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ProP Is Required for the Survival of Desiccated *Salmonella enterica* Serovar Typhimurium Cells on a Stainless Steel Surface

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Consumers trust commercial food production to be safe, and it is important to strive to improve food safety at every level. Several outbreaks of food-borne disease have been caused by *Salmonella* strains associated with dried food. Currently we do not know the mechanisms used by *Salmonella enterica* serovar Typhimurium to survive in desiccated environments. The aim of this study was to discover the responses of *S.* Typhimurium ST4/74 at the transcriptional level to desiccation on a stainless steel surface and to subsequent rehydration. Bacterial cells were dried onto the same steel surfaces used during the production of dry foods, and RNA was recovered for transcriptomic analysis. Subsequently, dried cells were rehydrated and were again used for transcriptomic analysis. A total of 266 genes were differentially expressed under desiccation stress compared with a static broth culture. The osmoprotectant transporters *proP*, *proU*, and *osmU* (STM1491 to STM1494) were highly upregulated by drying. Deletion of any one of these transport systems resulted in a reduction in the long-term viability of *S.* Typhimurium on a stainless steel food contact surface. The *proP* gene was critical for survival; *proP* deletion mutants could not survive desiccation for long periods and were undetectable after 4 weeks. Following rehydration, 138 genes were differentially expressed, with upregulation observed for genes such as *proP*, *proU*, and the phosphate transport genes (*pstACS*). In time, this knowledge should prove valuable for understanding the underlying mechanisms involved in pathogen survival and should lead to improved methods for control to ensure the safety of intermediate- and low-moisture foods.

Salmonella has the ability to survive under low-moisture conditions for extended times and is one of the main biological hazards for dry-food manufacturers (1). *Salmonella* has been shown to survive for 8 months in halva, for 10 weeks when desiccated on paper discs, and for 12 months in peanut-flavored candy, while storage at 4°C under desiccation on plastic has resulted in 100 weeks of survival (2–5). This bacterium has previously been linked with many food-borne outbreaks associated with low-moisture food products, such as toasted cereal, infant formula, chocolate, and peanut butter (6–12).

Although some studies have found that exposure to low water activity (a_w) can increase the tolerance of *Salmonella* to other, unrelated stresses, including heat and biocidal compounds (13), comparatively little is known about the bacterial mechanisms that ensure its survival under desiccated conditions (14). It is generally thought that organisms enter a dormant state with reduced metabolic activity that maintains viability for several months to years. When moisture is reintroduced, the risk of cross-contamination increases, posing a threat to food safety.

The source of contamination for low-moisture foods is often linked to production environments and manufacturing equipment that allow bacteria to persist for extended periods in a dried state (6, 7, 15). However, the mechanisms employed by cells to survive in such harsh environments, where little to no water is available, have not yet been thoroughly characterized. Studies have reported that the formation of filaments, the production of curli fimbriae, and cellulose can aid long-term persistence under low-moisture conditions (16, 17).

Recently, three studies examining the transcriptome of *Salmonella* under two different conditions have been published. First, Deng et al. assessed the transcriptomic response of *Salmonella enterica* serovar Enteritidis to peanut oil, a low-moisture liquid environment (18). The bacterial cells were found to exist in a metabolically dormant state, with <5% of the genome being transcribed (18). Second, Gruzdev et al. studied the transcriptome of an auxotrophic mutant, *Salmonella enterica* serovar Typhimurium SL1344, which had been desiccated on a plastic surface for 22 h (19). The most highly transcribed genes in that study were those involved in ribosomal structure and potassium ion transport (19). Third, Li et al. examined the transcriptomic responses of two *Salmonella* isolates to desiccation at a very low relative humidity (RH) (11%) (20). In that case, fatty acid metabolism was significantly upregulated after 2 h of desiccation on paper discs (20). From these studies, it is clear that a diverse range of responses can be induced in response to desiccation, depending on the precise experimental conditions involved.

The aim of this study was to examine the transcriptomic response of *Salmonella* Typhimurium ST4/74 desiccated on a stainless steel surface. A limited set of conditions was chosen for desiccation, and we acknowledge that this study represents only one potential condition during typical manufacture of low-moisture food products. These results provide an improved understanding

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of this bacterium's response upon transition into a dried state on an industrially relevant contact surface. Several *S*. Typhimurium genes required for survival of desiccation under these conditions were identified; among these, ProP is of critical importance.

MATERIALS AND METHODS

Bacterial strains and construction of static growth curves. *Salmonella* Typhimurium ST4/74 was used in this study. Strain ST4/74 was originally isolated from the bowel of a calf with salmonellosis (21), and it is the prototrophic parent of the *S*. Typhimurium strain SL1344 (22). Strain SL1344 is a histidine auxotroph and has been widely used for the study of *Salmonella* virulence and gene regulation (23). There are only 8 single nucleotide polymorphisms that differentiate strains ST4/74 and SL1344 (24). The growth medium was Luria-Bertani (LB) medium (pH 7.0) that had been filter sterilized (0.22-μm polyethersulfone membrane) using Stericup filter units (FDR-125-010Q; Millipore) into 1-liter Millipore disposable flasks. To avoid any batch-to-batch variations, the same batch of powdered medium constituents was used throughout this study.

Bacteria were resuscitated from storage on cryobeads (Technical Service Consultants Ltd., Heywood, Lancashire, England) at −80°C onto LB agar (Difco) and were incubated overnight at 37°C. Experiments were then carried out at 24°C using a static system. This static system was selected for use because it is representative of a storage environment the bacteria are likely to encounter in the food production setting. The method used for the production of a standard inoculum was similar to that described by Rolfe et al. (25). One colony from an LB agar plate was used to inoculate 10 ml of LB medium in a 25-ml disposable universal tube, and this culture was incubated statically at 24°C for 48 h. The culture was serially diluted 1:100, and 200 μl of the diluted culture was then used to inoculate 500 ml of fresh LB medium in a 1-liter Stericup flask (approximately 4×10^3 CFU/ml) and was incubated statically at 24°C. This bacterial culture would then serve as the standardized inoculum for subsequent experiments. Viable counts were used to measure cell numbers periodically. Serial decimal dilutions were carried out in sterile LB medium, and 20 μl of each dilution was plated in triplicate onto LB agar. The plates were incubated for 24 h at 37°C. The CFU/ml was then calculated from dilutions that produced 4 to 50 colonies per 20-μl droplet. It should be noted that because approximately 20 h was required for the cells to reach stationary phase, it was necessary to use two identical cultures that had been staggered by 12 h for each of the three independent replicates.

Viability of ST4/74 on stainless steel. Stainless steel (grade 304) was cut to dimensions of 5 cm by 0.7 cm by 0.1 cm. These stainless steel coupons were sterilized by soaking overnight in a 1% (wt/vol) solution of TriGene disinfectant. The stainless steel coupons were then placed in 70% ethanol for 3 h to remove any residue and were rinsed with sterile water. Ten coupons were then placed in an aluminum foil package and were heated to 200°C for 1 h. Dry heat has been used for sterilization previously (26, 27). Total viable counts were carried out to ensure the sterility of the stainless steel following this procedure (data not shown). The traditional method of autoclaving was not used to sterilize the stainless steel coupons, because the moist heat caused slight bends in some of the coupons. This process was repeated after each use.

A standard inoculum of *Salmonella* Typhimurium ST4/74 was prepared as described above and was grown to early stationary phase (ESP). Samples (10 ml) of ESP culture were centrifuged at $3{,}200 \times g$ for 10 min; the supernatant was removed; and the cell pellet was resuspended in 1 ml fresh LB medium. This cell suspension was spread in a zigzag fashion across the steel coupons with 100 μl of culture (approximately 4×10^8 CFU) on each coupon. The stainless steel coupons were allowed to dry in a sterile laminar flow cabinet at 24°C for 4 h, at ca. 45% relative humidity (RH). At 0, 1, 2, 3, and 4 h, one steel coupon was placed in 5 ml phosphate-buffered saline (PBS) and was vortexed at full speed for 1 min to remove cells from the steel (27). The coupon was removed from the PBS by using sterile tweezers, and the liquid was centrifuged for 5 min at $3{,}200 \times g$. The supernatant was removed; the pellet was resuspended in 1 ml PBS; the suspension was serially diluted 1:10; and 100 μl was spread onto LB agar to measure viability. To examine the effect of rehydration, at the end of the 4 h-period, one steel coupon was placed in sterile H_2O for 30 min before viable counts. This procedure was carried out in triplicate. The whole experiment was repeated on three separate, independent occasions.

RNA extraction from cells desiccated on stainless steel. To examine the transcriptome of *Salmonella* Typhimurium ST4/74 after 4 h of desiccation on stainless steel, an experiment was designed to extract sufficient RNA from dried cells for use in microarray experiments.

LB medium (500 ml) was inoculated as described above and was incubated at 24°C until the culture reached ESP. The stainless steel coupons were inoculated as described above with 100 μl of culture per coupon (a total of 1 ml per pack of 10 coupons). The bacteria were air dried on the steel for 4 h at 24°C in a laminar flow cabinet. After 4 h, the coupons from one pack (10 coupons) were placed in 20 ml RNAlater (Ambion) to stabilize the RNA, incubated for 30 min at 24°C, and vortexed at full speed intermittently until the cells had been removed. The resulting suspension was centrifuged for 15 min at $3{,}200 \times g$; the majority of the supernatant was discarded; and the cell pellet was first resuspended in the remaining liquid and then transferred to a 1.5-ml microcentrifuge tube. The suspension was centrifuged for 1 min at $20{,}800 \times g$, after which the supernatant was discarded and the pellet resuspended on ice in 1 ml TRIzol (Invitrogen). To examine the effect of rehydration on the cells, after the 4 h of drying, 10 stainless steel coupons were placed in 20 ml sterile H_2O for 30 min instead of RNAlater. A 10-ml sample of the original ESP culture was also harvested in a similar manner and was resuspended in 1 ml TRIzol on ice. Total RNA was then extracted as described previously by Kröger et al. and was resuspended in a final volume of 80 μl RNase-free water (24). Samples were treated with DNase I (catalog no. EN0521; Fermentas) and SUPERase·In RNase inhibitor (1 μl; catalog no. AM2694; Ambion). Samples were adjusted to a concentration of >1,300 ng/μl with RNase-free water.

A NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA) was used to quantify RNA concentrations. The Agilent 2100 Bioanalyzer (Agilent, Stockport, United Kingdom) was used to assess RNA quality according to the manufacturer's instructions. Ladders, gels, dye, and chips were taken from the Agilent RNA 6000 Nano kit (catalog no. 5067-1511).

Microarray preparation and transcriptome analysis. Microarray analysis was carried out using the SALSIFY2 array (Agilent microarray design identifier [AMADID] 037367; Agilent Technologies, Santa Clara, CA). In order to enable an optimum comparison between our samples here and those in future experiments, a common-reference (indirect) approach was used (28). This method avoids the use of dye swap experiments and can allow the comparison of expression profile results obtained under different growth conditions (28, 29). The methods used have been described previously (30). Using this approach, RNA produced under the defined environmental condition being tested is reverse transcribed to cDNA, which is fluorescently labeled with Cy3-dCTP using a random priming reaction. Genomic DNA (gDNA) was purified from *Salmonella* Typhimurium ST4/74 and was fluorescently labeled with Cy5-dCTP. This gDNA was then used as the standard reference in each experiment. Dye-labeled cDNA and gDNA were mixed, denatured, and hybridized to the array over an 18-h period at 65°C; then they were washed according to the manufacturer's instructions (Agilent). Microarray slides were cleaned with inert gas to remove any debris before scanning with an Agilent microarray scanner (Agilent Technologies, Santa Clara, CA). Scans were carried out at 5-μm resolution with green and red photomultiplier tube (PMT) values set to 100% and an extended-dynamic-range (XDR) value of 0.1. The images generated were saved as multi-image TIFF files. Feature extraction software (Agilent Technologies) was used to extract data, which were then analyzed using GeneSpring, version 7.3 (Agilent Technologies, Santa Clara, CA). Two biological replicates were carried out for each condition. Expression profiles were compared to that of the control early-stationary-phase culture. Differentially expressed genes were iden-

tified using a *t* test ($P < 0.05$) with a 5-fold-change cutoff. Only genes that were detected by two or more probes above the 5-fold cutoff were considered.

qRT-PCR. Quantitative real-time PCR (qRT-PCR) was used to validate the results obtained from microarray experiments. The primers used are listed in Table S1 in the supplemental material. From the microarray results, two upregulated (*proV*, *hisD*) and two downregulated (*fliZ*, *ssaG*) genes were chosen for analysis, and their expression profiles were normalized against that of the nondifferentially expressed housekeeping gene *gapA*. qRT-PCR was carried out using the Qiagen QuantiTect SYBR green RT-PCR kit in a Mastercycler ep realplex system, with a total-RNA concentration of 50 ng for each sample. Three biological replicates were carried out, and the fold change was calculated using the $2^{-\Delta\Delta CT}$ method (31).

Construction of deletion mutants and P22 transduction. The lambda Red method for gene deletions was used for the construction of gene knockouts as described previously (32). Approximately 70-mer oligonucleotide primers were designed to contain 50 bases that were specific to the start and end of the gene/operon of interest. Additional nucleotides were chemically added to the 3′ ends of both the forward (5′-GTGTAGG CTGGAGCTGCTTC-3′) and reverse (5′-CATATGAATATCCTCCTTA-3′) primers and served as sites for PCR amplification of the antibiotic resistance gene from the pKD3 or pKD4 plasmid. The primers used for the generation of mutants are listed in Table S2 in the supplemental material. The mutations were confirmed using the primers listed in Table S2 and were verified by sequencing. The mutations were then reintroduced into a wild-type *Salmonella* Typhimurium ST4/74 background via transduction with phage P22 HT105/1 *int*201 (33) using previously described protocols (34). Antibiotic resistance markers were removed using the pCP20 plasmid as described previously (32). The following mutations were constructed in ST4/74: Δ*proP* (strain CFS-0004), Δ*proU* (CFS-0005), and Δ*osmU* (CFS-0006). The Δ*rpoE* mutation was constructed previously in *S.* Typhimurium SL1344 (mutant strain JVS-1028) as described elsewhere (35). This deletion was subsequently transduced into ST4/74, yielding strain JH3630 (see Table S2 in the supplemental material).

Longer-term survival of deletion mutants. Growth curves were generated for isogenic mutant strains, by using the method outlined above for the reference strain *Salmonella* Typhimurium ST4/74, to determine the point of ESP. ESP cultures of mutants were then dried onto stainless steel as outlined above and were examined for viability over a period of 6 weeks. Student *t* tests were carried out to determine if there were any statistically significant differences (cutoff *P* value, <0.05 by a two-tailed test) in desiccation survival between the wild-type strain ST4/74 and the deletion mutants.

Microarray data accession number. Data from this study have been deposited in NCBI's Gene Expression Omnibus with GEO accession number GSE46763.

RESULTS

Experimental design, bacterial growth, and desiccation. A static, room temperature (24°C), ca. 45% RH growth condition was chosen to represent growth during storage at room temperature and subsequent desiccation. The experimental design involved bacterial cells at early stationary phase (ESP) in order to limit variability between independent experiments (see Fig. S1 in the supplemental material). Following growth to ESP, bacterial cells were removed and were subsequently dried down onto stainless steel coupons. It has been stated previously that *Salmonella* is likely to enter a dormant state under reduced-moisture conditions, since it lacks the water necessary to support biological reactions (18). Furthermore, after extended periods of desiccation, important signals may be missed due to RNA degradation. A 4-h period of drying on the steel was chosen as the point at which the transcriptome was to be examined, since it was hypothesized that the mechanisms used by bacteria in the initial stages of desiccation may be critical for their long-term survival. Over this period, a cell loss of approximately 0.74 log CFU/ml was observed (data not shown). A further 0.72 log CFU/ml decrease in numbers, which could be attributed to osmotic shock, was observed after 30 min of rehydration (data not shown).

Identification of genes differentially expressed under desiccated conditions. To examine the response of *S.* Typhimurium ST4/74 to desiccation, transcriptomic analysis was carried out on bacterial cells desiccated onto stainless steel for 4 h. These results were compared to those for a liquid-culture control with the same bacteria.

Desiccation stress (4 h after inoculation onto steel) caused differential expression of 266 genes (>5-fold change, with a *P* value of <0.05 [see Data set S1 in the supplemental material]). Of these, 79 genes were upregulated and 187 were downregulated. Some of the most highly upregulated genes expressed following desiccation are involved in the uptake of osmoprotectants, including *proP* and the *proVWX* operon. The OsmU ABC transporter system (the STM1491 to STM1494 genes, known as *osmVWXY*) was also upregulated under desiccation stress (36). This system is involved in the uptake of osmoprotectant molecules and is induced by osmotic stress using NaCl (36). Genes involved in trehalose biosynthesis (*otsAB*) were upregulated >11-fold over their level of expression in liquid culture. The global regulator *rpoE* and genes encoding sigma E-regulatory proteins (*rseA* and *rseB*) were upregulated 6- to 8-fold by desiccation stress.

An increase in the expression of genes involved in histidine biosynthesis (*hisABCDGH*) was observed, suggesting that this amino acid may play an important role in survival at low moisture. Genes involved in leucine biosynthesis, cysteine biosynthesis, and fatty acid metabolism were also upregulated under desiccation. A number of genes involved in the formation of Fe-S clusters (including *iscA*, *iscS*, and *fdx*), as well as the manganese superoxide dismutase gene *sodA*, which are induced under iron-limiting conditions (37, 38), were also upregulated. The *nhaA* gene, which encodes a Na^+/H^+ antiporter, was upregulated; this antiporter is associated with high-salinity environments and is used by bacteria to remove excess sodium from the cell (39).

The transcriptomic data showed that the majority of the differentially expressed genes were downregulated during desiccation (187 genes). These included genes involved in chemotaxis, including *cheM*, *tcp*, and *tsr*, and the flagellar genes *fliS*, *fliT*, and *fliZ*. Several other genes, involved in amino acid transport and metabolism, anaerobic metabolism, and carbohydrate transport and metabolism, were downregulated. The functions of more than half of the genes that showed >5-fold downregulation were unknown. Full gene lists are provided in Data set S1 in the supplemental material.

Identification of genes differentially expressed following rehydration of previously desiccated cells. Food production facilities are commonly maintained as moisture-free environments to improve food safety. However, water may enter a facility via several routes, such as leaks in the pipe infrastructure, or during wet-cleaning of the facility. In an effort to mimic this scenario of moving from a desiccated to a hydrated condition, bacterial cells that had been dried onto stainless steel were rehydrated and were subjected to transcriptomic analysis. Comparison with the static planktonic culture revealed differential expression of 138 genes (>5-fold-change, with a *P* value of <0.05), of which 44 were up-

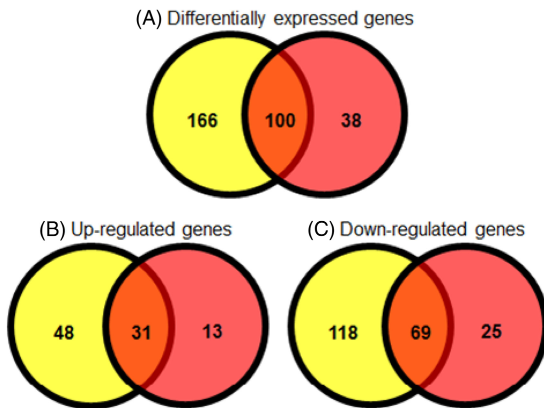

FIG 1 Comparison of statistically significantly ($P < 0.05$) differentially expressed genes with >5-fold changes. Yellow circles represent desiccated samples, and red circles represent rehydrated samples. (A) Genes differentially expressed with >5-fold changes; (B) genes upregulated >5-fold; (C) genes downregulated >5-fold.

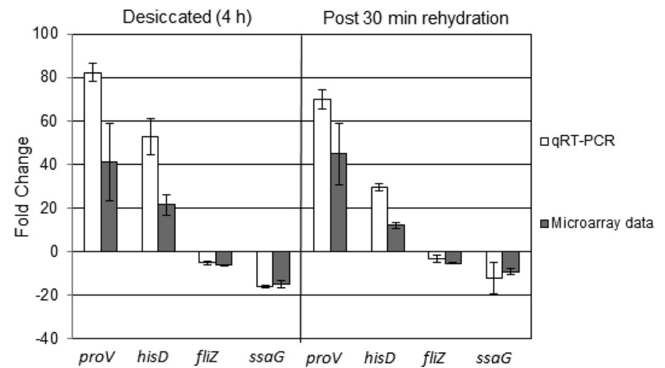

FIG 2 Validation of microarray data using qRT-PCR. The housekeeping gene *gapA* was used to normalize the data, and the fold change was calculated using the $2^{-\Delta\Delta CT}$ method.

regulated while 94 were downregulated (see Data set S2 in the supplemental material). One hundred of these genes (72.4%) were also differentially expressed after desiccation (Fig. 1). The osmoprotectant transporter genes *proP* and *proVWX* remained highly expressed in cells after rehydration. Trehalose-biosynthetic genes (*otsAB*) and the *osmU* ABC transporter were no longer overexpressed postrehydration, suggesting that these genes are specifically required for survival in low-moisture environments. Phosphate transport genes (*pstABCS*) showed increased expression in rehydrated cells. As in cells in a desiccated state, the global regulator *rpoE* was upregulated following rehydration. A number of small RNAs were also upregulated, including *ryeF* and sTnc800, which were found solely after rehydration.

Several genes that were downregulated in desiccated cells also showed the same pattern of expression postrehydration; these included chemotaxis genes and those involved in anaerobic metabolism. Rehydration restored the expression levels of flagellar genes and the iron storage genes *ftn* and *bfr*. Other genes that were downregulated significantly following desiccation and returned to normal transcriptional levels upon rehydration included the cell division activator *cedA* and the RNase E regulator *menG* (24, 40).

Transcriptomic data were confirmed by quantitative real-time PCR (qRT-PCR) (Fig. 2). This showed statistically significant changes ($P < 0.05$) in the expression of genes tested in both desiccated and rehydrated samples, compared with the liquid-culture control. The same pattern of expression observed for the selected genes in the transcriptomic arrays was also obtained by qRT-PCR, thereby validating the data derived from microarrays.

Physiological systems required for survival of desiccation. The realization that the *proP*, *proU*, and *osmU* genes were overexpressed in low-moisture environments led us to hypothesize that osmoprotectant transport systems could play a critical role when bacteria are dried. We tested this hypothesis by constructing a series of deletion mutants: the Δ*proP*, Δ*proU*, Δ*osmU*, and Δ*rpoE* mutants. All deletion mutants showed statistically significant ($P <$ 0.05) decreases in viability from that for wild-type ST4/74 upon desiccation (Fig. 3). The greatest survival defect was seen for the *S.* Typhimurium Δ*proP* mutant, which became undetectable after 4 weeks of desiccation.

DISCUSSION

Salmonella strains have been the causative agents of several food-borne outbreaks due to low-moisture foods (7, 12, 15, 41, 42). Recently, two studies examining the transcriptomes of *Salmonella* species desiccated onto an abiotic surface have been published (19, 20). These are the first insights into transcriptional mechanisms contributing to survival under desiccation stress. A comparison of genes upregulated under desiccation stress from this study and those reports is shown in Fig. 4. From these comparative data, several differentially regulated genes are shared between these studies, but interestingly, the majority of upregulated genes identified are unique to each investigation. These discrepancies may arise from the different methodologies used in the different studies, including such factors as the surface used for desiccation, the length of the desiccation period, or the *Salmonella* serotype used. First, Gruzdev et al. examined the transcriptome after 22 h on a plastic surface (19). In that study, *Salmonella* suspensions from a stationary-phase culture were air dried on 90-mm petri dishes in a biosafety cabinet at 25°C for 22 h and were compared to a control culture held at the same temperature in a 50-ml tube (19). Therefore, signals that might have been present at an earlier point of desiccation (such as that examined in this study) might have been absent after 22 h due to the short life of RNA. Second, Li et al. examined *Salmonella enterica* serovar Tennessee after 2 h of desiccation on filter paper (20). Stationary-phase cultures were dried onto filter paper discs and were kept dry for 2 h, after which RNA was isolated and compared to that from the liquid control (20). The different serotype, time point, and surface examined may have contributed to the differences observed.

In this study, we investigated the response of a prototrophic *Salmonella* Typhimurium strain, ST4/74, to desiccation on industrial-grade stainless steel at the transcriptomic level. We identified key signals that were upregulated upon drying, and we subsequently confirmed their contributions to survival by using mutant studies. The effect of rehydrating dried cells with water was also compared, since water is often the vector by which bacteria can disseminate quickly and efficiently throughout the production environment (43). To our knowledge, this is the first study to examine, at a transcriptional level, the nature of the effect(s) of desiccation on an industrially relevant surface as well as the events that follow rehydration. Our findings are broadly summarized in a schematic shown in Fig. 5. The processes shown in Fig. 5a are

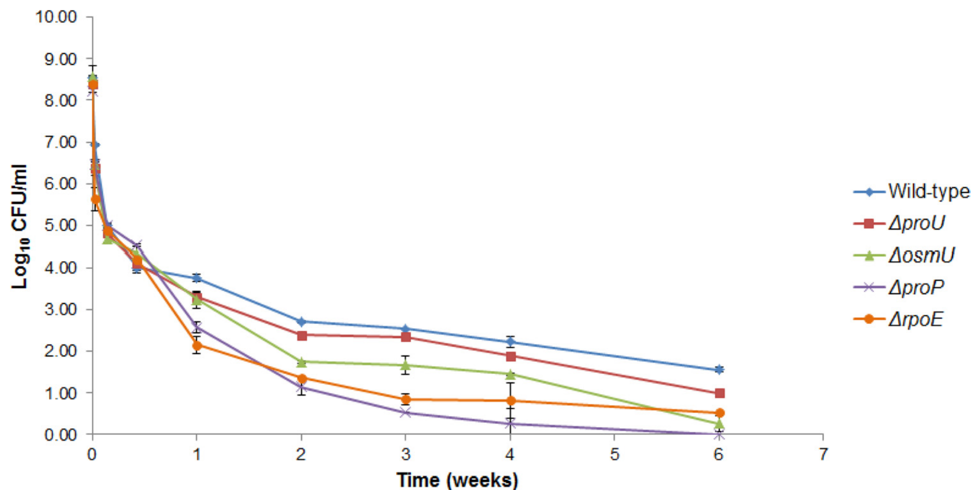

FIG 3 The ProP, ProU, and OsmU osmoprotectant systems and the global regulator RpoE are required for optimal survival of desiccation. All deletion mutants showed significant decreases in viability from that for the wild type ($P < 0.05$). The Δ*proP* mutant showed the greatest survival defect.

related to signals to preserve the osmotic stability and homeostasis of the bacterial cell. Osmoprotectant influx via three transporters is increased in order to protect the cell from the deleterious effects of osmotic shock. The level of trehalose biosynthesis is significantly increased via the metabolism of glucose. Due to this energy requirement, fatty acid catabolism increases, producing components from which energy can be derived. The upregulation of Fe-S clusters was also noted, and this is likely linked to the increased demands for energy made by the terminal electron transport chain. Upregulation in the synthesis of some key amino acids, including histidine, leucine, and cysteine, was also observed. Motility-related gene expression was downregulated, a feature that would be consistent with the requirement for bacterial cells to shut down unnecessary energy demands. In contrast, Fig. 5b depicts changes occurring in the cell after rehydration. Osmoprotectant solute transport systems appear less active, and trehalose biosynthesis is no longer upregulated. Similarly, the need for fatty acid catabolism is diminished. Interestingly, an increase in the phosphate transport level occurred; this would support the thesis that the bacterial cell requires phosphate elements for the synthesis of key building blocks, such as nucleic acids necessary for growth. Levels of expression of motility-associated genes have also begun to return to normal. The effects of these changes are discussed in more detail below.

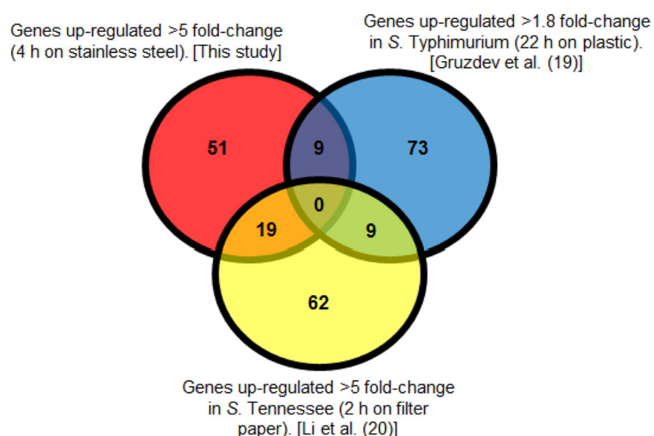

FIG 4 Comparison of the genes identified as upregulated under desiccation by this study with those found upregulated by Gruzdev et al. (19) and by Li et al. (20).

When bacteria are exposed to desiccation stress, the water activity in these cells is lowered. To combat the loss of water, and to maintain survival, bacteria accumulate low-molecular-weight compatible solutes known as osmoprotectants (44). Examples of some known osmoprotectants include L-proline, betaine (*N*-trimethylglycine), choline, and trehalose (44). Three transporters of osmoprotectant solutes were recorded as upregulated 6.77- to 50.83-fold (see data set S1 in the supplemental material). These included *proP*, *proU* (*proVWX*), and *osmU* (*osmVWXY*; STM1491 to -94). The ProP system belongs to the major facilitator superfamily (MFS) of permeases, while both the ProU and OsmU systems are known ABC transporters (36, 45, 46). All three transporters have been shown to promote survival under NaCl stress in a broth culture system (36, 47). Unlike ProP and ProU, the OsmU transporter has a low affinity for L-proline and glycine betaine, and it is possible that OsmU recognizes an alternative, as yet uncharacterized substrate (36, 44, 48–50). Our study demonstrated that these genes are also required for survival on stainless steel, which is representative of a typical surface used in food production, and that the loss of any of the three transporters reduces the viability of *S*. Typhimurium ST4/74. It was noted that *proP* was critically important for survival. This observation may explain in part why the ST4/74 Δ*proU* and Δ*osmU* mutants remain viable for the duration of the survival assay, given that the ProP system continues to function. Interestingly, of these three systems, the *proP*-encoded transporter was the least upregulated (6.77-fold change), but it was nevertheless essential for persistence.

As mentioned above, Gruzdev et al. examined the transcriptome of *S*. Typhimurium SL1344 dehydrated on a plastic surface. That study did not identify any upregulated osmoprotectant transport systems (19). This discrepancy could possibly be accounted for by the different experimental protocol and controls used, since the transcriptome was measured at 22 h postdesiccation, and some of these earlier signals may have been absent/lost. Li et al. reported that both the ProU transport system and some of

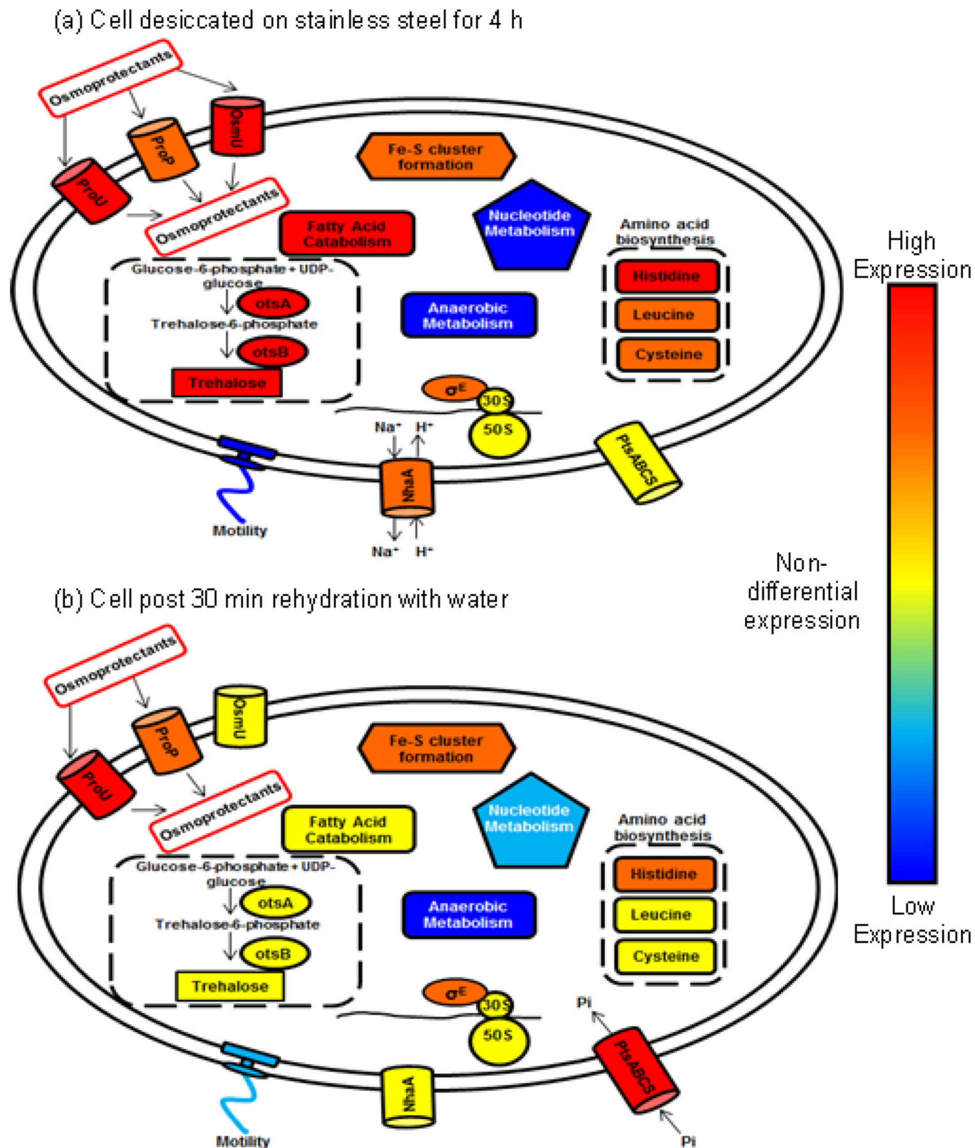

FIG 5 Proposed model of cellular changes occurring during desiccation of a cell on steel for 4 h (a) and during rehydration of a desiccated cell with water (b). Each symbol represents a range of genes involved in particular physiological or regulatory processes. Blue indicates decreased expression; yellow indicates genes that were expressed at similar levels in the planktonic control bacteria; and red indicates increased expression.

the genes of the OsmU transport system were upregulated when *Salmonella* cells were desiccated on paper discs, which supports our findings (20).

Our data highlight the importance of both *proP* and *proU* in *Salmonella* Typhimurium ST4/74 following rehydration in water. Although the *osmU* operon (STM1491 to -94) was highly upregulated under low-moisture conditions, it appeared to be redundant after rehydration. Deletion of *osmU* resulted in a marked reduction in long-term viability from that of the wild-type strain, indicating the functional importance of *osmU* in desiccation survival (Fig. 3). OsmU has been reported to alleviate growth inhibition in a Δ*proP* Δ*proU* double mutant; however, deletion of *osmU* alone can increase resistance to high salt levels and virulence due to an increase in the production of trehalose (36, 51). It is possible, therefore, that the regulation of *osmU* and the regulation of the *otsAB* genes, involved in trehalose production, are somehow linked, in a manner as yet undefined. While trehalose-biosynthetic genes were upregulated under desiccation stress, a situation similar to that for *osmU*, they returned to basal levels following rehydration in water. The induction of the cytoplasmic trehalase (encoded by *treF*) under desiccation stress suggests that bacteria catabolize trehalose to produce glucose, an efficient source of metabolic energy (52). Trehalose production in desiccated *Salmonella* cells has been measured previously (20). The results from that study showed a significant increase in trehalose production in an *S.* Tennessee isolate that was more tolerant to desiccation than an *S.* Typhimurium LT2 control (20). This finding further confirms the role of the *otsAB* genes in desiccation tolerance.

Models of osmoregulation in enteric bacteria suggest that in order to initiate the transport of osmoprotectants, an increase in K^+ transport is required to stimulate the transcription of the genes required, either directly or via the formation of potassium gluta-

mate (53–56). Balaji et al. reported that an alternative series of events, involving the upregulation of both *proU* and *proP* prior to the activation of a potassium ion transport channel, such as *kdpFABC*, may occur in *Salmonella* Typhimurium (56). The data from our study would appear to support this hypothesis, since no genes involved in potassium transport were found to be upregulated above 5-fold after 4 h. In contrast, however, Gruzdev et al. reported that the genes most highly upregulated after 22 h of desiccation were those of the *kdpFABC* operon, which would coincide with this alternative activation model (19).

A study of *Escherichia coli* showed that the overproduction of histidine biosynthesis gene products can lead to osmosensitivity (57). Conversely, upregulation of histidine biosynthesis was observed under desiccation stress and also, to some extent, after rehydration with water. Similar increased expression was reported by Gruzdev et al. after the dehydration of *Salmonella* Typhimurium on a plastic surface (19). These observations suggest that increasing the amount of proteins that contain histidine could provide the bacteria with some form of stabilization or protection under low-moisture conditions. Gruzdev et al. further explored this observation by deleting *hisD* to eliminate histidine biosynthesis; however, the tolerance of the deletion mutant to dehydration was comparable to that of the auxotrophic reference strain (19). Furthermore, the choice of the model bacterium in the latter study may be somewhat unfortunate, and the results consequently difficult to interpret, given that this particular reference strain is a histidine auxotroph. Further investigation into the role of this amino acid in desiccation tolerance may be warranted.

In our study, a gene encoding an alternative sigma factor, *rpoE*, was found to be upregulated both under desiccation stress and after rehydration. This sigma factor, σ^E, plays a key role in the regulation of genes involved in cell envelope stress, together with genes that respond to heat, starvation, osmotic shock with NaCl, and desiccation (18, 19, 35, 58–60). σ^E clearly plays a key role when cells are exposed to desiccation stress on stainless steel coupons, since the long-term survival of a Δ*rpoE* deletion mutant of *S.* Typhimurium was significantly reduced from that of the wild type ($P < 0.05$) (Fig. 3).

Previous studies have indicated that the formation of filaments and the production of curli fimbriae can aid the survival and long-term persistence of *Salmonella* species and *E. coli* under low-moisture conditions (16, 17, 61–64). However, no genes for the biosynthesis of curli fimbriae (*csgDEFG* or *csgBAC*) or cellulose (*bcsABZC*) were upregulated following 4 h of desiccation in our study. Moreover, none of these genes were upregulated in the studies reported by Gruzdev et al. and Li et al. (19, 20). This observation may indicate that while the formation of filaments may be important for survival in a low-moisture broth-based system, this may not be the case when bacteria are dried onto an abiotic food contact surface. White et al. demonstrated that bacteria lacking the ability to produce two main components found in a *Salmonella* biofilm, cellulose and curli fimbriae, exhibit a phenotype of susceptibility to desiccation (17). Since none of these genes were found to be upregulated under desiccation, it is possible that isolates capable of forming these structures prior to drying have a greater ability to attach to surfaces but that curli fimbriae and cellulose are not required once drying has occurred.

When cells were rehydrated with water after initially being desiccated, the expression of several of these genes, including *proP*, *proU*, and *rpoE*, as well as a number of Fe-S cluster-related genes (*iscA*, *iscS*, *fdx* and *nifU*), is required. An inorganic phosphate ABC transporter encoded by *pstSCAB* was observed to be upregulated only when dried cells were rehydrated with water. Phosphate is essential for the synthesis of nucleic acids and phospholipids and for phosphorylation-based signaling pathways within the bacterial cell (25). The induction of this transporter may suggest that these bacteria have entered the early stages of a lag phase, as they attempt to scavenge a central building block of the cell (25). We speculate that this occurrence may prime bacteria for growth if and when nutrients again became available.

Desiccation stress caused a marked reduction in a number of genes involved in motility and chemotaxis. Similar results have been observed previously (20). This inhibition was partially maintained postrehydration, suggesting that the cells are expending energy elsewhere. However, the expression of some flagellum-encoding genes did return to normal levels. It is possible that motility would be induced at a later stage to aid in dissemination throughout the aqueous environment.

Conclusion. The mechanism(s) used by *Salmonella* to promote its survival under low- and intermediate-moisture conditions is poorly understood. Our study investigated the response of *Salmonella* Typhimurium ST4/74 to desiccation and subsequent rehydration at a transcriptomic level. Several features were identified that contributed to desiccation survival, including the importance of the ProP, ProU, and OsmU osmoprotectant transporters. In addition, other cellular responses were observed, including the increase in trehalose production and histidine biosynthesis and the upregulation of an alternative sigma factor encoded by *rpoE*. While this study provides early information on the initial response of *Salmonella* Typhimurium to desiccation, further characterization to elucidate the importance of other upregulated systems is warranted so as to gain a more holistic understanding of the process(es) involved in desiccation tolerance. This investigation constitutes the first in-depth study of the processes occurring within a previously dried cell upon the reintroduction of moisture. The findings of our study may aid manufacturers of low-moisture foods in the design of effective control strategies by, for example, using the insight into desiccation stress to optimize cleaning and sanitation regimes. Such approaches could be aimed at the elimination of desiccated *Salmonella* cells from the production environment, thereby improving food safety and protecting public health.

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