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Article

Unique Posttranslational Modification Sites of Acetylation, Citrullination, Glutarylation, and Phosphorylation Are Found to Be Specific to the Proteins Partitioned in the Triton X-114 Fractions of *Leptospira*

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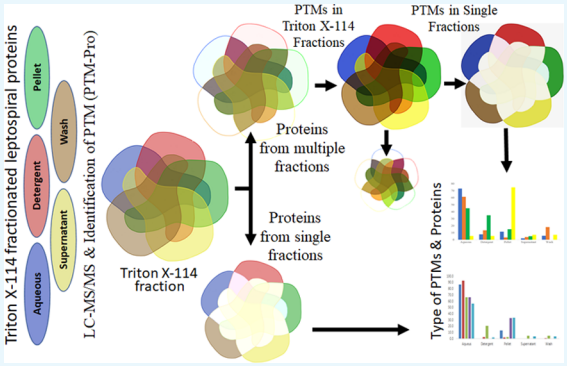
ABSTRACT: Posttranslational modifications (PTMs) are decisive factors in the structure, function, and localization of proteins in prokaryotic and eukaryotic organisms. However, prokaryotic organisms lack subcellular organelles, and protein localization based on subcellular locations like cytoplasm, inner membrane, periplasm, and outer membrane can be accounted for functional characterization. We have identified 131 acetylated, 1182 citrullinated, 72 glutarylated, 5 palmitoylated, and 139 phosphorylated proteins from Triton X-114 fractionated proteins of *Leptospira*, the pathogen of re-emerging zoonotic disease leptospirosis. In total, 74.7% of proteins were found exclusively in different Triton X-114 fractions. Additionally, 21.9% of proteins in multiple fractions had one or more PTM specific to different Triton X-114 fractions. Altogether, 96.6% of proteins showed exclusiveness to different Triton X-114 fractions either due to the presence of the entire protein or with a specific PTM type or position. Further, the PTM distribution within Triton X-114 fractions showed higher acetylation in aqueous, glutarylation in detergent, phosphorylation in pellet, and citrullination in wash fractions representing cytoplasmic, outer membrane, inner membrane, and extracellular locations, respectively. Identification of PTMs in proteins with respect to the subcellular localization will help to characterize candidate proteins before developing novel drugs and vaccines rationally to combat leptospirosis.



INTRODUCTION

Once restricted to agricultural practice, leptospirosis is becoming a re-emerging threat to public health even in the urban population. As part of urbanization, an increase in slum areas that host reservoir animals, together with frequent natural calamities like rain and flood, increased the occurrence of leptospirosis outbreaks.[1,2] One of the most widespread neglected tropical zoonosis is caused by pathogenic *Leptospira* transmitted through an animal vector, particularly rodents.[3,4] The global burden of the disease in terms of Disability Adjusted Life Years (DALY) reports ∼2.90 million from 1.03 million estimated cases per year with 58,900 lethalities.[5] Such health statistics greatly impact the country's economy significantly. Apart from this, in the lower- and middle-income countries, including regions of Africa where leptospirosis is getting misdiagnosed with other febrile illnesses, commonly malaria, the real picture of leptospirosis is unnoticed.[5,6]

Proteomics on *Leptospira* showed 2957 proteins accounting the highest percentage of the total proteome.[7] Due to the lack of characterization of many leptospiral proteins, their significance in pathogenicity and survival in the host is unknown. Posttranslational modification (PTM) of proteins can influence the structure, function, activity regulation, localization, and biomolecular interactions.[8−10] In *Pseudomonas aeruginosa*, it was found that modified ribosomal proteins and elongation factor TU play a crucial role in the early host colonization, and succinylated lysine of LsaB in the organism is known to degrade the host protein (i.e., collagen, fibrin) as well as inactivate the complement system.[11] Similarly, in *Leptospira interrogans*, LIC 11848 (OmpL32) methylation in glutamic acid (Glu) was found to enhance the bacterial virulence process;[12] multi-PTM profile established in *L. interrogans* lai showed 155 methylated, 46 acetylated, and 32 phosphorylated proteins and demonstrated that the protein modification systems are similar to eukaryotes.[13] Even though *Leptospira* is a primitive prokaryotic organism, it

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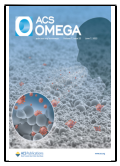

has a protein modification mechanism similar to eukaryotic organisms, evidenced by phosphorylation and arginine methylation.[13] Further, Stewart and colleagues demonstrated three major PTMs in two cellular fractions of *Leptospira biflexa*, i.e., methylation and acetylation of lysine residues concerning their subcellular location.[14] Studies on lysine modification of LipL32 showed increased bacterial diversity and stability.[15]

The Triton X-114 fractions aqueous, detergent, pellet, wash, and supernatant represent subcellular locations such as cytoplasmic and periplasmic proteins, outer membrane proteins, inner membrane proteins, surface, and secretory proteins, respectively.[7,16−18] Our earlier study analyzed Triton X-114 fractionated leptospiral proteins, showing a quantitative difference in the distribution across subcellular locations.[7,17] This report comprises the analysis of proteomic data and identification of PTMs, their distribution across the subcellular fractions that can lead to a better understanding of the protein function, and their stability in subcellular locations.

METHODOLOGY

Workflow of the Study. The PTM identification and analysis of *Leptospira* was carried out to find acetylation, citrullination, glutarylation, palmitoylation, and phosphorylation in *Leptospira* and to know their subcellular localization with respect to the modified site and type of modification. The entire study was carried out as per the workflow illustrated in Figure S1.

***Leptospira* Strain and Culture.** *L. interrogans* serogroup Icterohaemorrhagiae serovar copenhageni strain Fiocruz L1-130 was received from the repository of ICMR-Regional Medical Research Centre, a WHO Collaborative Centre for research in Leptospirosis, Port Blair, India. Leptospires were cultured in Ellinghausen−McCullough−Johnson−Harris (EMJH) medium (Difco Laboratories, Franklin Lakes, NJ) with 1% BSA as a supplement. The culture was incubated at 30 °C until the growth of the mid-log phase was reached.

Enrichment of Subcellular and Extracellular/Secretory Proteins. The mid-log phase *Leptospira* culture (4 replicates of 25 mL culture with ∼3.5×10^8 cells/mL each) was harvested by centrifugation at 2500*g* for 30 min at 4 °C. The remaining supernatant was consecutively centrifuged at 6000*g* for 30 min followed by 12,000*g* for another 30 min to remove any *Leptospira* left that can minimize the presence of cells in the supernatant from which the secretory/extracellular proteins were separated. The harvested cell pellet was washed three times with PBS containing 5 mM $MgCl_2$ followed by centrifugation at 2500*g* for 5 min at room temperature. The wash supernatant was transferred to another vial and recentrifuged at 12,000*g* for 30 min to remove any trapped viable cells, and the supernatant was termed as "wash fraction", which denotes surface proteins. The secretory proteins present in the supernatant were enriched with ProteoMiner (Bio-Rad). The supernatant was dialyzed against PBS to obtain ideal pH and salt concentration, which helped the proteins bind with ProteoMiner. The slurry from ProteoMiner was washed two times with PBS, mixed with 100 mL of the supernatant, and kept at 4 °C overnight for protein binding. The beads were recovered from the supernatant and repacked in the ProteoMiner column and washed with 100 μL of PBS. Finally, elution was performed using a 2 × 20 μL elution reagent (8 M urea, 2% CHAPS).[7,17]

The cell pellet obtained after washing was further treated with Triton X-114 extraction buffer (10 mM Tris-Cl (pH 8), 1% Triton X-114, and 150 mM NaCl) and incubated at 4 °C overnight. The extract was centrifuged at 12,000*g* for 30 min at 4 °C and resulted in two distinct fractions, i.e., cell pellet (named as "pellet fraction") and the supernatant used to separate aqueous and detergent phases. The concentration of the detergent soluble supernatant was raised to 2% (vol/vol) by the addition of sufficient amount of Triton X-114, mixed thoroughly, kept at 37 °C for 1 h, and centrifuged at 2000*g* for 5 min to isolate the upper aqueous from the bottom detergent phase. The insoluble proteins in the pellet fraction were extracted with another extraction buffer (10 mM Tris-Cl (pH 8), 8 M urea, protease inhibitor, 1% sodium dodecyl sulfate) including proteins from cytoplasmic cylinders. The extracted protein samples of all fractions, including aqueous, detergent, pellet, supernatant, and wash, were estimated for their protein concentration using the BCA method (Pierce, BCA protein assay kit #23225, Thermo Scientific)[19] and kept at −20 °C for further use.

Mass Spectrometry Analysis. *In-Solution Digestion.* In-solution digestion of the protein sample of each fraction was performed according to the method described previously.[7,17] In this method, 250 μg of the protein sample was taken and subjected to reduction with 10 mM dithiothreitol (DTT) and then alkylated with 20 mM iodoacetamide (IAA). The cell lysate was further acetone precipitated to remove SDS added to the extraction buffer and to concentrate the proteins into a pellet. Finally, the digestion of proteins was carried out using trypsin (Modified sequencing grade; Promega Corporation, Madison, WI) at 37 °C for 16 h. Formic acid (0.1%) was used to stop the reaction.

Basic pH/Reverse-Phase Liquid Chromatography-Based Fractionation. The peptides were vacuum dried and fractionated using the basic pH/reverse-phase liquid chromatography (bRPLC) method, and separated using a Hitachi LaChrom Elite HPLC System (Hitachi High-Technologies Corporation, Tokyo, Japan) equipped with an XBridge C18 column (130 Å, 5 μm, 250 mm × 4.6 mm; Waters Corporation, Milford, MA) with a linear increase in the gradient from 5 to 100% of 10 mM TEABC with 90% acetonitrile over 120 min. Initially, 96 fractions were obtained, which were then concatenated to six fractions and dried before being desalted with C18 cartridges. The desalted peptides were then vacuum dried and stored at −80 °C until LC-MS/MS analysis.

Tandem LC-MS/MS Analysis. The desalted peptides were analyzed using an Orbitrap Fusion Tribrid Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA) linked to the EASY-nLC 1200 liquid chromatography system (Thermo Fisher Scientific). The peptides were resuspended in 0.1% formic acid and loaded onto a 2 cm trap column (nanoViper, 3 μm C18 Aq; Thermo Fisher Scientific). The peptides were then separated using a 15 cm analytical column (nanoViper, 75 μm silica capillary, 2 μm C18 Aq) at a flow rate of 300 nL/min. The solvent was set to a linear gradient of 5−35% solvent B (80% acetonitrile in 0.1% formic acid) over 90 min through a run time of 120 min. MS analysis was performed in the data-dependent mode on an Orbitrap ion trap mass analyzer with a scan range of 400−1600 m/z (mass resolution of 120,000 at 200 m/z) and the maximum injection time was 10 ms. For MS/MS analysis, data were acquired in the top-speed mode with 3 s cycles and subjected to high-energy collision dissociation with 32% normalized collision energy. MS/MS scans were carried out at a range of 100−1600 m/z using an Orbitrap mass analyzer at a resolution of 30,000 at 200 m/z and the maximum injection time was 200 ms.

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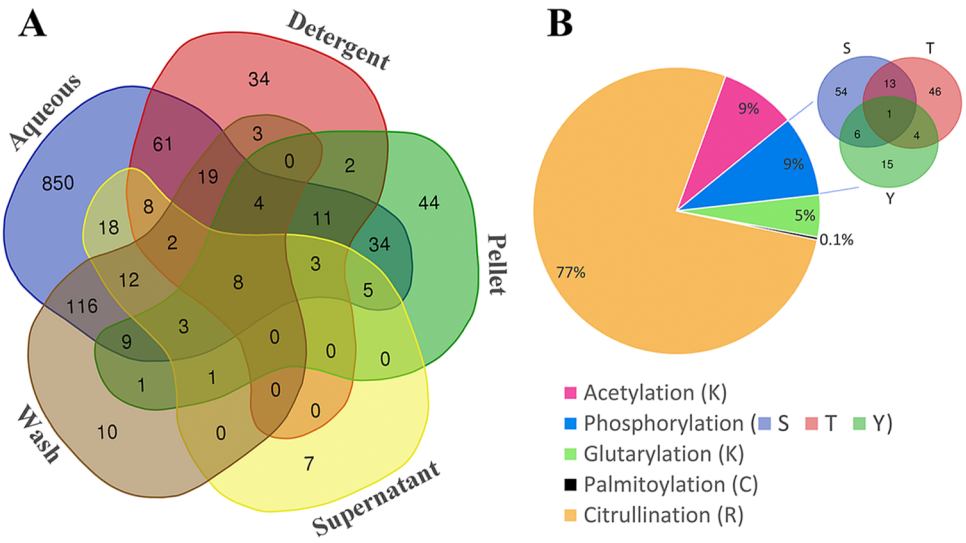

Figure 1. Identification of PTM-containing proteins from *Leptospira*: (A) Venn chart showing the number of PTM-containing proteins distributed across Triton X-114 fractions. (B) Pie chart showing different PTMs found in *Leptospira*. Further details are shown in Table S1.

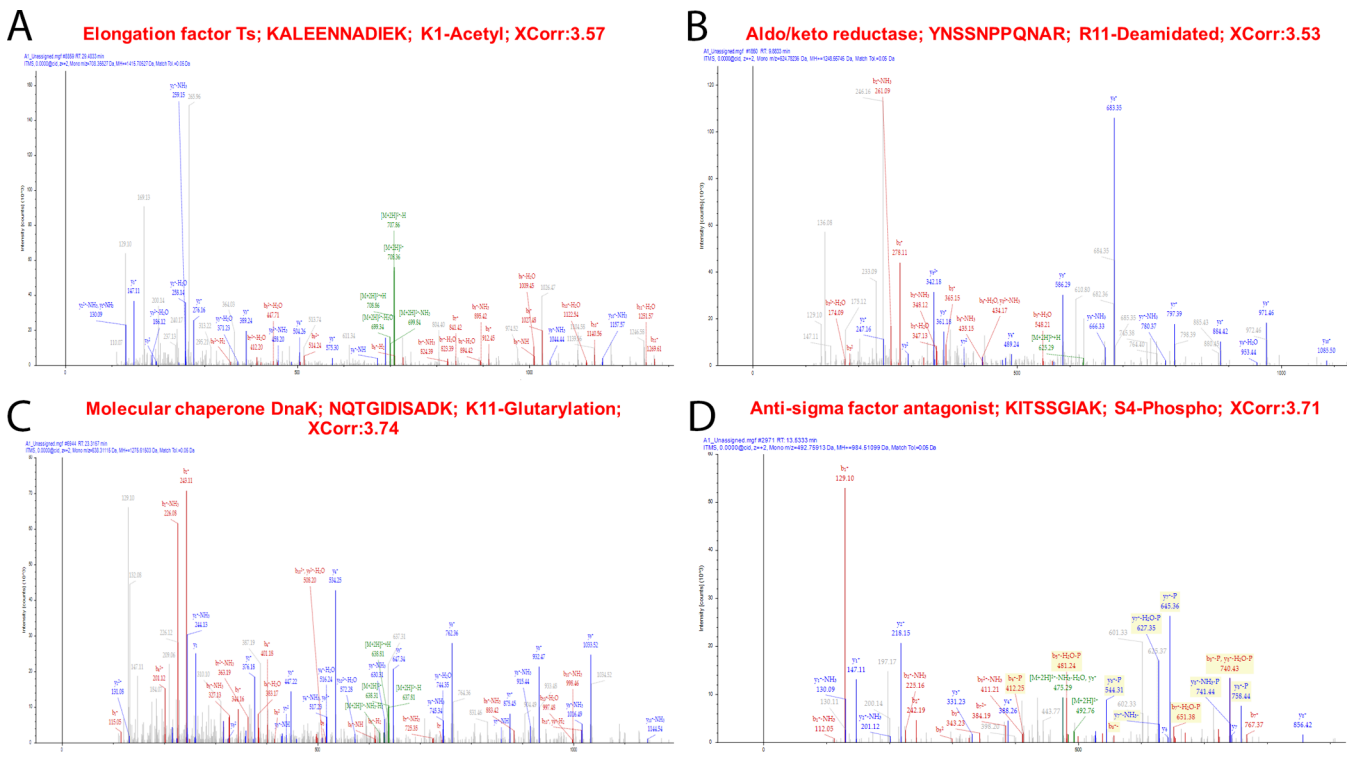

Figure 2. MS/MS spectra for peptides containing modified amino acids: (A) peptides representing acetylated lysine in elongation factor Ts, (B) citrullinated (deamidated) arginine in Aldo/keto reductase, (C) glutarylated lysine in molecular chaperone DnaK, and (D) phosphorylated serine in the anti-sigma factor antagonist. Additional spectra are shown in Figures S2–S19.

Proteomic Data Analysis. The mass spectrometry-derived data from all of the LC-MS/MS analyses were searched against *L. interrogans* serogroup Icterohaemorrhagiae serovar copenhageni (strain Fiocruz L1-130) obtained from the NCBI (3667 protein entries). The database was also added with sequences of commonly encountered protein contaminants such as BSA, trypsin, and keratins (115 contaminant entries). The MS data were analyzed using the SEQUEST-HT and Mascot search algorithms in the Proteome Discoverer software suite, version 2.2 (Thermo Fischer Scientific, Bremen, Germany) with the following search parameters: (a) trypsin as the proteolytic enzyme (with up to one missed cleavage); (b) fragment mass tolerance of 0.05 Da; (c) precursor mass tolerance of 10 ppm; (d) oxidation of methionine as a dynamic modification, and (e) carbamidomethylation of cysteine as a static modification. The peptide-to-spectrum match scoring function was identified with 1% false discovery rate (FDR) at the peptide level.

Retrieval of Data. Label-free raw mass spectrometry data (LC-MS/MS) from our early study were utilized, i.e., PXD016204 and PXD026044.[7,17] The raw files with precursor and fragment masses were assigned for peptide similarity search (peptide spectral matches—PSMs) using a suitable proteome

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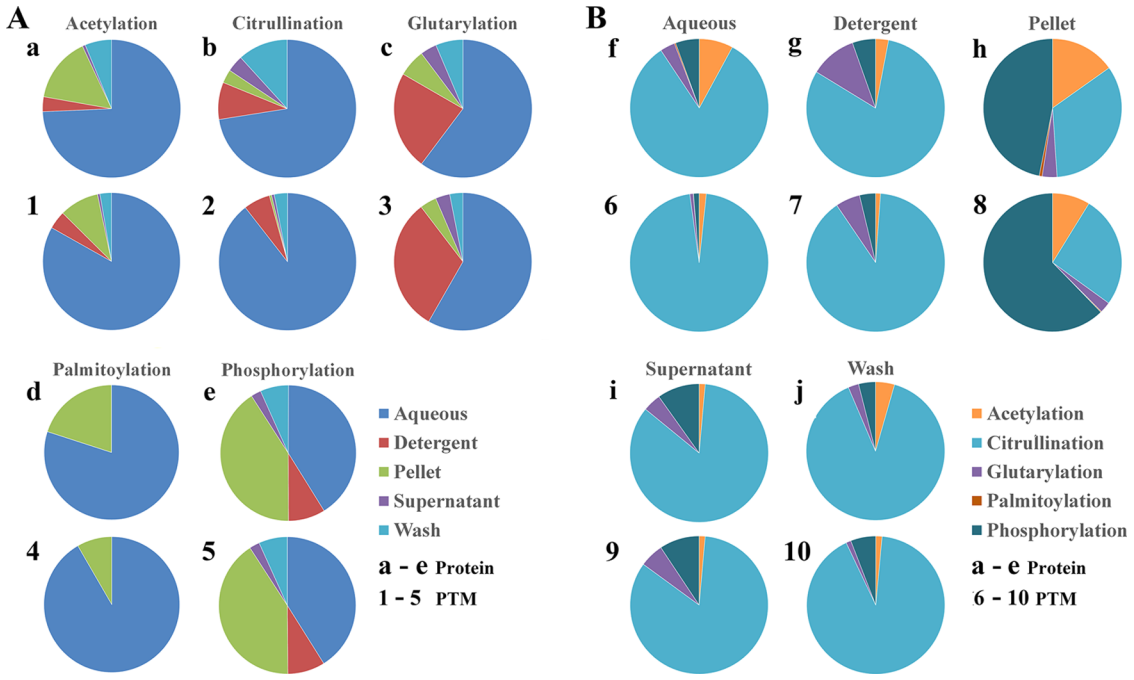

Figure 3. Distribution of PTMs across Triton X-114 fractions: (A) Pie charts showing the distribution of PTM-containing proteins (A, a−e) and PTMs (A, 1−5) across Triton X-114 fractions and (B) pie charts showing the types of PTM-containing proteins (B, f−j) and PTMs (B, 6−10) identified within Triton X-114 fractions. Details on the values and number of proteins present in each group are shown in Tables S2 and S3.

database. The unassigned MS/MS data from the proteome search was retrieved and compared with the reference database through multi-PTMs.

Peptide Identification. The retrieved mass spectrometry datasets were examined against the *L. interrogans* serovar Copenhageni (strain Fiocruz L1-130) protein database taken from NCBI RefSeq. The searches were accomplished with the Mascot search engine by Proteome Discoverer (V 2.2) software suite (Thermo Scientific, Bremen, Germany) and SEQUEST-HT. Here, trypsin was used as an enzyme in the search parameters, and also acetylation (+42.011 Da) of N-terminal and oxidation (+15.995 Da) of methionine (M) were set as a dynamic modification. Carbamidomethylation (+57.021 Da) of cysteine residues (C) was fixed as a static modification to analyze the total proteome. The unallocated MS/MS spectra obtained from whole proteome analysis were subjected to the reference database with precise PTMs. The PTMs such as phosphorylation (+79.966 Da) of serine (S), threonine (T), and tyrosine (Y); citrullination (+0.984 Da) of arginine (R); palmitoylation (+238.230 Da) of cysteine (C); glutarylation (+114.032 Da) of lysine (K); and acetylation (+42.011 Da) of lysine (K) were set as dynamic modifications. Carbamidomethylation (+57.021 Da) of cysteine (C) was set as a static modification. The mass error was set as 10 ppm and 0.05 Da for the precursor and fragment ions. To determine the probably modified site, ptmRS node was utilized. The PSMs and peptides that qualified 1% FDR were used for further analysis.

Bioinformatics Analysis. The peptide sequences coupled with their group accession ids and respective ptmRS probabilities were subjected to posttranslational modification profiling (PTM-Pro) at a minimum cut-off of 75% (version 2.0), an online platform to predict high confident PTMs.[20]

RESULTS

Identification of PTM Proteins. The LC-MS/MS data of Triton X-114 fractionated proteins of *Leptospira* submitted to PRIDE PXD016204[7] and PXD026044[17] were analyzed using Proteome Discoverer 2.4. The reassigned spectra are available in the Proteome Xchange Consortium (http://www.proteomecentral.proteomexchange.org) via the PRIDE partner repository under the dataset identifier PXD030370. The LC-MS/MS identified 29,066 peptides (including multiple identifications) with modified amino acids composed of 3348 unique peptides. These 3348 unique peptides represented 1266 modified proteins. This highest protein identification was a result of Triton X-114 fractionation and high-resolution LC-MS/MS.

Out of the modified proteins, 1163 (91%) were from aqueous, 155 (12.3%) from the detergent, 125 (9.9%) from the pellet, 67 (5.3%) from the supernatant, and 188 (14.9%) were from wash fractions in which 850 (73%), 34 (22%), 44 (35%), 7 (10%) and 10 (5%) proteins were found exclusively identified from aqueous, detergent, pellet, supernatant, and wash, respectively (Figure 1A and Table S1). Based on the type of protein modification, there were 131 acetylation on K, 1182 citrullination on R, 72 glutarylation on K, 5 palmitoylations on C, and 139 phosphorylation found as 74 on S, 64 on T, and 26 on Y (Figure 1B). Representative spectra showing the identification of PTMs are shown in Figure 2.

Distribution of PTMs in Triton X-114 Fractions. A detailed analysis was made based on the number of PTM-containing proteins and the number of PTMs (number of PTM sites) identified from the LC-MS/MS data. The analysis showed that the number of proteins and PTMs were comparable within each fraction, as shown in Figure 3 and Tables S2 and S3. The highest citrullination identified in the Triton X-114 fraction was 89% in the wash fraction, which accounted for 12% of the total citrullination in *Leptospira*. In contrast, the aqueous fraction

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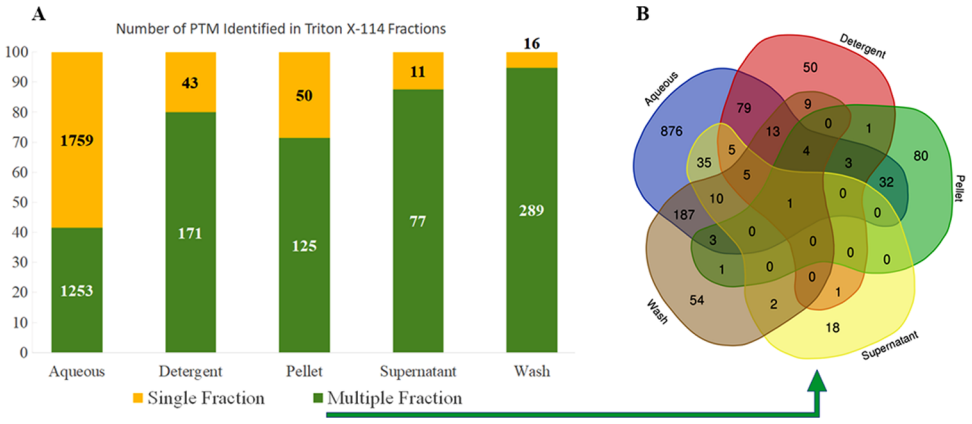

Figure 4. Distribution of PTMs identified in Triton X-114 fractions of *Leptospira*: (A) Column chart shows the number of PTMs identified in Triton X-114 fractions of *Leptospira*. The orange portion consists of PTMs from those proteins found to be unique to Triton X-114 fractions (Figure 1A). The green portion consisting of PTMs belonging to the proteins found in multiple fractions (Figure 1A) was further analyzed for Triton X-114 fraction-specific PTMs of the proteins, and (B) Venn chart shows the distribution of PTMs. Details on the groups of proteins depicted in the Venn chart, PTM sites, and type of modifications are shown in Table S2.

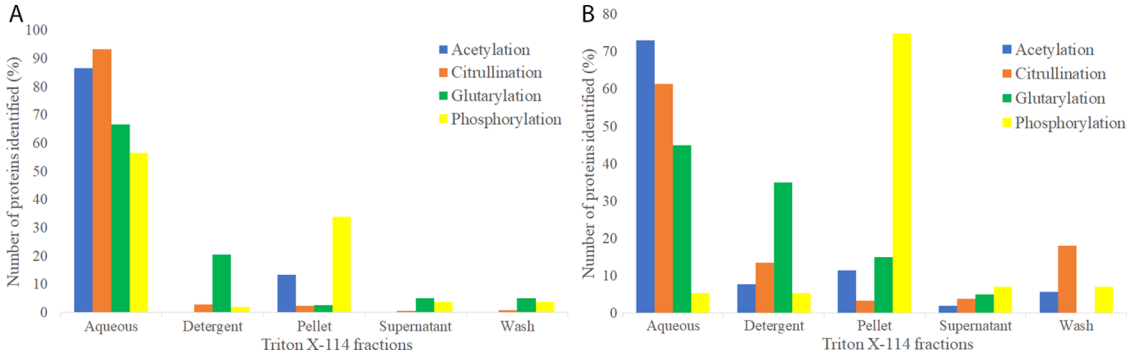

Figure 5. Proteins containing unique PTMs: The column chart shows the distribution of proteins with respect to the type of PTM for those proteins showing the unique presence in various Triton X-114 fractions based on (A) whole protein or (B) modification sites. Details on the values and number of proteins present in each group are shown in Table S4.

contained 83%, which is 72% of the citrullinated proteins identified in *Leptospira*. The pellet fraction contains only 34% citrullinated proteins that account for 3.2% of total citrullination. Within the Triton X-114 fraction, acetylation was found highest at 7.9% in the pellet, which accounted for 74% of the acetylated proteins in *Leptospira*. Similarly, the highest glutarylation in a fraction was found at 11% in the detergent, which accounted for 23% of the total glutarylation, while it was 60% in aqueous that constituting only 3.5% in the fraction. In the case of phosphorylation, the highest at 46% number of phosphorylated proteins in a fraction was the pellet fraction, which accounted for 40% of the total phosphorylated proteins.

Distribution of PTMs of Proteins Found in Multiple Fractions. The PTMs of proteins found in multiple fractions were analyzed to see PTMs specific to any Triton X-114 fractions. The result represented in the Venn diagram (Figure 4) shows 876 PTMs found only in aqueous, 50 in the detergent, 80 in the pellet, 18 in the supernatant, and 54 in the wash. These 1078 PTMs represent 277 proteins, which are 21.9% of the PTM proteins identified. The remaining 391 PTMs found in multiple Triton X-114 fractions contain 43 PTM proteins representing only 3.4% of the total modified proteins found.

Identification of Unique PTMs and PTM Types Concerning Triton X-114 Fractions. On further analysis, it was observed that there are differences in the number of unique PTM-containing proteins in various Triton X-114 fractions based on their PTM type (Figure 5). Although acetylation is present in multiple fractions, It was high in aqueous. Similarly, glutarylation was high in the detergent, and the highest percentage of phosphorylation accounted for in the pellet fraction. Even though citrullination was found to be the highest PTM in *Leptospira*, with respect to the Triton X-114 fraction-specific PTMs, citrullination was found to be less in number.

DISCUSSION

Like in eukaryotes, bacterial PTMs are also widely studied and appreciated to have important and diverse functional roles (Forrest and Welch, 2020). Although the specific purpose of PTMs is not clear, it has profound effects on protein stereochemistry, activity, stability, molecular interaction, and localization.[9,21] Considering PTMs in pathogenic *Leptospira*, it was found to be regulated in response to environmental stimuli including host conditions.[22] In this circumstance, the PTM-containing OMPs were predicted to be responsible for immune evasion (e.g., OmpL32 and LipL32).[15,22] Earlier studies on PTMs in *Leptospira*, were mostly focused on cell-associated protein alterations and the modification associated with proteins secreted into the extracellular milieu was unattended. In this regard, along with Triton X-114-based subcellular locations, extracellular proteins were also considered as they were shown to have an essential role in bacterial pathobiology and virulence.

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The PTM analysis of Triton X-114 fractionated leptospiral proteins showed 42.74 and 34.75% proteins bearing PTMs with respect to the previously identified 2957[7,17] proteins out of the 3667 protein entries of the species in NCBI, respectively. Protein modifications indicate active proteins and their functional capabilities.[23] Our previous results showed a quantitative difference in protein content across subcellular locations in *Leptospira*.[7,17,24] The active form of protein can be assessed with the type of modification, subcellular location, coexpressed molecules, interacting molecules, and the environment. Hence, identifying the PTM content of a protein at the subcellular level is more beneficial to finding the role and characteristics of the protein than the PTMs identified from the total protein extract.

The major PTMs identified in the analysis were citrullination in total and Triton X-114 fraction-wise samples. Stabilization of protein integrity and structure is one of the primary functions of citrullination.[25−27] Further, new evidence suggests that citrullination can play a significant role in regulating gene expression and cellular metabolism.[28] Though citrullination was not reported earlier in *Leptospira*, 5667 deamidations of asparagine and glutamine as dynamic modifications are available in the supplementary files of the proteomic analysis work on *Leptospira biflexa*.[14] Another PTM associated to a greater degree with the aqueous fraction was acetylation, which is higher in aqueous. Acetylation plays a significant role in primary and secondary metabolism, virulence, transcription, and translation.[29,30] Acetylated lysyl residues of bacterial proteins can change the cellular physiology that can play roles in transcription, translation, cellular metabolism, and virulence.[30,31] The detergent fraction was mainly associated with glutarylation. Glutarylation is a crucial PTM, associated with a range of cellular metabolism, translation, and subcellular localizations.[32] The pellet fraction contained phosphorylation as the highest modification found in serine, threonine, and tyrosine residues. Phosphorylations of serine, threonine, and tyrosine are well-known bacterial transcriptional regulators that regulate gene expression and signal transduction signaling networks.[33] Protein phosphorylation affects the protein activity by adding phosphate moieties on relevant amino acids.[34] In bacterial species, protein kinases and phosphatases play an important role in host−pathogen interactions through phosphorylation of key molecules.[35] In this regard, phosphorylation can indicate functionally active proteins, which is vital to understanding the metabolic state of the organism at the time of the experiment.

Our results showed that acetylation and citrullination are predominant in the cytoplasmic, wash, and supernatant, typically nonmembrane or aqueous-based fractions. However, a previous study reported that acetylation was predominately observed in the membrane-associated fraction (equivalent to detergent + pellet fractions in this study). At the same time, phosphorylation was found in the aqueous soluble protein fraction (equivalent to the aqueous fraction in this study) from *L. biflexa*.[14] Due to the difference in the methodology, it is not easy to compare with the Triton X-114 fractions in the present study. However, the terms "aqueous soluble" and "membrane protein" fraction are applicable to both works related to subcellular protein identification. In the previous work, whether there was a membrane protein solubilization step between the wash of the pellet and two-dimensional (2D) separation, as it is essential to know whether the membrane proteins got analyzed in 2D, was not mentioned.[14] Similarly, in the previous study, bands of similar size were found in the Western blot to detect acetylation in samples of membrane-associated and soluble protein fractions, as shown in Figure 4B, but in MS/MS PTM analysis, acetylation was not identified.[14] In addition, as an inconclusive result shown in the case of phosphoprotein identification, phosphorylation was identified in the Pro-Q Diamond stained gel (Figure 4C in Stewart et al.), while phosphorylation in the soluble fraction was not identified in mass spectrometry data except in the membrane-associated fraction (table in the Supporting material of Stewart et al.) despite considerably higher sensitivity of mass spectrometry.[14] Earlier studies on *Borrelia burgdorferi*, where >90% of acetylation was found in the cytoplasm, *Bacillus amyloliquefaciens*, where 89.4% of acetylation was in the cytoplasm, and *Helicobacter pylori*, where ∼24% of phosphoproteins over-represented the membrane proteins (cytoplasmic and outer membrane), also support our observations.[36−38] The glutarylation was higher in the outer membrane proteins and phosphorylation in inner membrane proteins. It is to be noted that the specific modifications related to subcellular fractions may have a particular role either in stabilizing the molecule in the respective subcellular environment or may be functionally active. Although many proteins and PTMs were identified in the supernatant and wash, specific PTMs relating to the fraction were less in these extracellular fractions. Interestingly, the wash fraction prepared by simple rinsing of *Leptospira* using PBS could extract the loosely bound proteins from the surface and these proteins contained many different PTMs as the surface protein fraction. These PTM-containing proteins from the outer membrane, surface, and secretion may be useful as diagnostic and vaccine candidates.

Of 1266 PTM proteins, 945 (74.68%) were found exclusively in various Triton X-114 fractions. In addition to that, there were 277 proteins (21.9%) bearing unique PTMs specific to different Triton X-114 fractions. Altogether, it was found that 1222 (96.6%) proteins had PTM specificity against Triton X-114 fractions. There were only four proteins not found in the aqueous fraction. The distribution of PTM proteins either exclusively or with specific PTM present in different Triton X-114 fractions showed the relation of PTMs in subcellular localization. As there are no cellular organelles in prokaryotes, protein modification generally happens in the cytoplasm, and in this regard, common PTMs may be found between the target fraction and the aqueous fraction. It is also found that 377 peptides represented 39 proteins shared between aqueous and other Triton X-114 fractions that indicate their cytoplasmic origin. Altogether, it was observed that the cytoplasm represented by the aqueous fraction forms the primary source of modified proteins, and specific modifications targeted to the assigned locations get enriched in those locations. It may also be possible to identify the subcellular location of proteins based on the position and type of PTM without subcellular fractionation, which could predict active forms for research and diagnosis.

CONCLUSIONS

Leptospirosis is a re-emerging zoonotic disease. There is no reliable vaccine or diagnostic tool available. Whole proteome analysis of *Leptospira* has been carried out together with Triton X-114 fractionation to achieve high-resolution and subcellular localization to identify candidate molecules for anti-leptospirosis measures. As part of the characterization of the proteins, PTM analysis was carried out to find active molecules and their subcellular location. The results showed specificity in type and position of PTM with respect to the Triton X-114 fractions that

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represent the various subcellular location of proteins in *Leptospira*.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c01245.

Workflow of the experiment and analysis (Figure S1) and MS spectra of PTM (Figures S2−S19) (PDF)

Data related to relevant figures (Tables S1−S4) (PDF)

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Notes

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