Secretome and Comparative Proteomics of *Yersinia pestis* Identify Two Novel E3 Ubiquitin Ligases That Contribute to Plague Virulence

Authors

Shiyang Cao, Yuling Chen, Yanfeng Yan, Songbiao Zhu, Yafang Tan, Tong Wang, Yajun Song, Haiteng Deng, Ruifu Yang, and Zongmin Du

Correspondence

dht@mail.tsinghua.edu.cn;
ruifuyang@gmail.com;
zmduams@163.com

Graphical Abstract

In Brief

Rapid adaption to the environments is critical for microbes to establish infection. Quantitative proteome and secretome analyses of *Y. pestis* grown under conditions mimicking its two typical natural niches were performed to understand the adaption strategies of this deadly pathogen. We identified three secreted proteins that can be translocated into host cells and further demonstrated that two of them show strong E3 ubiquitin ligase activity and contribute significantly to the virulence of *Y. pestis*.
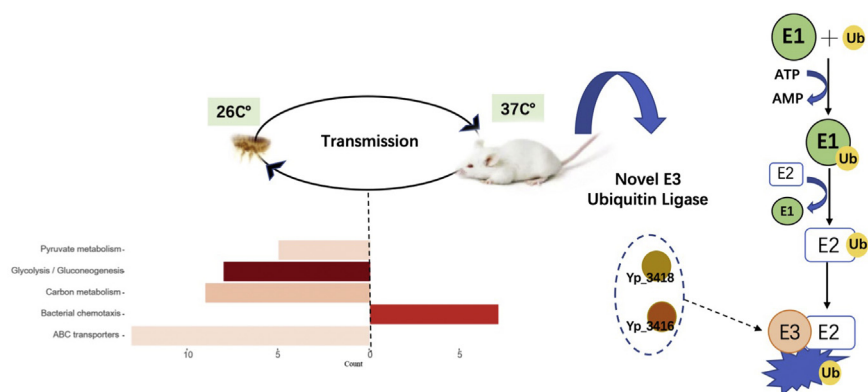
Highlights

- Quantitative proteome analysis of *Y. pestis* was performed to understand its adaption strategies.

- A secretome of *Y. pestis* containing 308 proteins has been identified.

- YP_3416 and YP_3418 exhibited strong E3 ubiquitin ligase activity.

- *yp_3416~3418* deletion mutant of *Y. pestis* showed significant virulence attenuation in mice.

Secretome and Comparative Proteomics of *Yersinia pestis* Identify Two Novel E3 Ubiquitin Ligases That Contribute to Plague Virulence

Shiyang Cao[1,‡], Yuling Chen[2,‡], Yanfeng Yan[1], Songbiao Zhu[2], Yafang Tan[1], Tong Wang[1], Yajun Song[1], Haiteng Deng[2,*], Ruifu Yang[1,*], and Zongmin Du[1,*]

Plague is a zoonotic disease that primarily infects rodents *via* fleabite. Transmission from flea to host niches requires rapid adaption of *Yersinia pestis* to the outer environments to establish infection. Here, quantitative proteome and secretome analyses of *Y. pestis* grown under conditions mimicking the two typical niches, *i.e.*, the mammalian host (Mh) and the flea vector (Fv), were performed to understand the adaption strategies of this deadly pathogen. A secretome of *Y. pestis* containing 308 proteins has been identified using TMT-labeling mass spectrometry analysis. Although some proteins are known to be secreted, such as the type III secretion substrates, PsaA and F1 antigen, most of them were found to be secretory proteins for the first time. Comparative proteomic analysis showed that membrane proteins, chaperonins and stress response proteins are significantly upregulated under the Mh condition, among which the previously uncharacterized proteins YP_3416~YP_3418 are remarkable because they cannot only be secreted but also translocated into HeLa cells by *Y. pestis*. We further demonstrated that the purified YP_3416 and YP_3418 exhibited E3 ubiquitin ligase activity in *in vitro* ubiquitination assay and *yp_3416~3418* deletion mutant of *Y. pestis* showed significant virulence attenuation in mice. Taken together, our results represent the first *Y. pestis* secretome, which will promote the better understanding of *Y. pestis* pathogenesis, as well as the development of new strategies for treatment and prevention of plague.

Yersinia pestis is the etiological agent of plague that has claimed millions of death in history. This lethal pathogen still poses a huge threat by causing sporadic plague outbreak every year and being a bioterrorism agent that can be potentially misused by terrorist (1, 2). Plague is a natural focus disease that primarily infects rodents, and humans are the accidental hosts (3). After entering the host, for instance, by fleabite, a typical transmission route of plague, *Y. pestis* is readily taken up by the host professional phagocytes and those engulfed by the macrophages can survive and replicate in this shield niche (4, 5). During transmission process, *Y. pestis* has to adapt itself to the adverse host environments through orchestrating expression of virulence factors and then spills out of the macrophages, where it becomes resistant to phagocytosis and initiates an extracellular life. As a facultative intracellular pathogen, *Y. pestis* predominantly survives and replicates extracellularly in the host. Transition between the two drastically different environments demands a rapid adaption of *Y. pestis* to external conditions, especially those in mammalian hosts to avoid being eliminated before establishing a successful infection.

Expressions of *Y. pestis* virulence factors are finely tuned in a temperature-dependent manner and significantly induced at the mammalian host temperature (6–9). One of the essential virulence mechanisms of *Y. pestis* is the pCD1-encoded type III secretion system (T3SS) that can deliver virulent effectors named *Yersinia* Outer Proteins (Yops) into eukaryotic cells to hijack the host cellular signaling pathway (10–12). Mammalian host temperature (37 °C) strongly induces the expression of *Yersinia* T3SS, and the contacts between the bacteria and the host cells trigger Yops delivery into eukaryotic cells. In *in vitro* growth, the temperature of 37 °C and the low calcium concentration of culture medium mimic the host environments and strongly induce the expression and delivery of Yop effectors. Expressions of the critical virulence factors other than T3SS, such as the adhesion PsaA and F1 capsular antigen, are also highly expressed at 37 °C controlled by a temperature-dependent regulatory mechanism.

Pathogenic bacteria express a lot of exported proteins to interplay with their surrounding environments. Tjalsma *et al.* firstly coined the term of "secretome" in the study of *Bacillus subtilis* (13, 14), which defined the secretome as all of the secreted proteins and secretory machinery of a bacteria. Agrawal *et al.* (15) proposed to define the "secretome" as the global group of secreted proteins into the extracellular space

by a living creature or its constituent parts. Bacterial secreted proteins are thought to be involved in the bacteria–bacteria communications and bacteria–host interactions, which can be potentially used as diagnostic markers, vaccination immunogens, and therapeutic targets ([16], [17]). To define the secretome of *Y. pestis* is of considerable significance for the better understanding of how *Y. pestis* interacts with the host and eludes the immune surveillance.

Here, in order to understand the physiological and functional adaptions of *Y. pestis* in fleas and mammals, quantitative proteomics of *Y. pestis* grown under conditions mimicking the two typical niches, termed as Mh (mammalian host) and Fv (flea vector), were analyzed and compared. Abundances of 373 proteins (98 up- and 275 down-regulated) in the culture supernatant of *Y. pestis* were significantly different between the two conditions, whereas only 31 proteins (24 up- and 7 down-regulated) were significantly altered in bacterial cells. A secretome of *Y. pestis* containing over 300 proteins was defined, and 112 and 73 of them were secreted exclusively in Mh and Fv conditions, respectively. The secreted proteins that were increased in abundance in Mh condition might contribute to the virulence of *Y. pestis*. Among them, YP_3416 and YP_3418 were demonstrated to be translocated into HeLa cells during infection and exhibited E3 ubiquitin ligase activity in *in vitro* ubiquitination assay. Deletion of the *yp_3416~3418* locus resulted in significant virulence attenuation in mice, suggesting that they could play some important roles in the virulence of plague.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions

Y. pestis 201 strain belongs to the biovar Microtus, which is highly virulent to mice, but avirulent to humans ([18]). *ΔyscI* is a mutant of *Y. pestis* strain 201, which is impaired in T3SS assembly and substrate secretion ([19]). *Y. pestis* strains were grown in TMH medium without calcium at 37 °C or TMH medium with 2.5 mM calcium at 26 °C to mimic the conditions when *Y. pestis* infects the mammalian host (Mh) or resides in the flea vectors (named Fv as the counterpart of Mh), respectively ([20]). Antibiotics were added to the culture media when needed at the following concentrations: 100 mg/ml ampicillin, 20 mg/ml streptomycin, 10 mg/ml tetracycline, and 20 mg/ml kanamycin.

Experimental Design and Statistical Rationale

Y. pestis strains were grown under Mh or Fv condition and proteins isolated from the culture supernatant (P_{CS}) or bacterial cells (P_{BC}) were subjected to quantitative proteomic analysis based on TMT (tandem mass tag) labeling with three independent biological replicates. For the comparative proteomics between the two culture conditions mimicking the two typical niches, proteins isolated from the culture supernatant (P_{CS}) and bacterial cells (P_{BC}) of *Y. pestis* strain 201 grown under Mh and Fv conditions were used as experimental and control group, respectively. For the secretome study, proteins isolated from P_{BC} and P_{CS} of *Y. pestis* strain 201 grown in Mh or Fv conditions were used as experimental and control group, respectively. For the proteomics analysis of the potential T3SS substrates, proteins

isolated from P_{CS} of *ΔyscI* and *Y. pestis* strain 201 grown under the Mh conditions were used as experimental and control group, respectively. The proteomic results were further analyzed by using R (version 4.0) and RStudio software (version 1.3). Welch's two-sample *t*-test was used to compare the means of scaled abundances of each protein, adjusted by Benjamini–Hochberg method for multiple comparison ([21]). Adjust *p*-value <0.05 was defined as significant difference in protein abundance. The cutoff threshold for filtering differentially regulated proteins was determined by applying population statistics to the biological replicates ([22], [23]).

Functional Annotation and Analysis

Functional categories were assigned to each protein according to the genome annotation of *Y. pestis* CO92 and the up- or down-regulated proteins were counted by the functional categories. KEGG pathway enrichment and GO term in biological process, cellular compartment, and molecular function enrichment analysis were performed using web-based DAVID bioinformation tools (http://david.abcc.ncifcrf.gov) ([24]), and the significance of the enrichment was tested by Fisher's exact test. The volcano plot, GO dotplot, and KEGG barplot were drawn using ggplot2 R package.

Sample Preparations and Protein Extraction

An aliquot of glycol suspension (stored at –80 °C) of *Y. pestis* strain 201 was inoculated into 5 ml fresh TMH medium with 2.5 mM calcium and grown at 26 °C to the log phase. This seed culture was inoculated into 200 ml fresh TMH medium with 2.5 mM calcium, and the culture was grown at 26 °C until the optical density at 600 nm (OD_{600}) reached approximate 1.0. Bacterial cultures were separated equally into two portions, followed by centrifugation at 1700 *g* for 10 min and carefully removing of the supernatant to collect the bacterial cells. One-half of the bacterial cell pellets was resuspended in fresh TMH medium with 2.5 mM calcium and cultured at 26 °C (mimicking Fv condition); the other half was resuspended in fresh TMH medium without calcium and cultured at 37 °C (mimicking Mh condition). After 4 h of cultivation, the cultures were centrifuged at 1700*g* for 10 min and the supernatants were carefully transferred to a precold clean tube. The supernatants were added with protease inhibitor cocktail (cOmplete ULTRA Tablets, Roche Diagnostic GmbH), then filtered through 0.22 μm sterilized filters (Millipore) to remove any bacterial cell. The supernatants were concentrated to 300 μl by centrifugation through an Amicon Ultra-15 (3000-Da molecular weight cutoff) centrifugal filter device (Millipore). The bacterial pellets were resuspended in lysis buffer (8 M urea in PBS, pH 7.4) and lysed by sonication in ice bath. The concentrations of protein samples from the supernatants and the cell pellets were determined by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

For the study of the substrate secretion of *Y. pestis* T3SS, *Y. pestis* strain 201 and *ΔyscI* were cultured under the Mh and Fv conditions and protein samples from the culture supernatants and cell pellets were extracted and the protein concentrations were determined as described above.

Equal amounts of proteins (100 μg) of the secreted proteins or the bacterial cells were used to perform the quantitative proteomics analysis. Proteins were reduced by 5 mM dithiothreitol and alkylated by 12.5 mM iodoacetamide. The samples were diluted by PBS to the urea concentration of 1.5 M, followed by digestion with trypsin of a 1:100 protease/protein ratio at 37 °C overnight. The samples were desalted by Sep-Pak columns. Peptides from different samples were labeled with TMT reagents (Thermo, Pierce Biotechnology) according to the manufacturer's instruction. The TMT-labeled peptides were mixed as the Figure 1*A* shown and desalted by Sep-Pak column.
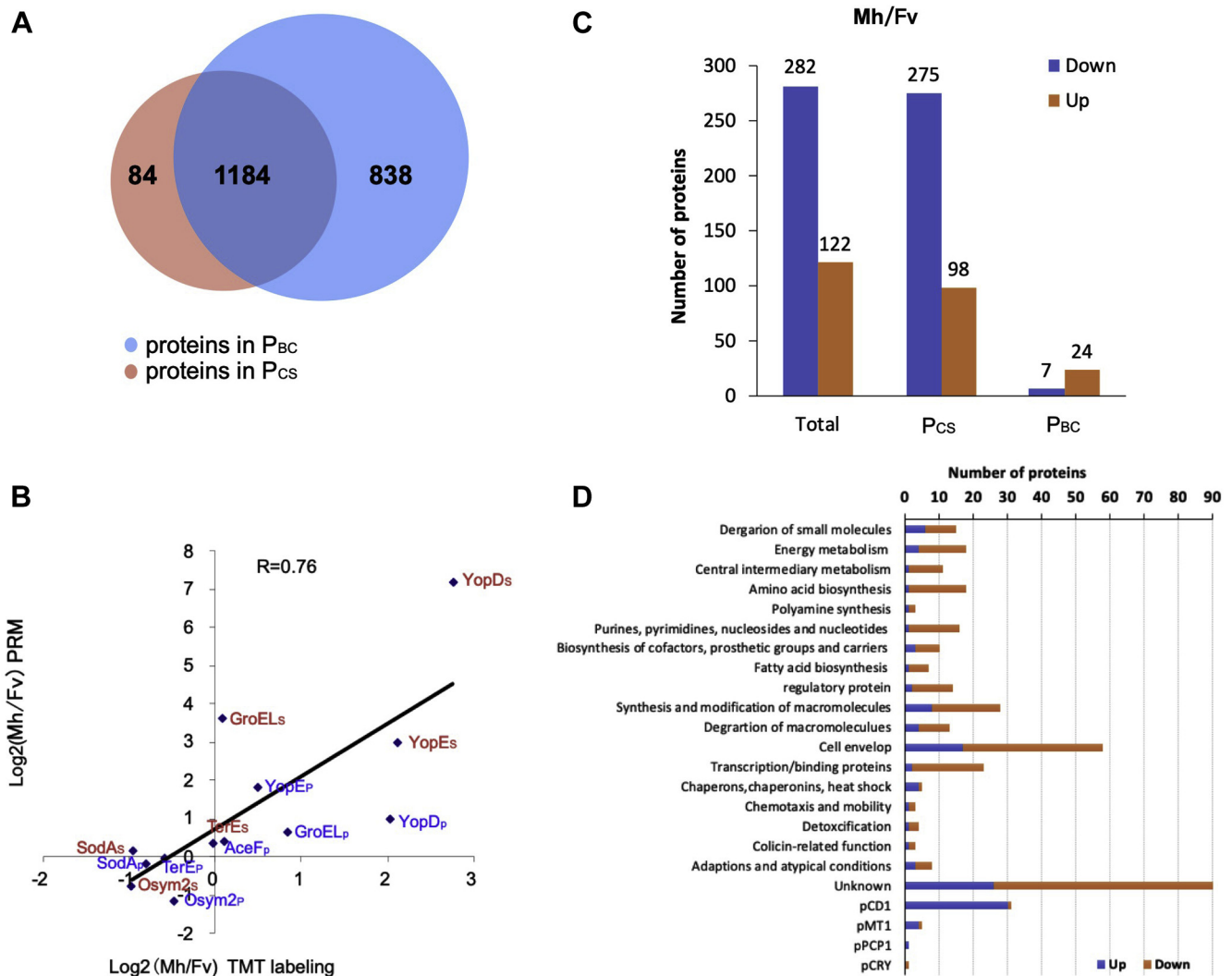
FIG. 1. **Comparative proteomics analysis of *Y. pestis* 201 strain grown under the Mh and Fv conditions.** *A*, Venn diagram of proteins identified in the bacteria cells (P_{BC}) or the culture supernatants (P_{CS}) of *Y. pestis* 201 strain. *B*, validation of protein abundance alterations using PRM MS analysis. Abundance of seven proteins (AceF, SodA, YopD, YopE, GroEL, TerE, and OsmY2) in an independent set of P_{CS} and P_{BC} samples were determined using PRM MS analysis. Subscript S and P following a protein name indicates the abundance in P_{CS} and P_{BC}, respectively. The correlation between the protein abundances obtained from the PRM and TMT-labeling-based MS analysis was calculated (R = 0.76) by linear regression model. *C*, the numbers of proteins that were significantly differently regulated under the two conditions in P_{BC}, P_{CS}, or total proteins ($P_{BC} + P_{CS}$) were shown. *D*, the significantly up- or down-regulated proteins ($p < 0.05$) under the two conditions were classified according to the functional categories with the protein numbers of each category labeled.

LC-MS/MS Analysis

The peptides were prefractionated by a UPLC3000 system with an XBridgeTM BEH300 C18 column. Mobile phase A is H_2O adjusted by ammonium hydroxide to pH 10, and mobile phase B is acetonitrile in ammonium hydroxide pH 10. Peptides were separated with the followed gradients: 8% to 18% phase B, 30 min; 18% to 32% phase B, 22 min 48 fractions were collected, dried by a speedvac, combined into 12 fractions, and redissolved in 0.1% formic acid. For quantitative proteomic analysis, the TMT-labeled peptides were separated by a 60-min gradient elution at a flow rate of 0.250 μl/min with an EASY-nLCII integrated nano-HPLC system (Proxeon), which is directly interfaced with a Q Exactive mass spectrometer (Thermo Fisher Scientific). The analytical column

was a fused silica capillary column (75 μm ID, 150 mm length; packed with C-18 resin). Mobile phase A consisted of 0.1% formic acid, and mobile phase B consisted of 100% acetonitrile and 0.1% formic acid.

The Q Exactive mass spectrometer was operated in the data-dependent acquisition mode using the Xcalibur 2.1.3 software, and there was a single full-scan mass spectrum in the Orbitrap (300–1800 m/z, 70,000 resolution) with automatic gain control (AGC) target value of 3e6. A data-dependent acquisition method was used to collect generated MS/MS spectra at 17,500 resolution with AGC target of 1e5 and maximum injection time (IT) of 60 ms for top 20 ions observed in each mass spectrum. The isolation window was set at 2 Da width, the dynamic exclusion time was 60 s, and the normalized collisional energy (NCE) was set at 30.

Protein Identification and Quantification

The generated MS/MS spectra were searched against the *Y. pestis* 91001 database (downloaded from NCBI on OCT 27, 2020, GenBank assembly accession: GCA_000007885.1) using the SEQUEST searching engine of Proteome Discoverer (PD) Software package (version 1.4, Thermo Scientific). The search criteria were as follows: full tryptic specificity was required; one missed cleavage was allowed; carbamidomethylation (C) and TMTsixplex (K and N-terminal) were set as the fixed modifications; the oxidation (M) was set as the variable modification; precursor ion mass tolerances were set at 10 ppm for all MS acquired in an orbitrap mass analyzer; and the fragment ion mass tolerance was set at 20 mmu for all MS2 spectra acquired. The peptide false discovery rate (FDR) was calculated using Percolator provided by PD. When the q value was smaller than 1%, the peptide spectrum match was considered to be correct. False discovery was determined based on peptide spectrum match when searched against the reverse, decoy database. Peptides only assigned to a given protein group were considered as unique. The FDR was set to 0.01 for protein identification. Relative protein quantification was performed by using PD according to the manufacturer's instructions on the six reporter ion intensities per peptide. Quantitation was carried out only for proteins with two or more unique peptide matches. Protein ratios were calculated as the median of all peptide hits belonging to a protein. Quantitative precision was expressed as protein ratio variability.

Parallel Reaction Monitoring Analysis of Target Proteins

The target proteins were picked up in the results of quantitative proteomics analysis, and parallel reaction monitoring (PRM) analysis was used to confirm the alterations in abundance of the target proteins. Equal amounts of proteins from the culture supernatants and cell pellets were separated by SDS-PAGE, and corresponding bands of the target proteins were cut to perform in-gel digestion. The peptide samples of target proteins were analyzed in mass spectrometry, and three peptides from each target protein were selected for the PRM analysis. The selecting criteria of peptides were sequence length of 6 to 15 amino acids and excluded those with any modifications and missed cleavages sites. The peak area of 3 b/y ions from each peptide was used to calculate the peptide ratio, and the ratios of three peptides were used to determine the target protein ratio in different samples. The samples used for PRM analysis were digested in-gel without desalting. 100 μm × 2 cm C18 column from Thermo was used as the trapping column, and 75 μm × 15 cm C18 column from Thermo was used as the analytic column. The gradient with flowrate of 0.3 μl/min was as follows: 0 to 5 min, 4% ACN; 5 to 42 min, ACN from 4% to 25%; 42 to 47 min, ACN from 25% to 47%; 47 to 55 min, 90% ACN; 56 to 60 min, 4% ACN. For the full-scan mass spectrum, the resolution was set as 70,000, and AGC target was 3e6 with injection time of 20 ms. For the PRM scan, the resolution was set as 17,500, and AGC target was 1e6 with injection time of 60 ms. The isolation window was set at 2.5 m/z width, and the NCE was set at 27. For the data analysis, the peak area of each ion was integrated manually in Xcalibur software 2.1.3, used for calculating the protein ratio of experimental sample compared with control.

In Vitro Ubiquitination Assays

Prepare a prereaction mixture containing 4 μl dH$_2$O, 3 μl FLAG-Ubiquitin, 5 μl 10× reaction buffer, 1 μl E1, 2 μl UbcH5a, 2 μg GST-tagged YP_3416, YP_3417 or YP_3418 or their mutants at room temperature. Add 2 μl of 10× Mg^{2+}-ATP solution to initiate conjugation reaction and incubate the reaction mixtures at 37 °C for 30 min. After terminating the reaction by addition of DTT, samples were added with 5 μl 5× SDS loading buffer and separated by running 4 to 15% SDS-PAGE gels. Proteins were visualized using a mouse monoclonal FLAG

M2 antibody, followed by incubation with IRDye 800CW-conjugated goat anti-mouse secondary antibodies. Images of the immunoblotting results were taken by an Odyssey SA imaging system (LI-COR Biosciences).

Construction of the Y. pestis *Mutants and the Complemented Strains*

Mutant cassettes of *yp_3416* and *yp_3418*, which consisted of ~300 bp homologous sequences flanking the mutated genes at both sides of DNA fragments, were generated by spliced overlap extension PCR and cloned into pDS132. The recombinant plasmids were subsequently transformed into *E. coli* S17λpir. Then, the *yp_3416* and *yp_3418* deletion was introduced into the *Y. pestis* 201 strain by conjugation and allelic exchange. The *blaTEM*-1 coding sequence was cloned into pBBR1MCS to generate pBBR1-TEM, and then ~300 bp of upstream sequences and the coding sequences of *yp_3416*, *yp_3417*, *yp_3418* or *yopE* were amplified and inserted directly upstream of the *blaTEM* gene in pBBR1-TEM, generating the corresponding TEM reporter plasmids, which were then electroporated into the wild-type 201 strain.

TEM-1 β-Lactamase-Based Translocation Assay

Y. pestis strains harboring various reporter plasmids were grown at 26 °C to OD$_{600nm}$ = 1.0 and shifted to 37 °C for 3 h' incubation prior to infection. HeLa cells were seeded into 96-well plate with black walls and clear bottoms (Invitrogen). After reaching 60~70% confluence, HeLa cells were infected with *Y. pestis* strains harboring the TEM reporter plasmids at an MOI of 20. CCF2-AM substrate was loaded at 1 μg/ml to the infected HeLa cells according to the manufacturer's instructions (Invitrogen), and the plates were incubated at 37 °C and analyzed by SpectraMax M5 (Molecular Devices) at 10-min intervals with an emission wavelength of 409 nm and detection wavelengths of 447 and 520 nm (25).

Animal and Cell Infection Experiments

The wild-type *Y. pestis* strain 201 and the null mutants of *yp_3416*, *yp_3418* and *yp_3416~yp_3418* were grown in LB medium at 26 °C. For each strain tested, ten female BALB/c mice (6–8 weeks old) were infected with 100 CFU (colony forming unit) bacteria *via* intranasal (*i.n.*) route of infection, and the infected animals were observed daily for 14 days. Survival rates were calculated for each animal group and the statistical significance of differences between the wild-type strain and the mutants was determined by the log-rank (Mantel-Cox) test using GraphPad Prism 6.0 software. All animals were handled in strict accordance to the Guidelines for the Welfare and Ethics of Laboratory Animals of China, and all the animal experiments were approved by the Institutional Animal Care Committee of Beijing Institute of Epidemiology and Microbiology (protocol number IACUC-DWZX-2020-058).

RESULTS

Comparative Proteome Analysis of Y. pestis *Grown Under the Conditions Mimicking Its Two Typical Niches*

Two typical niches of *Y. pestis* are the proventriculus of flea vector (26 °C) and the mammalian host environments (37 °C). The adverse environments and the body temperature of 37 °C in mammalian hosts considerably impact the physiology as well as the virulence of *Y. pestis*. In this study, *Y. pestis* strain 201 was grown at 26 °C in TMH with calcium (Fv condition) or 37 °C in TMH without calcium (Mh condition), and proteins in the culture supernatants (P$_{CS}$) or bacterial cells (P$_{BC}$) were

extracted, TMT-labeled, and analyzed by MS. Total of 2022 proteins in bacterial cells and 1268 in culture supernatants were detected, and 1184 proteins could be quantitatively measured in both bacterial cells and culture supernatants (Fig. 1*A*, supplemental Table S1). In total, 2106 proteins were identified in P_{CS} and P_{BC}, representing 52.32% proteins of the *Y. pestis* 91001 genome (4025 CDSs with proteins, GenBank assembly accession: GCA_000007885.1).

Abundances of ten target proteins in P_{CS} and P_{BC} samples were confirmed using PRM (parallel reaction monitoring)-based targeted MS analysis. Six proteins (SodA, YopD, YopE, GroEL, TerE and OsmY2) were successfully measured in both culture supernatant and bacterial cell pellets, and AceF was only detectable in culture supernatant (Fig. 1*B*), while the other three proteins (PsaA, YP_3608, and YP_0175) were failed to be detected probably due to the relatively low abundances. The correlation analysis results showed that PRM results correlate well with that of TMT labeling (supplemental Table S2), suggesting that the data acquired by the TMT-labeling-based quantitative proteomics analysis were reliable.

Abundance of 98 proteins was significantly increased and 275 decreased ($p < 0.05$), in the culture medium of *Y. pestis* grown under the Mh condition compared with the Fv condition (Fig. 1*C*), while only 31 proteins in P_{BC} appeared to be altered in abundance, indicating that the secreted proteins were more profoundly influenced by the environmental conditions. These results implied that secreted proteins might play vital roles in adapting to the various environmental conditions, including the adverse mammalian host environments, thus contributing to the pathogenesis of *Y. pestis*.

Differentially regulated proteins (supplemental Table S3) under the two culture conditions were classified according to their functional classifications (Fig. 1*D*). A number of proteins with significantly altered abundance belong to the categories of cell envelope (58), synthesis and modification of macromolecules (26), transcription/binding proteins (24), amino acids and metabolism (18), or encoded by the plasmid pCD1 (27) and PMT1 (5). Among them, virulence factors including T3SS components, F1 capsular antigen, PsaA, and KatY were the most significantly upregulated proteins (supplemental Table S3), which is consistent with the previous reports that expression of virulence factors of *Y. pestis* was elevated at 37 °C (6). Membrane proteins or lipoproteins including OmpC, NlpD, LPP, chaperonins (GroES and GroEL), and stress-response-related proteins (HdeB and HtpG) were significantly upregulated under the Mh condition (supplemental Table S3). The most significantly decreased proteins are those involved in amino acids biosynthesis (17) and macromolecules synthesis/modification (21), which is also in line with the growth restriction phenotype of *Y. pestis* grown at 37 °C without calcium (28). Notably, over 100 unannotated proteins were significantly differentially expressed under the two culture conditions, which deserves further investigation to clarify their roles in adaptive response to the adverse host environments.

Defining the Secretome of Y. pestis

Proteins showing significantly higher abundance ($p < 0.05$) in P_{CS} than in P_{BC} under Mh or Fv conditions were considered to be secreted by *Y. pestis*. All the proteins that fulfill this criteria have a $|\text{Log}_2 \text{FC}| \geq 0.77$ (about $1.7 \times \text{FC}$) at least. Thus, the influence of the proteins simply released from a few disrupted bacterial cells that possibly existed in *Y. pestis* cultures can be excluded, because the vast majority of this kind of proteins should be present in bacterial cells and have a very low P_{CS}/P_{BC} abundance ratio. Total of 308 proteins of *Y. pestis* were defined as the secretome of *Y. pestis* (supplemental Table S4), 123 of which were found to be secreted proteins under both the Mh and Fv conditions (Fig. 2*A*). However, 112 and 73 proteins were identified to be secreted proteins specifically under Fv or Mh conditions, respectively, suggesting that the constitutions of *Y. pestis* secretome under the two typical conditions are drastically distinct from each other.

Functional classification analysis of the proteins listed in Fv-secretome showed that the cellular compartment GO term of periplasmic space, extracellular region, outer membrane, pore complex (Fig. 2*B*) were significantly enriched among others ($p < 0.05$) (Fig. 2*C*). Proteins in Mh-secretome were also significantly enriched in GO term of periplasmic space, extracellular region, as well as pathogenesis. The enrichments of GO terms mentioned above hinted that large amounts of proteins listed in Fv- or Mh-secretome were outer membrane proteins, exported proteins, or virulent effectors. Indeed, some of the proteins listed in *Y. pestis* secretome are the known secreted proteins, including Yop effectors, the known T3SS secreted substrates, F1 capsular protein, PsaA, and so on (supplemental Fig. S1, *A* and *B*, supplemental Table S4). Others include outer membrane proteins, periplasmic proteins, ABC transporter-associated proteins (such as Livk2, RbsB1, RbsB5, RbsB10, and Tbp1), autotransporters (YapA, YapH, MisL), adhesions (FhaB2), type IV secretion system component (virB1, 2, and 5) (supplemental Table S4). However, more than half of the proteins listed in secretome were irrelevant to the functional categories mentioned above and were firstly shown to be potentially exported out of *Y. pestis*.

Interestingly, KEGG pathway of glycolysis/gluconeogenesis and carbon metabolism were significantly enriched in the secreted proteins under the Fv, but not the Mh condition (Fig. 2*B*), whereas the biological process GO term of pathogenesis and bacterial chemotaxis pathway were significantly enriched in the secreted protein under the Mh condition. These results indicated that the secretome of *Y. pestis* under the different conditions is highly regulated in response to the environmental stimuli to promote its growth and survival. Gram-negative bacteria including *Y. pestis* can produce outer membrane vesicles (OMVs) containing biologically active
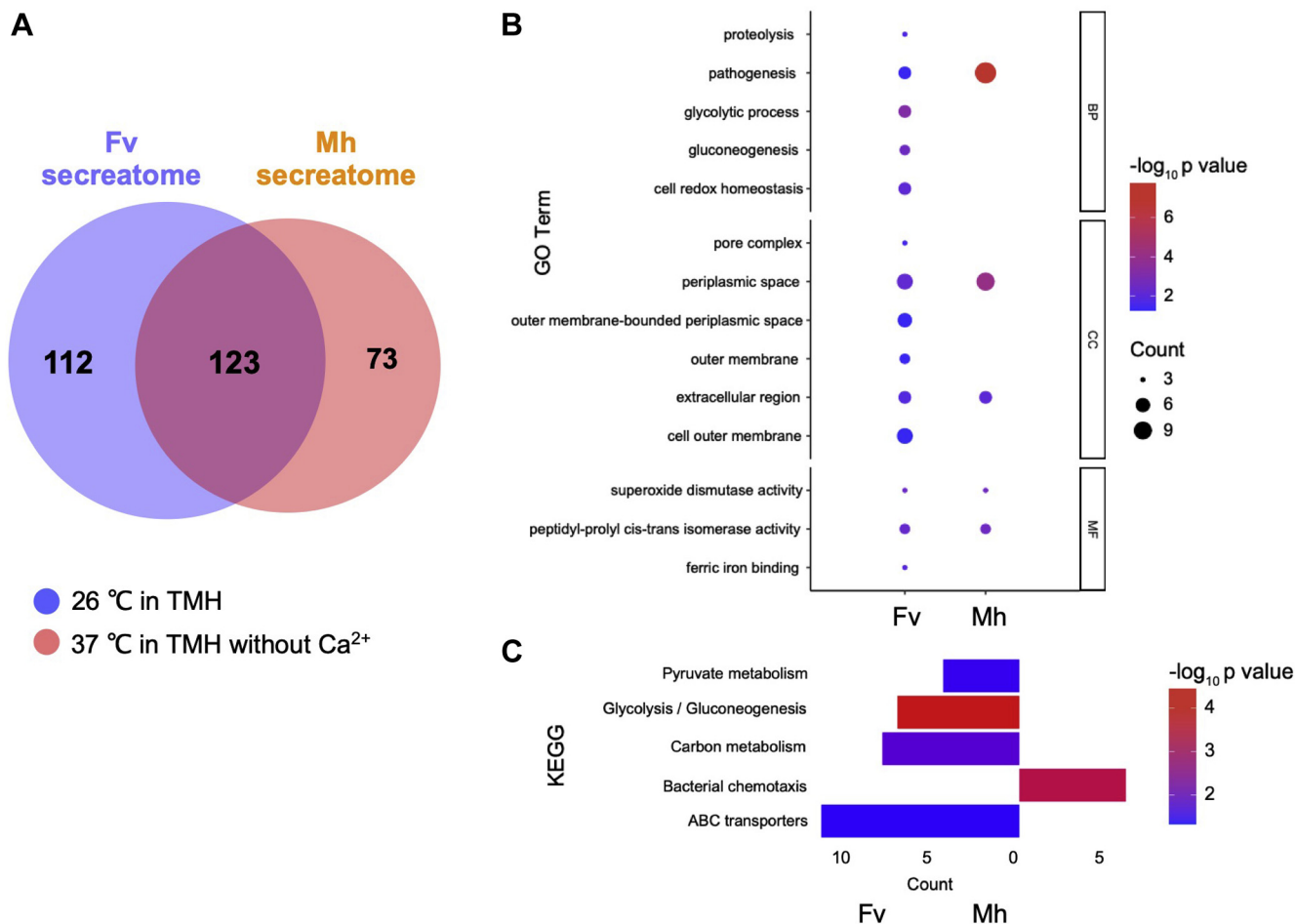
FIG. 2. **Secretome analysis of *Y. pestis* 201 strain grown under the Mh or Fv conditions.** *A*, Venn diagram of proteins that have been identified in the culture supernatants of *Y. pestis* 201 strain grown under the Mh or Fv conditions. GO term and KEGG pathway enrichment analysis of proteins listed in Fv-secretome and Mh-secretome. The *dotplot* showed the significance of the $-\log_{10}$(*p*-values)(color) and the number of proteins (size) in the enriched GO-terms (*B*). The *barplot* showed the significance of the $-\log_{10}$(*p*-values)(color) and the number of proteins (length) in the enriched KEGG pathways (*C*).

products, such as proteins, cell wall components, and toxins (29). Our sample preparation procedures extract all the proteins with molecular weight over 3 kDa from the culture supernatants including those contained in the OMVs. We speculate that a substantial number of proteins could be exported to extracellular milieu *via* OMVs, which greatly enriched the diversity of proteins comprising the secretome of Gram-negative bacteria.

Define the Secreted Substrates of Yersinia T3SS

In order to identify the possible novel substrates secreted by T3SS, we compared the proteins in culture supernatant of *Y. pestis* and *ΔyscI*, a mutant defective in substrates secretion *via* T3SS (19). YscI is a rod protein that is essential for the assembly and secretion of *Y. pestis* T3SS. P_{CS} of *Y. pestis* strain 201 and *ΔyscI* grown under the Mh condition that strongly induces the T3SS both at the transcription and secretion levels were filter-concentrated and subjected to TMT labeling and MS analysis.

The abundance of 52 proteins in the culture supernatant of the *Y. pestis* 201 strain was significantly increased ($p < 0.05$) in comparison to *ΔyscI*, and 17 well-established T3SS substrates were among the list as expected (Fig. 3*A*, supplemental Table S5). The rest 35 proteins including 34 chromosomally encoded proteins and one pMT1 plasmid encoded protein YP_pMT006, were significantly reduced due to the mutation of YscI, suggesting they might be the possible substrates of T3SS. However, whether they could be translocated *via* T3SS machine required further experimental validation. Remarkably, secretion of YP_3416, YP_3417, and YP_3418, three proteins encoded by an operon of *yp_3416~3418*, was significantly impaired in *ΔyscI* (Fig. 3, *A* and *B*).

YP_3416~YP_3418 Are Delivered Into HeLa Cells During Infection

Among the secreted proteins detected in P_{CS} samples, proteins encoded by *yp_3416~3418* locus are noticeable
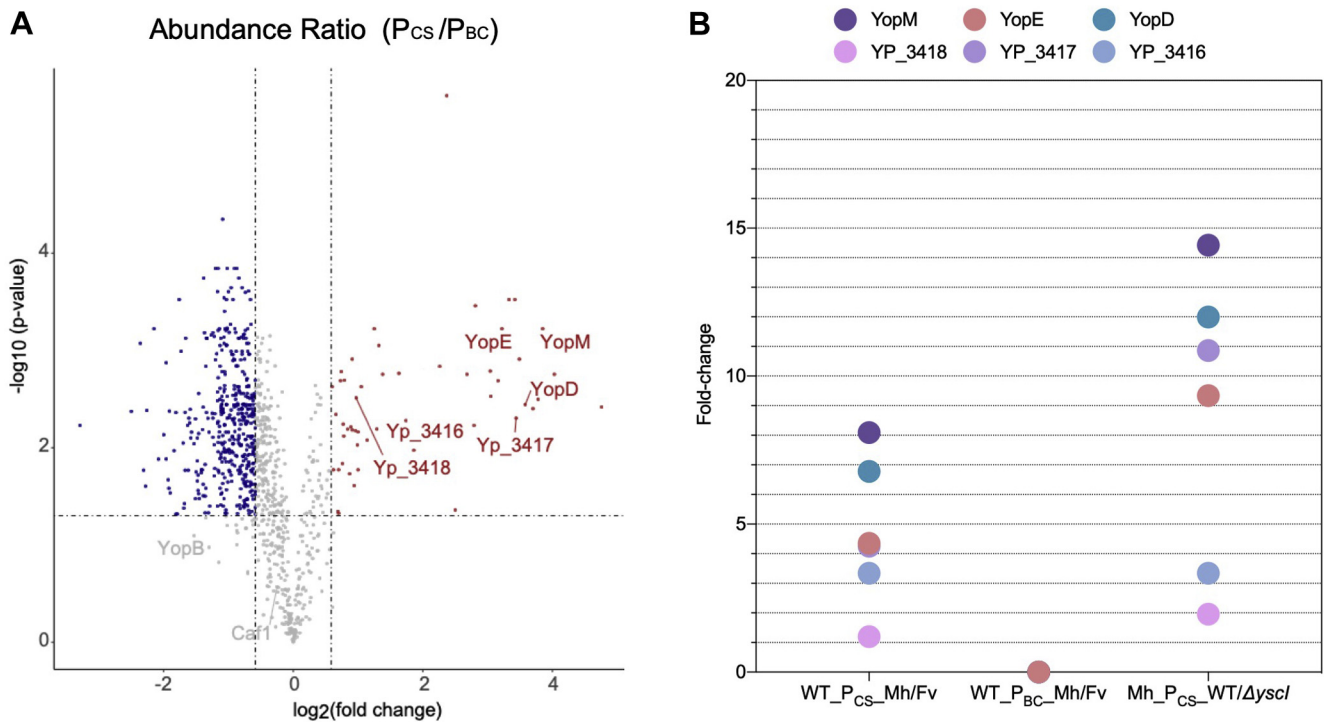
FIG. 3. **Comparative analysis of the secreted proteins between the wild-type *Y. pestis* and *ΔyscI* grown under the Mh condition.** *A*, volcano plot showed statistical significance $-\log_{10}$ (*p*-value) plotted against $\log_2$ (fold change) for the proteins with significantly different abundances between the P_{CS} samples of the wild-type *Y. pestis* and *ΔyscI*. Significance of the differences was determined by Benjamini–Hochberg multiple test and the adjust *p*-value <0.05 was considered to be significant. The significantly higher abundance of the T3SS components, including YopD, YopM, and YopE, and Yp_3416, Yp_3417, and Yp_3418, was illustrated. *B*, fold change in proteins abundance in different experiments was shown. WT_P_{CS}_Mh/Fv and WT_P_{BC}_Mh/Fv represent the comparison between proteins in P_{CS} or P_{BC} samples of *Y. pestis* strain 201 grown under Mh and Fv conditions, respectively. Mh P_{CS}_WT/*ΔyscI* represents the comparison between proteins in P_{CS} samples of *Y. pestis* strain 201 and *ΔyscI* grown under Mh condition.

(supplemental Table S1). Their abundances were significantly higher under Mh condition and dramatically influenced by the defection in T3SS machine (Fig. 3*B*). These data indicated that both the production and secretion of YP_3416~YP_3418 could be significantly increased in the infected mammalian hosts, which might probably contribute to the pathogenesis of *Y. pestis*.

Therefore, we next determine whether they can be delivered into the eukaryotic cytosol during infection using a TEM-1 β-lactamase reporter assay. All the three proteins could be translocated into the infected HeLa cells, showing a translocation dynamics similar to that of YopE, an established Yop effector of *Y. pestis* (Fig. 4). Taken together, these results demonstrated that YP_3416~3418 could not only be secreted by *Y. pestis* but also delivered into the host cytosol, where they could possibly act as virulence factors through interaction with the host proteins during infection.

YP_3416 and YP_3418 Are E3 Ligases Belonging to Novel E3 Ubiquitin Ligase Family

Sequence alignment analysis revealed that *yp_3416~3418* encode three leucine-rich repeat (LRR) proteins that are highly homologous to SspH2, a T3SS effector from *Salmonella*, and IpaHs from *Shigella* (26, 30, 31), which belong to the novel E3 ubiquitin ligase (NEL) family. Ubiquitin ligase activity of NEL family members depended on a conserved Cys residue at the C-terminal NEL domain. YP_3416 and YP_3418 contain both LRR and NEL domain containing the conserved active motif, whereas YP_3417 contains only LRR domain (supplemental Fig. S2). We then expressed and purified GST-tagged YP_3416, YP_3418 or ubiquitin ligase dead mutants YP_3416$_{C386A}$ and YP_3418$_{C408A}$ and determined their E3 ligase activities in *in vitro* ubiquitination assay. YP_3416 exhibited strong ubiquitin ligase activity, which was abrogated in the absence of E1, E2, or FLAG-Ub (Fig. 5*A*). YP_3418 exhibited E3 ligase activity similar to YP_3416; however, YP_3416$_{C386A}$ and YP_3418$_{C408A}$ lacking the conserved Cys residue critical for E3 ligase totally lost ubiquitin ligase activity (Fig. 5*B*). In line with the sequence alignment showing that YP_3417 lacks NEL domain, no ubiquitin-modified product was observed for YP_3417 (Fig. 5*B*). The ubiquitination catalyzed by Yp_3416 became saturated as soon as 10 min after the initiation of reaction, indicating a strong ubiquitin ligase activity of this protein (Fig. 5*C*).
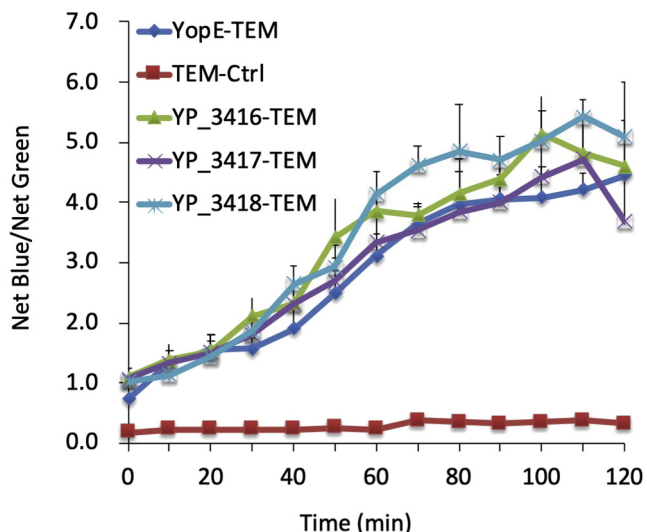
FIG. 4. **YP_3416~YP_3418 can be translocated into HeLa cells during infection.** *Y. pestis* strains harboring plasmids of pBBR1-*yp_3416-TEM*, pBBR1-*yp_3417-TEM*, pBBR1-*yp_3418-TEM*, or pBBR1-*yopE-TEM*, in which the expression of interested proteins was driven by their intrinsic promoters and C-terminally fused with TEM, were grown at 26 °C in LB medium and shift to 37 °C for another 3 h to induce T3SS expression. HeLa cells at 70~80% confluence were infected with the aforementioned strains at an MOI of 20. HeLa cells were loaded with CCF2-AM substrate solution and analyzed by SpectraMax M5. Similar results were obtained in three independent experiments, and only the representative results from one experiment were shown. The average values of Net blue/Net green ±SD from triplicate samples were measured and plotted against the time points of infection.

YP_3416~YP_3418 Contribute to Virulence of Y. pestis

To evaluate the role of YP_3416~3418 in the pathogenesis of *Y. pestis*, the null mutants of *yp_3416*, *yp_3418*, and *yp_3416~yp_3418* were subjected to virulence assay. Mice were challenged by intranasal route with the wild-type *Y. pestis* or the mutants and consecutively observed for 14 days. Mice infected with the *Δyp_3418* exhibited a slight delay in death time compared with those infected with the wild-type strain, and *yp_3416* mutant attenuated more significantly. *Δyp_3416~3418* lacking the entire gene locus showed the most significant virulence attenuation among the three tested strains (Fig. 6). These data demonstrated that *yp_3416~yp_3418* locus contributes to the pathogenesis of *Y. pestis*, and the underlying mechanisms are worth further investigations.

DISCUSSIONS

Y. pestis is a flea-borne bacterial pathogen that recently evolved from the enteropathogenic *Yersinia pseudotuberculosis*. Rapid adaption to and survival in the adverse environments are crucial for *Y. pestis* to circulate in the natural foci between the arthropod vectors and the reservoir rodents, as

well as to establish successful infection in mammalian host. After announcements of complete genome projects of *Y. pestis* (CO92, KIM and 91001 strains), proteomics studies for the *Y. pestis* proteins or outer membrane proteins were performed using 2D-gel electrophoresis coupled with MS, which greatly improved our understandings on the physiology and virulence of *Y. pestis* (27, 32–34). About 2000 proteins have been identified in *Y. pestis* 91001 strain grown at 26 °C in TMH medium using multiple strategies, including SDS-PAGE, 2D liquid chromatography peptide separation or intact protein separation combined with LTQ-FT (27). In this study, we took the advantage of rapid progress in mass spectrum techniques to analyze the secretome, as well as the comparative proteomics of *Y. pestis*, grown under the conditions mimicking its two typical niches, the mammalian host and the flea gut environments. The most significantly regulated proteins are the cell envelope-related proteins and virulence factors including T3SS components, F1 capsular antigen, PsaA, and KatY. It's worth noting that over 90 proteins without any functional annotation were significantly upregulated as well, which probably play some roles in the pathogenesis of *Y. pestis* and need further explorations.

The term of "secretome" in microbiology field first appeared only a dozen years ago (13, 14), which is defined as the group of secreted proteins into the extracellular space by a living creature. Proteins can be translocated out of the bacterial cell by various ways. For instance, proteins containing specific N-terminal signal peptides are translocated by general secretory (Sec) pathway or the twin-arginine translocation (Tat) pathway (35), while a significant fraction of the proteins without obvious signal peptide are transported *via* Type I~IV, autotransporters, or some uncharacterized mechanisms (36). Besides, Gram-negative bacteria can naturally secrete OMVs that contain a wide variety of substances including protein and nonprotein molecules (37–40), which also serve as a function analogous to secretion system and play critical roles in interacting with both prokaryotic and eukaryotic cells (41). Eddy *et al.* (29) have reported the proteomics of OMVs released by *Y. pestis* CO92 lacking pCD1 plasmid that encodes T3SS, and 270 proteins including virulence factors adhesion Ail, F1 antigen, and protease Pla were identified by using liquid chromatography–electrospray ionization (LC-ESI) MS, over-half of which are cytoplasmic proteins. By using bioinformatics methods, Yen *et al.* (42) have reported an *in silico* mapping of all the secretion systems in the genome of *Y. pestis* KIM strain. However, no experimentally defined secretome of *Y. pestis* has been reported till now. In this study, a total of 308 proteins were detected in the culture supernatant of *Y. pestis* 201 strain under Mh and Fv conditions, which we define as the secretome of *Y. pestis*. Of this secretome, 123 were identified to be secreted proteins under both the Mh and Fv conditions, while 112 and 73 were specifically identified under Fv or Mh conditions, respectively. The constitutions of *Y. pestis* secretome under the two typical environmental conditions are significantly distinct from each other, implying a critical role of bacterial secretome in
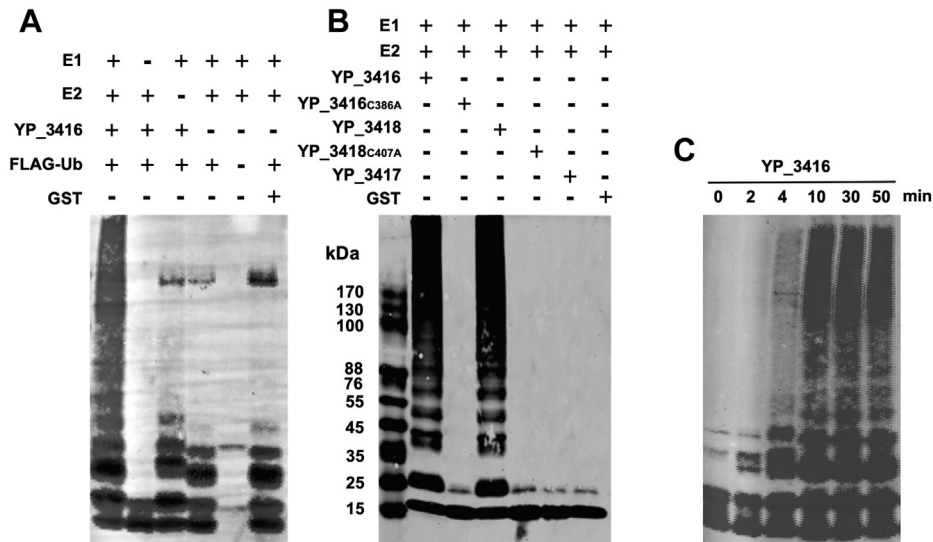
FIG. 5. **YP_3416 and YP_3418 of *Y. pestis* 201 exhibit E3 ubiquitin ligase activity.** *A*, Yp_3416 exhibits E3 ubiquitin ligase activity. Purified recombinant GST-YP_3416 was incubated with the FLAG-Ubiquitin and the mixture containing E1, UbcH5a, reaction buffer, and Mg^{2+}-ATP solution at 37 °C for 30 min. Reaction mixtures without one of the components as indicated or using GST instead of GST-YP_3416 served as controls. All the samples were separated in 4 to 15% SDS-PAGE and immunoblotted with mouse monoclonal FLAG M2 antibody to detect ubiquitin conjugates. *B*, the E3 ubiquitin ligase activity of YP_3416 and YP_3418 dependents on the conserved Cys on the NEL domain. The individual purified GST-tagged wild-type or YP_3416$_{C386A}$ and YP_3418$_{C407A}$ were determined for their ubiquitin ligase activity as described in (*A*). Disruption of the conserved Cys at the NEL domain of YP_3416 and YP_3418 resulted in the loss of catalase activity. *C*, dynamic analysis of ubiquitin conjugation reaction of YP_3416. The purified GST-YP_3416 was incubated with ubiquitination reaction mixture at 37 °C for 2, 4, 10, 30, or 50 min, and protein samples were SDS-PAGE separated and analyzed by immunoblotting as described above.
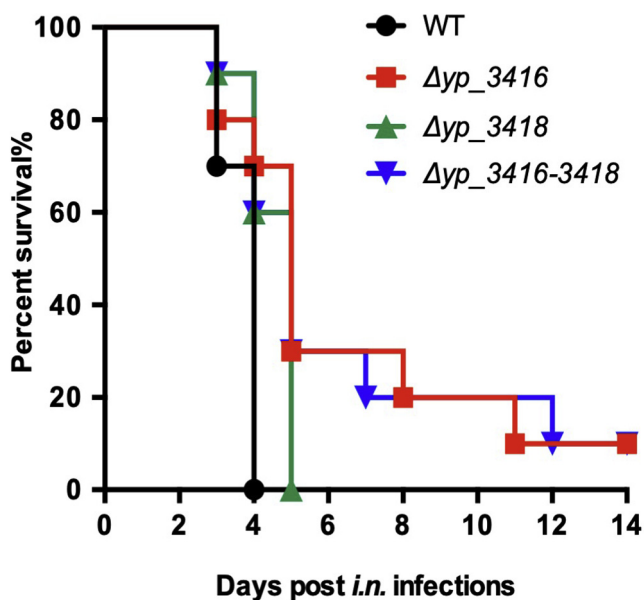

FIG. 6. **The *yp_3416~3418* deletion mutants of *Y. pestis* 201 attenuated in virulence for mice.** Groups of mice (n = 10) were infected with ~100 CFU of the wild-type, *Δyp_3416*, *Δyp_3418*, or *Δyp_3416~3418* of *Y. pestis* 201 strain *via i.n.* route of infection. Significance of the differences between the mice infected with the wild-type and the various mutants was determined by Log-rank (Mantel-Cox) test (wild-type *versus Δyp_3416* p = 0.0073, wild-type *versus Δyp_3418* p = 0.0075, wild-type *versus Δyp_3416~3418* p = 0.0075) using GraphPad Prism 6.0 software.

adaption to adverse environments. Some of the proteins in secretome are predicted as exported proteins or the known substrates of the well-established secretion mechanisms, such as Yop effectors, Caf1, Pla, YapA, and YapH, etc, while more than half of them are functionally unknown and identified as the secreted protein for the first time, a larger number of which we assumed to be probably secreted *via* OMVs. Proteomic analysis of OMVs has been reported for over 40 Gram-negative bacteria, which usually consist of hundreds of bacterial proteins (43–46). The most frequently identified proteins from OMVs contain cytoplasmic proteins including GroEL, GroES, elongation factors, DNA-directed RNA polymerases, ribosomal proteins, ATP synthase, in addition to the outer membrane protein/lipoproteins or some periplasmic component (43). In this study, we indeed found GroEL, GroES, elongation factors Tu and Ts, DNA-directed RNA polymerases RpoA, B, C, and Z, ATP synthase AtpA, D, and G, and various ribosomal proteins (supplemental Table S4) in the secretome of *Y. pestis*, which is also in line with the proteome of OMVs of *Y. pestis* CO 92 lacking pCD1.

It's noteworthy that the products of *yp_3416 ~ yp_3418* gene locus are the secreted proteins that are expressed at elevated levels under the condition mimicking the mammalian host environment. We further illustrated that the two proteins, YP_3416 and YP_3418, encoded by the gene locus are E3 ligase that can be translocated into the infected HeLa cells and *yp_3416* and *yp_3418* mutants are attenuated in virulence in mice. In preparation of this paper, we noticed that our

results are consistent with a recent published work carried out in *Y. pestis* KIM5 showing that *y3397*, *y3399*, and *y3400* (the counterparts of *yp_3416~yp_3418* in strain 201) encode proteins that can be translocated into the host cytosol (47). They also showed that mice intravenously infected with an equal mixture of *Y. pestis* KIM5 and the isogenic mutant lacking *y3397~y3400* yielded a significant overrepresentation of the parental strain. In-depth study is needed to clarify the contribution of those newly identified virulence factors to the pathogenesis of *Y. pestis*.

REFERENCES

1. Yang, R., and Butler, T. (2016) Discovery of the plague pathogen: Lessons learned. *Adv. Exp. Med. Biol.* **918**, 27–33
2. Butler, T. (2009) Plague into the 21st century. *Clin. Infect. Dis.* **49**, 736–742
3. Perry, R. D., and Fetherston, J. D. (1997) Yersinia pestis–etiologic agent of plague. *Clin. Microbiol. Rev.* **10**, 35–66
4. Pujol, C., Grabenstein, J. P., Perry, R. D., and Bliska, J. B. (2005) Replication of Yersinia pestis in interferon gamma-activated macrophages requires ripA, a gene encoded in the pigmentation locus. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 12909–12914
5. Lukaszewski, R. A., Kenny, D. J., Taylor, R., Rees, D. G., Hartley, M. G., and Oyston, P. C. (2005) Pathogenesis of Yersinia pestis infection in BALB/c mice: Effects on host macrophages and neutrophils. *Infect. Immun.* **73**, 7142–7150
6. Han, Y., Zhou, D., Pang, X., Song, Y., Zhang, L., Bao, J., Tong, Z., Wang, J., Guo, Z., Zhai, J., Du, Z., Wang, X., Zhang, X., Huang, P., and Yang, R. (2004) Microarray analysis of temperature-induced transcriptome of Yersinia pestis. *Microbiol. Immunol.* **48**, 791–805
7. Suomalainen, M., Lobo, L. A., Brandenburg, K., Lindner, B., Virkola, R., Knirel, Y. A., Anisimov, A. P., Holst, O., and Korhonen, T. K. (2010) Temperature-induced changes in the lipopolysaccharide of Yersinia pestis affect plasminogen activation by the pla surface protease. *Infect. Immun.* **78**, 2644–2652
8. Hinnebusch, B. J., Fischer, E. R., and Schwan, T. G. (1998) Evaluation of the role of the Yersinia pestis plasminogen activator and other plasmid-encoded factors in temperature-dependent blockage of the flea. *J. Infect. Dis.* **178**, 1406–1415
9. Bolin, I., Portnoy, D. A., and Wolf-Watz, H. (1985) Expression of the temperature-inducible outer membrane proteins of yersiniae. *Infect. Immun.* **48**, 234–240
10. Cornelis, G. R., Boland, A., Boyd, A. P., Geuijen, C., Iriarte, M., Neyt, C., Sory, M. P., and Stainier, I. (1998) The virulence plasmid of *Yersinia*, an antihost genome. *Microbiol. Mol. Biol. Rev.* **62**, 1315–1352
11. Viboud, G. I., and Bliska, J. B. (2005) *Yersinia* outer proteins: Role in modulation of host cell signaling responses and pathogenesis. *Annu. Rev. Microbiol.* **59**, 69–89
12. Galan, J. E., and Wolf-Watz, H. (2006) Protein delivery into eukaryotic cells by type III secretion machines. *Nature* **444**, 567–573
13. Tjalsma, H., Antelmann, H., Jongbloed, J. D., Braun, P. G., Darmon, E., Dorenbos, R., Dubois, J. Y., Westers, H., Zanen, G., Quax, W. J., Kuipers, O. P., Bron, S., Hecker, M., and van Dijl, J. M. (2004) Proteomics of protein secretion by Bacillus subtilis: Separating the "secrets" of the secretome. *Microbiol. Mol. Biol. Rev.* **68**, 207–233
14. Tjalsma, H., Bolhuis, A., Jongbloed, J. D., Bron, S., and van Dijl, J. M. (2000) Signal peptide-dependent protein transport in Bacillus subtilis: A genome-based survey of the secretome. *Microbiol. Mol. Biol. Rev.* **64**, 515–547
15. Agrawal, G. K., Jwa, N. S., Lebrun, M. H., Job, D., and Rakwal, R. (2010) Plant secretome: Unlocking secrets of the secreted proteins. *Proteomics* **10**, 799–827
16. Zubair, M., Khan, F. A., Menghwar, H., Faisal, M., Ashraf, M., Rasheed, M. A., Marawan, M. A., Dawood, A., Chen, Y., Chen, H., and Guo, A. (2020) Progresses on bacterial secretomes enlighten research on Mycoplasma secretome. *Microb. Pathog.* **144**, 104160
17. Kulp, A., and Kuehn, M. J. (2010) Biological functions and biogenesis of secreted bacterial outer membrane vesicles. *Annu. Rev. Microbiol.* **64**, 163–184
18. Zhou, D., Han, Y., Dai, E., Song, Y., Pei, D., Zhai, J., Du, Z., Wang, J., Guo, Z., and Yang, R. (2004) Defining the genome content of live plague vaccines by use of whole-genome DNA microarray. *Vaccine* **22**, 3367–3374
19. Cao, S. Y., Liu, W. B., Tan, Y. F., Yang, H. Y., Zhang, T. T., Wang, T., Wang, X. Y., Song, Y. J., Yang, R. F., and Du, Z. M. (2017) An interaction between the inner rod protein YscI and the needle protein YscF is required to assemble the needle structure of the Yersinia type three secretion system. *J. Biol. Chem.* **292**, 5488–5498
20. Straley, S. C., and Bowmer, W. S. (1986) Virulence genes regulated at the transcriptional level by Ca2+ in Yersinia pestis include structural genes for outer membrane proteins. *Infect. Immun.* **51**, 445–454
21. Benjamini, Y., and Hochberg, Y. (1995) Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B* **57**, 289–300
22. Gan, C. S., Chong, P. K., Pham, T. K., and Wright, P. C. (2007) Technical, experimental, and biological variations in isobaric tags for relative and absolute quantitation (iTRAQ). *J. Proteome Res.* **6**, 821–827
23. Suriyanarayanan, T., Qingsong, L., Kwang, L. T., Mun, L. Y., Truong, T., and Seneviratne, C. J. (2018) Quantitative proteomics of strong and weak

biofilm formers of Enterococcus faecalis reveals novel regulators of biofilm formation. *Mol. Cell. Proteomics* **17**, 643–654

24. Huang da, W., Sherman, B. T., and Lempicki, R. A. (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* **4**, 44–57

25. Marketon, M. M., DePaolo, R. W., DeBord, K. L., Jabri, B., and Schneewind, O. (2005) Plague bacteria target immune cells during infection. *Science* **309**, 1739–1741

26. Quezada, C. M., Hicks, S. W., Galan, J. E., and Stebbins, C. E. (2009) A family of Salmonella virulence factors functions as a distinct class of autoregulated E3 ubiquitin ligases. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 4864–4869

27. Zhou, L., Ying, W., Han, Y., Chen, M., Yan, Y., Li, L., Zhu, Z., Zheng, Z., Jia, W., Yang, R., and Qian, X. (2012) A proteome reference map and virulence factors analysis of *Yersinia pestis* 91001. *J. Proteomics* **75**, 894–907

28. Fowler, J. M., and Brubaker, R. R. (1994) Physiological basis of the low calcium response in Yersinia pestis. *Infect. Immun.* **62**, 5234–5241

29. Eddy, J. L., Gielda, L. M., Caulfield, A. J., Rangel, S. M., and Lathem, W. W. (2014) Production of outer membrane vesicles by the plague pathogen Yersinia pestis. *PLoS One* **9**, e107002

30. Miao, E. A., Scherer, C. A., Tsolis, R. M., Kingsley, R. A., Adams, L. G., Baumler, A. J., and Miller, S. I. (1999) Salmonella typhimurium leucine-rich repeat proteins are targeted to the SPI1 and SPI2 type III secretion systems. *Mol. Microbiol.* **34**, 850–864

31. Rohde, J. R., Breitkreutz, A., Chenal, A., Sansonetti, P. J., and Parsot, C. (2007) Type III secretion effectors of the IpaH family are E3 ubiquitin ligases. *Cell Host Microbe* **1**, 77–83

32. Chromy, B. A., Choi, M. W., Murphy, G. A., Gonzales, A. D., Corzett, C. H., Chang, B. C., Fitch, J. P., and McCutchen-Maloney, S. L. (2005) Proteomic characterization of Yersinia pestis virulence. *J. Bacteriol.* **187**, 8172–8180

33. Pieper, R., Huang, S. T., Robinson, J. M., Clark, D. J., Alami, H., Parmar, P. P., Perry, R. D., Fleischmann, R. D., and Peterson, S. N. (2009) Temperature and growth phase influence the outer-membrane proteome and the expression of a type VI secretion system in Yersinia pestis. *Microbiology* **155**, 498–512

34. Pieper, R., Huang, S. T., Clark, D. J., Robinson, J. M., Alami, H., Parmar, P. P., Suh, M. J., Kuntumalla, S., Bunai, C. L., Perry, R. D., Fleischmann, R. D., and Peterson, S. N. (2009) Integral and peripheral association of proteins and protein complexes with Yersinia pestis inner and outer membranes. *Proteome Sci.* **7**, 5

35. Beckwith, J. (2013) The Sec-dependent pathway. *Res. Microbiol.* **164**, 497–504

36. Holland, I. B. (2004) Translocation of bacterial proteins–an overview. *Biochim. Biophys. Acta* **1694**, 5–16

37. Kaparakis-Liaskos, M., and Ferrero, R. L. (2015) Immune modulation by bacterial outer membrane vesicles. *Nat. Rev. Immunol.* **15**, 375–387

38. Jung, A. L., Stoiber, C., Herkt, C. E., Schulz, C., Bertrams, W., and Schmeck, B. (2016) Legionella pneumophila-derived outer membrane vesicles promote bacterial replication in macrophages. *PLoS Pathog.* **12**, e1005592

39. Koeppen, K., Hampton, T. H., Jarek, M., Scharfe, M., Gerber, S. A., Mielcarz, D. W., Demers, E. G., Dolben, E. L., Hammond, J. H., Hogan, D. A., and Stanton, B. A. (2016) A novel mechanism of host-pathogen interaction through sRNA in bacterial outer membrane vesicles. *PLoS Pathog.* **12**, e1005672

40. Elmi, A., Watson, E., Sandu, P., Gundogdu, O., Mills, D. C., Inglis, N. F., Manson, E., Imrie, L., Bajaj-Elliott, M., Wren, B. W., Smith, D. G., and Dorrell, N. (2012) Campylobacter jejuni outer membrane vesicles play an important role in bacterial interactions with human intestinal epithelial cells. *Infect. Immun.* **80**, 4089–4098

41. Ellis, T. N., and Kuehn, M. J. (2010) Virulence and immunomodulatory roles of bacterial outer membrane vesicles. *Microbiol. Mol. Biol. Rev.* **74**, 81–94

42. Yen, Y. T., Bhattacharya, M., and Stathopoulos, C. (2008) Genome-wide in silico mapping of the secretome in pathogenic Yersinia pestis KIM. *FEMS Microbiol. Lett.* **279**, 56–63

43. Lee, J., Kim, O. Y., and Gho, Y. S. (2016) Proteomic profiling of gram-negative bacterial outer membrane vesicles: Current perspectives. *Proteomics Clin. Appl.* **10**, 897–909

44. Kwon, S. O., Gho, Y. S., Lee, J. C., and Kim, S. I. (2009) Proteome analysis of outer membrane vesicles from a clinical Acinetobacter baumannii isolate. *FEMS Microbiol. Lett.* **297**, 150–156

45. Choi, D. S., Kim, D. K., Choi, S. J., Lee, J., Choi, J. P., Rho, S., Park, S. H., Kim, Y. K., Hwang, D., and Gho, Y. S. (2011) Proteomic analysis of outer membrane vesicles derived from Pseudomonas aeruginosa. *Proteomics* **11**, 3424–3429

46. Avila-Calderon, E. D., Lopez-Merino, A., Jain, N., Peralta, H., Lopez-Villegas, E. O., Sriranganathan, N., Boyle, S. M., Witonsky, S., and Contreras-Rodriguez, A. (2012) Characterization of outer membrane vesicles from Brucella melitensis and protection induced in mice. *Clin. Dev. Immunol.* **2012**, 352493

47. Schesser Bartra, S., Lorica, C., Qian, L., Gong, X., Bahnan, W., Barreras, H., Jr., Hernandez, R., Li, Z., Plano, G. V., and Schesser, K. (2019) Chromosomally-encoded Yersinia pestis type III secretion effector proteins promote infection in cells and in mice. *Front. Cell. Infect. Microbiol.* **9**, 23