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Analysis of differentially expressed proteins in Yersinia enterocolitica-infected HeLa cells

Navatha Alugubelly[2], **Kamil Hercik**[2], **Peter Kibler**[1], **Bindu Nanduri**[2], and **Mariola J. Edelmann**[1,*]

[1]Department of Microbiology and Cell Science, University of Florida, Gainesville, Florida, USA

[2]Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University, Mississippi State, USA

Abstract

Yersinia enterocolitica is a facultative intracellular pathogen and a causative agent of yersiniosis, which can be contracted by ingestion of contaminated food. *Yersinia* secretes virulence factors to subvert critical pathways in the host cell. In this study we utilized shotgun label-free proteomics to study differential protein expression in epithelial cells infected with *Yersinia enterocolitica*. We identified a total of 551 proteins, amongst which 42 were downregulated (e.g. Prostaglandin E Synthase 3, POH-1 and Karyopherin alpha) and 22 were upregulated (e.g. Rab1c and RhoA) in infected cells. We validated some of these results by western blot analysis of proteins extracted from Caco-2 and HeLa cells. The proteomic dataset was used to identify host canonical pathways and molecular functions modulated by this infection in the host cells. This study constitutes a proteome of *Yersinia*-infected cells and can support new discoveries in the area of host-pathogen interactions.

Keywords

label-free proteomics; *Yersinia enterocolitica* infection; pathway modeling; integrin signaling; protein ubiquitination

3. Introduction

Y*ersinia enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis* are pathogenic bacteria, which are of concern due to their biomedical significance. While *Y. pseudotuberculosis* and *Y. pestis* are close from the evolutionary standpoint, *Y. enterocolitica* has a more distant phylogenetic relationship with the other two species [1]. Y*ersinia* pathogens are facultative intracellular pathogens, which translocate their virulence factors, which modify the eukaryotic proteome to interfere with the anti-microbial responses [2], but at the same time the host cell also modifies expression of its proteins to defy the bacterial infection.

*Correspondence should be addressed to: Mariola J. Edelmann, medelmann@ufl.edu. Department of Microbiology and Cell Science, University of Florida, PO Box 110700, Bldg 981, Museum Rd., Gainesville, FL 32611-0700, United States of America.

8. Conflict of interest statement

The authors have declared no conflict of interest.

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Proteomics has been utilized to understand the molecular basis of pathogenesis in several bacterial infections, including *Listeria monocytogenes* [3–5], *Salmonella enterica* Typhimurium [6, 7], and *Mycobacterium tuberculosis* [8]. Proteomics has also been a useful tool in characterization of proteins encoded by *Yersiniae* [9–15]. Furthermore, systems biology-integrated analysis of bacterial proteome, genome and metabolome was performed on *Y. pestis* and *Y. pseudotuberculosis* [16]. Finally, two-dimensional gel electrophoresis combined with protein identification was used to identify differentially expressed proteins in human monocytes infected with *Y. pestis* and *Y. pseudotuberculosis*, but only 29 proteins have been identified by using this approach [17]. However, label-free proteomics has never been utilized to analyze differential protein expression in cells infected with *Y. enterocolitica*.

The purpose of our study was to characterize the epithelial cell response to *Y. enterocolitica* infection by label-free proteomics. The tested non-ionic detergents used for protein extraction, n-dodecyl-beta-D-maltoside and NP-40, gave comparable results in uninfected cells although n-dodecyl-beta-D-maltoside led to a slightly higher number of protein identification reproduced in two biological replicates. Next, we analyzed the proteome of *Y. enterocolitica*-HeLa cells and compared it to the proteome of uninfected cells. Analysis of the canonical pathways and molecular functions was also performed. The differential expression of selected proteins was confirmed by western blot analysis of proteins extracted from infected HeLa cells, but also from colonic epithelial Caco-2 cells infected with wild-type and virulence plasmid-cured *Y. enterocolitica* mutant. This is the first report of proteomics analysis of host protein networks altered in response to *Y. enterocolitica* infection, and it can enable further investigation of the host-pathogen interactions.

4. Materials and Methods

4.1. Overall experimental design

Two different proteomic experiments were performed. First, to test protein extraction methods two biological replicates were used. Second, for analysis of differentially expressed proteins in infected cells three biological replicates were used. Statistical testing was done by using Fisher's exact test. Independent and complementary validation experiments (western blot analysis) were also performed.

4.2. Culture conditions

Yersinia enterocolitica 8081 wild-type (pYV) and virulence plasmid-cured mutant (8081c) [18] were grown in tryptic soy broth (TSB) overnight at 27°C with aeration. HeLa cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS) and incubated at 37°C under 5% CO_2 environment. Caco-2 cells were also maintained similarly, but medium was supplemented with 20% FBS.

4.3. Infection conditions

The overnight culture of wild-type *Y. enterocolitica* or 8081c mutant grown aerobically in TSB at 27°C was diluted to OD_{600} of 0.05 and incubated at 27°C. At OD_{600} of 0.25 the

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temperature was changed to 37°C. Bacteria were grown until OD_{600} reached 0.5 (~1.5 hour), centrifuged at 5,000 × g for 10 minutes, washed with phosphate buffered saline (PBS) and resuspended in cell culture medium. One hour before infection, the fresh DMEM medium (Gibco, USA) lacking antibiotics and FBS was added to HeLa or Caco-2 cells. Cells were infected at multiplicity of infection (MOI) of 20:1. One hour post-infection cells were washed with PBS. DMEM medium containing gentamicin (50 μg/ml) was added to eliminate extracellular bacteria, cells were incubated for three hours, and collected by scraping in cold PBS.

4.4. Gentamicin survival assay

2.5×10^5 HeLa cells per well were plated on a 6-well plate and incubated overnight. On the next day, cells were washed with warmed PBS and counted. 2 mL of DMEM medium with no antibiotics or FBS was added to HeLa cells and the cells were incubated for an hour, followed by infection with *Yersinia* as described above (MOI of 20:1). One hour post-infection the cells were washed with PBS, and DMEM medium containing gentamicin (50 μg/ml) was added to eliminate extracellular bacteria. Cells were incubated for three hours, after which they were washed three times with warm PBS. Next, cells were counted by detaching them from the cell culture dish by using trypsin, staining with trypan blue and counting by using an automatic TC20 cell counter (Bio-Rad, USA) to establish the total number of HeLa cells. Alternatively, cells well (three biological replicates were used) were lysed as follows: sterile 500 μL Triton-X (0.1% in PBS) was added to each and incubated for 5 minutes, after which PBS was added to bring the final volume to 1 mL. Each well was thoroughly rinsed with the lysis buffer and pipetted into a sterile microcentrifuge tube. Biological triplicates of samples were diluted in LB medium, dilutions of 10^{-3}, 10^{-4}, and 10^{-5} were plated on LB agar (100 μ//each), incubated at 27 °C for two days, and counted to establish the colony forming units (CFUs).

4.5. Cell lysis and western blot analysis

The cells were lysed in two different buffers, n-dodecyl-beta-D-maltoside lysis buffer (150 mM NaCl, 20 mM $MgCl_2$, 50 mM Tris-HCl pH 7.4, 0.5% n-dodecyl-beta-D-maltoside), and NP-40 lysis buffer (150 mM NaCl, 20 mM $MgCl_2$, 50 mM Tris-HCl pH 7.4, 0.5% NP-40). Both buffers were supplemented with 1 mM serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) and lysis was carried out for 30 minutes on ice. The cell debris was removed by centrifugation at 21,000 × g, at 4°C for 10 minutes. The protein concentration was measured and the crude protein extract was subjected to trypsin-based digestion and proteomics, or western blot analysis. Crude protein extracts containing the same amount of protein were resolved by 4–12% polyacrylamide gel electrophoresis (PAGE) and then transferred by western blotting to a polyvinylidene difluoride (PVDF) membrane. The proteins of interest were detected using primary antibodies against RhoA (1:2,000 dilution, Cell Signaling Technology, USA), Rab1c (1:500 dilution, Santa Cruz Biotechnology, USA), Prostaglandin E Synthase 3 (1:1,000 dilution, Santa Cruz Biotechnology, USA), and POH-1 (1:125 dilution, Life Technologies Corporation, USA) and following secondary conjugated antibodies: goat anti-rabbit-HRP (1:7,000 dilution, Santa Cruz Biotechnology, USA) and goat anti-mouse-HRP (1:6,000 dilution, Santa Cruz Biotechnology, USA), and visualized using enhanced chemiluminiscent substrate Luminata Forte Western HRP Substrate

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(Milipore). Anti-beta-actin antibody (Sigma-Aldrich, USA) was used as a loading control. All antibodies were diluted with 1% milk in Tris buffered saline (TBS) containing 0.1% Tween-20.

4.6. Mass Spectrometry

Proteins (100 μg per sample) were precipitated via chloroform/methanol extraction exactly as we described previously [19]. Protein pellets were suspended in 100 μl of 100 mM Tris-HCl pH containing 6 M urea. Samples were reduced with 5 μl 200 mM dithiothreitol (DTT) for 45 minutes at room temperature, followed by alkylation with 20 μl 200 mM iodoacetamide (IAA) for 45 minutes at room temperature, and addition of 20 μl 200 mM DTT for 45 minutes to quench the remaining IAA. The urea concentration was then reduced by adding 775 μl of milliQ-H_2O. Finally, proteins were digested with trypsin (Sequencing Grade Modified Trypsin, Promega) at 1:50 ratio for 18 hours at 37°C. The reaction was stopped by adjusting pH of solution to <6 by addition of acetic acid. The samples were then purified by using C18 SepPak columns (Waters, USA). The peptide samples were dissolved in 98% milliQ-H_2O, 2% acetonitrile, 0.1% formic acid. An equivalent of 1 μg of protein content was used for mass spectrometric analysis by in-line HPLC (Dionex, USA) and a linear trap (LTQ Velos) mass spectrometer by using conditions we previously described [20].

4.7. Data analysis

Tandem mass spectra were extracted by Proteome Discoverer (Thermo Scientific, USA), version 1.4. 0.288. Charge state deconvolution and deisotoping were performed and analysis was done by using Sequest (Thermo Fisher Scientific, USA; ver. 1.4.0.288) and X! Tandem (ver. CYCLONE 2010.12.01.1). The databank used was Uniprot human reference proteome (68,949 entries) assuming trypsin as a digestive enzyme. Sequest and X!Tandem were searched with a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 2.0 Da (error mass distribution in ppm is shown in the Supplementary Figure 1), which are typical tolerances used when a linear ion trap mass spectrometer is utilized [21]. Oxidation (methionine) and carbamidomethylation (cysteine) were specified in Sequest and X! Tandem as variable modifications. Glu->pyro-Glu (n-terminus), ammonia-loss (n-terminus), gln->pyro-Glu (n-terminus) were also specified in X! Tandem as variable modifications. Scaffold (ver. 4.4.1; Proteome Software Inc., USA) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications achieved False Discovery Rate (FDR) <0.35% by the Scaffold Local FDR algorithm, and protein identifications were accepted at >95.0% probability (assigned by the Protein Prophet algorithm [22]) with a minimum 1 peptide present in a protein. Proteins that could not be differentiated were grouped to satisfy the principles of parsimony. Similar databank search by using *Yersinia* proteome database (NCBI) was performed but no protein entries were found. Although the bacteria itself were most likely not lysed by using the used protein extraction buffer, there was a possibility that the bacterial proteins were secreted inside the host cell by the use of *Yersinia*'s secretory system, yet most likely due to their lower abundance no *Yersinia*-derived proteins were identified.

The spectral counting label-free quantitation was performed in Scaffold (Proteome Software, USA), in which we used the sum of weighted spectra associated with a protein (where the

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weight is a measure of uniqueness of a given spectrum in terms of its presence in other proteins, this method is briefly described here [23]). This analysis was performed only on proteins, which were identified in minimum two replicates (with minimum 4 exclusive spectra per protein in total), and which passed the Fisher's Exact Test with a p-value equal or lower than 0.05. Normalization was applied to calculate the fold ratio and a minimum value of 0.2 was used for the samples, in which a protein was not identified. The minimum fold difference was 1.5 for both up- and down-regulated proteins (see Supplementary Table 1 for more information).

4.8. Gene ontology and network analysis

The Database for Annotation, Visualization and Integrated Discovery (DAVID) resource (ver. 6.7) was used for functional annotation. GO terms with a p value<0.005 were taken into consideration, with FDR <5.0% and minimum 10 genes in each category. Statistical significance was determined by EASE Score Threshold (Maximum Probability), which is a modified Fisher Exact p-value along with the FDR correction [24]. The number of molecules was plotted for each GO term in Excel.

The datasets were compared in Blast2Go (ver 3.0.10) [25] by using Fisher's Exact Test with Multiple Testing Correction of FDR (Benjamini and Hochberg) to establish whether GO terms are enriched in a test group when compared to a reference group while testing effect of a detergent on the subcellular localization of identified proteins. The network analysis was performed by Ingenuity Analysis Pathway (ver. 21901358) software to evaluate the Canonical Pathways, Molecular Functions and Networks; Benjamini and Hochberg multiple testing correction was used. The top two networks were merged, and EIF2 signaling and integrin signaling were overlaid with this network on the basis of the high coverage of identified proteins within the associated canonical pathways (5 for integrin pathway; 9 for EIF2 pathway).

5. Results and discussion

5.1. Proteomic analysis of subcellular localization of proteins obtained by lysis with non-ionic detergents

Although there are many detergents available for cell lysis, non-ionic detergents are claimed to be more compatible with the high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS; [26]). In this study, we lysed HeLa cells with two non-ionic detergents, which were compared to each other, n-dodecyl-beta-D-maltoside and NP-40. The number of the identified proteins and subcellular localizations of the identified proteins yielded by each lysis buffer was evaluated. The n-dodecyl-beta-D-maltoside led to identification of slightly higher number of proteins in comparison to NP-40 (Fig. 1A). DAVID [24] and Blast2GO [25] tools were used for GO term (subcellular localization) analysis of the identified proteins (Fig. 1B). Fisher's Exact Test established that there were no statistically significant differences in GO terms related to subcellular localization of the proteins in one dataset versus another.

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5.2. Analysis of differentially expressed proteins in epithelial HeLa and Caco-2 cells infected with *Yersinia enterocolitica*

To quantify the changes in host protein expression during infection with *Y. enterocolitica*, we infected epithelial HeLa cells for four hours. We tested whether the used infection conditions yield satisfactory number of infected cells by using a gentamicin survival assay. The percentage of infected HeLa cells after 4 hours of infection period was 69.5 % +/ − 8.8%. Label-free quantitative proteomic analysis by HPLC-MS-MS/MS led to identification of 551 proteins (FDR<0.35%), amongst which 22 were significantly upregulated (e.g. Rab1c, SUMO-3, and RhoA), and 42 were downregulated (e.g. Karyopherin, POH-1, and Prostaglandin E synthase) in infected cells (Fig. 2A). A subset of these proteins was independently validated by western blot analysis (Fig. 2B). Also, Caco-2 colonic epithelial cells, a frequently used cell line to study host-pathogen interactions in *Yersinia* infection [27, 28], were used to further confirm these results (Fig. 2C). Caco-2 cells were infected with wild-type *Y. enterocolitica* or *Y. enterocolitica* 8081c mutant lacking the virulence plasmid. Upregulation of RhoA and downregulation of POH-1 and Prostaglandin E synthase 3 were observed in Caco-2 cells infected with wild-type *Yersinia* (Fig. 2C), but protein levels of Prostaglandin E synthase 3 and POH-1 were not affected in cells infected with the 8081c mutant. It could either be caused by an attenuated infection or by one of the virulence factors, which needs to be tested further. Possible functions of some of the identified proteins in regulation of *Yersinia* infection are discussed below.

Rab1c is a small GTPase with unknown functions, and a significant homologue of Rab1b (Rab1) responsible for maintaining transport between endoplasmic reticulum and Golgi complex [29]. Rab1 is hijacked by *Legionella pneumophila*'s secreted proteins (SidM, SidD, and LepB), which leads to inactivation of Rab1 [30]. In *Salmonella* infection, knockdown of Rab1 causes attenuated replication of this bacterium in HeLa cells due to the interference with the autophagic process [31]. To date, no specific function of Rab1 in *Yersinia* infection has been proposed, but up-regulation of Rab1 could potentially lead to increased autophagy of *Yersinia* [32].

RhoA is another small GTPase, which controls the actin cytoskeleton assembly and is crucial in regulation of phagocytosis, or integrity of the epithelial cell monolayer. RhoA is known to regulate the host cell response to *Yersinia* infection, and there are several *Yersinia* virulence factors that bind and alter the function or activity state of this protein [33]. Moreover, during infection with *Y. enterocolitica* RhoA accumulates on membranes of HeLa cells, which corroborates our results [34].

SUMO-3, SUMO-1 and SUMO-2 are ubiquitin-like proteins that form a covalent post-translational modification on proteins [35]. SUMO-3 and SUMO-2 are often referred to as SUMO-2/3 due to their similarity. SUMO molecules modify the components of the NF-kappaB pathway, and together with ubiquitin they lead to the TNF-alpha-mediated activation of NF-kappaB [36]. It has been recently shown that that during infection with another gram-negative bacterium, *Salmonella* Typhimurium, the overall SUMOylome was significantly reduced at early stages of infection [37]. Moreover, sumoylation is required for restriction of epithelial invasion and pro-inflammatory transcriptional response in *Shigella flexneri* infection [38]. It was initially reported that *Yersinia*'s virulence factor YopJ inhibits

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conjugation of SUMO-1 to host proteins [39], but it was subsequently shown that this protein rather functions as an acetyltransferase [40, 41], therefore its desumoylating function was questioned. However, it cannot be completely ruled out that YopJ directly or indirectly leads to removal of SUMO modification from protein substrates during infection [39].

Prostaglandin E Synthase 3 (p23) is a component of the cyclooxygenase (COX)-1 pathway of prostaglandin E2 (PGE2) biosynthesis [42]. As such, it is involved in a variety of processes, which include regulation of immune responses [43]. Documentation of downregulation of this synthase in epithelial cells infected with *Y. enterocolitica* could point towards novel function of eicosanoids in the host-pathogen interactions.

Karyopherin alpha belongs to the family of karyopherins (importins), which are adaptor proteins that recognize the nuclear localization signals critical in the protein export to the nucleus [44]. Karyopherin alpha is involved in the NF-kappaB p50/p65 heterodimer translocation upon TNF-alpha stimulation [45]. Together with Importin beta 1, Karyopherin alpha activates a large number of genes encoding pro-inflammatory cytokines and chemokines, and other proteins critical in host responses to infection [46]. Down-regulation of karyopherin alpha in response to *Yersinia* infection is interesting, as *Yersinia* evades immune response via inhibition of key inflammatory regulators by its virulence factors [47], some of which might be also regulated by karyopherin alpha [46]

Finally, POH-1 (RPN11, PSMD14) is a Zn^{2+}-dependent deubiquitinating enzyme important in proteolysis as a component of the proteasome [48]. POH-1 is most likely involved in cleavage of the ubiquitin chains from protein substrates prior to their processing by the proteasome [49]. Although POH-1's precise function in *Yersinia* infection is cryptic, proteasome-dependent degradation of certain virulence proteins has been described, e.g. YopE [50].

5.3. Network analysis

The canonical pathways altered in infected cells were EIF2, RAN, regulation of eIF4/p70S6K signaling, and mTOR signaling pathways (Fig. 3A). EIF2 pathway regulates translation initiation in response to stress. This pathway is important in the functions of bacterial virulence factors, including *Yersinia* protein kinase A (YpkA) and *Yersinia* outer protein J (YopJ) [51] as described below. Specifically, EIF2 signaling is disrupted by *Yersinia* virulence factor YopJ, which thereby alters the expression of pro-inflammatory cytokines and bacterial invasion [52]. Another pathway significantly altered in infected cells was RAN (Ran) pathway. Ran is a small GTPase predominantly localized to nucleus and it has important functions in nucleo-cytoplasmic transport [53]. *Yersinia* infection also affected proteins involved in such molecular functions as death and survival, cellular movement, cell cycle, gene expression, carbohydrate metabolism, lipid metabolism, small molecule biochemistry, as well as cellular function and maintenance (Fig. 3B). Inducing cell death is a common tactic used by bacterial pathogens for effective spread in the host, and *Yersinia* is not an exception [54]. It would be interesting to establish whether *Yersinia* promotes cell death in epithelial cells by targeting any of the identified molecules specifically. For example, *Yersinia* is already known to modify Cdc42 via its toxin CNF-gamma, which activates several other GTPases, and this is proposed to lead to stronger pro-inflammatory

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responses [55, 56]. Cdc42 and Ezrin (both present in our dataset) are GTPases involved in modification of actin cytoskeleton and they both regulate cell viability [57, 58].

Moreover, the differentially regulated proteins were placed into two networks (Fig. 3C and Fig. 4) to illustrate significant relationships between these molecules. One of the canonical pathways associated with this network was integrin signaling, which role in *Yersinia* infection is already known [59], [60]. Moreover, several molecules in this network were associated with “signaling by Rho family of GTPases” canonical pathway, such as Cdc42 (downregulated in infected cells) and RhoA (up-regulated in infected cells), both of which are small GTPases controlled by guanine nucleotide dissociation inhibitors (GDI), which prevent GTPase activation [61]. *Yersinia* encodes YopO (YpkA) that mimics host guanidine nucleotide dissociation inhibitors, and it has affinity for Rac1 and RhoA but not for Cdc42 [62, 63]. Also, cytotoxic necrotizing factor-Y (CNF-gamma) activates Rac1 and Cdc42, but not RhoA [55]. Another interesting interaction found in the second identified protein network (Fig. 4) is association of the differentially expressed proteins to the protein ubiquitination pathway. Additionally, this network predicted binding of a ubiquitin-like protein SUMO-3 (up-regulated in infected cells) to several proteins identified as downregulated in infected cells, including NONO, EPRS, MATR3, TRIM28, and XRCC5. It would be interesting to establish whether SUMO-3 modification is regulated during infection with *Yersinia* and identify its specific protein substrates, since it is clear that this ubiquitin-like modifier affects critical regulators of the host immune responses [36, 38, 39]. The function of SUMO modification in *Yersinia enterocolitica* infection of epithelial cells will be a subject of future studies, especially in the light of a study, which describes the role of SUMO in intracellular survival of another gram-negative bacterium, *Salmonella* Typhimurium [37].

6. Concluding remarks

Label-free quantitative proteomics has been applied to understand the cell host response to various bacterial pathogens [3–8], but this is the first description of differentially expressed proteome of *Y. enterocolitica*-infected and uninfected HeLa cells, which is likely to contribute to a better understanding of *Y. enterocolitica* pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

TSB	Tryptic soy broth
DMEM	Dulbecco’s modified Eagle medium

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FBS	Fetal bovine serum
PBS	Phosphate buffered saline
MOI	Multiplicity of infection
PVDF	Polyvinylidene difluoride
TBS	Tris buffered saline
DTT	Dithiothreitol
IAA	Iodoacetamide

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2. Statement of significance of the study

We describe a proteome of *Yersinia enterocolitica*-infected HeLa cells, including description of specific proteins differentially expressed upon infection, molecular functions as well as pathways altered during infection. This proteomic study can lead to a better understanding of *Y. enterocolitica* pathogenesis in human epithelial cells.

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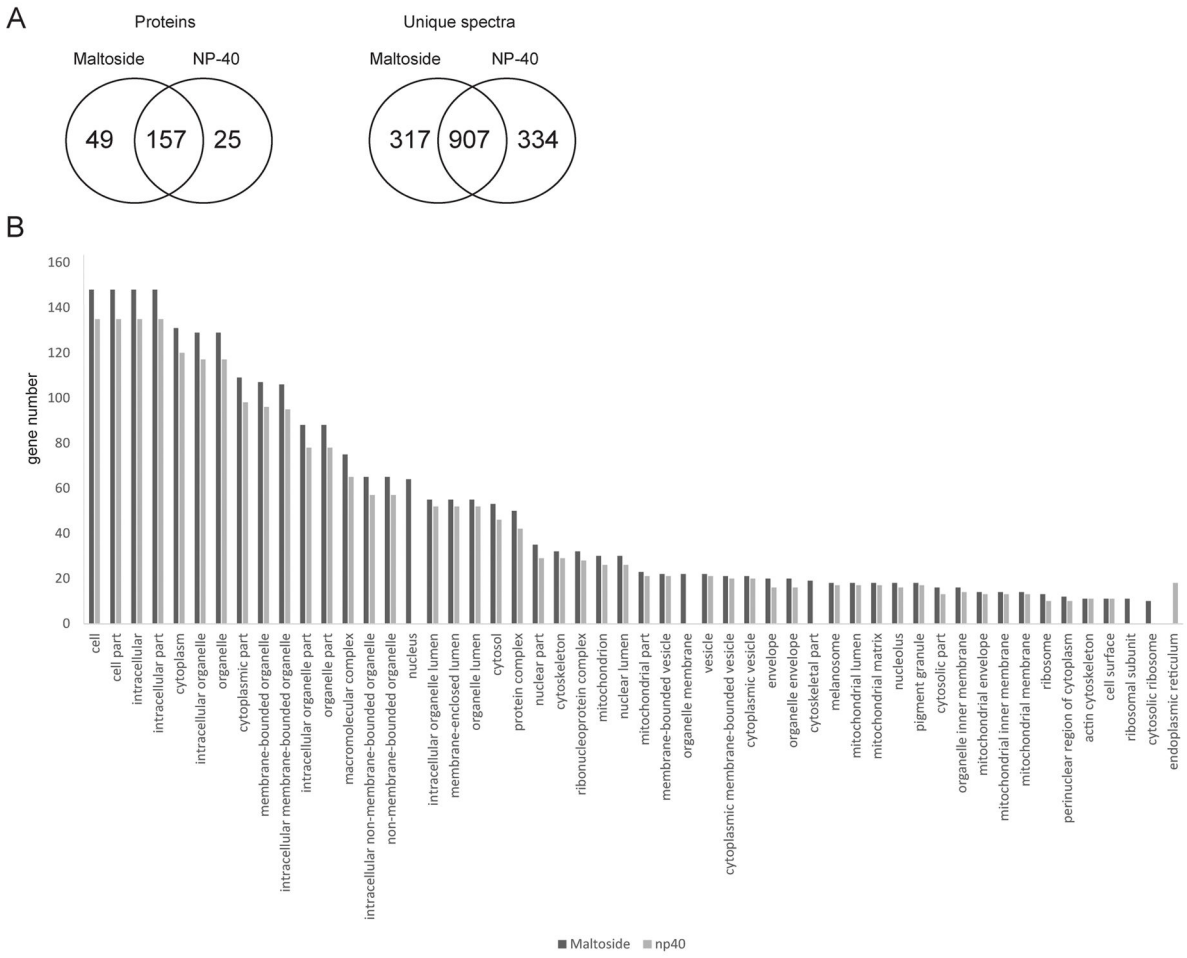

Figure 1. Comparison of proteomics results obtained from HeLa cells lysed by using two different buffers

(A). The Venn diagrams illustrate the number of proteins identified in at least two of the analyzed biological replicates, and unique spectra detected in each sample type. **(B).** GO term analysis of subcellular localizations of unique proteins. Proteins identified in each sample type were subjected to the GO term analysis by using The Database for Annotation, Visualization and Integrated Discovery (DAVID).

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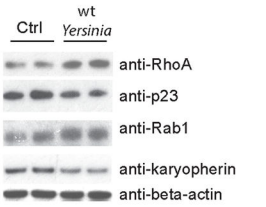
A

Fold change	Uniprot ID	Gene Symbol	Entrez Gene Name	Location	Protein type
16	DEST_HUMAN	DSTN	destrin (actin depolymerizing factor)	Cytoplasm	other
16	RAB1C_HUMAN	RAB1C	RAB1C, member RAS oncogene family pseudogene	Other	other
11	F2Z393_HUMAN	TALDO1	transaldolase 1	Cytoplasm	enzyme
9.3	AK1C3_HUMAN	AKR1C3	aldo-keto reductase family 1, member C3	Cytoplasm	enzyme
8.8	HINT1_HUMAN	HINT1	histidine triad nucleotide binding protein 1	Nucleus	enzyme
8.6	RL27_HUMAN	RPL27	ribosomal protein L27	Cytoplasm	other
7.5	RL5_HUMAN	RPL5	ribosomal protein L5	Cytoplasm	other
6.9	MDHC_HUMAN	MDH1	malate dehydrogenase 1, NAD (soluble)	Cytoplasm	enzyme
6.8	A8MU27_HUMAN	SUMO3	small ubiquitin-like modifier 3	Nucleus	other
6.8	TPM4_HUMAN	TPM4	tropomyosin 4	Cytoplasm	other
5.5	RS8_HUMAN	RPS8	ribosomal protein S8	Cytoplasm	other
4.7	ERF1_HUMAN	ETF1	eukaryotic translation termination factor 1	Cytoplasm	translation regulator
3.1	1433T_HUMAN	YWHAQ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta	Cytoplasm	other
2.6	GSTP1_HUMAN	GSTP1	glutathione S-transferase pi 1	Cytoplasm	enzyme
2.5	B4DR52_HUMAN	HIST2H2BF	histone cluster 2, H2bf	Nucleus	other
2.5	RHOA_HUMAN	RHOA	ras homolog family member A	Cytoplasm	enzyme
2.5	SF01_HUMAN	SF1	splicing factor 1	Nucleus	transcription regulator
2.2	S10AB_HUMAN	S100A11	S100 calcium binding protein A11	Cytoplasm	other
1.9	TCPQ_HUMAN	CCT8	chaperonin containing TCP1, subunit 8 (theta)	Cytoplasm	enzyme
1.9	K7ELW0_HUMAN	PARK7	parkinson protein 7	Nucleus	enzyme
1.6	ENOA_HUMAN	ENO1	enolase 1, (alpha)	Cytoplasm	enzyme
1.5	PRDX1_HUMAN	PRDX1	peroxiredoxin 1	Cytoplasm	enzyme
-1.429	B4DPJ8_HUMAN	CCT6A	chaperonin containing TCP1, subunit 6A (zeta 1)	Cytoplasm	other
-1.667	CALX_HUMAN	CANX	calnexin	Cytoplasm	other
-1.667	PDIA1_HUMAN	P4HB	prolyl 4-hydroxylase, beta polypeptide	Cytoplasm	enzyme
-1.667	PHB_HUMAN	PHB	prohibitin	Nucleus	transcription regulator
-1.667	TRAP1_HUMAN	TRAP1	TNF receptor-associated protein 1	Cytoplasm	enzyme
-1.667	XRCC5_HUMAN	XRCC5	X-ray repair complementing defective repair in Chinese hamster cells 5 (double-strand-break rejoining)	Nucleus	enzyme
-2	A8MXP9_HUMAN	MATR3	matrin 3	Nucleus	other
-2	NACAM_HUMAN	NACA	nascent polypeptide-associated complex alpha subunit	Cytoplasm	transcription regulator
-2	NP1L1_HUMAN	NAP1L1	nucleosome assembly protein 1-like 1	Nucleus	other
-2	PRDX6_HUMAN	PRDX6	peroxiredoxin 6	Cytoplasm	enzyme
-2.5	AT1A1_HUMAN	ATP1A1	ATPase, Na+/K+ transporting, alpha 1 polypeptide	Plasma Membrane	transporter
-2.5	AT5F1_HUMAN	ATP5F1	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit B1	Cytoplasm	transporter
-2.5	DNJA1_HUMAN	DNAJA1	DnaJ (Hsp40) homolog, subfamily A, member 1	Nucleus	other
-2.5	B3KSH1_HUMAN	EIF3F	eukaryotic translation initiation factor 3, subunit F	Cytoplasm	translation regulator
-2.5	E7EQR4_HUMAN	EZR	ezrin	Plasma Membrane	other
-2.5	SCPDL_HUMAN	SCCPDH	saccharopine dehydrogenase (putative)	Cytoplasm	other
-3.333	AHNK_HUMAN	AHNAK	AHNAK nucleoprotein	Nucleus	other
-3.333	E7EUU4_HUMAN	EIF4G1	eukaryotic translation initiation factor 4 gamma, 1	Cytoplasm	translation regulator
-3.333	IMA1_HUMAN	KPNA2	karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	Nucleus	transporter
-3.333	PSDE_HUMAN	PSMD14	proteasome 26S subunit, non-ATPase 14	Cytoplasm	peptidase
-3.333	RS7_HUMAN	RPS7	ribosomal protein S7	Cytoplasm	other
-3.333	C9K0U8_HUMAN	SSBP1	single-stranded DNA binding protein 1, mitochondrial	Cytoplasm	other
-3.333	TIF1B_HUMAN	TRIM28	tripartite motif containing 28	Nucleus	transcription regulator
-3.333	XPO1_HUMAN	XPO1	exportin 1	Nucleus	transporter
-5	ARF1_HUMAN	ARF1	ADP-ribosylation factor 1	Cytoplasm	enzyme
-5	AT2A2_HUMAN	ATP2A2	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	Cytoplasm	transporter
-5	SYEP_HUMAN	EPRS	glutamyl-prolyl-tRNA synthetase	Cytoplasm	enzyme
-5	TFR1_HUMAN	TFRC	transferrin receptor	Plasma Membrane	transporter
-10	THIM_HUMAN	ACAA2	acetyl-CoA acyltransferase 2	Cytoplasm	enzyme
-10	CALU_HUMAN	CALU	calumenin	Cytoplasm	other
-10	CDC42_HUMAN	CDC42	cell division cycle 42	Cytoplasm	enzyme
-10	NSUN2_HUMAN	NSUN2	NOP2/Sun RNA methyltransferase family, member 2	Nucleus	enzyme
-10	TEBP_HUMAN	PTGES3	prostaglandin E synthase 3 (cytosolic)	Cytoplasm	enzyme
-10	DHSO_HUMAN	SORD	sorbitol dehydrogenase	Cytoplasm	enzyme
-10	TMEDA_HUMAN	TMED10	transmembrane p24 trafficking protein 10	Cytoplasm	transporter
-11.111	IF2A_HUMAN	EIF2S1	eukaryotic translation initiation factor 2, subunit 1 alpha, 35kDa	Cytoplasm	translation regulator
-11.111	B4DDD8_HUMAN	HARS	histidyl-tRNA synthetase	Cytoplasm	enzyme
-11.111	LC7L2_HUMAN	LUC7L2	LUC7-like 2 pre-mRNA splicing factor	Other	other
-11.111	TBB3_HUMAN	TUBB3	tubulin, beta 3 class III	Cytoplasm	other
-14.286	ATPD_HUMAN	ATP5D	ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit	Cytoplasm	transporter
-14.286	NONO_HUMAN	NONO	non-POU domain containing, octamer-binding	Nucleus	other
-14.286	RAB8A_HUMAN	RAB8A	RAB8A, member RAS oncogene family	Plasma Membrane	enzyme

B



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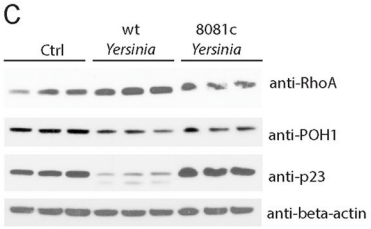

Figure 2. Differential protein expression in HeLa cells infected or not infected with *Yersinia enterocolitica*

(A). HeLa cells were infected with *Y. enterocolitica* and analyzed by label-free quantitative proteomics. Significantly regulated proteins are listed, along with their fold change (infected/control), identification number, symbol, Entrez gene name, localization (suggested by Ingenuity Pathway Analysis software) and protein function. **(B)–(C)** HeLa cells were infected with *Y. enterocolitica* wild-type **(B)** and Caco-2 cells were infected with wild-type or plasmid-cured mutant **(C)** for 4 hours. The extracted proteins were analyzed by SDS-PAGE and western blotting. Anti-beta-actin antibody was used as a loading control.

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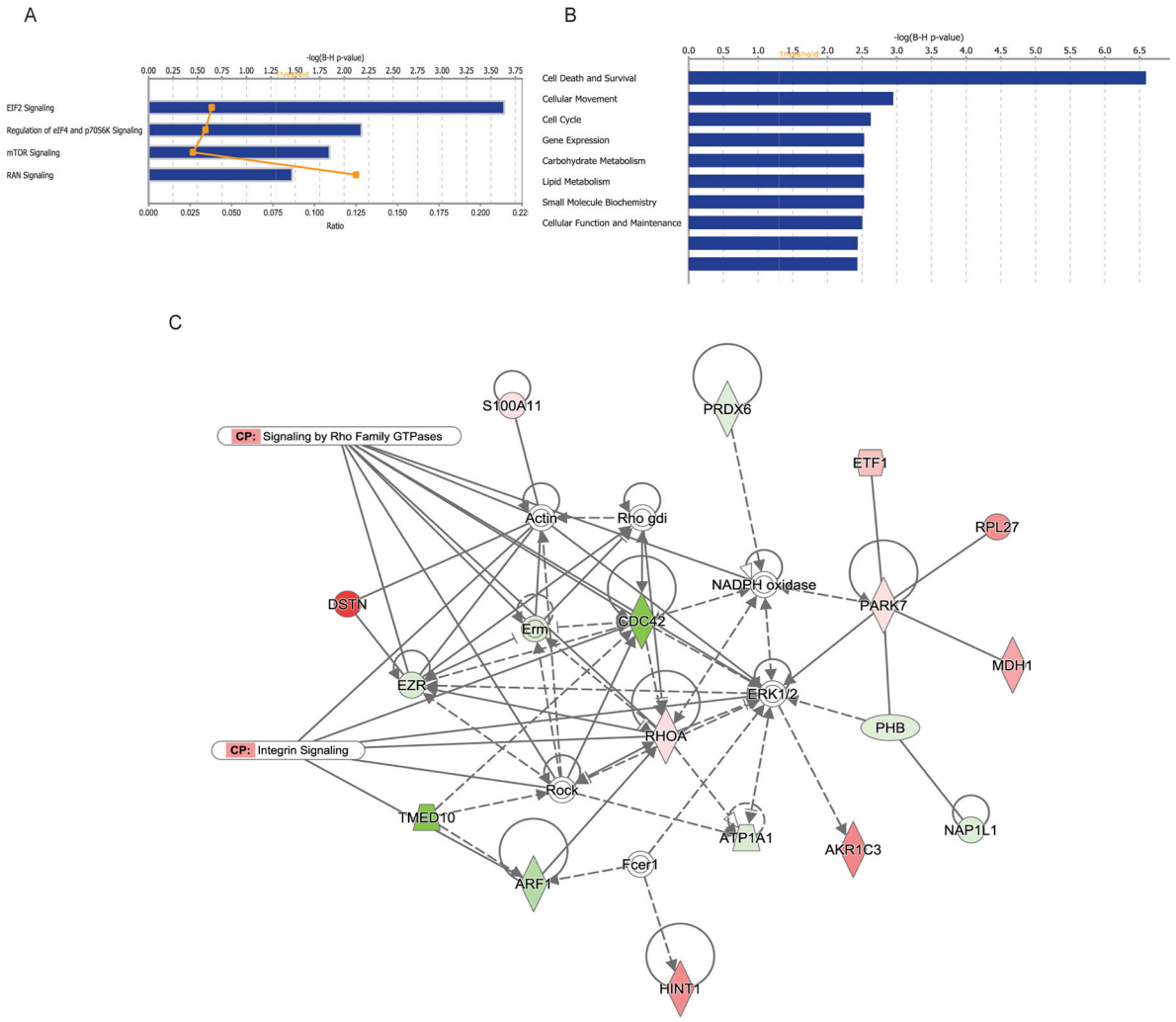

Figure 3. Network and pathway analysis of differentially regulated proteins in HeLa cells subjected to *Yersinia* infection

(A)–(B). Differentially expressed proteins with protein level affected by *Yersinia* infection (Fig. 2A) were analyzed by Ingenuity Pathway Analysis to identify canonical pathways **(A)** and molecular functions **(B). (C)**. The top protein network identified from differentially regulated proteins in infected cells is associated with the integrin signaling and Signaling by Rho family of GTPases. Expression of proteins is indicated by colors (red for downregulated and green for upregulated proteins, white for proteins that were not on a differentially expressed protein list). Different shades of color represent the level of regulation.

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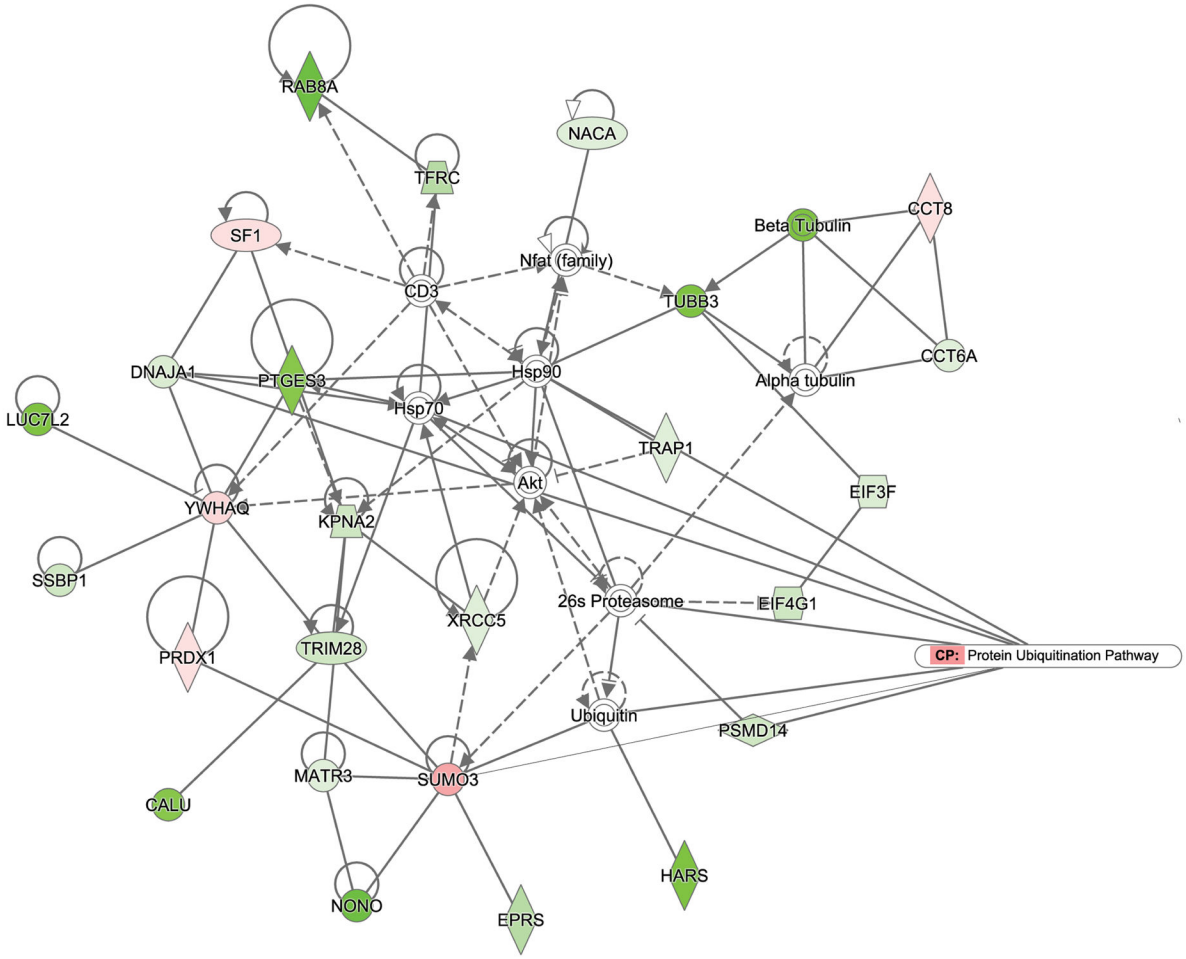

Figure 4. Protein network analysis of differentially regulated proteins in HeLa cells subjected to *Yersinia* infection

The second top network identified from differentially regulated proteins in *Yersinia*-infected HeLa cells is associated with the protein ubiquitination pathway. Expression of proteins is indicated by colors (red for downregulated and green for upregulated proteins, white for proteins that were not on a differentially expressed protein list). Different shades of color represent the level of regulation.