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iTRAQ-Based Global Proteomic Analysis of *Salmonella enterica* Serovar Typhimurium in Response to Desiccation, Low Water Activity, and Thermal Treatment

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ABSTRACT In this study, the changes in the global proteome of *Salmonella* in response to desiccation and thermal treatment were investigated by using an iTRAQ multiplex technique. A *Salmonella enterica* serovar Typhimurium strain was dried, equilibrated at high (1.0) and low (0.11) water activity (a_w), and thermally treated at 75°C. The proteomes were characterized after every treatment. The proteomes of the different treatments differed in the expression of 175 proteins. On the basis of their proteomic expression profiles, the samples were clustered into two major groups, namely, "dry" samples and "moist" samples. The groups had different levels of proteins involved in DNA synthesis and transcription and in metabolic reactions, indicating that cells under either of the a_w conditions need to strictly control energy metabolism, the rate of replication, and protein synthesis. The proteins with higher expression levels in moist samples were flagellar proteins (FlgEFGH), membrane proteins, and export systems (SecF, SecD, the Bam complex), as well as stress response proteins, suggesting that rehydration can trigger stress responses in moist cells. Dry samples had higher levels of ribosomal proteins, indicating that ribosomal proteins might be important for additional regulation of the cellular response, even when the synthesis of proteins is slowed down. At both a_ws, no differences in protein expression were observed between the thermally treated samples and the nonheated cells. In conclusion, our study indicates that the preadaptation to a dry condition was linked to increased thermal tolerance, while reversion from a dry state to a moist state induced a significant change in protein expression, possibly linked to the observed loss of thermal tolerance.

IMPORTANCE *Salmonella enterica* is able to survive in dry environments for very long periods. While it is well known that the initial exposure to desiccation is fundamental to trigger thermal tolerance in this organism, the specific physiological and molecular processes involved in this cross-protection phenomenon have not been fully characterized. Several studies have focused on the low-a_w transcriptome of this pathogen when inoculated in different food matrices or on abiotic surfaces, but proteomic analyses have not been reported in the literature. Our study investigated the changes in proteomic expression in *Salmonella enterica* serovar Typhimurium during desiccation, exposure to low a_w, and thermal treatment. A better knowledge of the systems involved in the response to desiccation and thermal tolerance, as well as a better understanding of their interplay, is fundamental to identify the most effective combination of interventions to prevent *Salmonella*'s contamination of foods.

KEYWORDS *Salmonella*, proteomics, iTRAQ, low water activity, thermal process, desiccation, foodborne pathogens

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Salmonella enterica is the main etiological agent of bacterial foodborne disease outbreaks and hospitalizations (1). This bacterium is ubiquitous in the environment and can survive, if not thrive, under diverse and stressful conditions, ranging from acidic to basic pHs, at low and high temperatures, and under low-moisture conditions, defined by a water activity (a_w) lower than 0.6 (2–4). Because of its ability to tolerate harsh treatments and sublethal environmental stresses, this bacterium can contaminate food production facilities, thus posing a relatively high risk for the cross-contamination of food products (5).

The increasing number of reports on *Salmonella* outbreaks linked to the consumption of dry foods has raised scientific awareness on its ability to survive under low-a_w conditions for extended periods of time. *Salmonella* has been shown to survive for an extended time in different dry food matrices, such as peanut butter (6), skim milk powder (7), whole black peppercorns and cumin seeds (8), and flour (9). In addition to food matrices, *Salmonella* has also been shown to survive for more than 100 weeks on plastic abiotic surfaces at 5°C (10) and on stainless steel discs for at least 30 days at 25°C (11). These findings highlight the ability of *Salmonella* to persist in multiple dry foods and environments, potentially leading to contamination events and subsequent outbreaks.

One of the main ordeals this microorganism faces when exposed to dry environments is desiccation. During desiccation, as water evaporates, hydrophobic interactions weaken, which leads to the instability of protein structure and, eventually, to protein denaturation. This results in damage to the cellular membranes (12). As a result, the cell activates a complex system of cellular responses aimed to minimize these damages (12). The intracellular accumulation of osmoprotectants, low-molecular-weight solutes such as betaine and glycine, is the first line of defense deployed to retain intracellular water (13). Among these solutes, trehalose appears to play an important role in the desiccation response. This disaccharide has been found to decrease intracellular fluidity through a process called vitrification. Vitrification decreases the diffusion and, therefore, the accumulation rates of reactive oxygen species (ROS), thus slowing the deterioration of cellular components (14, 15). In a transcriptional study by microarray on desiccated *Salmonella*, Li et al. (16) observed an upregulation of *otsB*, a gene involved in trehalose biosynthesis. Supporting this observation, they also measured a significant increase in trehalose concentration after cells had been equilibrated to an a_w of 0.11 for 5 days (16). More recently, a study investigating the transcriptome of desiccated cells found that the ProU and OsmU systems, involved in cellular osmoregulation, were upregulated under low-a_w conditions and in dried cells (17).

It has been widely observed and generally accepted that the exposure of *Salmonella* to desiccating conditions triggers cross-protection against other environmental stressors (18, 19). This cross-response enables *Salmonella*'s survival during prolonged heat treatments (19–22). Although the molecular network behind this cross-protection in *Salmonella* remains largely unknown, it has been hypothesized that the absence of water results in a reduction of intracellular molecular mobility, leading to the stabilization of the structure of the ribosomal subunits (23). The destabilization of the ribosome subunits is thought to be one of the main causes for bacterial inactivation during exposure to elevated temperatures (24, 25). There are other factors that influence *Salmonella*'s ability to survive thermal exposure, such as the extracellular matrix produced by the cells during biofilm formation (26) and the components of the food matrix in which the cells are exposed to the treatment (27). In fact, biofilm-forming strains of *Salmonella enterica* serovar Enteritidis inoculated in wheat flour had greater thermal tolerance than strains that did not produce biofilms (26). In peanut butter, the higher fat content and lower carbohydrate content corresponded to an increased heat resistance of *Salmonella* (27).

There is a broad consensus in the literature that osmotic, thermal, and oxidative stress response systems might overlap and determine *Salmonella*'s ability to adapt to dry conditions and develop thermal tolerance (18). A recent study in *Escherichia coli* reported that genes that are part of the oxidative stress regulons, *oxyR* and *soxRS*, were

induced during exposure to osmotic stress and/or a high temperature (28). Moreover, the adaptation to acidic conditions also induced higher thermal tolerance in *Salmonella* cells, via modifications of the membrane composition (29). The thermal shock response in *Salmonella* is mainly regulated by two sigma factors (30–32), namely, σ^H (*rpoH*), an inducer of cytoplasmic chaperone synthesis, and σ^E (*rpoE*), a regulator of the extracytoplasmic response responsible for the detection and repair of misfolded outer membrane proteins (OMPs) in the periplasm (30, 33–36). Interestingly, it has been shown that the interplay between σ^E and σ^H requires also the general stress response σ^S (*rpoS*) (37). Clearly, these observations show that cellular responses to different stresses, such as those encountered during desiccation and thermal treatment, require a concerted activation of multiple and partially overlapping regulons. Because of the breadth of the network of regulators and targets involved, the extent of this response has yet to be fully characterized.

In the past decade, numerous transcriptional studies on desiccated and thermally treated *Salmonella* have elucidated some aspects of this phenomenon. However, there is a lack of information at the posttranscriptional level. This would provide an important addition to our understanding of the final steps of the response activated by the cell. In this work, we used the isobaric tags for relative and absolute quantification (iTRAQ) to characterize the proteomic profiles of *Salmonella* cells affected by desiccation, exposure to very low a_w, and thermal treatment. iTRAQ is based on the use of isobaric tags, which bind to the N-terminal amines and the side chains of lysine residues, thus ensuring the labeling of every peptide in the digested mixture (38, 39). The tags uniquely identify up to 8 different samples, enabling the analysis of multiple samples at the same time (multiplex) (39).

RESULTS

PCA and hierarchical clustering. The initial global analysis using the software Scaffold Q+ identified 734 differentially expressed proteins ($P < 0.05$) between the pooled samples used as the control and those of the 6 conditions tested (before drying, after drying, after an additional equilibration to an a_w of 0.11, after thermal treatment at 75°C at an a_w of 0.11, after an additional exposure to an a_w of 1.0, and after thermal treatment at 75°C at an a_w of 1.0) (see Table S1 in the supplemental material). For all 734 proteins, the fold change in expression compared to that before drying was calculated (see Materials and Methods). A primary principal-component analysis (PCA) on the entire set of proteins resulted in a component 1 of 92.5% and a component 2 of 6.47% (Fig. 1A). The initial analysis revealed a clear separation between the "dry" samples ("after drying," "a_w 0.11," and "a_w 0.11 thermally treated" samples) and the "moist" samples ("a_w 1.0" and "a_w 1.0 thermally treated" samples) (see Table 4 for the labeling scheme) but no separation was observed among the 734 proteins (Fig. 1B).

We selected the proteins contributing to the differences between the conditions by first performing a hierarchical clustering (see Fig. S1) with intracluster ordering based on the first principal component from the PCA. To better hone in on the sources of the dry versus moist vectorial separation, we removed those clusters of proteins that did not show variations between the two groups of samples (dry and moist) from the subsequent analyses. We obtained a final number of 175 proteins (Table S1). A PCA (Fig. 1C) on this smaller set of proteins resulted in a better separation between the 2 major groups, namely, dry and moist, with the first two components of 61.6% and 34.1%, respectively. Two clearly distinct sets of proteins and 6 outliers were distinguishable (Fig. 1D). A two-way hierarchical clustering performed on this group of 175 proteins identified 13 clusters in the two major groups, dry and moist (Fig. 2A), which were separated in two sets and 4 outlier clusters in the PCA (Fig. 2B). The hierarchical clustering also showed that the proteomes of dried and low-a_w samples clustered together and were separated from the high-a_w samples (Fig. 2A).

Cluster analysis. After the hierarchical clustering, each of the 13 clusters was further characterized, and the clusters were divided into 2 major sets (Fig. 3). All the clusters in which the protein expression levels in the dry samples were lower than the expression

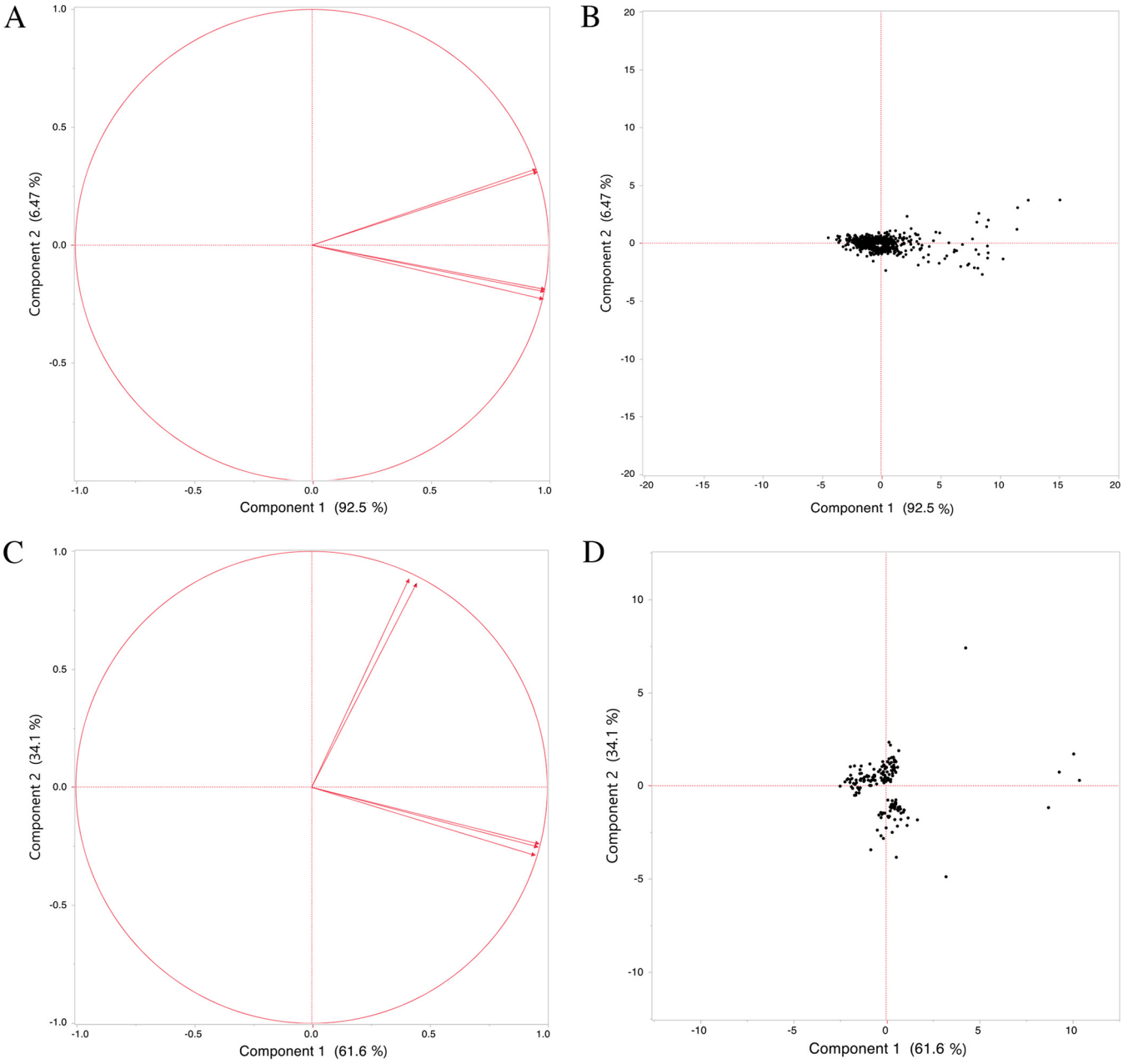

FIG 1 Two-dimensional PCA plots representing the distributions of the 5 different *Salmonella* serovar Typhimurium samples based on differential protein analysis (A and C) and the differentially expressed proteins (B and D). Initial PCA plots showing the distribution of the different samples (top to bottom: a_w 1.0, a_w 1.0 thermally treated, a_w 0.11, a_w 0.11 thermally treated, after drying) (A) and the distribution of the 734 differentially expressed proteins (B). Final PCA plots showing the distribution of the different samples (top to bottom: a_w 1.0, a_w 1.0 thermally treated, after drying, a_w 0.11, a_w 0.11 thermally treated) (C) and the separations of the 175 proteins into two major groups and 6 outliers (D).

levels in the moist samples were included in the "L" set, whereas all the clusters in which the protein expression levels were higher in the dry samples than in the moist samples were assigned to the "H" set.

Set L included six clusters (clusters 1 to 6), for a total of 120 proteins and 68.8% of the total (Table 1). The largest cluster was cluster 4, containing 50 proteins (28.6% of the total), while the smallest cluster was cluster 6, with only one protein (YcgM). This cluster was originally identified as an outlier in our PCA, because the fold changes in expression between after drying and before drying, as well as between the a_w of 0.11 and before drying, were positive (0.12 $\log_2$ and 0.22 $\log_2$ fold change, respectively) rather than negative, as in the rest of the set L. In our analysis, we decided to include this

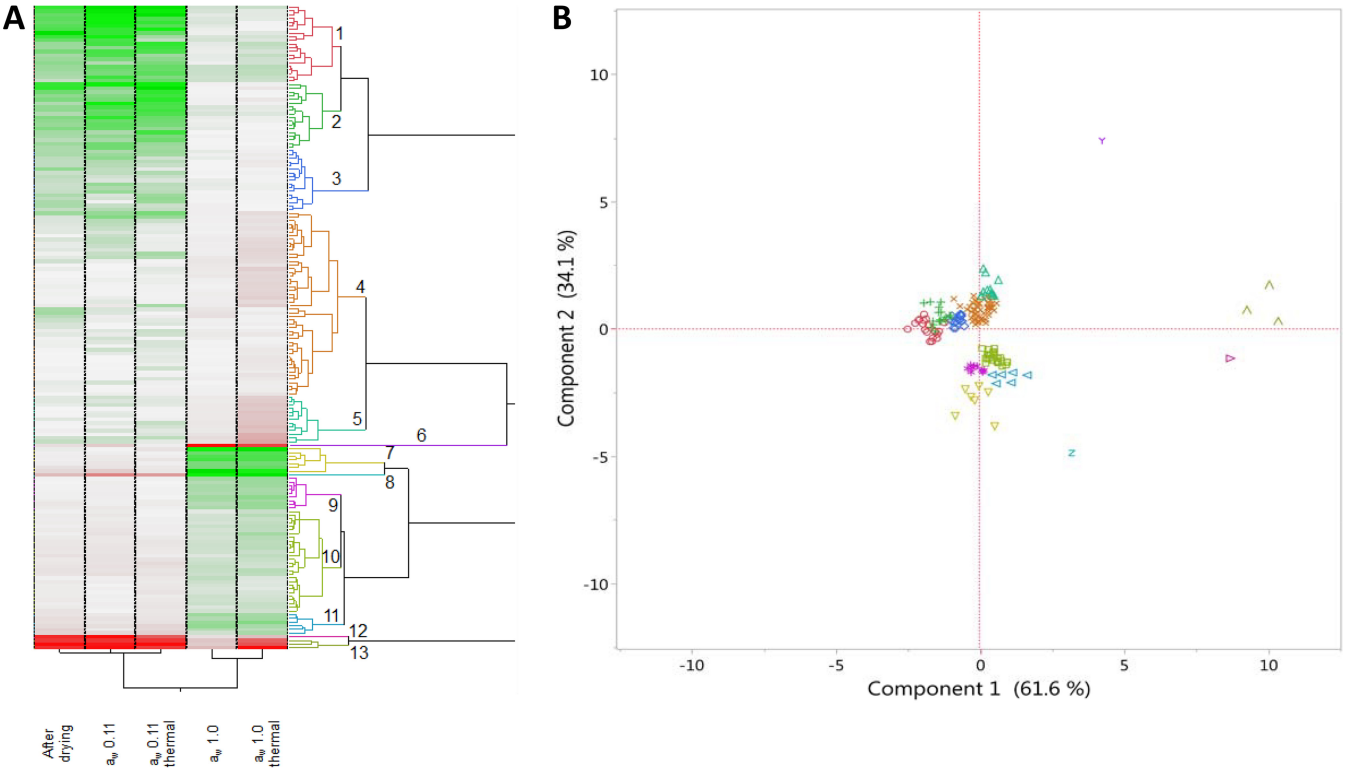

FIG 2 Hierarchical clustering heatmap of 175 proteins differentially expressed in *Salmonella* serovar Typhimurium cells subjected to 5 different treatments (A) and PCA plot of the proteins grouped in the 13 clusters (B). (A) The hierarchical clustering shows a division between the dry samples on the left (after drying, a_w 0.11, and a_w 0.11 thermally treated) and the moist samples on the right (a_w 1.0 and a_w 1.0 thermally treated). Different colors and numbers indicate the 13 clusters identified. The expression levels of the proteins in the treated samples compared to those in the before drying control are indicated by a green/red scale, where green is low expression and red is high expression. (B) The PCA plot shows the distribution, based on component 1 and 2, of the proteins in the 13 clusters. Proteins are identified by different color-coded symbols representing the different clusters they belong to, as identified by hierarchical clustering.

cluster in set L, since our focus was to characterize the variations in protein expression patterns between the two main groups of samples, dry and moist, as these groups were identified by the PCA as the main source of variation in protein expression levels. The *P* values were calculated for each cluster in set L as a statistical indication of the difference between the two sample groups. In set L, the *P* values ranged from 2.7×10^{-86} for cluster 4 to 4.7×10^{-2} for cluster 6 (Table 1).

TABLE 1 Distribution of 175 differentially expressed proteins in *Salmonella* serovar Typhimurium cells identified by hierarchical clustering analysis

Set[a]	Cluster	No. of proteins	% of total	*P* value (dry vs moist)
L	1	21	12	1.5×10^{-27}
	2	18	10.3	1.5×10^{-39}
	3	17	9.7	3.0×10^{-35}
	4	50	28.6	2.7×10^{-86}
	5	13	7.4	3.1×10^{-18}
	6	1	0.6	4.7×10^{-2}
H	7	7	4	4.4×10^{-16}
	8	1	0.6	2.0×10^{-4}
	9	9	5.1	6.2×10^{-29}
	10	28	16	7.8×10^{-52}
	11	6	3.4	6.4×10^{-16}
	12	1	0.6	5.0×10^{-3}
	13	3	1.7	1.0×10^{-3}

[a]Sets were identified on the basis of a significantly lower (L) or higher (H) protein expression in the dry samples (after drying, a_w 0.11, a_w 0.11 thermally treated) than in the moist samples (a_w 1.0, a_w 1.0 thermally treated).

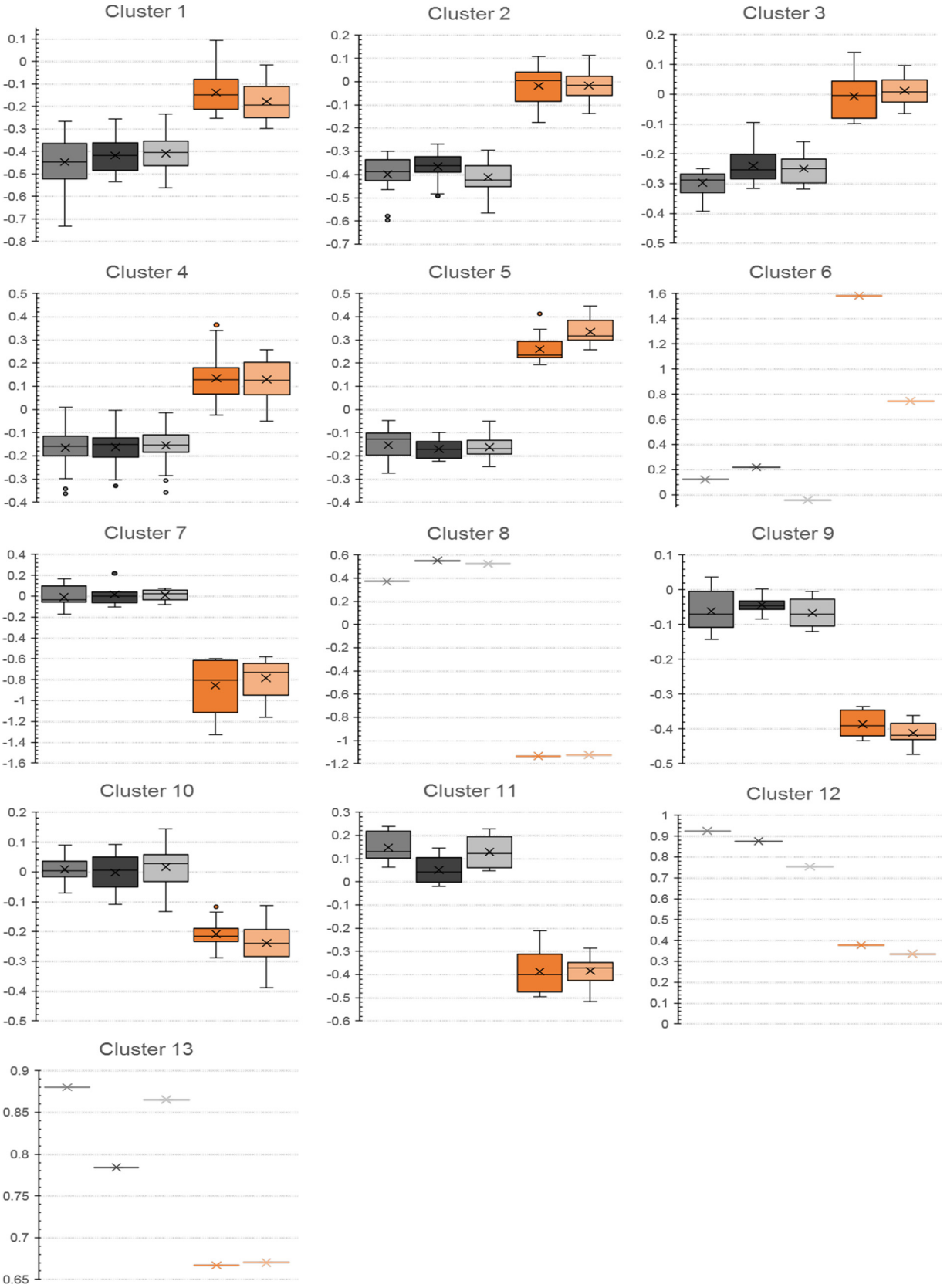

FIG 3 Differential protein expression levels of *Salmonella* serovar Typhimurium cells distributed into clusters identified by two-way hierarchical clustering. The dry sample group is divided into after drying (gray), a_w 0.11 (dark gray), and a_w 0.11 thermally treated (light gray), while the moist sample group is divided into a_w 1.0 (orange) and a_w 1.0 thermally treated (light orange). The protein expression levels are expressed on the *y* axes as $\log_2$ fold change between each sample and the before drying sample. In each box, the x represents the mean and the horizontal line represents the median.

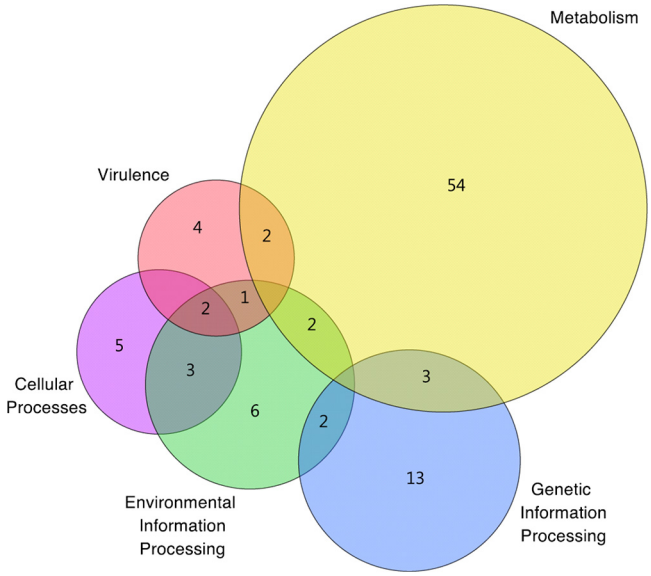

FIG 4 KEGG Orthology classes. Venn diagram representing the division in functional classes based on the KEGG Orthology database analysis of the 175 proteins differentially expressed in the 5 samples.

Set H included the remaining 7 clusters, all of which presented higher protein expression levels in the dry samples than in the moist samples. The largest cluster was cluster 10, with 28 proteins (16% of the total), while the smallest clusters were 8, 12, and 13 (1, 1, and 3 proteins, respectively). These clusters were originally identified as outliers in our PCA. Cluster 8, though characterized by higher expression levels for the dry samples than for the moist samples, presented the largest variation between the two sample groups (1.61 $\log_2$ fold change, with mean $\log_2$ fold changes of 0.48 and -1.13 for the dry and the moist samples, respectively). Clusters 12 and 13 were different from the other clusters in set H, because the fold changes for both sample groups were positive, indicating a higher expression than that before drying, while the rest of the clusters had at least one sample group whose fold change was lower than that before drying (negative $\log_2$ fold change values). Also for set L, the *P* values calculated between the two sample groups were all statistically significant and ranged from 7.8×10^{-52} for cluster 10 to 5×10^{-3} for cluster 12 (Table 1).

Differentially expressed proteins. The role of 175 proteins in the global cellular physiology was determined by assigning them to functional categories as defined by the KEGG Orthology database. Of the 175 proteins, 78 proteins were not assigned to any orthology group. The remaining 97 proteins were classified on the basis of five functional groups: (i) metabolism, (ii) genetic information processing, (iii) environmental information processing, (iv) cellular processes, and (v) virulence. The largest category was metabolism, with 61 proteins (Fig. 4), followed by genetic and environmental information processing (18 and 16 proteins, respectively). Some proteins belonging to various different pathways were classified in multiple functional categories (e.g., the flagellin proteins FljB and FliC were classified in the environmental information processing, cellular processes, and virulence categories).

Table 2 shows a selected group of proteins belonging to set L (proteins more abundant in the a_w 1.0 group than in either after drying or a_w 0.11 samples). Among these, there were several metabolism-related proteins, in particular, TreA, a periplasmic trehalase that catalyzes the hydrolysis of trehalose into two molecules of glucose (40), and MogA, MoaB, and MoaC, which are involved in the biosynthesis of the molybdenum cofactor and are an important prosthetic group for several molybdenum-based enzymes (41, 42). A large number of transporters, e.g., the copper-exporting ATPase CopA (43), the zinc/cadmium exporter ZntA (44), and several ABC transporters, as well

TABLE 2 Selected group of proteins with greater expression level patterns in *Salmonella* serovar Typhimurium in moist samples than in dry samples

Accession no.	Protein description	Name	Cluster
Metabolism			
A0A0F6B297_SALT1	Periplasmic trehalase	TreA	4
A0A0F6AWC4_SALT1	Molybdenum cofactor biosynthesis protein	MogA	4
MOAC_SALTY	Molybdenum cofactor biosynthesis protein	MoaC	4
A0A0F6AYV4_SALT1	Molybdenum cofactor biosynthesis protein	MoaB	5
Transporters			
COPA_SALTY	Copper-exporting P-type ATPase A	CopA	1
A0A0F6BA72_SALT1	Maltose ABC transporter periplasmic protein	MalE	2
A0A0F6B7A6_SALT1	Putative ABC transporter ATP-binding protein	YhbG	4
A0A0F6AZ15_SALT1	Putative ABC transporter periplasmic-binding protein	YliB	4
Q8ZLE5_SALTY	Zinc/cadmium transporting protein	ZntA	4
Replication, transcription, and translation			
TATD_SALTY	3′–5′ ssDNA/RNA exonuclease	TatD	1
TUS_SALTY	DNA replication terminus site-binding protein	Tus	3
A0A0F6AZA8_SALT1	Serine-tRNA ligase	SerS	3
A0A0F6B9J7_SALT1	D-Aminoacyl-tRNA deacylase	YihZ	3
A0A0F6B4G4_SALT1	Glutamyl-tRNA synthetase	GltX	4
RRAA_SALTY	Regulator of RNase activity A	RraA	5
Stress response			
A0A0F6B7D2_SALT1	Stringent starvation protein A	SspA	1
Q7CPU8_SALTY	Oxidative stress defense protein	YggE	1
Q8ZLD4_SALTY	Glutathione oxidoreductase	GorA	2
A0A0F6B2F5_SALT1	Zinc-dependent endoprotease	HtpX	3
BASS_SALTY	Sensor protein part of the PmrA/PmrB system	BasS	4
USPG_SALTY	Universal stress protein G	UspG	4
DPS_SALTY	Starvation/stationary phase protein	Dps	4
Motility			
FLGE_SALTY	Flagellar hook protein	FlgE	4
FLGG_SALTY	Flagellar basal-body rod protein	FlgG	4
FLJB_SALTY	Phase 2 flagellin	FljB	4
A0A0F6B013_SALT1	Flagellar basal body protein	FlgF	5
A0A0F6B015_SALT1	Flagellar L-ring protein	FlgH	5
FLIC_SALTY	Flagellin	FliC	5
Membrane and protein export			
A0A0F6AZA5_SALT1	Outer membrane lipoprotein carrier protein	LolA	1
A0A0F6B274_SALT1	Outer membrane lipoprotein	LolB	1
A0A0F6AWM8_SALT1	LPS assembly protein	LptD	5
A0A0F6B4T1_SALT1	Outer membrane protein assembly factor BamB	YfgL	2
Q8ZMW8_SALTY	Outer membrane protein assembly factor BamD	YfiO	4
A0A0F6AX28_SALT1	Outer membrane protein assembly factor BamA	YaeT	4
Q8ZRD7_SALTY	Protein translocase subunit SecD	SecD	4
A0A0F6AXN1_SALT1	Membrane protein translocase subunit SecF	SecF	4

as several flagellar components (FlgE, FlgF, FlgG, and FlgH) and two flagellin components (FliC and FljB) were also part of set L.

Various membrane proteins and membrane protein transporters were more abundant in moist samples than in dry samples. More specifically, BamA, BamB, and BamD, components of the outer membrane protein assembly complex Bam (45, 46), SecD and SecF, components of the Sec translocon, LolA and LolB, a chaperone and an outer membrane assembly protein, respectively, involved in the transport of lipopolysaccharides (LPS) to the outer membrane (47, 48), and LptD, required for LPS transport across the outer membrane (45, 49), were all significantly higher in expression under moist conditions than under dry conditions.

Among the proteins involved in replication, transcription, and translation were TatD, a magnesium-dependent exonuclease involved in DNA degradation during apoptosis as well as in response to H_2O_2-induced DNA repair (50), Tus, a DNA-binding protein that

TABLE 3 Selected group of proteins with greater expression level patterns in *Salmonella* serovar Typhimurium cells in dry samples than in moist samples

Accession no.	Protein description	Name	Cluster
Metabolism			
A0A0F6B9R6_SALT1	Fructose-1,6-bisphosphatase	GlpX	10
Q8ZP45_SALTY	Aldehyde-alcohol dehydrogenase	AdhE	10
A0A0F6B200_SALT1	Aconitate hydratase	AcnA	13
Replication, transcription, translation, and post-translational regulation			
A0A0F6AZM9_SALT1	Ribosome modulation factor	Rmf	7
A0A0F6B5K2_SALT1	DNA-binding protein	StpA	7
SYW_SALTY	Tryptophan-tRNA ligase	TrpS	7
A0A0F6B282_SALT1	Ribosome-binding ATPase	YchF	9
A0A0F6B771_SALT1	Translation initiation factor IF-2	InfB	9
DNAJ_SALTY	Chaperone protein DnaJ	DnaJ	9
SYGB_SALTY	Glycine-tRNA ligase	GlyS	9
Q8ZMN7_SALTY	DNA helicase	STM2767	10
SYT_SALTY	Threonine-tRNA ligase	ThrS	10
UVRD_SALTY	DNA helicase II	UvrD	10
Q8ZLJ1_SALTY	Putative RNase R	YhgF	10
SYA_SALTY	Alanine-tRNA ligase	AlaS	10
A0A0F6AYJ6_SALT1	Negative modulator of initiation of replication	SeqA	11
RHLB_SALTY	ATP-dependent RNA helicase RhlB	RhlB	11
Ribosomal			
A0A0F6B9S9_SALT1	50S ribosomal protein L31	RpmE	7
RS12_SALTY	30S ribosomal protein S12	RpsL	7
RL34_SALTY	50S ribosomal protein L34	RpmH	8
A0A0F6B7N0_SALT1	30S ribosomal protein S3	RpsC	11
A0A0F6B7N3_SALT1	50S ribosomal protein L2	RplB	11
RL25_SALTY	50S ribosomal protein L25	RplY	11
Virulence			
A0A0F6B5V0_SALT1	Secreted effector protein	SipA	10

is part of the Tus-TerB DNA replication termination complex (51), and RraA, an RNase regulator (52). In set L, we also identified proteins required for tRNA biogenesis (e.g., SerS, YihZ, and GltX).

Proteins identified as stress response proteins were found to be more abundant in set L, including GorA, a glutathione oxidoreductase (53), and Dps, a ferritin-like protein that protects DNA from damage under starvation and during long-term stationary phase (54). Both of these genes are involved in oxidative and starvation stress responses. Other proteins of note in set L that were differentially expressed included HtpX, a zinc-dependent inner membrane endoprotease under the control of CpxR-CpxA (55, 56), the stringent starvation protein SspA (57, 58), and the sensor protein BasS, part of the PmrAB two-component system (59).

Set H, which included proteins whose expression was higher in dry samples than in the moist samples (Table 3), presented a very different functional profile compared to that of set L. Among set H proteins were those belonging to metabolic pathways, e.g., GlpX, fructose-1,6-bisphosphatase involved in gluconeogenesis (60), AdhE, an alcohol dehydrogenase that is part of the fermentative pathways (61), and AcnA, an aconitase that is part of the Krebs cycle (62), as well as proteins involved in tRNA charging of different amino acids, e.g., TrpS for tryptophan, GlyS for glycine, ThrS for threonine, and AlaS for alanine. Proteins involved in DNA replication and repair (DnaJ and UvrD), replication regulation (SeqA), transcriptional regulation (StpA), and the degradation of mRNAs (RhlB) were more abundant in the dry samples. Similarly, several ribosomal proteins, including the 50S ribosomal proteins L2 (RplB), L25 (RplY), L31 (RpmE), and L34 (RpmH) and the 30S ribosomal proteins S3 (RpsC) and S12 (RpsL), as well as the virulence factor SipA, a *Salmonella* pathogenicity island 1 (SPI-1)-encoded effector protein, were more abundant in dry samples.

DISCUSSION

The effects of desiccation on *Salmonella* have been studied with transcriptomic techniques by several groups (16–18, 63–66). Unfortunately, transcriptomic analysis does not provide a clear picture of the actual physiological state of the cell, since additional regulatory pathways can take place after transcription. For this reason, proteomic characterizations of desiccated cells can provide a better insight on the last phase of the cell response to desiccation and, therefore, on the cross-protection to thermal treatment. In this study, we present the results of a global proteomic comparative analysis of *Salmonella enterica* serovar Typhimurium cells dried, exposed to both high and low a_w, and thermally treated.

In general, the largest differences in protein expression patterns were mainly observed between the two groups of samples, namely, dry and moist, with protein abundances very similar between the after drying and the a_w 0.11 samples (both thermally treated and not thermally treated). It is important to remember that both dry and moist samples underwent a drying step before being equilibrated to the respective a_w, meaning that even the moist sample cells had to adapt to desiccation before being reexposed to moisture. Therefore, for these samples, we most likely observed the effect of the rehydration on desiccated cells. This, together with the observation that differences between thermally treated samples and samples not thermally treated were small and limited to few proteins at both a_ws (see Fig. S2A and B in the supplemental material), indicates that the preadaptation to desiccation and low a_w is responsible for *Salmonella* tolerance to heat treatment. The principal-component analysis (PCA) can provide some information regarding the source of variability between the treatments observed. However, due to the nature of the PCA, we can only speculate what the sources of variability described by component 1 and component 2 are. Considering the clustering of the proteins in the PC 1 and 2 plots (Cartesian and vectorial), their expression levels, and their distributions in the 6 different treatment samples, we believe that the source of variability described by component 2 is the relative expression levels of the proteins between dry and moist samples compared to that of the control. Indeed, proteins with higher expression levels in moist samples grouped together in the upper quadrants of the plot, whereas proteins with higher expression in dry samples clustered in the bottom quadrants (Fig. 1D and 2B). Component 1 most likely describes the variability of the expression levels of the treated samples compared to the values from before drying (expression levels were calculated as a ratio of the expression level in one specific treated sample and the expression level in the sample before drying).

Protein levels were consistently higher in the sample before drying than in any of those from the five treatments tested. This can be easily explained by considering that the cells were grown in rich medium. Although at the time of the sampling the cells were in stationary phase and in a nutrient-depleted medium, it is probable that nutrients were being supplied by lysed cells, making the medium much more nutritious than the barren microbeads. As a result, the metabolic rates and protein synthesis of the cells before drying were likely higher than in the other samples.

In a nutrient-depleted environment, such as those on the microbeads under both a_w conditions, cells need to strictly regulate the rate of replication and, consequently, their cell division rate. The higher abundance of DNA replication and repair proteins, as well as of transcriptional and translational regulators, in the dry samples confirmed what we observed in our previous transcriptomic analysis of samples at a_w values of 0.11 and 1.0 (65). One of the effects of desiccation and low a_w is damage to DNA molecules, e.g., covalent modifications and breaks in the double helix (67), as well as the upregulation of DNA repair genes, which has been described in many microorganisms, including *Deinococcus radiodurans* (68) and *Bradyrhizobium japonicum* (69).

In our experiments, we observed a higher expression of SeqA in dry samples than in moist samples. SeqA was first discovered as a DNA-binding protein able to bind to the hemimethylated origin of replication *oriC* in *E. coli*, sequestering the DNA site from

DnaA and preventing the reinitiation of replication (70–73). This indicates that the generation time of *Salmonella* was slowed under these conditions. In *Salmonella*, *seqA*-negative mutants have shown higher sensitivity to H_2O_2 and bile salts (74), and *in vitro* assays suggest that mutations in this gene negatively affect *Salmonella* pathogenicity, decreasing its adhesion and invasion abilities (75).

We observed a shift in metabolism between the two groups of samples. TreA, responsible for the hydrolysis of trehalose into two molecules of glucose in the periplasm (76), was found to be more abundant in moist samples than in dry samples. This finding contradicts what was previously described at the transcriptomic level by Li et al. (16). In their study, Li et al. detected an increase in the expression of *treA* in *S. enterica* serovar Typhimurium LT2 desiccated for 2 h on filter paper at an a_w of 0.11 compared to that in cells spotted on filters and not desiccated (16). Some of the differences between their observations and ours might be due to the different durations of the experiments or to differences in the handling of the cells. In fact, we observed the samples over a long-term exposure to low a_w, while Li et al. observed the changes in the first hours of adaptation. Similarly, the use of different media for the cell resuspension prior to inoculation on the beads might be the reason for different observations. In contrast to that by Li et al., we decided to use water rather than 0.1 M phosphate-buffered saline (PBS) because the resuspension of bacterial cells in PBS before inoculation may trigger the activation of an osmotic response due to the increase in solute concentration during desiccation (64).

Trehalose accumulation is a well-known component of the desiccation defense mechanism. Trehalose works both as an osmoprotectant induced by osmotic stress and as a membrane stabilizer by replacing water clathrates around macromolecules, thus preventing desiccation damage (77, 78). This last function is thought to be due to the structure of the α,α-(1→1) glycosidic bond between the two molecules of glucose, which enables this molecule to form clam shell structures, thus facilitating interactions between the sugar and the lipid head groups of the membrane (79). In our case, we hypothesize that when equilibrating to a high a_w after desiccation, the membranes return to their hydrated state, and trehalose accumulation in the phospholipid layer is no longer necessary. Besides, at a high a_w, when the stress from desiccation ceases and the metabolic rate can increase, the cell needs to activate catabolism to produce ATP; under these conditions, accumulating trehalose would waste an important energy and carbon resource. Supporting this hypothesis, we found that the expression of GlpX, an enzyme converting fructose 1,6-bisphosphate to fructofuranose 6-phosphate in gluconeogenesis (60), was lower in the moist than in the dry samples.

The correct assembly and transport of outer membrane proteins (OMPs) are required for membrane integrity and for cell division. The presence of membrane protein transporters and assembly complexes at higher levels in moist samples than in dry samples supports the idea that cells exposed to an a_w of 1.0 are more metabolically active and have activated a series of responses to favor cellular growth and replication. Larger amounts of YaeT (BamA), YfgL (BamB), and YfiO (BamD) were detected in moist samples than in dry samples. These proteins, together with NlpB (BamC), whose expression was not significantly different between the two groups of samples, and SmpA (BamE), which was not identified in any sample, form the Bam complex, which is required for the assembly and the transport of outer membrane proteins (OMPs) in *Salmonella* (80, 81). The higher abundance of three of the five proteins of the Bam complex in moist samples indicates the importance of the entire Bam complex in ensuring a correct membrane assembly during adaptation and survival at a high a_w. Similarly, HtpX was found to be more abundant in samples equilibrated to an a_w of 1.0 (but not at an a_w of 1.0 when thermally treated). HtpX is a membrane protein with proteolytic activity (55), is involved in membrane protein degradation on the cytoplasmic side of the inner membrane, and is under the control of the CpxA-CpxR regulon in response to the accumulation of misfolded proteins under stress conditions (56).

At a_w of 1.0, the expression of flagella was higher than in dry conditions. The suppression of flagella has been previously described for *Salmonella* under desiccating

conditions, as well as for other microorganisms, such as *Bradyrhizobium japonicum* (69). It has been suggested that the downregulation of chemotaxis and motility during prolonged desiccation is associated with the cell's need to preserve energy by shutting down dispensable functions, enabling the redirection of ATP toward essential cellular functions (14, 82).

Surprisingly, various ribosomal proteins, including 30S and 50S subunit proteins, were more abundant in dry than in moist samples. The rate of cellular growth is strictly related to the rate of proteins synthesis (83). Since the rate of protein synthesis per ribosomal unit has been shown to be constant and independent of growth rate (84), it has been suggested that the number of ribosomal units is what determines the rate of protein synthesis (84–88). Considering that the growth rate is almost zero under desiccating conditions, we would expect less synthesis of ribosomal units and, therefore, fewer ribosomal proteins. Protein degradation with the purpose of energy recycling is mainly under the activity of 3 degradation systems, ClpXP, Lon, and ClpAP (89), and none of these proteins was differentially expressed in dry samples compared to moist samples. The role of ribosomal proteins in extraribosomal functions has been partially characterized in eukaryotic cells (90–92), and in *E. coli* the ribosomal protein L4 has been shown to bind RNase E and, consequentially, modulate mRNA composition in response to environmental stresses (93). A very intriguing possibility is that ribosomal proteins might play a specific role in modulating the adaptation to low a_w through specific extraribosomal functions; therefore, their expression levels might be regulated independently from the protein synthesis rate required for cellular growth. An alternative explanation is that ribosomal proteins might be degraded at a higher rate in moist cells to recycle amino acids for the synthesis of other essential proteins. This hypothesis is supported by our data showing lower expression of ribosomal proteins in both dry and moist samples than in the before drying sample. However, this explanation conflicts with our observations of a higher abundance of the degradosome component RhlB in dry samples than in moist samples and the higher abundance of RraA in moist samples than in dry samples, suggesting higher protein degradation in dry samples.

Our previous analysis of the *Salmonella enterica* serovar Typhimurium transcriptome at a low a_w (65) detected the upregulation of six virulence-related genes (*sscA*, *sseA*, *sopD*, *sseD*, *mgtC*, and *mviN*). The importance of two of them, *sopD* and *sseD*, in surviving desiccation and low a_w was confirmed by specific *ad hoc* mutants. Similarly, in the analysis of the proteome, we found a higher expression in dry samples than in moist samples of the virulence protein SipA, an SPI-1 type 3 secretion system (T3SS) secreted effector that induces the uptake of *Salmonella* cells by the host cells by stabilizing the cytoskeleton actin filaments (94). SipA and SopD are cosecreted by the SPI-1 T3SS and have correlated functions in promoting host cell invasion and uptake, together with 4 other effectors, SopA, SopB, SopE, and SopE2 (95, 96). SseD is also part of the T3SS injection mechanism (97). Our observation partially supports the transcriptome and mutant analyses (65), in which we revealed a role of the two virulence genes, *sopD* and *sseD*, in *Salmonella enterica* serovar Typhimurium desiccation survival, and might also indicate that SPI-1 T3SS and the related effectors are involved in the response to desiccation and low-a_w conditions, a role that has yet to be described.

More stress response proteins (e.g., SspA, GorA, Dps, and BasS) were found in set L than in set H, indicating that the adaptation to moisture after being dried induces a general multiple-stress response system. This is very interesting, and partially unexpected, since many studies in the literature report the activation of diverse stress response systems when the cells undergo desiccation (16–18, 63, 64), which lead us to expect higher expression of stress-response-related proteins in dry samples than in moist samples. GorA and Dps were among the stress response proteins identified in set L. In *E. coli*, both of these proteins are part of the OxyR regulon and respond to oxidative stress induced by H_2O_2 during exponential phase (53, 98). During stationary phase, the expression is controlled by RpoS (99), although for GorA, the control might be an indirect effect (99). Dps has a dual function of DNA protection: as a regulator by its DNA

binding activity, and as a chelator as a ferritin-like protein. Dps binds Fe(II) and facilitates the oxidation of Fe(II) by H_2O_2 by sequestering H_2O_2 and Fe(II), thus avoiding the hydroxyl radical formation by the Fenton reaction (54). The higher abundance of these proteins in moist samples might be an indication that rehydration in an aerobic environment causes the formation of ROS. In particular, rehydration might facilitate the spontaneous dismutation of O_2^-, a byproduct of aerobic respiration, into H_2O_2. O_2^- is also converted to H_2O_2 by superoxide dismutase (SOD) (100). Two SODs (SodA, a manganese-dependent SOD, and SodC1, a zinc- and copper-dependent SOD) were among the 734 proteins initially identified, but their expression was not different between the two groups of samples. O_2^- also reduces Fe(III) to Fe(II), which then reacts with H_2O_2 in the Fenton reaction, producing hydroxyl radical $OH^{\cdot}$ (100). Therefore, the accumulation of O_2^- leads to the accumulation of H_2O_2 and $OH^{\cdot}$ (100). These data taken together might explain why the Fe(II)-chelating protein Dps was more abundant in moist samples than in dry samples.

The stringent starvation protein SspA was more abundant in moist samples than in dry samples. In *E. coli*, SspA, together with SspB, whose expression was not different between the 2 groups of samples, has been found to act as a global regulator that activates cellular defense systems in response to nutrient starvation through the inhibition of the global transcriptomic repressor H-NS (58), whose expression did not differ between the dry and the moist samples. This suggested that long-term starvation plays an important role in the regulation of the proteomic profile observed in cells after a week of high-a_w equilibration.

Our study is a global proteomic analysis of both desiccated and thermally treated *Salmonella* cells performed using the iTRAQ method. Our analysis clearly showed that preadaptation to desiccation can trigger thermal tolerance, as the cellular proteomic profiles of nontreated and thermally treated samples did not differ. The analysis of the protein expression patterns clearly revealed that once dried, *Salmonella* cells do not have major changes in proteomic expression when equilibrated to a low a_w and thermally treated, while a major cell adjustment is required to readapt to high-a_w conditions.

MATERIALS AND METHODS

Bacterial strains and culture preparation. The strain used in this study was *Salmonella enterica* serovar Typhimurium strain ATCC 14028. The stock cultures were prepared in a 5:1 solution of Luria-Bertani broth (LB; BBL, Detroit, MI) and glycerol and stored at −55°C; the working cultures were prepared in 0.01 M glucose-supplemented LB (LBglc) at 37°C as previously described (65).

Inoculations, desiccation, and thermal treatment on micro glass beads. The procedure followed for bead inoculation, drying, and equilibration to a_ws of 0.11 and 1.0 was the same as previously described (65). Briefly, overnight bacterial cultures were collected by centrifugation, washed with distilled sterile water (DSW), and inoculated on micro glass beads (150 to 250 μm; Corpuscular Inc., Cold Spring, NY) (named "before drying"). The beads were dried for 4 days at 38.5 ± 0.5°C (named "after drying") and then equilibrated to a_ws of 0.11 and 1.0 for 7 days at 25°C. We determined the thermal death kinetics (calculated as δ-values using the Weibull model [101]) at 75°C for cells equilibrated at a_ws of 1.0 and 0.11. The δ-value for the a_w 1.0 cells, as expected, was shorter than for cells at an a_w of 0.11 (29 s [standard error of the mean, ± 4 s; adjusted R^2, 0.96] and 51 min [standard error of the mean, ±7 min; adjusted R^2, 0.95], respectively). To compare the proteomes of cells under the same conditions at both a_ws, we thermally treated the samples at 75°C for 29 s for cells equilibrated to an a_w of 1.0 and for 51 min for cells equilibrated to an a_w of 0.11.

After every treatment, the beads were snap-frozen using liquid nitrogen and the samples were immediately transferred to a −80°C freezer, where they were stored less than 4 weeks before being processed for extraction and liquid chromatography-mass spectrometry (LC-MS) at the University of Minnesota Center for Mass Spectrometry and Proteomics (College of Biological Sciences, St. Paul, MN).

An experimental workflow summary is described in Fig. S3 in the supplemental material.

Protein preparation, proteolytic digestion, and iTRAQ labeling. Aliquots of 400 μl of extraction buffer [7 M urea, 2 M thiourea, 0.4 M triethylammonium bicarbonate (TEAB), pH 8.5, 20% acetonitrile, and 4 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP)] were added to frozen glass bead samples. The samples were vortexed for 15 s and incubated at 37°C for 1 h, and then methyl methanethiosulfonate (MMTS) was added to each sample to a final concentration of 8 mM. The samples were vortexed briefly and incubated at room temperature for 30 min. After incubating, the samples were centrifuged at 12,000 × *g* for 10 min, and the supernatant of each sample was transferred to a new 1.5-ml snap-cap microcentrifuge tube. A Bradford assay was performed to determine the concentration of each sample. Proteolytic digestion, iTRAQ 8-plex labeling, and high-pressure liquid chromatography (HPLC) fractionation were carried out as previously described (102). Two sets of iTRAQ 8-plex (each for biological replicates) were prepared with replicate internal controls (pooled samples) in each iTRAQ set. Every sample was labeled with isobaric

TABLE 4 Labeling scheme for the samples of *Salmonella* serovar Typhimurium cells subjected to drying, two water activity levels, and thermal treatment in iTRAQs 1 and 2

iTRAQ 8-plex	iTRAQ label at *m/z*:							
	113	114	115	116	117	118	119	121
No. 1 Sample	Pooled control	a_w 0.11 thermal	a_w 1.0 thermal	Pooled control	a_w 0.11	a_w 1.0	Before drying	After drying
No. 2 Sample	a_w 0.11	Pooled control	a_w 1.0	a_w 1.0 thermal	After drying	Pooled control	Before drying	a_w 0.11 thermal

tags (Table 4), and then samples within each iTRAQ set were multiplexed together and processed according to Anderson et al. (102).

Liquid chromatography-mass spectrometry. Fractions obtained from the first dimension of LC separation were analyzed by online capillary LC-nanoelectrospray-MS on an Orbitrap Velos MS system (Thermo Fisher Scientific, San Jose, CA) (103) with the following exceptions. The higher-energy collision dissociation (HCD) activation time was 20 ms, the lock mass was not employed, and the dynamic exclusion settings were as follows: repeat count, 1; exclusion list size, 500; exclusion duration, 30 s; exclusion mass width (high and low), 15 ppm; and early expiration was disabled.

Database searching. Tandem mass spectra were extracted by Proteome Discoverer software (v. 2.1.0.81; Thermo Fisher Scientific, San Jose, CA, USA). Charge state deconvolution and deisotoping were not performed. All tandem mass spectrometry (MS/MS) samples were analyzed using Sequest v. 2.1.0.81 (Thermo Fisher Scientific, San Jose, CA, USA), which was set up to search *Salmonella enterica* serovar Typhimurium (strains LT2 and ATCC 14028) protein FASTA sequences (downloaded from UniProt on 21 June 2016) after concatenation with the common lab contaminants (from the common Repository of Adventitious Proteins [cRAP] database available at http://www.thegpm.org/crap/) for a total of 6,068 protein sequences. Sequest was searched with a fragment ion mass tolerance of 0.100 Da and a parent ion tolerance of 50 ppm. The methylthio group of cysteines was specified in Sequest as a fixed modification, while pyroglutamic acid, deamidation of asparagine, oxidation of methionine, dioxidation of methionine, iTRAQ 8-plex of lysine, and peptide N terminus were specified as variable modifications.

Criteria for protein identification. Scaffold (v. Scaffold_4.7.3; Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they were established at greater than 97.0% probability to achieve a false discovery rate (FDR) of less than 1.0% by the scaffold local FDR algorithm. Protein identities were accepted if they were established at greater than 99.0% probability to achieve an FDR of less than 1.0% and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (104). Proteins that contained similar peptides and were not differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Quantitative data analysis. Scaffold Q+ (v. Scaffold_4.7.3; Proteome Software Inc., Portland, OR) was used to quantitate label-based quantitation (iTRAQ) peptide and protein identifications as described above. Normalization was performed iteratively (across samples and spectra) on intensities, as described in reference 105. The medians were used for averaging. Spectra data were log-transformed, pruned of those matched to multiple proteins and those missing a reference value, and weighted by an adaptive intensity weighting algorithm. Of 41,277 spectra in the experiment at the given thresholds, 38,914 (94%) were included in quantitation. A total of 1,435 proteins were identified.

For the global analysis, differentially expressed proteins in the different samples compared to the average of the pooled control samples were determined by Scaffold Q+ by applying a permutation test with the significance threshold set at a P value of <0.05. Subsequently, the fold change of each protein between every sample and the before drying sample was calculated by dividing the fold change of the proteins in each sample (calculated by averaging the fold change ratios for both quantitations) by the average fold change ratio of the before drying sample (wet cells-beads inoculation step) used as the reference. The fold change ratios were then converted to $\log_2$ ($\log_2$ fold change).

Principal-component analysis and hierarchical clustering. Principal-component analysis and two-way hierarchical clustering were performed using the JMP Pro 13.0.0 software (SAS, Cary, NC, USA). The hierarchical clustering was performed using the Ward method and represented as a two-way clustering dendrogram using distance as the scale.

Statistical analyses. The experiments were performed in duplicates, with each biological duplicate performed on a different day. For each biological duplicate, three different technical replicates were collected and mixed together for protein extraction. Statistical analysis among the protein expression levels in the different samples was performed as described above in "Quantitative data analysis." The P values between the different sample groups described in Table 1 were determined using a two-tailed Student's t test assuming equal variance for all experiments.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00393-18.

SUPPLEMENTAL FILE 1, PDF file, 1.3 MB.

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