following the decrease in calcium from 4 mM to 0 mM calcium at both 26°C (data not shown) and 37°C (panel C). Temperature change from 26°C to 37°C at both 4 mM calcium (panel D) and 0 mM calcium (data not shown) showed an increase in Pla of 2.1-fold.

Differential protein expression following change in calcium concentration. To characterize protein expression changes due to the low-calcium response, we examined the soluble *Y. pestis* proteome with and without supplementation of calcium. 4 mM calcium was used to mimic the calcium concentration present within the flea midgut and to prevent the LCR. BVA analysis showed 94 protein spots were differentially expressed when comparing *Y. pestis* growth at 0 mM and 4 mM calcium at both temperatures.

Figure 4 shows representative 2-D DIGE multiplexed overlay images comparing differential protein expression due to calcium shift at 26°C (panel A) and 37°C (panel B). Red spots represent proteins with increased expression in samples with no added calcium,

while blue spots show proteins with increased expression in the presence of 4 mM calcium Green spots represent proteins with increased expression in the pooled standard as compared to the Cy3- (red) and Cy5-labeled (blue) samples, and therefore, green spots represent proteins with greater protein expression at 37°C (panel A) or 26°C (panel B). White spots represent proteins that are equally expressed between the Cy3-, Cy5-labeled and pooled standard sample.

Differential protein expression following change in temperature. To mimic temperatures in the natural *Y. pestis* lifecycle, bacteria were grown at 26°C, representing the ambient temperature of the flea, and at 37°C, representing the natural body temperature of a mammalian host. Comparison of the samples grown at the two different temperatures revealed 267 differential protein spots.

Figure 5 shows representative 2-D DIGE multiplexed overlay images comparing differential protein expression due to temperature without supplemental calcium (panel A) and with 4 mM calcium (panel B). Red spots represent proteins that were increased at 26°C, while blue spots represent proteins that showed increased expression at 37°C. Green spots represent proteins with increased expression in the pooled standard as compared to the Cy3- (red) and Cy5-labeled (blue) samples, and therefore, green spots represent proteins that were increased at 4 mM calcium (panel A) or 0 mM calcium (panel B). White spots represent proteins that are equally expressed between the Cy3-, Cy5-labeled and pooled standard sample.

When differential expression due to temperature was separated from the effect of calcium, 139 proteins were differentially expressed at 0 mM calcium and 68 proteins were differentially expressed at 4 mM high calcium.- Fewer protein expression differences were due to calcium changes (Figure 4) than for temperature changes (Figure 5), revealing that the classically observed increase in protein expression due to the LCR is also dependent upon the temperature change that occurs during interaction with the host.

Differential protein expression following change from flea to mammalian physiological conditions. The samples grown in 4 mM calcium at 26°C and 37°C, and 0 mM calcium at 37°C represent the flea, host plasma, and intracellular host *in vitro* growth conditions, respectively. Images in Figure 5B reveal the proteomic alterations following transition of the bacteria from flea to host plasma physiological conditions. The relatively low number, 68, of differentially expressed proteins suggests that entry into the host bloodstream does not involve a significant alteration of the *Y. pestis* proteome. However, 375 differential protein spots were detected between the flea and host intracellular growth conditions, suggesting that the majority of differential expression occurs after entry in the blood stream and upon interaction with host cells.

Figure 6 shows the 2-D DIGE multiplexed overlay image of the soluble proteome of *Y. pestis* grown under the flea and host intracellular conditions, indicating protein spots that were further analyzed by mass spectrometry. Due to the relevance of these two growth conditions in *Y. pestis* virulence, further Decyder DIA analysis was performed. From DIA, protein spots exhibiting greater than 2.4-fold differences, or two standard deviations, between the flea and host intracellular culture conditions were excised for protein identification by trypsin digest, peptide mapping and mass spectrometry. Of the 43 protein spots that were subjected to peptide mapping, 24 unique proteins were identified including four known virulence factors. Other proteins identified represent potential novel virulence determinants. The arrows in Figure 6 delineate the position of the protein spots that were identified, and Table 1 lists the identified proteins and levels of differential expression between the flea and host growth conditions including both host blood and intracellular physiology.

DISCUSSION

Differentially expressed protein detected by 2-D DIGE comparing temperature and calcium concentrations relevant to the physiological environments that *Y. pestis* encounters in the flea vector and host intracellular and bloodstream are listed in Table 1. Several proteins were identified in multiple protein spots suggesting these proteins undergo post-translation modifications such as proteolysis or phosphorylation. For some of the proteins detected in multiple spots, the proteolytic or post-translational modification processes are well established. Most notably, the seven protein spots identified as KatY have been previously described (25). In addition, the two protein spots identified as murine toxin (Ymt), correspond to known Ymt species (44). Finally, multiple protein spots of Cpn60 were detected, representing cleavage possibly required for the role of Cpn60 as a chaperone for multiple *Y. pestis* proteins. Identified differentially expressed proteins and interpretation with regard to virulence follow.

Virulence factors

The differentially expressed *Y. pestis* proteins identified in this study include four known virulence factors, Pla, F1 antigen, Ymt, and KatY. These proteins were determined to be upregulated in the transition from experimental culture conditions mimicking the flea to the mammalian host physiological conditions. Other virulence factors that are known to be differentially expressed under virulence inducing growth were not identified here. Three likely possibilities exist for the absence of other Yops in this study. First, it is known that Pla proteolyzes Yops (65), which could result in the degradation, and therefore lack of detection, of the Yops under the experimental growth conditions used. Secondly, it is known that the *in vitro* stimulation of the Type III secretion mechanism results in secretion of Yops into the culture media (43); however, this study did not address extracellular proteomic changes. Finally, many of the Type III secretion components, in particular Syc (specific Yop chaperone) proteins, are small in molecular weight (13.5–19 kDa) and may not be detected under the 2-DE conditions used (12.5% acrylamide gels), which preferentially resolve 20-120 kDa sized proteins. The differentially expressed virulence factors detected, Pla, F1 antigen, Ymt and KatY, are discussed in turn.

Plasminogen activator (Pla): Pla, an outer membrane protease with fibrinolytic and coagulase activity, is believed to contribute to the highly invasive nature of *Y. pestis* (41, 63, 64). Pla as been demonstrated to play a role in virulence through Pla knockout experiments that failed to cause disease in mice (76). The fibrinolytic activity of Pla is believed to contribute to virulence by facilitating bacterial metastasis, allowing access of *Y. pestis* to the viscera (34). The fibrinolytic activity of Pla is temperature-dependent, increasing from 26°C to 37°C (41), and the increased activity is likely due to the amount of Pla protein, as synthesis continues at 37°C in calcium deficient media while the expression of most other proteins is inhibited (42). In previous gene expression studies, neither temperature-dependent transcription or translation were observed for the *pla* gene, suggesting the temperature-dependence of the fibrinolytic activity may be due to temperature-induced modifications or conformational changes in the Pla protein (12, 41). Here, Pla expression was shown to be temperature-dependent, as a 2.4-fold increase was detected at 37°C in the presence of 4 mM calcium compared to 26°C with 4 mM calcium, reflecting the change in conditions from the flea midgut to host plasma.

A functional role for Pla has been proposed following *Y. pestis* entry into host plasma, as Pla has been reported to enhance bacterial attachment to the mammalian extracellular matrix (34, 66). Notably, the Pla protein shares homology with other proteolytic plasminogen activators, and in addition to plasminogen, the Pla protease cleaves complement component C3 (59, 65, 66). Further, Pla maintains high plasmin concentration in the host by degrading α-2 antiplasmin. Since Pla is able to bind several non-collagenous matrix proteins and is involved in the degradation of these proteins, a possible role for Pla is aiding *Y. pestis* entry into host cells.

Pla was also reported to play a pivotal role in bacterial invasion of HeLa cells resulting in altered signal transduction and cytoskeletal rearrangement (7). Here, Pla was determined to be upregulated 2.8-fold when *Y. pestis* was grown under conditions that the bacteria would encounter upon transition from the flea to host intracellular conditions. Protein kinases, including protein tyrosine kinases, have been implicated as mediators in this Pla-mediated invasion, and it is well known thatProtein tyrosine kinases are regulated

by protein tyrosine phosphotases. For example, YopH, a protein tyrosine phosphotase and known virulence factor, is responsible for altered host signaling and cytoskeletal rearrangement (5, 10, 54). Since YopH and Pla have been found to function in altered host signaling and cytoskeletal arrangement, YopH may be a regulator in a protein kinase pathway following Pla-mediated invasion. Consequently, Pla may serve multiple roles in *Y. pestis* virulence upon interaction with the host including degradation of host proteins and mediating YopH activity.

F1 antigen (Caf1): It is known that *Y. pestis* resistance to phagocytosis coincides with development of the F1 capsule (13, 14), of which Caf1 is a major constituent. Caf1 has been previously reported as a virulence factor due to its role in inhibiting invasiveness of HeLa cells (19). Interestingly, this protein contains potential antigenic determinants that may stimulate T-cells, and host-pathogen interaction studies have shown that the resistance to phagocytic uptake of *Y. pestis* by the host is due to the expression of Caf1 (11). The protein spot identified as Caf1 in this study (Figure 6, spot 19) agrees with the theoretical molecular weight and charge of the Caf1 dimer. Although native Caf1 is a homo-oligomer of megadalton size, the mature Caf1 protein monomer contains 149 amino acids, after cleavage of a 21 amino acid leader sequence, and has a molecular weight of 15.5 kDa and has a calculated pI of 4.3.

Here, there was a 2.2-fold increase detected in Caf1 expression in host plasma growth conditions compared to flea vector conditions, consistent with the formation of the F1 capsule under similar growth conditions (74). The 2.4-fold increase in Caf1 protein expression in host intracellular conditions compared to flea vector conditions is also consistent with existing studies. For example, Caf1 was shown to be stable over several hours following pulse-chase experiments using radioactively labeled *Y. pestis* grown at 37°C in calcium deficient media (42). Further, the temperature regulation of Caf1 induction was demonstrated to be at the transcription level, using a luciferase construct where the *caf1* gene was over-expressed 20- to 40-fold at 37°C compared to 26°C (20). The increased gene expression is consistent with protein expression changes later found at 37°C (61). Here, decrease in calcium concentration had no effect on Caf1

protein expression. Our studies show that temperature increase resulted in a 2.1-fold increase at 37°C compared 26°C growth, consistent with reports of temperature-dependent Caf1 expression (42).

Murine toxin (Ymt): Murine toxin (Ymt) is a phospholipase D superfamily member, containing two phospholipase D motifs (58), which hydrolyzes host phospholipids upon invasion. Two protein spots identified as Ymt were upregulated in host conditions compared to flea midgut conditions in this study. The first Ymt species identified, spot 7 of Figure 6, showed a 1.8-fold increase in host plasma compared to flea midgut conditions, and a 2.3-fold increase in host intracellular compared to flea midgut conditions. The gel location of this protein spot is in agreement with the full length, soluble form of Ymt, with a molecular weight of ~67 kDa and theoretical pI of 5.6. Although the active form of Ymt is a dimer, with an apparent molecular weight of ~125 kDa {Du, 1995 #84}, the monomeric molecular weight of this protein by SDS-PAGE is approximately 63-68 kDa, while the cDNA predicts a 61.1 kDa protein. Ymt is known to have three start methionines at amino acids 1, producing a soluble species, and at amino acids 42 and 56, producing insoluble species. It appears that Ymt species starting at the first methionine was identified based on solubility, molecular weight and pI. Previously, full length *ymt* gene expression was shown to be greater at 26°C than at 37°C using a lux promoter (20). However, there is conflicting data on the temperature regulation for the Ymt protein (15), where Ymt was shown to be increased at higher temperature. Results from the present study showing a 1.9-fold increase in expression due to solely a temperature increase, further suggest temperature-dependent expression for Ymt at the protein level.

In addition to the three Ymt monomer species and the native dimer, smaller breakdown products of Ymt exist. Initially, Ymt was shown to be a fully active adrenergic antagonist in a mouse model reported to exist as a polymer comprised of 12 kDa subunits (20). A 24 kDa is believed to be a subunit of the native oligomer containing two chains of the 12 kDa product (44). It was also reported that the 12 kDa motif may be the minimally necessary component of Ymt for penetration and disruption of host cell function by interfering with the reception of β-adrenergic agonsists {Montie, 1981 #62}

(44). . The second Ymt protein spot identified in this study (Figure 6, spot 29) is consistent in size and charge with the 24 kDa C-terminal truncated form, which contains only one phospholipase D motif. It is possible that, one phospholipase D domain may be sufficient for Ymt activity explaining the functional activity of smaller Ymt species.

Catalase-peroxidase (KatY): KatY, one of two catalases in *Y. pestis*, binds LcrF, a transcription activator responsible for the upregulation of Yops, and has a theoretical molecular weight of 81.4 kDa. The first 23 amino acids of the KatY open reading frame denote a leader sequence, and thus, after cleavage of the leader, full length KatY is a 78.8 kDa protein having a calculated pI of 6.43. In this study, KatY was identified in seven distinct protein spots. The presence of seven species of KatY is consistent with reports that suggest KatY proteolysis is necessary for activity (25). Previously, KatY was detected in both the cytoplasmic and the periplasmic fractions of *Y. pestis* (53) and is considered to be released from the periplasm during the low calcium response (25). The presence of multiple KatY proteins may be due modification due to or required for cellular location.

The average upregulation of the seven KatY spots in host plasma compared to flea midgut condition was 5.2-fold, while the average upregulation in host intracellular conditions compared to flea midgut condition was 5.0-fold. Three of the KatY protein spots (Figure 6, spots 1, 2, and 3) represent different charge isomers of the full-length KatY protein, α-KatY. The first seven amino acids of KatY (Met-Ala-Met-Asn-Asp-Glu-Glu) can result in false translational starts giving rise to the β form (25). This smaller β isoform of KatY, starts at amino acid 250 and codes for a 53.6 kDa species with a pI of 5.87 (25). β-KatY is 488 amino acids in length, starting just downstream of the second peroxidase motif found in the full-length protein. Several different modifications could be responsible for the different charge of the two protein isoforms found for β-KatY. Spots 8 and 9 of Figure 6 are consistent in both molecular weight for the β isoform, however these two spots appear to be charge isomers.

The γ and δ forms of KatY, 36 and 34 kDa respectively, are Pla-mediated degradation products of the larger α and β isoforms. Spots 26 and 38 of Figure 6 presumably are γ–KatY (36 kDa) and δ–KatY (34 kDa) respectively. Pla-mediated fragments of KatY correlate with increased expression of Pla also detected in this study. KatY expression has not previously been shown to be regulated by calcium, as it is expressed in both calcium containing and deficient medium. However, KatY has been shown to be temperature dependent with increased expression at 37°C, and KatY expression occurs almost immediately after temperature shift. The increased expression for full length KatY is also consistent with an earlier study that showed increased KatY expression by chip-based mass spectrometry analysis of *Y. pestis* cultured under the same physiologically relevant growth conditions used here 37°C (70).

Putative virulence factors

Outer membrane porin protein, OmpA: The *Y. pestis* outer membrane porin protein, OmpA, was found to be upregulated almost 8.0-fold in host plasma compared to flea midgut conditions and 12.3-fold in host intracellular compared to flea midgut conditions. While listed here as a putative virulence factor, several lines of evidence suggest that OmpA is directly implicated in virulence. First, in host-pathogen interaction studies, OmpA was detected in monocyte cell lysates following exposure to *Y. pestis* (data not shown). Although protein carryover from sample preparation could not be ruled out, the presence of OmpA inside host cells suggests that the protein may be injected into host cells. It is also known that OmpA can induce an immunogenic response, triggering dendritic cell migration to infected lymph nodes (31). A mutant OmpA strain of *E. coli* (*ompA::*Tn*phoA* insertion mutant, E58) was found to be significantly less virulent in both embryonic chicken and neonatal rat models (75). The mutant strain also exhibited increased sensitivity to serum bactericidal activity compared to the parent *E. coli* K1 strain E44, suggesting a role for this protein in evasion of host defenses.

N-acetylmuramoyl-L-alanine amidase (AmpD3): AmpD3, a putative N-acetylmuramoyl-L-alanine amidase, was found to be upregulated 2.3-fold in host plasma compared to flea midgut conditions and 2.7-fold in host intracellular compared to flea

midgut conditions. The N-acetylmuramoyl-L-alanine amidase activity of AmpD3 cleaves the amide bond between the lactyl group of the muramic acid residue and the α-amino group of the L-alanine residue of the stem peptide of certain bacterial cell wall glycopeptides {Genereux, 2004 #127}. Bacterial proteins possessing N-acetylmuramoyl-L-alanine amidase activity are often autolysins, able to cleave muramyl-containing glycopeptides on the surface of the same bacteria. Two examples of autolysins from pathogens that possess N-acetylmuramoyl-L-alanine activity include the *atl* gene product in *Staphylococcus aureus* {Oshida, 1995 #128}, and the AmiC protein in *Bacillus anthracis* {Mesnage, 2002 #129}. Autolysins are believed to contribute to virulence via pathogen adhesion and amplification of host inflammatory response {Gupta, 1995 #130}{Milohanic, 2001 #131}, suggesting a potential role for AmpD3 in *Y. pestis* virulence.

An alternative mechanistic interpretation for the increase in AmpD3 found in this study is signaling of extracellular environmental change upon interaction with the host. AmpD, also an N-acetylmuramoyl-L-alanine amidase like AmpD3, is known to degrade muramyl peptides aiding in cell wall and extracellular matrix recycling (46). It is known that bacterial cell wall recycling can alert bacteria of environment changes which crucial for bacterial cell survival and proliferation (51). Therefore, the bacterial cell wall recycling function of AmpD3 may serve to alert *Y. pestis* of a change in physiological environment. The increase in AmpD3 during the change in temperature and calcium concentration encountered during transition from flea to mammalian host, could signal other expression changes crucial for bacterial survival. Moreover, another known *Y. pestis* virulence factor, pesticin which was not identified in this study, also possesses muramidase activity (73), and it is known that muramoyl peptides act as cytotoxins. This further suggests that AmpD3 may play a role in virulence via cytotoxicity. Although, the exact function of AmpD3 was not examined here, future studies with gene knockouts may provide evidence as to the exact mechanistic role of AmpD3 in virulence.

α-enolase: α-enolase, a cytoplasmic enzyme active in the glycolytic pathway, was found to be upregulated 4.4-fold in host plasma compared to flea vector conditions

and 4.9-fold in host intracellular compared to flea midgut conditions. This protein has been suggested to function as a plasminogen or fibrinylase both *in vitro* and *in vivo* (21), and expression of α-enolase on the surface of the outer membrane of Gram-negative bacteria in thought to provide access to host plasminogen (60). The bacterial α-enolase and host plasminogen complex aid in the dissemination of invasive pathogens by degradation of fibrin blots, laminin and fibronectin (26, 36). In fact, it is known that the human pathogen, *Staphylococcus pneumoniae*, binds both human plasminogen and plasmin using α-enolase (9). It is possible that *Y. pestis* α-enolase may function via a similar mechanism. α-enolase has also been shown to be a multifunctional protein, possessing heat shock, cytoskeletal binding, and transcriptional modulation activities (for review, 48). α-enolase has also been directly implicated in pathophysiology (48, 49) including during bacterial infection (8, 48). Thus, expression increases in both α-enolase and Pla under host-like growth conditions are consistent with literature reported, and these proteins may work in concert to prevent the host immune clotting response and permit increased growth and dissemination of *Y. pestis* in the host.

Metabolic Proteins

A number of the differentially expressed proteins that were identified here are enzymes involved in sugar metabolism. Expression increases in physiological host conditions compared to flea vector conditions were detected for phosphoglycerate kinase, 6-phosphogluconate dehydrogenase, D-3-phosphoglycerate, succinyl-CoA synthetase, ribulose-5-phosphate epimerase and glyceraldehyde-3-phosphate dehydrogenase-α, with decreases detected for fructose-bisphosphate aldolase class II and maltose binding protein periplasmic protein precursor. The differential expression of these proteins suggests a model whereby different types and / or amounts of sugars are utilized in the transition from the flea-like to the mammalian host-like growth conditions. Notably, in *E. coli* ribulose-5-phosphate epimerase is essential for growth on single pentose sugars, and deletion of the *rpe* gene caused the loss of catalytic activity rendering the mutant strain unable to utilize single pentose sugars (38). The specific utilization of sugars in different milieus is suggestive of a metabolic adaptive mechanism that may help promote virulence. Alternatively, glycolytic proteins may play another role in virulence, similar to

α-enolase which was also increased in host compared to flea growth conditions. As discussed, α-enolase possesses plasminogen and fibrinylase activity and is implicated in the invasiveness of *Y. pestis*. Multiple functions have now been attributed to the former housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase -α, separate from its role in glycolysis. These alternate functions include a role in apoptosis, microtubule binding, and membrane transport and fusion {Sirover, 1999 #123}, which may be responsible for the upregulation of the protein noted in this study. Moreover, glyceraldehyde-3-phosphate dehydrogenase-α has been reported to function as a plasminogen activator (50, 62), and thus may alsocontribute to the invasive nature of *Y. pestis*.

Protein Production/Chaperones

The Type III secretion mechanism employed by *Y. pestis* is believed to be the most complex protein translocation process in biological systems (57). In order for the Type III secretion system to initiate and produce all the necessary proteins for the delivery of virulence factors into the host cell, significant protein production machinery must be activated. Therefore, the upregulation of elongation factor 1B (EF1B), and ribosomal proteins including 50S L3, 50S L6, and 30S S1, in host physiological (plasma and intracellular) conditions compared to flea midgut conditions found in this study, is likely due to increased protein production of virulence factors under the host intracellular growth conditions that induce virulence *in vitro*. The increased expression of several ribosomal subunit proteins and EF1B suggest involvement of EF1B with the ribosome, potentially regulating *Y. pestis* virulence factors. Exonuclear protein production during temperature and calcium changes in the media could quickly result in virulence factor protein production from existing transcripts. Since translation factors are known to interact with the ribosome, the increased expression of EF1B and ribosomal proteins with a concomitant increase in total protein supports this theory. Other theories suggest that EF1B may be an effector protein. Since the α isoform of EF1B prevents binding of amino acids to tRNA (for review of bacterial elongation factors (4)), EF1B may target the host ribosomal network to alter host protein production. Alternatively, since EF1B activates EF-Tu (EF1A) from the GDP-bound state to a GTP form, EF1B may be

translocated into the host cell to target host G protein signal molecules causing altered host signal transduction.

It is well known that chaperone proteins in *Y. pestis* aid the formation and injection of virulence factors into the host cell via the Type III secretion system. Chaperone proteins are also expressed by bacteria for protection against the stresses of the *in vivo* environment (67). Three chaperone proteins, ClpB, HtpG, and Cpn60, showed increased expression in host physiological (plasma and intracellular) compared to flea vector conditions. Two known isoforms of ClpB were identified here with a 2.6- and 1.7-fold increase in host plasma, and 2.9- and 2.3-fold increase in host intracellular compared to flea midgut conditions, respectively. HtpG was co-identified in the same protein spot as phosphoglycerate kinase. While it is not possible to determine if both or only one of these proteins was differentially expressed, together they demonstrated a 3.1-fold increase in host intracellular compared to flea midgut conditions. Future studies aimed at resolving this gel region are needed to elucidate the individual expression levels. Cpn60 was found in ten distinct spots, potentially due to post-translational regulation for multiple chaperone functions or sample processing (2). Nine spots were upregulated an average of 4.3-fold and 1 spot was downregulated 1.2-fold in host intracellular compared to flea midgut conditions.

ClpB is known to play a role in the regulation of the virulence factor invasin and bacterial motility in *Y. enterocolitica* (6). Further, ClpB and HtpG participate in cellular protein folding under mild heat-shock conditions (69). HtpG has also been shown to function as an immunogen and may contribute to the virulence of the periodontal pathogen, *Porphyromonas gingivalis* (35). Both HtpG and Cpn60 are heat shock proteins and expression increases could be due to increased protein production as well as increased temperature of the mammalian host culture conditions. However, Cpn60 may have a more prominent role in virulence determination as this protein possesses multiple chaperone functions in different cellular locations. The identification of multiple forms of Cpn60 is consistent with a potential role in stabilizing multiple folding intermediates of nascent proteins formed during *Y. pestis* production of virulence proteins. Moreover,

known regulation of Cpn60 provides an explanation for the identification of Cpn60 in multiple protein spots(3). There are numerous proteolysis sites in Cpn60, including multiple sites for thermolysin (55), and the *E. coli* homolog of Cpn60, GroEL, has been shown to be sensitive to chymotrypsin cleavage resulting in multiple isoforms, including a major, stable N-terminal species (26 kDa) and C-terminal species (44 kDa). Another C-terminal cleavage product starting at residue 268 and other intermediate species of GroEL exist (29). The multiple species of Cpn60 identified here may also be due to an alternative function based on the fact that the Cpn60 chaperonin homolog of *Helicobacter pylori*, hsp60, acts as an antigen in the pathogenesis of gastric mucosa-associated lymphoma (2, 77). Thus it is possible that Cpn60 may serve not only as a chaperone for virulence factors, but that Cpn60 could be processed by degradation to a species capable of modulating the host immune response.

Membrane Proteins

Five of the proteins found to be differentially expressed in this study are associated with the *Y. pestis* inner and outer membranes including Pla, maltose binding protein periplasmic protein precursor, Ymt, KatY, and OmpA (25, 33). The finding of multiple membrane-associated proteins in the soluble protein fraction suggests a subset of membrane-bound proteins may be solubilized by the methods used in this study and can be examined by 2-D DIGE without the need for more complex membrane fractionation and sample processing {Hixson, In Prep #106}. Alternatively, the presence of these proteins in the soluble fraction could be a result of subcellular relocation into more soluble compartments of *Y. pestis* during virulence inducing growth conditions, as noted for an *E. coli* membrane chemoreceptor that migrates from the cell membrane to the cytosol following upregulation (37). While these results are consistent with virulence induction and significant membrane alteration to facilitate Type III secretion (18), further study of the membrane fraction and bacterial protein translocation is required understand the full involvement of membrane-associated proteins in the complex virulence paradigm of *Y. pestis*.

CONCLUSION

This study represents the first comprehensive 2-D DIGE proteomic analysis of *Y. pestis* cultured under virulence-inducing physiologically relevant growth conditions mimicking the flea vector and mammalian host. The proteomic map of *Y. pestis* constructed provides knowledge of expression levels for known virulence factors, putative virulence factors, metabolic proteins, and potential novel virulence determinants. Future proteomic studies addressing secreted and membrane-bound *Y. pestis* proteins, using altered gel formulations to examine the lower molecular weight *Y. pestis* proteome which includes several known virulence factors, and experiments to control the proteolytic activity of Pla will more fully address the *Y. pestis* proteome. The data and methods established from this study are already guiding proteomic comparisons of variant and mutant strains of *Y. pestis*. For example, comparison of virulent strains of *Y. pestis*, including clinical isolates that possess over 1000-fold differences in pathogenicity as determined in animal models, may identify novel virulence determinants and thus new therapeutic targets for plague (22). From a mechanistic point, while inferences can be made as to the function of differentially expressed proteins identified from this and future studies, knockouts and mutant strains of *Y. pestis* are also required to address the biological function of putative virulence proteins. From a biodefense viewpoint, differentially expressed proteins identified from proteomic characterization of virulence induction have practical use for early, rapid and specific detection of *Y. pestis*. The proteomic studies of *Y. pestis* reported here paves the way for future comparison of clinical isolates, strains with different virulence attributes, as well as mutant and knockout strains, which will provide an improved mechanistic understanding of virulence, lead to pathogen signatures for detection, and identify new therapeutic targets for plague.

Figure legends:

Figure 1. 2-D DIGE images of the *Y. pestis* soluble proteome from four growth conditions. Panel A, representative soluble proteomic map of *Y. pestis* grown at 26°C without calcium. Panel B, representative soluble proteomic map of *Y. pestis* grown at 26°C with 4mM calcium. Panel C, representative soluble proteomic map of *Y. pestis* grown at 37°C without calcium. Panel D, representative soluble proteomic map of *Y. pestis* grown at 37°C with 4 mM calcium.

Figure 2. DeCyder BVA output images showing three differentially expressed proteins identified. Comparison of three proteins between the flea midgut and host intracellular conditions for Pla (A), OmpA (panel B), and fructose-bisphosphate aldolase class II (panel C). Each panel shows three types of data output from the DeCyder software. Zoomed regions of the gel image containing each protein spot under two growth conditions are shown in the top panels. Borders for the selected spots are shown in magenta. The three-dimensional fluorescence intensity profiles of the individual spot volumes are shown in the middle panels, highlighting the differential expression for the selected spots. The bottom panels show a graph of the normalized spot volumes from replicate gels. The circles displayed for each condition on the graphs represent a single normalized spot volume from one gel, and lines are plotted corresponding to the averaged values for the particular growth condition. This data is representative of proteins identified in Table 1.

Figure 3. DeCyder BVA output images of plasminogen activator, Pla, for the four growth comparisons. Panel A shows a 2.8-fold increase in Pla expression between flea and intracellular host conditions. Panel B shows a 2.4-fold increase between flea midgut and host plasma conditions. Panel C shows no detectable increase in Pla expression between 4 mM calcium and 0 mM calcium growth. Both 26°C and 37°C samples were examined together and are represented in this panel. Panel D shows a 2.1-fold increase between 26°C and 37°C growth conditions. Both 4 mM and 0 mM calcium samples were examined together. Each panel shows three types of data output from BVA. Zoomed

regions of the gel image containing Pla under two growth conditions are shown in the top panels. Borders for the selected spots are shown in magenta. The three-dimensional fluorescence intensity profiles of the individual spot volumes are shown in the middle panels, highlighting the differential expression of Pla across the experiment. The bottom panels show a graph of the normalized spot volumes from replicate gels. The circles displayed for each condition on the graphs represent a single normalized spot volume from one gel, and lines are plotted corresponding to the averaged values for the particular growth condition.

Figure 4. 2-D DIGE overlay image of the *Y. pestis* soluble proteome showing differential protein expression following changes in calcium concentration. Panel A shows an overlay comparing *Y. pestis* cells grown in 0 and 4 mM calcium at 26°C. Blue represents protein spots that are more highly expressed at 4 mM calcium. Red represents protein spots that are more highly expressed at 0 mM calcium concentration. Green represents protein spots that are more highly expressed in the pooled standard, which contains both calcium and temperature growth conditions. Since only the pooled standard contains 37°C samples, these spots represent proteins that are upregulated at 37°C, as red and blue samples contain only 26°C samples. Panel B shows an overlay 2-D DIGE image comparing 0 and 4 mM calcium concentration protein samples at 37°C. Blue spots represent protein spots that are more highly expressed at 4 mM calcium. Red spots represent protein spots that are more highly expressed at 0 mM calcium concentration. Green represents protein spots that are more higly expressed in the pooled standard. Since only the pooled standard contains 26°C samples, these spots are more highly expressed at 26°C, as red and blue samples contain only 37°C samples. White protein spots in Panels A and B represent proteins with unaltered expression across the gel.

Figure 5. 2-D DIGE overlay image of the *Y. pestis* soluble proteome showing differential protein expression following changes in temperature. Panel A shows an overlay comparing 26°C and 37°C protein samples in 0 mM calcium concentration samples. Blue spots represent protein spots that are more highly expressed at 37°C. Red spots represent protein spots that are more highly expressed at 26°C. Green represents protein spots that

are more highly expressed in the pooled standard, which contains both calcium and temperature growth conditions. Since only the pooled standard contains 4 mM calcium samples, these spots represent proteins that are upregulated in 4 mM calcium, as red and blue samples contain only 0 mM calcium. Panel B shows an overlay comparing 26°C and 37°C protein samples in 4 mM calcium concentration. Blue spots represent protein spots that are more highly expressed at 37°C. Red spots represent protein spots that are more highly expressed at 26°C. Green represents protein spots that are more highly expressed in the pooled standard. Since only the pooled standard contains 0 mM calcium samples, these spots represent proteins that are more highly expressed at 0 mM calcium, as red and blue samples contain only 4 mM calcium. White protein spots in Panels A and B represent proteins with unaltered expression across the gel.

Figure 6. 2-D DIGE overlay image of the *Y. pestis* soluble proteome showing differential protein expression between the flea midgut and host intracellular *in vitro* growth conditions. Red spots represent protein spots that are upregulated under conditions mimicking the flea midgut relative to the host intracellular conditions. Blue spots represent protein spots that are upregulated in the host intracellular conditions relative to the flea midgut. Green spots represent protein spots that are higher in the pooled standard, representing differential expression in at least one of the conditions not directly compared in this image. White spots represent proteins with unaltered expression across the gel. Yellow arrows with numbers show the location of the differentially expressed proteins that were identified by mass spectrometry.

Table 2. Differentially expressed *Y. pestis* proteins related to virulence.

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