Identification of genes involved in *Salmonella* tolerance to desiccation stress

Thesis Submitted to

The Robert H. Smith Faculty of Agriculture, Food and Environment

Hebrew University of Jerusalem

For The Degree of "Master of Agriculture Science"

By

Tami Hazin

October, 2013

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Abstract

In recent years there was increase in numbers of Salmonellosis outbreaks associated with consumption of food products with low water content, such as chocolate, peanut butter, snacks, dried milk, and spices. Concurrently, it was reported that *Salmonella* can survive under dry conditions on various surfaces, including soil and food products for prolonged periods. The mechanisms of *Salmonella* tolerance to desiccation stress remain poorly understood. In order to limit *Salmonella* survival on dry surfaces it is important to understand the underlining mechanisms. Consequently, the main objective of this research was to identify *Salmonella* genes and mechanisms involved in the developement of desiccation stress tolerance.

In the first phase of the study, we have characterized several factors that affect survival of *Salmonella* in dry conditions. We found that rapid drying diminishes the ability of bacteria to survive in a dry state for prolonged period. We also found that exponentialy grown bacteria are more susceptible to drying compared to stationary phase bacteria. We have shown that cultures grown with shaking are more tolerant to desiccation stress compared to standing cultures. In order to identify specific proteins that confer desiccation tolerance to *Salmonella*, proteomic analysis was done in both shaking and standing cultures. The analysis revealed that the expression of several proteins was repressed in standing culture, including the following: superoxide dismutase (SodB), glutathione-S-transferase (Gst), bacterioferritin (Bfr), DNA gyrase inhibitor (SmbC), and hydrolase oxidase (YbgI). In order to examine the involvement of these proteins in *Salmonella* tolerance to desiccation stress, knockout mutants were generated in each of the corresponding genes and tested for desiccation tolerance. Only *Δbfr* mutant, which encodes a bacterioferritin, involved in cellular antioxidative activity, was found to be compromised in it's desiccation tolerance. Other mutants showed survival capacity comparable to that of WT strain.

Since, during growth in standing culture bacteria encounter limited oxygen concentrations, we have further examined survival of bacteria grown under anaerobic conditions on agar plates. We found that anaerobicaly-grown bacteria are more susceptible to drying compared to aerobically grown cells.

We have also shown the importance of the cellular capacity to neutralize free radicals in desiccation stress tolerance. Cells with low total oxidative scavanging capacity (TOSC) were more susceptible to drying than cells with high TOSC. Inhibition of catalases by sodium azide

reduced the survival of *Salmonella* upon drying, indicating the role of H_2O_2 scavengers in cellular defense to desiccation. However, mutation in *katE* gene, encoding a *Salmonella* catalase, which reduces tolerance to hydrogen peroxide, had no effect on bacterial survival under desiccation. Interestingly, mutations in *katG* gene encoding another catalase and in *ahpCF* gene encoding alkylperoxidase, also didn't affect bacterial tolerance neither to H_2O_2 challenge nor to desiccation stress. It is known that mutation in one of these genes is compensated by overexpression of the other gene and that double mutation is needed to reduce H_2O_2 scavenging activity and tolerance to H_2O_2. Indeed, a double mutation in *ahpCF/katG* resulted in reduced bacterial tolerance to both hydrogen-peroxide and desiccation stresses. Mutation in *oxyR*-global H_2O_2 stress regulator also resulted in reduced bacterial tolerance to both hydrogen-peroxide and desiccation stresses. This finding support the notion of involvement of cellular H_2O_2 stress response in desiccation tolerance.

In summary, our study support previous findings on possible link between bacteria oxidative stress response and desiccation tolerance and underscroes the important role of oxidative scavenging capacity in desiccation stress tolerance. In order to understand common mechanisms that are involved in cellular response to both stresses, further studies are needed. It is important to measure the nature and concentrations of the free radicals that are generated during desiccation stress in order to build an appropriate mechanistic model. Further research is required to investigate conrtribution of genes involved in oxidative damage-repair to desiccation tolerance. Since, addition of antioxidants to processed foods is quite common in the food industry for enhancing the freshness and improve the nutritous value of the food, the effects of added antioxidants on *Salmonella* survival in dry foods, should be investigated as well.

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List of Abbreviations

Amp- Ampicillin

CFU- Colony Forming Unit

CLSM- Confocal Laser Scanning Microscope

Cm- Chloramphenicol

Kan- Kanamycin

LB- Luria Bertani

LPS- Lipopolysaccharide

MS- Mass spectrometry

NCBI- National Center for Biotechnology Information

O.D- Optical Density

OPS- O- polysaccharide

PCR- Polymerase Chain Reaction

RH- Relative humidity

ROS- Reactive oxygen species

RPM- Rounds per minute

SDDW- sterile double distilled water

SDS PAGE- Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

STm- *Salmonella enterica* serovar Typhimurium

TAE- Tris Acetic Acid EDTA buffer

TOSC- Total oxidative scavenging capacity

WT- Wild type

1. Introduction

1.1 Salmonella and Salmonellosis outbreaks

Salmonella is a Gram-negative, rod-shaped, facultative anaerobic, motile bacterium. The *Salmonella* genus belongs to the family *Enterobacteriaceae* and comprises of two species: *S. enterica* and *S. bongori*. *S. enterica* includes more than 2,500 different serotypes, many of which are causative agents of Salmonellosis in both humans and animals (Daoust et al., 1997; Su and Chiu, 2007). Salmonellosis is a food-borne disease transmitted by ingestion of contaminated food. The major symptoms of Salmonellosis include inflammation of the gastrointestinal tract- gastroenteritis, accompanied with fever, diarrhea and vomiting, which occurs 6 to 48 hour after infection and lasts for 4 to 7 days (Crum, 2008). The symptoms are most severe in people with compromised immune system, such as patients with chronic diseases, infants and the elderly. In some cases Salmonellosis requires hospitalization due to severe diarrhea, dehydration and fever. In the U.S *Salmonella* infections cause 15,000 hospitalizations, over 150,000 visits to the physician, and 400 deaths, annually (Mead et al., 1999; Kennedy et al., 2004; Scallan et al., 2011). Salmonellosis outbreaks cause heavy economic burden; in the U.S total patient-related costs associated with Salmonellosis were estimated to be approximately 1 billion dollar per year (Trevejo et al., 2002; Kennedy et al, 2004). In addition to the health aspects, there are economic losses associated with food industry through recall of products and lost reputation and income.

Most Salmonellosis outbreaks caused as a result of consumption of contaminated animal products such as eggs, poultry, meat, dairy products as well as via contaminated fruit and vegetables or water (Tauxe, 1991; Alteksure et al., 1997; Angulo et al., 1997; Brandl, 2006). Some of the large Salmonellosis outbreaks were associated with consumption of food products with low water content such as chocolate, peanut butter, snacks, dried milk, and spices (Rowe et al., 1986; Shohat et al., 1996; Weber et al., 2005; Isaacs et al., 2005; MMWR, 2007). *Salmonella* have been occasionally detected also in granola, pistachio, and corn flakes (Anonymous, 2008; Anonymous, 2009; Anonymous, 2010a).

In the food industry reducing of available water in products is a long-established method for controlling bacterial growth, as most pathogenic species cannot proliferate and survive in low water-activity products (Fennema, 1985). However Salmonellosis outbreaks associated with consumption of dry foods suggest that this approach is not always useful against *Salmonella*. In

addition, it was found that *Salmonella* can survive on dry surfaces, such as plastic, glass and stainless steel (de Cesare et al., 2003), thereby increasing the chances of cross contaminations in the food processing environment.

1.2 Desiccation induced damage of bacteria cells

Desiccation is one of the common and severe stresses bacteria encounter outside the host. Desiccation tolerance is characterized by the ability of cells to undergo nearly absolute dehydration without being killed (Potts, 1994).

Water is essential for life, it has a central role in the maintenance of cellular microenvironment, stabilize macromolecules and membranes, essential for proteins folding and maintaining their spatial structure. Deficit of water molecules causes conformational changes in proteins and loss of function. Damage to enzymes involved in the electron transport chain leads to accumulation of free radicals inside the cell and consequent lipid peroxidation, proteins denaturation and DNA mutagenesis (Billi and Pots, 2002). Dehydration of membrane bilayer increases van der Waals interactions between adjacent phospholipids and cause an increase in the phase transition temperature (Tm), resulting in the transition to a gel phase at environmentally relevant temperatures. Membranes with a higher Tm will pass to the gel phase first and will separate from those with lower Tm, resulting in proteins exclusion and aggregation (Bryant et al., 2001; Billi and Pots, 2002). After rehydration, membranes undergo a further phase transition, which results in increase of membrane permeability, leading to leakage of solutes and cell death (Beney et al., 2004).

1.3 Mechanism of survival of prokaryotes in dry condition

In the early stages of dehydration cellular water content is still sufficient to allow maintenance of osmotic balance with the environment by increasing the concentration of various organic solutes (Wood et al., 2001). Mostly these are solutes required for physiological activity of the cell, such as amino acids and sugars. A common feature of both prokaryotes and eukaryotic anhydrobiotes is increased synthesis of disaccharides, such as trehalose and sucrose. According to the water replacement theory, during dehydration disaccharides replace water molecules around proteins (forming hydrogen bonds with polar residues) and membrane lipids and as a result prevent denaturation of proteins and transition of the membrane to gel phase (Hershkovitz et al., 1991; Wood et al., 2001; Mclntyre et al., 2007). Another hypothesis

suggests that during water loss, trehalose and sucrose form glass (vitrification) and thus stabilize the cytoplasm, keeping it in an amorphous state, preventing diffusion of various molecules and oxidative reactions that can cause cellular death (Crowe et al., 1998; Billi and Potts, 2002).

Mclntyre et al. (2007) have shown that *Rhizobium leguminosarum* mutants defective in trehalose synthesis genes (*treY, otsA*) lost the ability to produce trehalose and showed decreased survival following desiccation. Increased expression of trehalose was observed in stationary phase of growth and in response to various stresses, including osmotic stress, and was linked to increased desiccation tolerance of *Escherichia coli* (Welsh and Herbert, 1999; Mclntyre et al,. 2007). Welsh and Herbert, (1999) hypothesized that the better survival of stationary phase bacteria depends not only on trehalose synthesis, but also on the expression of other genes controlled by sigma factor *rpoS*. Sigma factor *rpoS* is a stationary phase transcription regulator that controls the expression of multiple stress-related genes, including genes associated with starvation, oxidative stress, and trehalose synthesis (*otsAB* and *treA*). Humann et al. (2008) found the involvement of other regulatory genes: *relA, rpoE2, hpr* and the DNA repair gene *uvrC* in tolerance of the soil bacterium, *Sinorhizobium meliloti* to desiccation. Mutants that were defective in these genes were found to be less tolerant to desiccation than the wild type strain. Cytryn et al. (2007) examined genes expression of another soil bacterium, *Bradyrhizobium japonicum* in response to desiccation stress. Microarray analysis revealed a significant increase in expression of transcription regulators, genes associated with metabolism and transport of sugars; chaperones; genes related to oxidative stress, DNA repair, synthesis of pili and flagella and genes involved in synthesis of extracellular polysaccharides.

Desiccation stress response was also studied in the human pathogen *Cronobacter sakazakii* a member of the *Enterobacteriaceae* family. It was found that during desiccation *Cronobacter* increases expression of genes that encode cold-shock protein (CspC), DNA repair enzymes, heat-shock proteins (GroES, HSP, ClpB), oxidative stress related proteins (such as superoxide dismutase and alkyl hydroperoxide reductase), as well as proteins involved in organic solute transport, such as glutamine-binding protein and outer membrane proteins, such as OmpA, and OmpC (Osalii and Forsythe, 2009).

1.4 Desiccation and oxidative stress

Hydration shell of proteins' surface is essential for their appropriate folding and functioning (Levy et al., 2004). Removal of water molecules leads to conformational changes and consequent dysfunction of proteins, including enzymes of the electron transport chain, which result in electron leakage and reactive-oxygen species (ROS) formation (Billi and Potts, 2002; Fredrickson et al., 2008).

It has been found that the red algae, *Porphyra columbina* exposed to desiccation had induced formation of ROS, such as H_2O_2 and superoxide anions ($O_2^{\cdot -}$) (Contreras-Porcia et al., 2011). Concurrently, increased activity of several antioxidant enzymes, such as catalase (CAT), glutathione reductase (GR), dehydroascorbate reductase (DHAR), and peroxiredoxin (PRX) was observed (Contreras-Porcia et al., 2011).

Accumulation of ROS molecules causes oxidative stress, which impairs cell metabolism and leads to protein denaturation, lipid-peroxidation, DNA breaks and mutations (Billi and Potts, 2002). Several studies suggest that desiccation tolerance and long term survival in the dry state may depend on the ability of cells to scavenge free radicals and to protect themselves from oxidative stress damages (Kranner et al., 2005; Fredrickson et al., 2008; Contreras-Porcia et al., 2011). Fredrickson et al. (2008) suggests that survival of the environmentally resistant bacterium, *Deinococcus radiodurans* in dry conditions depends on its ability to limit protein oxidation during dehydration.

Shirkey et al. (2001) detected the presence of an active oxidative stress-response protein, Fe-SOD (superoxide dismutase) in 13-year-dried cells of the cyanobacterium, *Noctos commune*.

Transcriptional analysis performed in independent studies on two bacterial species: the gammaproteobacterium, *Bradyrhizobium japonicum* and the actinomycete, *Rhodococcus jostii RHA1* exposed to desiccation, revealed a common response (Cytryn et al., 2007; Leblanc et al., 2008). Besides up-regulation of genes involved in the synthesis of compatible solutes and transcriptional regulators, genes involved in cell protection from oxidative damage were also up-regulated. *B. japonicum* up-regulated superoxide dismutases (*chrC* and *sodF*), and *R. jostii RHA1* up-regulated catalase (*katG)* and the *dps1* gene, which is known to protect DNA from oxidative damage in various bacterial genera (Martinez et al., 1997; Cytryn et al., 2007; Leblanc et al., 2008). Further studies are needed to establish the contribution of these genes to bacterial fitness (survival) under desiccation stress.

1.5 Survival of Salmonella in dry conditions

Salmonella colonizes the intestinal tracts of multiple hosts, including human and other mammals, poultry, and reptiles (Uzzay et al., 2000). Once excreted from its host, *Salmonella* encounters environmental stresses, such as starvation, desiccation, osmotic stress, changes in pH, temperature and radiation. A critical stress *Salmonella* may encounter outside the host environment is desiccation. It has been reported that *Salmonella* can survive in dry conditions on various surfaces, soil and food products for several weeks to several years (Zibilske and Weaver, 1978; de Cesare et al., 2003; Hiramatsu et al., 2005; Bernstein et al., 2007).

Several studies show involvement of lipopolysaccarides (LPS) in *Salmonella* tolerance to desiccation. It has been suggested that because of their water-retaining qualities, polysaccharides may act as a bacterial 'water reservoir' by slowing down the rate of cellular water loss (Billy and Potts, 2002; Garmiri et al., 2008).

Garmiri et al. (2008) found that mutants (*waaL (rfaL), wbaP (rfbP), waaC (rfaC), waaP (rfaP), wzx (rfbx), waaK (rfaK)*) lacking O-polysaccharide element (OPS) of LPS were sensitive to desiccation. Gibson et al. (2006) found that mutations in *yih*Q and *yih*O genes, involved in O-antigen capsule assembly and translocation, decrease the survival of *Salmonella* during desiccation stress.

White et al. (2006) found that *Salmonella* colonies with rdar (red-dry and rough) morphotype, characterized by the expression of cellulose and thin aggregative fimbriae (Tafi) display enhanced tolerance to desiccation. Mutants lacking fimbriae and cellulose *(*Δ*bcsA* and Δ*agfD*), didn't form rdar morphotype and were found to be more sensitive to desiccation.

Hiramatsu et al. (2005) found that survival of *Salmonella* on dry food significantly increased in the presence of sucrose. Bullifient et al. (2000) found that induced trehalose biosynthesis contributes to *Salmonella* survival during desiccation.

Gruzdev et al. (2011) showed that desiccated *S. enterica* serovar Typhimurium (STm) has acquired higher tolerance to multiple stressors compared to non-desiccated cells. Dried STm cells were significantly more resistant to most stressors, including ethanol, sodium hypochlorite, didecyl dimethyl ammonium chloride, hydrogen peroxide, NaCl, bile salts, dry heat and UV irradiation, but less tolerant to acetic and citric acids than non-desiccated cells.

Deng et al. (2012) studied *S. enterica* serovar Enteritidis global gene expression in peanut oil, a low water activity (a_w) food product, by RNA sequencing. RNA seq analysis revealed that

Salmonella cells in peanut oil were in a physiologically dormant state expressing <5% of their genome compared to 78% in LB broth-grown bacteria. Among the few detected transcripts in peanut oil, were genes involved in heat- and cold-shock response (*grpE, cspA*), DNA protection (*dps*) and regulatory genes (rpoH, rpoE) (Deng et al., 2012).

In summary, while information regarding desiccation tolerance in *Salmonella* is accumulated in recent years, the mechanisms of cellular adaptation of *Salmonella* to desiccation still remain poorly understood, and further investigation is needed.

1.6 Research objectives

Salmonella is able to survive in dry conditions on various surfaces, soil and food products for several weeks to several years (Zibilske and Weaver, 1978; de Cesare et al., 2003; Hiramatsu et al., 2005; Bernstein et al., 2007). This feature makes *Salmonella* a threat to the food industry and therefore further knowledge regarding desiccation tolerance is needed in order to limit entry of the pathogen into the food supply chain. Consequently, the objectives of this research were:

1. To identify environmental and cellular factors that affect *Salmonella* desiccation tolerance and long term survival in the air-dried state.
2. To identify additional genes that are involved in *Salmonella* tolerance to desiccation.

2. Materials and methods

2.1 List of materials

2.1.1 Chemical reagents, enzymes and kits

Chemical reagents, enzymes and kits used in this study are detailed in Tables 1-3.

Table 1: List of reagents

Acetic acid	Gadot, Israel
Agarose I / TBE Blend 1.0%	Amresco, Solon, OH, USA
Ampicillin	Sigma, St. Louis, MO, USA
Anaerobic System, AnaeroGen	Oxoid Ltd, Hampshire, UK.
Anaerobic indicator	Oxoid Ltd, Hampshire, UK.
Arabinose	Sigma, St. Louis, MO, USA
Bacto agar	Difco, Sparks, MD, USA
Bacto tryptone	Difco, Sparks, MD, USA
Bacto yeast extract	Difco, Sparks, MD, USA
Beta-Mercaptaethanol	Promega, Madson, WI, USA
Chloramphenicol	Sigma, St. Louis, MO, USA
EDTA (Ethylenediaminetetraacetic	Analar, BDH Chemicals, Poole, Dorset
Electroporation Cuvette 0.1 mm	USA Scientific Ocala, FL.
Ethanol, Absolute, AR	Gadot, Netanya, Israel
Ethidium Bromide (EtBr) 10 mg/ml	Sigma, St. Louis, MO, USA
Guanidine Hydrochloride (GuHCl)	Sigma, St. Louis, MO, USA
Glucose	Merck, Darmstadt, Germany
Hank's balanced salt solution (HBSS)	Beit Haemek, Israel
Hydrogen Peroxide 30%	Merck, Darmstadt, Germany
Instant Blue (protein stain)	Expedeon, Cambridgeshire, UK
Kanamycin	Sigma, St. Louis, MO, USA
LDS Sample Buffer	Expedeon, Cambridgeshire, UK
Luria Bertani (LB) Broth	Difco, Sparks, MD, USA
Luria Bertani (LB) Agar	Difco, Sparks, MD, USA
Paraquat dichloride x-hydrate pestanal	Sigma chemicals, St. Louis, MO, USA
Petri dishes	Miniplast, Ein-Shemer, Israel
RunBlue (SDS running buffer)	Expedeon, Cambridgeshire, UK
Safe View (DNA staining)	ABM , Richmond, Canada
Saline	0.85% NaCl
SDS precast gel (12%)	Expedeon, Cambridgeshire, UK
SOB (Super Optimal Broth) medium	Pronadisa, Madrid, Spain

Sodium azide	Sigma, St. Louis, MO, USA
Sodium chloride NaCl	Biolab, Jerusalem, Israel
Sodium phosphate NaH_2PO_4	Merck, Darmstadt, Germany
Sterile polypropylene tube (15, 50 ml)	Lancon,Petaluma, CA, USA
Streptomycin	Sigma, St. Louis, MO, USA
Sucrose	Duchefa Biochemie, Haarlem, The
TAE (Tris-Acetate-EDTA) buffer	40mM Tris, 20 nM acetic acid, 1mM EDTA
Tris base	Sigma, St. Louis, MO, USA
Water (ultra pure)	Beit-Haemek, Israel
Xylose lysine deoxycholate (XLD)	Acumedia, Baltimore, MD, USA
96 well plate	Greiner bio-one, Frickenhansen, Germany
Amicon Ultra-4 3K device	Millipore, Tullagreen, Ireland

Table 2: List of commercial kits, enzymes and markers

FastDigest NheI Restriction enzyme	Thermo Fisher Scientific, Waltham, USA
FastDigest SalI Restriction enzyme	Thermo Fisher Scientific, Waltham, USA
T4 DNA Ligase	Thermo Fisher Scientific, Waltham, USA
Live/dead bacterial viability kit	Invitrogen, Oregon, USA
DNA Purification kit	Promega, Madison, USA
DNA Clean and Concentrator kit	Zymo, Irvine, USA
Zyppy Plasmid Miniprep	Zymo, Irvine, USA
BCA protein assay kit	Thermo Fisher Scientific, Waltham, USA
Go Taq Green Master Mix	Promega, Madison, USA
Primers	Hylabs, Rehovot , Israel

Precision plus protein Marker	Bio-Rad, Hercules, CA, USA
ΦX174 DNA/*Hae*III Marker	Fermentas Inc., Hanover MD, USA
Lambda DNA/*Hind*III Marker	Fermentas Inc., Hanover MD, USA

Table 3: List of equipment

Cell density meter 595 nm	Biowave CO8000, WPA, Cambridge, UK
Eppendorf centrifuge 5147C	Eppendorf, Engelsdorf, Germany
Electroporator, Bio-Rad Micropulser	Bio-Rad, Hercules, CA, USA
ELISA reader, ELx 800UV	Bio-Tek Instruments, Winooski, VT, USA
pH meter, PHM 210	Radiometer, Copenhagen, Denmark
Vortex, Mini-Gennie	Biofan, Latvia
Climate-controlled incubator Climacell	MMM Group; Munich, Germany
Gel electrophoresis machine Dual Run & Blot Unit (protein separation)	Expedeon, Cambridgeshire, UK
Water-activity meter	Novasina , Lahen, Switzerland
NanoDrop 2000	Thermo Scientific, Waltham, USA
Electrophoresis power supply 200/2.0	Bio-Rad, Hercules, CA, USA
Gel electrophoresis machine Mini-Sub cell GT cell (nucleic acid separation)	Bio-Rad, Hercules, CA, USA
PCR machine T-Gradient Thermoblock	Biometra, Gottingen, Germany

2.1.2 Stains and plasmids

S. enterica sv. Typhimurium (STm) ATCC 14208 was used in the study. This strain is a descendent of a strain isolated from chickens in 1960 and is virulent in both human and mice (García-Quintanilla and Casadesús, 2011). This strain is also a close relative of the commonly used laboratory strain, STm LT2, whose genome and proteome data is available on http://www.ncbi.nlm.nih.gov. Other strains and plasmids used in this study are detailed in Table 4.

Table 4: List of strains and plasmids.

Strain/Plasmid	**Genotype**	**Reference/source**
S. Typhimurium ATCC14208	WT	M. MCclelland (SDIBR)
S. Typhimurium ATCC14208 pKD46	Km ^{r} Amp^{r}, containing Red recombinase expression plasmid pKD46	This study
E. coli BW 25113 pKD4*6*	Amp^{r}, containing template plasmid pKD46	Datsenko & Wanner, 2000
E. coli BW 25113 pKD4	Km^{r}, containing template plasmid pKD4	Datsenko & Wanner, 2000
E. coli BW 25113 pKD3	Cm^{r}, containing template plasmid pKD4	Datsenko & Wanner, 2000
ATCC14208Δ*rfaL*	*rfaL* Km^{r} deletion mutant	M. McClelland (SDIBR)
ATCC14208Δ*rfbp*	*rfbp* Km^{r} deletion mutant	M. McClelland (SDIBR)
ATCC14208Δ*sodA*	*sodA* Km^{r} deletion mutant	M. McClelland (SDIBR)
ATCC14208Δ*bfr*	*bfr* Km^{r} deletion mutant	This study
ATCC14208Δ*oxyR*	*oxyR* Km^{r} deletion mutant	This study
ATCC14208Δ*soxR*	*soxR* Km^{r} deletion mutant	This study
ATCC14208Δ*gst*	*gst* Km^{r} deletion mutant	This study
ATCC14208Δ*katE*	*katE* Km^{r} deletion mutant	This study
ATCC14208Δ*ahpCF*	*ahpCF* Km^{r} deletion mutant	This study

ATCC14208Δ*katG*	*katG* Km^r deletion mutant	This study
ATCC14208Δ*ahpCF*Δ*katG*	*ahpCF/katG* (Km^r Cm^r) deletion mutant	This study
S. Typhimurium SL1344	Sm^r Nal^r	M. Brandl, USDA
S. Enteritidis *F-3 251/01*		Clinical isolate, NSRL*
S. Newport MB17		Alfalfa sprouts/M. Brandl, USDA
pKD46	Amp^r, Red recombinase expression plasmid	Datsenko & Wanner, 2000
pKD4	Km^r template plasmid	Datsenko & Wanner, 2000
pkD3	Cm^r template plasmid	Datsenko & Wanner, 2000

*NSRL, National Salmonella Reference Laboratory, Ministry of Health, Central laboratories, Israel.

2.1.2.1 Bacterial storage conditions

All strains were stored at -80ºC, in glycerol 40%.

2.1.2.2 Bacterial growth conditions

Bacteria were grown in either liquid culture or on agar plate, as follow:

a. Liquid culture: a single colony grown on LB agar (Lennox; Difco Laboratories) was inoculated into 5 ml of LB broth and incubated overnight at 37°C, with either shaking at 150 rpm or without any shaking in static conditions.

b. Agar plate culture: A fresh colony was spread on LB agar plate with a sterile swab and incubated overnight at 37°C. For anaerobic conditions cells were incubated during growth in anaerobic jar containing gas generator kit (Oxoid Ltd, Hampshire, UK) that generates anaerobic atmosphere by reducing the oxygen level to below 1%, and increasing carbon dioxide level to 10%.

2.2 Methodology

2.2.1. Growth curve

Growth curve of STm in shaking and static cultures was determined, as follow: 100 µl of STm overnight culture were inoculated into 5 ml of Luria-Bertani (LB) broth (1:50) and incubated at 37°C, with shaking at 150 rpm or in static conditions. The optical density at 595 nm was

measured hourly, during 9 hours and at 16 and 24 h using Cell density meter (Biowave CO8000, Cambridge, UK). In each time point, CFUs were also determined.

2.2.2 Examination of dehydration tolerance and long-term persistence of the cells

2.2.2.1 Dehydration tolerance assay

Cell cultures were grown overnight at 37°C, washed twice in sterile deionized water (SDDW) by centrifuging (3,800 × *g*, 5 min) at room temperature, and the final pellet was resuspended in 2 ml SDDW to a final concentration of 2.0×10^9 CFU/ml ($O.D._{595nm} = 1.0$).

Further cells were dried in two modes:

- ***Slow drying***: aliquots (50 µl) containing 10^8 CFU of SDDW-washed STm cells were placed into each well of a 96-well polystyrene plate (Greiner Bio-One, Frickenhausen, Germany) and air-dried in a climate-controlled incubator (Climacell, MMM Group; Munich, Germany) for 22 h at 25°C, 40% RH. The length of drying was chosen according to a_w measurements. Maximal dehydration of the cells was achieved after 22 h, where the a_w level reached a minimal value of 0.53 (Fig.3a).
- ***Rapid drying***: aliquots (50 µl SDDW) containing 10^8 CFU of washed STm cell were spotted onto the center of a 50 mm polysterene petri dish (Miniplast, Ein-Shemer, Israel, Cat. No.: 72050-01) and air-dried in a climate-controlled incubator at 25°C, 40% RH for 1.5 h. Maximal dehydration under these conditions was achieved as early as 75 minutes, where the a_w level reached a minimal value of 0.45 (Fig.3b).

Following dehydration, the dried cells were resuspended in SDW, serially diluted (1:10), plated on LB agar, and incubated for 24 h at 37°C. Dehydration tolerance was determined by counting viable cells (CFU) at the end of the dehydration period.

2.2.2.2 Long-term persistence assay

For examination of long-term persistence, both slowly and rapidly dried cells were stored during 1 month in a climate-controlled incubator at 25°C, 40% RH. Persistence was tested weekly by counting viable cells, as described above.

2.2.2.3 Viability Staining

Dried *Salmonella* cells were resuspended in 100 µl SDDW, centrifuged (3000 x g, 5 min) and the pellet was resuspended in 30 µl of staining solution according to the manufacturer's

instructions (Live/dead bacterial viability kit; Invitrogen, Oregon, USA). SDDW-washed fresh *Salmonella* cells (before dehydration) were stained similarly. The stained cells were visualized under a confocal laser-scanning microscope (Olympus IX81, Tokyo, Japan). The ratio of viable cells was calculated by dividing the number of viable (green) cells by the total number of bacteria, live (green) and dead (red). The data derived from three independent experiments each performed in triplicate. Each replicate includes data from four microscopic fields (x400).

2.2.3 Water activity (a_w) measurements

Cells were dried on both 96-well plate and in small petri dishes (50mm) and the water activity of the cells on both surfaces was measured using a water activity meter (model ms1; Novasina, Lahen, Switzerland). Since the entire 96-well plate was too large for the a_w meter, the plate was sawed into several small pieces, each compose of 4 intact wells. The inoculated wells were placed into the a_w meter, and the measurements were performed every 2 h during 26 h of dehydration, until no further reduction in a_w was obtained. Additional a_w measurements were made after 36 and 48 hours of drying. Determination of a_w in petri dish was performed by placing the inoculated plate into the a_w meter and measuring the a_w every 15 min during 2 h of dehydration, until no further reduction in a_w was obtained. The data derived from two independent experiments each performed in triplicate.

2.2.4 Oxidative challenge experiments

STm cells were grown overnight at 37°C, washed twice in SDDW by centrifugation (3,800 × *g*, 5 min) at room temperature, and the final pellet was resuspended in 2 ml SDDW to an $O.D._{595nm}$= 1.0, which corresponds to ~ 2.0×10^9 CFU/ml. 50 µl of washed cells were mixed with 50 µl of double-strength (120 mM) of either hydrogen peroxide or paraquat dichloride solution to achieve a 60 mM concentration in a final volume of 100 µl. The cells were incubated at room temperature for 1.5 hour. The challenge was terminated by 10-fold serial dilutions with SDDW (Gruzdev et al., 2011), and viable bacteria enumerated by plating.
Hydrogen peroxide and paraquat dichloride concentrations and exposure time were chosen on the basis of previous publications *(*Imlay and Linn, 1986; Tseng et al., 2002).

2.2.5 Chloramphenicol effect on bacterial tolerance to desiccation and H_2O_2 challenge

STm suspension of 2.0×10^9 CFU/ml was prepared as described above (section 2.2.4).
Washed cells (990 µl) were mixed with 10 µl of chloramphenicol (10 mg/ml) to achieve a final concentration of100 µg/ml and incubated for 45 min at room temperature. Desiccation challenge was performed by drying 50µl of pretreated cells in a climate-controlled incubator at 25°C, 40% RH for 1.5 h. H_2O_2 challenge was performed by mixing 50 µl of pretreated cells with 50 µl of double-strength (120 mM) hydrogen peroxide to achieve a final concentration of 60 mM in 100 µl. The cells were incubated at room temperature for 1.5 hour. Cm concentration and pretreatment time were chosen according to Imlay and Linn, (1986).

2.2.6 Sodium azide (NaN_3) effect on bacterial tolerance to desiccation and H_2O_2 challenge

STm suspension of 2.0×10^9 CFU/ml was prepared as described above. A volume of
990 µl of washed cells was mixed with 10 µl of sodium azide (100 mM) to achieve a final concentration of 1 mM and incubated for 1 min at room temperature. Desiccation and H_2O_2 challenges were performed as described above.
Sodium azide concentration and pretreatment time were chosen according to previous publications (Switala et al., 2002; Koren et al., 2010).

2.2.7 Molecular biology techniques and Genetic manipulations

2.2.7.1 Plasmid DNA isolation

Plasmids were isolated from *E. coli* with Plasmid Miniprep Kit (Zymo, Irvine, USA), according to manufacturer's protocol.

2.2.7.2 Purification of PCR products

PCR products were purified with DNA Clean and Concentrator kit (Zymo, Irvine, USA).

2.2.7.3 Chromosomal DNA isolation from colony

A single colony was resuspended in 100 µl of NaOH (100 mM), boiled for 1 min, chilled on ice for 2 min, neutralized by adding 16 µl of Tris-HCl pH-8.0 buffer (1 M), and centrifuged at 8,000 x g for 5 min. The supernatant (80 µl) was used as a template for PCR.

2.2.7.4 DNA gel electrophoresis

DNA samples were separated on agarose gel 1% (Amresco, Solon, USA), using TAE (TrisAcetate-EDTA) buffer containing Safe-view DNA stain (ABM, Richmond, Canada), at 90 V for 30 minutes.

2.2.7.5 Preparation of electrocompetent cells

- *Preparation of electrocompetent cells for plasmid DNA transformation*: Electrocompetent cells of STm were prepared as described by Choi et al., (2006). Briefly, cells were grown overnight in LB broth (5 ml), harvested by centrifuging (16,000 x g, for 2 min), washed twice in sucrose (300 mM), and the final pellet was resuspended in 100 µl of sucrose 300 mM.
- *Preparation of electrocompetent cells for linear DNA transformation*: STm cells containing plasmid pkD46 were grown overnight in LB broth 5 ml, supplemented with Amp 100 µg/ml, at 30°C with shaking (150 rpm). 500 µl of overnight culture were transferred to 50 ml LB broth, and incubated for 2.5 hours at 30°C with shaking (150 rpm). After 2.5 hours incubation, 50 µM of arabinose were added in order to induce λ red recombinase expression, the culture was incubated for additional period of time (1-2 h) till the optical density ($O.D_{595}$) of the culture reached 0.9. The cells were incubated at 4°C for 15 min, washed twice in 40 ml of ice cold 15% glycerol, and centrifuged at 4°C (3000 x g, 15 min). The pellet was resuspended in 1 ml of 15% glycerol, cells were centrifuged under the same conditions for another 5 min, and the final pellet was resuspended in120 µl of 15% glycerol.

2.2.7.6 Transformation

- *Transformation of plasmid DNA*: Plasmid transformation was done as described by Choi et al. (2006). About 500 ng of plasmid DNA were mixed with 200 µl of electrocompetent cells and the mixture was transferred to a 2 mm gap width electroporation cuvette. Electroporation was performed using a Bio-Rad Micropulser under the following conditions: pulse time- 5 ms; voltage- 2.5 kV; capacity- 25 µF; resistance- 200 Ω. The cells were then resuspended in LB (1 ml), incubated for 1 hour at 30°C and plated on LB agar, supplemented with the selective antibiotics. The plates were then incubated overnight at 30°C.

- *Transformation of linear DNA*: About 200 ng of DNA were mixed with 80 µl of electrocompetent cells and the mixture was transferred into a 1 mm gap width electroporation cuvette. Electroporation was performed as follow: pulse time- 5 ms; voltage- 1.8 kV; capacity- 50 µF; resistance- 600 Ω. The cells were then resuspended in 1 ml SOC medium (SOB with 20 mM glucose), and incubated for 1 hour at 37° C with shaking (225 rpm). The entire 1 ml was plated on LB agar, supplemented with selective antibiotics (Km 50 µg/ml or Cm 30 µg/ml), and incubated overnight at 37°C.

2.2.7.7 Primers used in this study

Primers used in the study and PCR conditions are detailed in Tables 5 and 6, respectively.

Table 5: List of primers used in the study

Gene	**Name**	**Sequence 5' to 3'**	**Reference**	**Gene ID[1]**
waaL	waal_F	CTCACCAGAACAGAACCTGG[2]	This study	1255237
waaL	waal_R	GATAAGTGATTGAGTCCTGA[2]	This study	
wbap	wbap _F	CCTCAAGACCAATTAGATAC[2]	This study	1253603
wbap	wbap _R	AGCGATTGATGCGTTCAGAT[2]	This study	
ahpCF	ahpC_F	CGTTAACTTACTCCTCAACG[2]	This study	1252128
ahpCF	ahpF_R	AGTTAACTGGCCTTGCAGGT[2]	This study	1252129
katG	katG_F	TTGCTCCTGGTGTATATCGT[2]	This study	1255633
katG	katG_R	AACGTCAGCGACTACCGAAT[2]	This study	
sodB	sodB_F	TCCTGTCGTTTAACGACAGG[2]	This study	1252949
sodB	sodB_R	TTGTCGCAGGTGGATTAACG[2]	This study	
bfr	bfr_F	CGAGAAGTGATGCAGGATGA[2]	This study	1254966
bfr	bfr_R	AAGTACCTCGCTGCCTTAGG[2]	This study	
smbC	smbC_F	TGATCACGCTGTCCTTGTGA[2]	This study	1253582
smb	smbC_R	GCATTGTACCTGCTTTATAG[2]	This study	

C				
soxR	soxR_F	GGCGAGTATAATTCCTCAAG[2]	This study	1255792
soxR	soxR_R	AGGTGAGCTCAGCCCGATGG[2]	This study	
gst	gst_F	ATCGCCGTCCTGATGCTGCT[2]	This study	1252969
gst	gst_R	GGCGGCAGTCTATGAGATTA[2]	This study	
oxyR	oxyR_F	AACCATGGCGGAAGCTTATC[2]	This study	1255651
oxyR	oxyR_R	CATTGCTATGCTACCTATCG[2]	This study	
katE	katE_F	TGTTCTATAGTTAGAGTGAT[2]	This study	1252836
katE	katE_R	CTCAAGGCAGCGATTGCGGA[2]		
ahpCF	ahpCF_Dac_F	ATGTCCTTAATTAACACCAAAATCAAACCTTTCAAAAACCAGGCGTTCAA**TGTAGGCTGGAGCTGCTTCG**[3]	This study	1252128
ahpCF	ahpCF_Dac_F	TTATGCGATTTTGGTGCGAATCAGATAATCAAAGGCGCTCAACGAGGCTT**CATATGAATATCCTCCTTAG**[3]	This study	1252129
katG	katG_Dac_F	ATGAGCACGACCGACGATACCCATAACACGTTATCCACTG**TGTAGGCTGGAGCTGCTTCG**[3]	This study	1255633
katG	katG_Dac_R	TTATTGCAGATCGAAACGGTCCAGGTTCATCACTTTCACC**CATATGAATATCCTCCTTAG**[3]	This study	
KatE	KatE_Dac_F	ATGTCGCATAATGAGAAATCCCCCCATCAGTCCCCCGTGC**CATATGAATATCCTCCTTAG**[3]	This study	1252836
KatE	KatE_Dac_R	TTATGCAGGAATCGCGTTAATTTTCCCCGCACGCGACCAG**TGTAGGCTGGAGCTGCTTCG**[3]	This study	
smbC	smbC_Dac_F	CGCAAGATAGCCGGTTTCCACATGGTCGGTCCATGGGAACACACCGTGAA**CATATGAATATCCTCCTTAG**[3]	This study	1253582
smbC	smbC_Dac_R	GCTGTACCGGAATATACATTTCAATATCCCAGTATCCATCTTCCATGCCG**TGTAGGCTGGAGCTGCTTCG**[3]	This study	

bfr	bfr_Dac_F	CTCAATAAACTATTGGGAAATGAGCTTGTCGCAATTAATCAGTATTTTCT**CATATGAATATCCTCCTTAG**[3]	This study	1254966
bfr	bfr_Dac_R	TTAATCGGTAACCTTAATTTGTGATTGCAGATAATTTTGCATACCAAGTT**TGTAGGCTGGAGCTGCTTCG**[3]	This study	
gst	gst_Dac_F	ATGAAACTGTTCTACAAACCGGGTGCCTGCTCTCTTGCTTCCCATATTAC**CATATGAATATCCTCCTTAG**[3]	This study	1252969
gst	gst_Dac_R	TTAATTCAGTCCTTCCGCTTTAAGCGCTGCCGCAACGGTCGGTCTTTTTG**TGTAGGCTGGAGCTGCTTCG**[3]	This study	
soxR	soxR_Dac_F	ATGGAAAAAAAATCTCCCCGTTTAAAAGCCTTACTGACGCCGGGGGAAGT**TGTAGGCTGGAGCTGCTTCG**[3]	This study	1255792
soxR	soxR_Dac_R	TTAATCATCTTCAAGCAGCCGGGCGCCCGTCCCGTGTTCGCCAAGCCTGT**CATATGAATATCCTCCTTAG**[3]	This study	
oxyR	oxyR_Dac_F	ATGAATATTCGTGATCTTGAATATCTGGTGGCGTTAGCCGAACATCGCCA**TGTAGGCTGGAGCTGCTTCG**[3]	This study	1255651
oxyR	oxyR_Dac_R	TTAAACCGCCTGTTTTAACGCCTTGTCGAAATGGCCATCCATTGCGCCAC**CATATGAATATCCTCCTTAG**[3]	This study	
kan	kt	CGGCCACAGTCGATGAATCC[4]	Datsenko & Wanner, 2000	15554333
kan	k2	CGGTGCCCTGAATGAACTGC[4]	Datsenko & Wanner, 2000	
cat	c1	TTATACGCAAGGCGACAAGG[4]	Datsenko & Wanner, 2000	15554330
cat	c2	GATCTTCCGTCACAGGTAGG[4]	Datsenko & Wanner, 2000	
oxyR	oxyR_Comp_F	GGGGCTAGCGTGATAATAATAAGGAGTATTGATTATGAATATTCGTGATCTTGAATATCTGGTG[5]	This study	1255651

oxyR	oxyR_Comp_R	GGGGTCGACTTATCATTAAACCGCCTGTTTTAACGCCTTG[5]	This study	
bfr	Bfr_Comp_F	GGGGCTAGCGTGATAATAATAAGGAGTATTGATTATGAAAGGTGATGTTAAAATCATAAATTAT[5]	This study	1254966
bfr	Bfr_Comp_R	GGGGTCGACTTATCATTAATCGGTAACCTTAATTT[5]	This study	
pbr	Pbr_F	AAATCTAACAATGCGCT[6]	This study	208959
pbr	Pbr_R	CACCTGTCCTACGAGTTGC[6]		

[1]All primers were designed using the *S.* Typhimurium LT2 genome sequence (www.ncbi.nlm.nih.gov), and the program Gene Runner, version 3.02 (Hastings Software, Inc., Westwood, NJ). [2] Primers for mutants' confirmation contain nearby gene specific sequences. [3]Primers for mutagenesis: regular fonts refer to the gene specific sequences, and bold fonts refer to sequences derived from pkD3 or pkD4. [4]Primers for mutants' confirmation: common primers of Km/Cm resistance genes (*kan*/*cat*), were derived from the sequence of plasmids pkD3 and pkD4. [5]Primers for complementation: underlined fonts refer to the recognition site of restriction enzymes, *Sal*1/*Nhe*1, regular fonts refer to gene specific sequences. [6]Primers for recombinants' confirmation contain nearby *tet* gene specific sequences, were designed according to the sequence of the pBR322 cloning vector.

Table 6: PCR conditions used in the study

# Reaction	**Reaction conditions**
1	95°C, 4 min, 35 x (94°C 30sec, 52°C 30 sec, 72°C 1.5 min), 72°C , 10 min, 4 °C
2	95°C, 5 min, 35 x (94°C 1 min, 58°C 1 min, 72°C 1.5 min) 72°C 10 min, 4°C

2.2.7.8 Site-specific mutagenesis

Knockout mutagenesis was generated by the lambda red recombinase procedure (Datsenko and Wanner, 2000), as shown in Fig. 1.

DNA fragments containing the Km/Cm resistance gene and the flanking regions of the gene to be mutagenized were generated, as depicted in Fig 1.1. Primers used to amplify the Km/Cm resistance genes from pkD3/pkD4 plasmids are detailed in Table 5. Each primer included 20 bp derived from the Km/Cm resistance gene ends and 50 bp derived from the specific gene to be

mutagenized. The DNA fragments were amplified by PCR using reaction #1 (Table 6) and further purified.

The amplified DNA fragment was electroporated into STm containing the red helper plasmid pkD46 which allows homologous recombination between a linear DNA containing an antibiotic resistance marker and the chromosomal DNA (Fig1.2). In order to confirm the presence of the mutation, colonies that grew on antibiotic-selective medium were isolated (Fig. 1.3), DNA was extracted and used as a template for amplification of the mutagenized gene using nearby locus-specific primers in combination with Km/Cm resistance gene primers (Table 5). The PCR conditions are depicted in Table 6 (reaction #2). Final confirmation was done by sequence analysis of the PCR products.

Site-specific mutagenesis was performed in 8 genes: *soxR, oxyR, bfr, gst, ahpCF, katE, katG, smbC*. Double mutation (*ahpCF* and *katG*) was generated as described above by first generating an *ahpCF* mutant using the Km resistance cassette, and then generating a *katG* mutation using theCm resistance cassette on the *ahpCF* background. Colonies that grew on both antibiotics Km and Cm, were amplified as described above and sequenced for confirmation of the mutations.

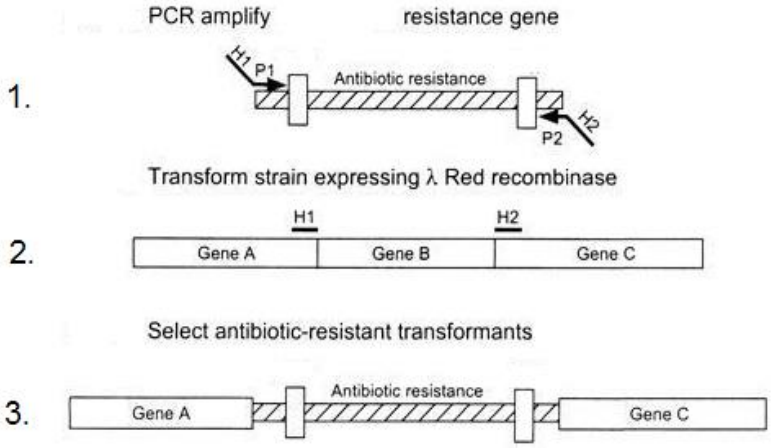


Figure 1. Gene mutagenesis strategy: 1. Generation of DNA fragment which encodes antibiotic resistance. Arrows represent primers. P1, P2 refer to pKD3/pKD4 region of primer and H1/H2 refer to the homologous gene regions of the primer. 2. Transformation: DNA fragment is transformed into lamda red recombinase containing cells, homologous recombination occurs. 3. The final product is shown: the gene is replaced with antibiotic resistance fragment.

2.2.7.9 Complementation

Plasmid pBR322 was used as a cloning vector. The plasmid contains both ampicillin (*bla*) and tetracycline (*tet*) resistance genes. An intact gene was inserted within the *tet* gene in the

restriction sites *SalI* and *NheI* (Table 2). The intact genes for complementation (*bfr* and *oxyR*) were amplified by PCR (reaction #2; Table 6). The details of the primers are listed in Table 5. Each primer included in addition to the specific gene sequences, restriction enzymes sites (*SalI/NheI*). Both plasmid and the amplified DNA fragments were cut with NheI and SalI according to the manufacturer's protocol, purified, and ligated with T4 DNA ligase.

After ligation, the recombinant plasmid was purified again, and transformed into electrocompetent cells. Colonies that grew on Amp-plates, were examined for Tet resistance, and those which lost ability to grow on Tet were chosen for further confirmation. The nature of the insertion was confirmed following PCR with primers prb322_F and prb322_R (Table 5; PCR reaction # 1, Table 6) and sequence analysis.

2.2.8 Proteomic analyses

2.2.8.1 Preparation of total protein extract

Protein extracts from *S.* Typhimurium ATCC14208 overnight cultures grown in 5 ml LB broth at 37°C, in shaking (150 rpm), or in static conditions were prepared. Cell cultures were washed twice in sterile deionized water (SDDW) by centrifuging (3,800 × *g*, 5 min) at room temperature, and the final pellet was resuspended in 5 ml SDDW to a final concentration of 2.0 × 10^9 CFU/ml, $O.D._{595nm}$= 1.0. Following centrifugation at 4,000 x *g* for 20 min, the pellet was lysed with 500 uL of lysis buffer (100 mM NaH_2PO_4, 10 mM Tris·Cl, 6 M GuHCl, pH 8). The lysate was centrifuged at 15,000 x *g* for 20 min to pellet cellular debris, and the supernatant containing the protein was used for further analysis. Proteins were concentrated using Amicon Ultra- 4 3K device, having a 3,000 molecular weight cutoff, according to manufacturer's instructions (Millipore, Ireland). Protein concentration was determined by the BCA protein assay (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's protocol.

2.2.8.2 SDS-PAGE

Protein samples were prepared according to the RunBlue protocol (Expedeon, Cambridgeshire, UK), as followed: protein extract (8 µg) was mixed with 20 µl of RunBlue LDS sample buffer (Table 1) and 2 µl beta-mercaptaethanol (Promega, Madison, WI, USA), and heated for 5 min at 80 ºC. Afterwards the samples were separated by SDS-PAGE using precasted SDS-PAGE 12% gel cassette and RunBlue SDS running buffer. The gel was run at 180 V for 40 min on gel

electrophoresis machine Dual Run & Blot Unit (Table 3). The gel was stained by InstantBlue stain (Expedeon, Cambridgeshire, UK).

2.2.8.3 Mass Spectrometry (MS) analysis

Selected bands were cut from SDS-PAGE gel and identified by Liquid Chromotography Mass Spectrometry (LC-MS), at the Smoler proteomic research center (Technion, Hafia, Israel). The samples were trypsinized and the peptides were analyzed by LC-MS on the Orbitrap mass spectrometer (Thermo). The data were analyzed using the Sequest 3.31 software versus the *Salmonella* database of the NCBI-NR database. The identification results were filtered according to the X-core value (cross correlation score), mass accuracy and the probability.

2.2.9 Total oxidant-scavenging capacity (TOSC) analysis

Total oxidant scavenging capacity (TOSC) of bacterial cells was measured by the luminol-dependent chemiluminescence (LDCL) assay, as described by Ginsburg et al. (2004).

The assay is based on the ability of anti-oxidative agents to reduce the luminescence generated by the luminescent mixture ('GO' (glucose oxidase) cocktail). The 'GO' cocktail comprises of luminol (10 µM), glucose oxidase (2.3 U/ml), sodium selenite IV (2mM), cobalt chloride (10 µM), Hank's balanced salt solution (HBSS) containing 1% glucose, pH 7.4). The cocktail generates an intense and steady peak of luminescence due to formation of hydrogen peroxide and hydroxyl radical, and the subsequent oxidation of luminol. Lumiescence reaction is detailed in Fig.2.

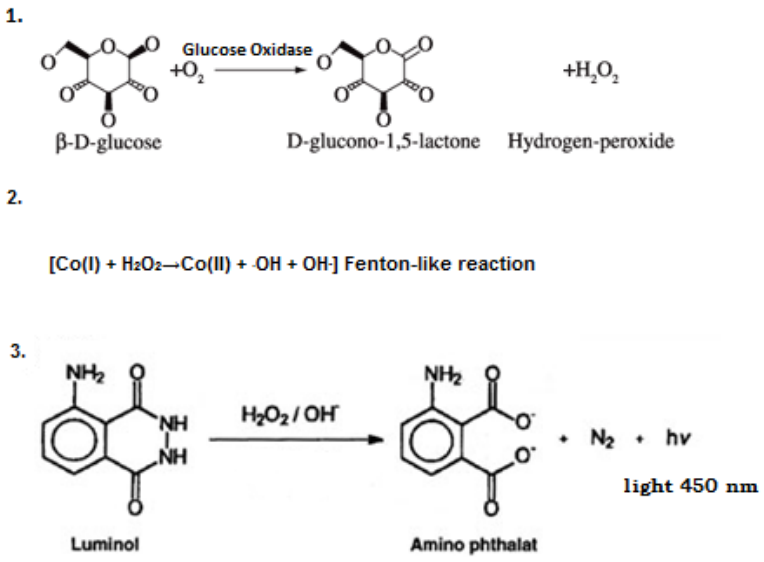


Figure 2.Chemical reaction leading to luminescence generation from 'GO' cocktail. (1) Glucose oxidase oxidize glucose and generates D-glucono-1,5-lactone and hydrogen peroxide. (2) Cobalt reacts with hydrogen peroxide to yield hydroxyl radical in a Fenton-like reaction. (3) Luminol is oxidized by hydrogen peroxide and hydroxyl radical, and the reaction results in the emission of light at 450 nm (blue).

For TOSC determination, bacteria (~10^9) were added to the 'GO' cocktail and the quenching of light, which is expressed as counts per minute (cpm), was monitored in 30 sec intervals for 10-20 minutes by a Lumac 2500 M Luminometer (Landgraaf, Netherlands). TOSC is described as time needed to quench the light (luminescence), that generated by free radicals (Fig. 2). It is a comparative method; the faster quenching of light indicates a higher TOSC, and *vice versa*.

2.2.10 List of websites and programs used in the study

Gene Runner version 3.02; Hastings Software, Inc., Westwood, NJ
National Center for Biotechnology Information - www.ncbi.nlm.nih.gov
MicrobesOnline: a portal for comparative and functional genomics-
http://www.microbesonline.org/

2.2.11 Statistical analysis

Statistical analysis was performed using unpaired student's t-test and One-way ANOVA with the Tukey-Kramer multiple-comparisons test, using GraphPad InStat version 3.06, (GraphPad Software, Inc., La Jolla, CA, USA). Unpaired student's t-test was used in experiments where only two groups were presented, while One-way ANOVA with the Tukey-Kramer multiple-comparisons test was used when more than two groups were presented. Differences were considered significant when $p<0.05$. Results are presented as the mean ± standard deviation.
All experiments were performed in triplicate and were repeated independently at least three times on different days.

3. Results

3.1 Characterization of STm dehydration

3.1.1 Kinetics of STm dehydration

In order to determine the dehydration kinetics, water activity (a_w) of STm cells during slow and rapid drying was measured. During slow drying maximal dehydration was achieved after 22 hours, where a_w level reached a minimal constant value of 0.53, a_w decreased gradually until 8 h, and then sharply between 8-12 h (Fig. 3a). During rapid drying, maximal dehydration was achieved as early as 75 min, where the a_w level reached a minimal constant value of 0.45. During the first 60 min no significant change in a_w was observed. However, a sharp decrease in a_w was observed during 15 minutes between 60-75 minutes of drying (Fig. 3b).

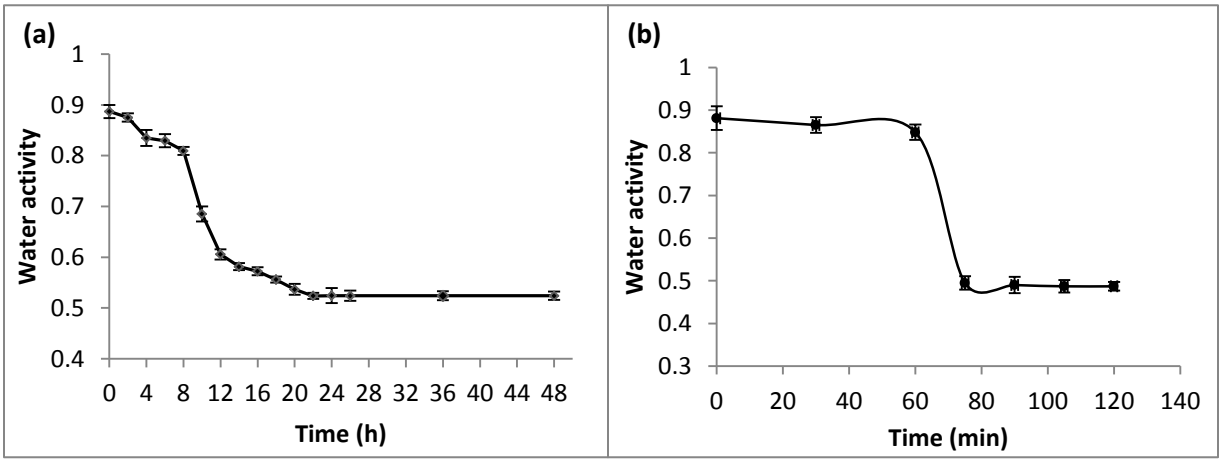


Figure 3. Dehydration kinetics of STm cells during slow and rapid drying. Aliquots (50 µl SDDW) containing ~ 10^8 CFU of washed STm cell were air-dried at 25°C, 45% RH. (a) Slow drying: cells were dried in a 96-well polystyrene plate for up to 48 h; The a_w of brand new polystyrene plate was 0.53. (b) Rapid drying: cells were dried on 50 mm polystyrene petri dish for 2 h; The a_w of a brand new petri dish was 0.45.

3.1.2 Effects of rapid and slow drying on dehydration tolerance and long-term persistence

To determine if rate of drying affect dehydration tolerance and long-term persistence during storage at 25C° 45% RH, the number of viable bacteria was determined immediately after rapid or slow drying (1.5 hour and 22 hours, respectively). The dehydration tolerance was not affected by the rate of drying (Fig. 4a), however long-term persistence was affected by the rate of drying. The number of viable cells after slow drying was significantly higher as compared with rapidly dried cells (Fig. 4b).

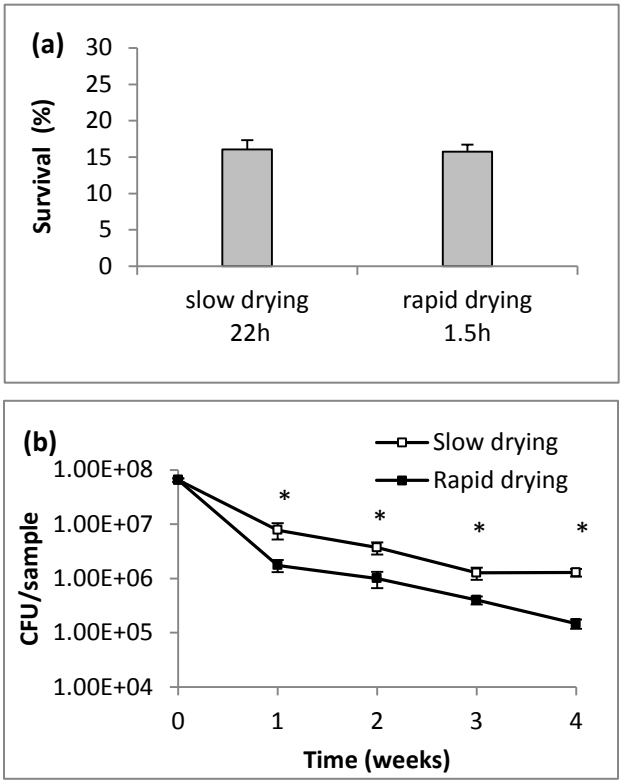


Figure 4. Effects of rapid and slow drying on STm survival immediately after dehydration, and during long-term storage. STm cells (10^8 CFU) were dried at 25C° 45% RH and the survival was examined immediately after desiccation (a) and weekly during 1 month of storage at 25°C, 45% RH (b). The data represents the average numbers of viable cells (CFU) from three independent experiments, each performed in triplicate. Error bars denote standard deviations of the means (*n*=9). Asterisks indicate significant difference ($p < 0.05$) in CFU between slow and rapidly dried cells.

3.1.3 Effects of growth phase on bacterial survival under desiccation stress

When grown under laboratory conditions, bacteria first enter a lag phase and then shift into a rapid growth phase (logarithmic). At high density, bacteria enter a stationary phase and become more tolerant to multiple stresses, including heat, oxidative-, acid- and osmotic-stresses (Ishihama, 1997). Accordingly, we have assumed that STm cells at the stationary phase of growth will be more tolerant to desiccation stress than logarithmic phase cells.

Indeed, STm logarithmic cells were found to be more susceptible to rapid drying compared to stationary phase cells, with a 2.3-log CFU reduction compared to 0.6-log reduction, respectively (Fig. 5a). However, after slow drying there was no difference in the survival of both logarithmic and stationary phase cells.

Since, cells were dried in SDDW, the survival of STm in SDDW without drying was also examined. Cells were incubated in SDDW for the same time periods that were used for drying (1.5 h for rapid drying; 22 h for slow drying). No change in CFU was observed in both logarithmic and stationary cells in SDDW (Fig. 5b).

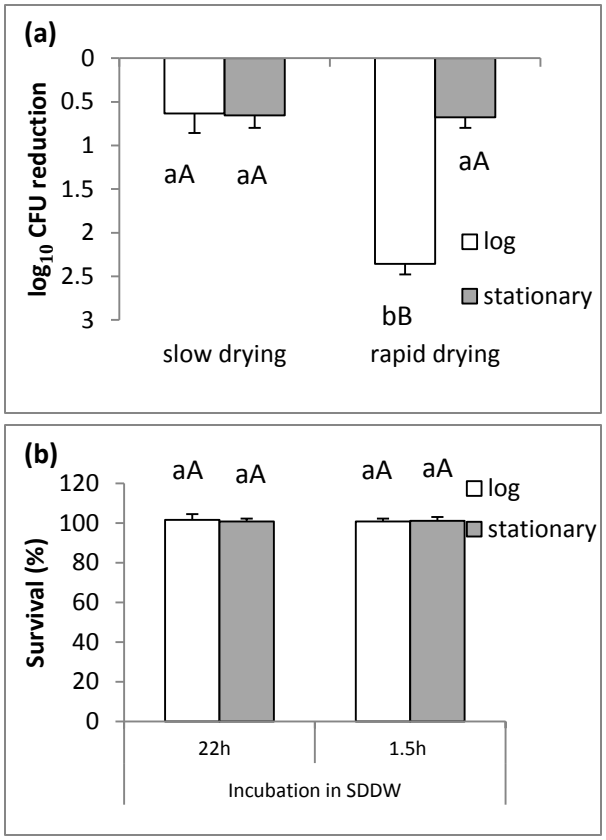


Figure 5. Survival of log- and stationary STm after slow or rapid drying in SDDW. (a) Survival after slow or rapid drying. (b) The survival of STm following incubation in SDDW. The data represent the mean $\log_{10}$ CFU reduction compared to the initial inoculums (10^8 CFU/sample) (a) or the average percentage of survival relative to the inoculum (100%) (b), from three independent experiments each performed in triplicate. Error bars denote standard deviations of the means (n=9). Different lower-case letters indicate significant difference between the two phases of growth for the same rate of drying/incubation period. Different capital letters indicate significant differences between the two rates of drying/ incubation periods at the same phase of growth ($p < 0.01$).

3.1.4 Desiccation tolerance of lipopolysaccharide (LPS) mutants

Garmiri et al. (2008) found that STm SJW1103 mutants lacking the O polysaccharide (OPS) element of the outer membrane lipopolysaccharide (LPS) (*waaL, wbaP , waaC, waaP, wzx, waaK*) were more sensitive to desiccation compared to the WT strain. In order to examine if desiccation tolerance in STm strain 14208 also requires an intact LPS, we have generated three of these mutants (*waaL, wbaP , waaP*) and tested their desiccation tolerance. Δ*waaL* mutant is defective in the ability to ligate O-antigen to lipid A core molecule, Δ*wbaP* is deficient in an enzyme that participates in O-antigen synthesis and release, and Δ*waaP* lacks an enzyme participating in LPS inner core synthesis. While two mutants (Δ*wbaP* and Δ*waaL*) were more susceptible to dehydration after rapid drying, no difference in the susceptibility of the three mutants was observed in slowly-dried cells (Fig. 6).

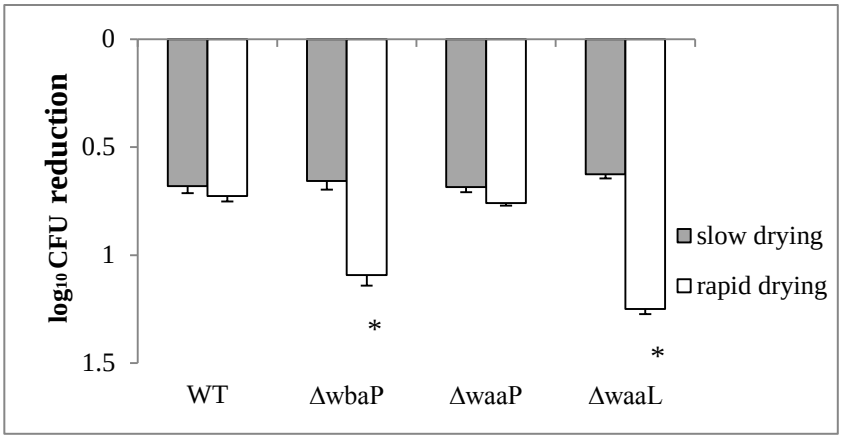
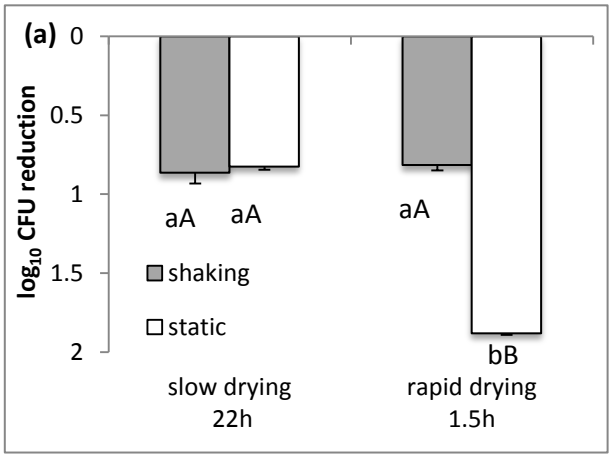


Figure 6. Survival of STm LPS mutants, Δ*wbaP*, Δ*waaP*, Δ*waaL* after slow and rapid drying. The data represent the mean $\log_{10}$ CFU reduction compared to the starting inoculums (10^8 CFU/sample), from three independent experiments, each performed in triplicate. Error bars denote standard deviations of the means (n=9). Asterisks indicate significant difference ($p < 0.01$) in log-reduction between the mutant and the WT strain.

3.1.5. Desiccation tolerance of STm cells grown in shaking and static cultures

In order to examine if the aeration conditions (static versus shaking) during growth of bacteria affect desiccation tolerance, we tested desiccation tolerance in bacteria grown to stationary phase under both static and shaking conditions (Fig. 7). STm grown under static culture conditions is more susceptible to rapid drying than cells grown in shaking culture, with 1.7-log CFU reduction in static cells compared to 0.7-log CFU reduction in shaking cultures. Interestingly, there was no significant difference in the survival between static and shaking cultures after slow drying. Similarly, 100 % survival was observed after incubation of the STm in SDDW after 1.5 and 22 hours (Fig. 7b).



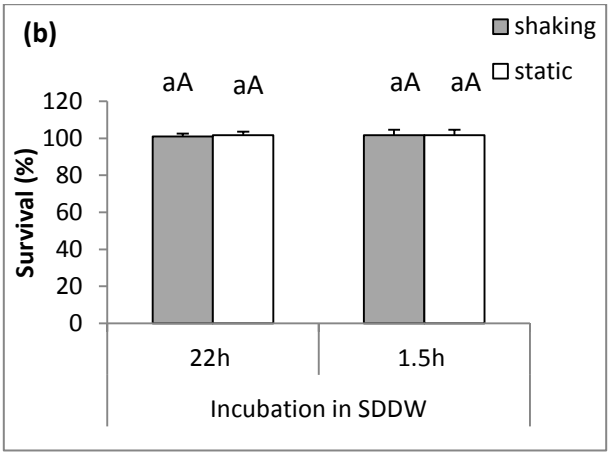


Figure 7. Effects of aeration during growth on STm survival after slow or rapid drying and in SDDW. STm was subjected to slow or rapid drying (a) or kept in SDDW for comparable times (b). The data represent the mean $\log_{10}$ CFU reduction compared to the initial inoculums (10^8 CFU/sample) (a) or the average percentage of survival relative to the inoculum (100%) (b), from three independent experiments each performed in triplicate. Error bars denote standard deviations of the means (n=9). Different lower case letters indicate significant difference between static and shaking cultures after same rate of drying (rapid or slow). Different capital letters indicate significant differences between slow- and rapid-drying of the same culture (shaking or static; $p < 0.05$).

3.1.6 Correlation between STm survival and dehydration rate

Since, a higher dehydration tolerance was documented in bacteria grown in shaking cultures only during rapid drying (Fig. 7), we have decided to study the underlined mechanism. As a first step, cell viability was monitored as function of the dehydration state of bacteria, represented by a_w. Fig. 8 shows that cells death follows similar trend as a_w reduction with maximal CFU reduction occurring between 60-90 min following the onset of drying, when water activity drops to its lower value. As shown before, cells grown under static cultures are more susceptible to dehydration than shaking cultures.

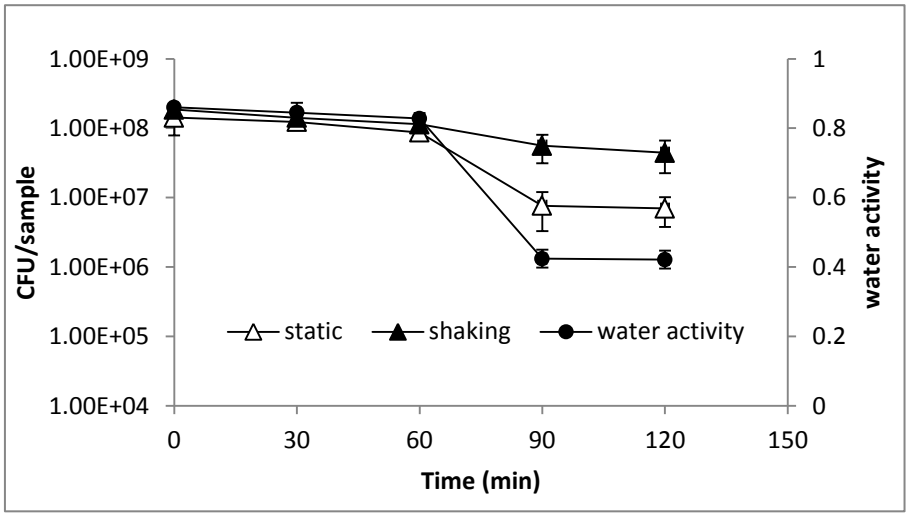


Figure 8. Survival of STm grown in shaking or static culture during rapid drying. Cells' viability and water activity measurements were performed during rapid drying, every 30 minutes, for up to 120 minutes. The data represent the average number of CFU per sample (50 µl) from three independent experiments, each performed in triplicate. Error bars denote standard deviations of the means (n=9).

3.1.7 Desiccation tolerance of static and shaking cultures in different strains of *Salmonella*

In order to test whether the higher susceptibility to dehydration of static cultures is widespread in other *S. enterica* strains, in addition to strain *S.* Typhimurium ATCC 14208, dehydration tolerance was also examined in other *Salmonella* strains, namely: *S.* Newport, MB17; *S.* Enteritidis, F-3 251/01; and *S.* Typhimurium, SL1344. The results are presented in Fig. 9. In all four strains, static cultures of *Salmonella* were more susceptible to dehydration than shaking cultures.

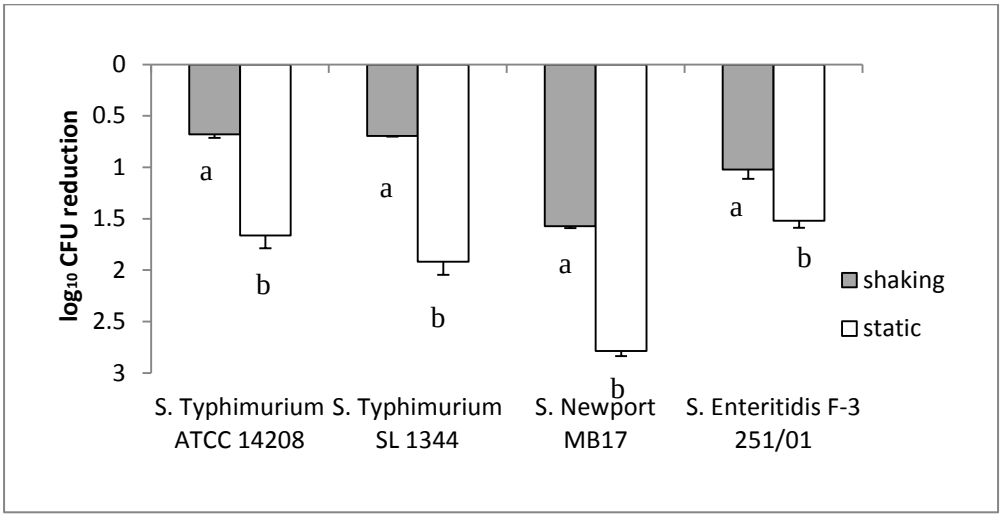


Fig. 9 Survival of *Salmonella* strains grown under static and shaking cultures following rapid drying. The data represent the mean $\log_{10}$ CFU reduction compared to time zero (before dehydration) from three independent experiments, each performed in triplicate. Error bars denote standard deviations of the means (n=9). For each strain, means followed by different letters indicate significant difference in dehydration tolerance between cells grown under shaking or static conditions ($p< 0.01$).

Stressed bacteria may enter a viable but not culturable (VBNC) state (Oliver, 2010). To test if the reduction in CFU resulted from loss of viability, or transition into the VBNC state, cells were stained using LIVE/DEAD method. Representative microscopic fields are shown in Fig. 10. Quantification of live and dead cells was performed as described in section 2.2.2.3 and the results are illustrated in Fig. 11.

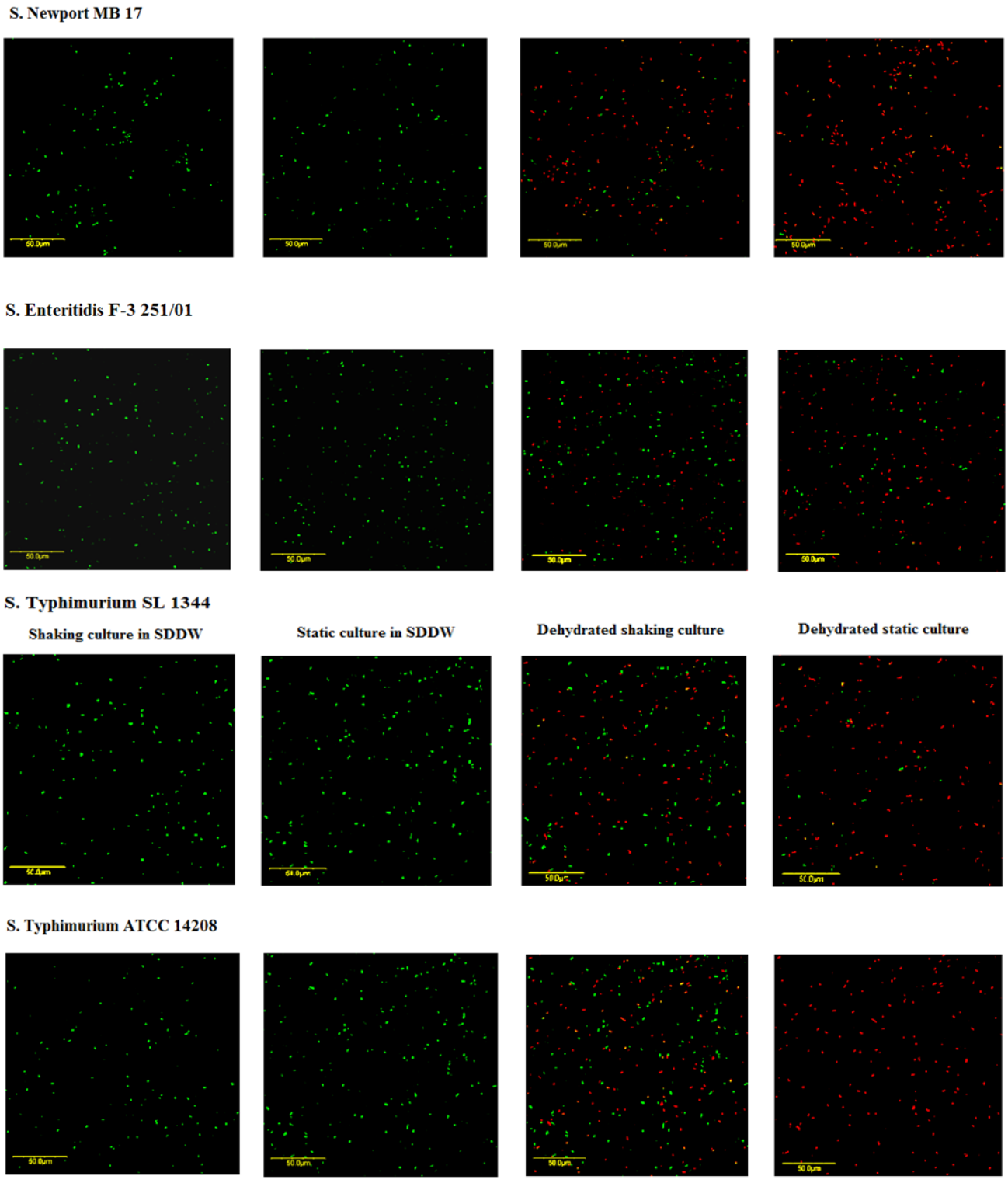


Figure 10. Visualization of viable and dead bacteria by confocal laser-scanning microscopy. Live (green) and dead (red) cells were visualized before dehydration and immediately after dehydration (1.5 h). Shown are representative images (scale: 50µm).

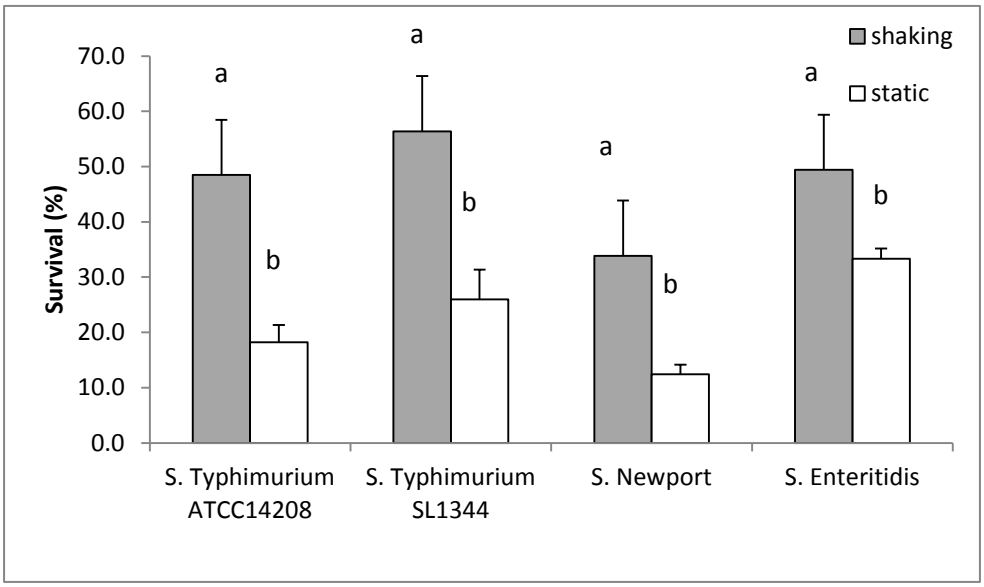


Figure 11. Survival of *Salmonella* strains after dehydration, according to LIVE/DEAD® staining. The columns represent the average percentage of surviving cells ±SD in three independent experiments, each performed in triplicate. Each replicate included data from four microscopic fields. Means followed by different letters indicate significant differences between shaking and static cultures ($P < 0.01$).

The results obtained from the viability staining experiments (Figures 10, 11) support those obtained with the CFU determination method (Fig. 9); static cultures of *Salmonella* are more susceptible to dehydration than shaking cultures. The reduction of CFU occurs as a result of cell death and not due to transition to the VBNC state.

3.2 Characterization of STm grown in shaking and static cultures

3.2.1 Growth curve analysis

Growth curves of static and shaking cultures were studied in order to examine whether both cultures were presented in the same growth phase when sampled (Fig.12). The growth kinetics of both cultures seems to be identical. Yet, at 24 h, there were about 3 times more bacteria in shaking- compared to static culture, even though both started with the same inoculum.

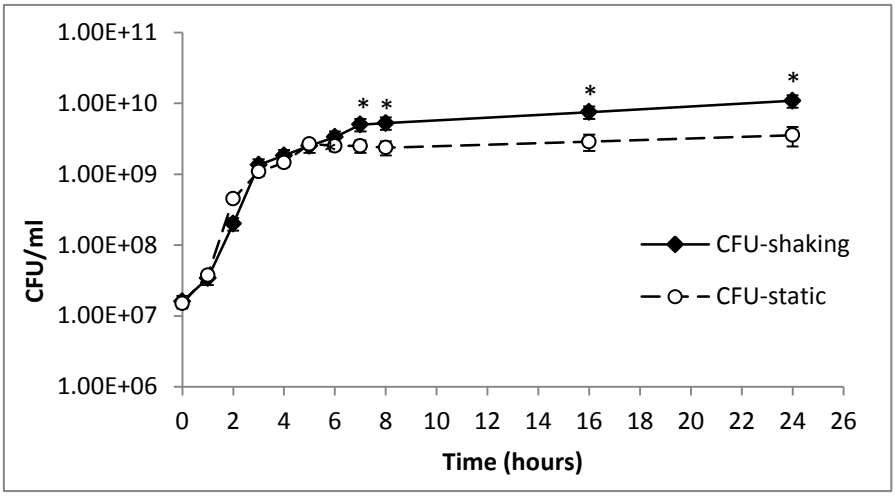
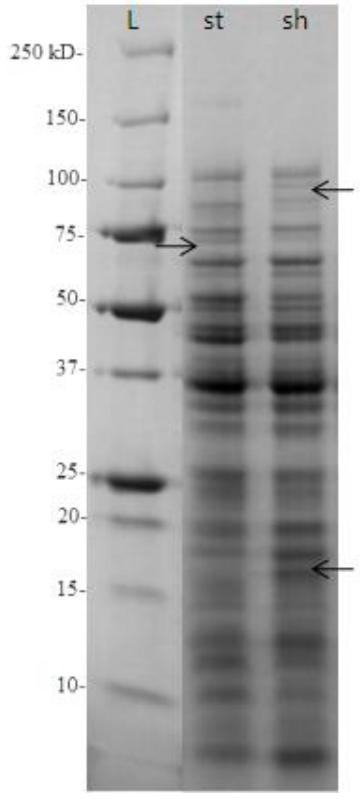


Figure 12. Growth curve of static- and shaking-cultures of STm. STm was grown in LB broth at 37°C for up to 24 h as described in section 2.2.1. At various intervals the culture was sampled and the number of viable bacteria (CFU) was determined. The average CFU/ml (± SD) from three independent experiments is represented. Asterisks indicate significant difference ($P < 0.05$) in CFU/ml between shaking and static cultures.

3.2.2 Protein analysis of shaking- and static cultures



It is possible that physiologic differences between cells grown in shaking- or static-cultures were responsible for the different dehydration tolerance. To test this possibility, one-dimensional protein analysis was performed. Total intracellular proteins of STm from static and shaking cultures were separated by SDS PAGE (Fig. 13) Three unique protein bands were observed. Two bands seems to be specific for cells grown in shaking culture, one of approximately 100 kDa and a second of about 17 kDa. One band, of approximately 75 kDa, appeared in static- but not in shaking-culture.

Figure 13. A representative SDS-PAGE of proteins from static and shaking cultures. Lane st- total proteins extracted from static culture, and lane sh showing proteins derived from shaking culture. The left lane contains molecular mass markers. Arrows depict bands that appeared in one of the two cultures.

3.2.3 Identification of proteins by mass spectrometry

The three specific protein bands were cut from the gel and subjected to MS analysis at the Smoler proteomic research center, Technion, Israel. Analysis of two of the bands (75 and 100 kDa) resulted in the same set of proteins in both shaking- and static-cultures. However, analysis of the 17 kDa band that was detected only in shaking culture lysate revealed a number of proteins (Table 7).

Table 7. Proteins identified by mass spectrometry in a 17 kDa band unique to shaking culture lysate.

Protein designation	**Protein function**
SodB	iron binding superoxide dismutase
Gst	glutathione-S- transferase
Bfr	bacterioferritin
SmbC	DNA gyrase inhibitor
YbgI	hydrolase oxidase

3.2.4 Desiccation tolerance of STm mutants

In order to test if the proteins differentially expressed in shaking culture confer enhanced desiccation tolerance, knockout mutations were generated in each of the following genes (*sodB, bfr, gst, smbC, ybgI*) and the desiccation tolerance of the mutants was compared to that of the WT strain (Fig. 14). A knockout mutation in the *bfr* gene (Δ*bfr*) but not in the other genes (*sodB, gst, ybgI* and *smbC*) resulted in ~ 30% survival compared to the WT strain.

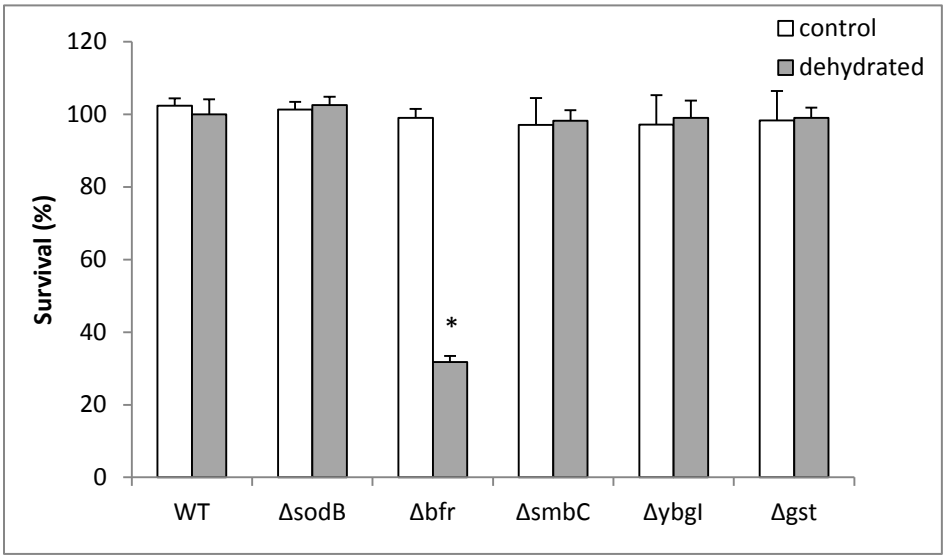


Figure 14. Survival of STm mutants following rapid drying or following incubation in SDDW (control). Survival is expressed as a percentage of bacteria that survived desiccation or incubation in SDDW relative the WT bacteria (considered as 100%). The data represent the mean survival ± SD from three independent experiments, each performed in triplicate. Asterisk indicates significant difference ($P < 0.05$) in survival between the mutant and the WT strain.

In order to confirm that single mutation is responsible for reduced desiccation tolerance genetic complementation was performed in Δ*bfr* mutant, as described in section 2.2.8.2. Indeed *bfr* complementation restored the desiccation tolerance of mutant back to the WT's level (Fig.15). Effect of the cloning vector pBR322 alone on WT desiccation tolerance was also examined. The WT strain transformed with pBR322 showed similar survival under desiccation as the WT strain (Fig.15). These results support the involvement of the *bfr* gene in desiccation tolerance of STm.

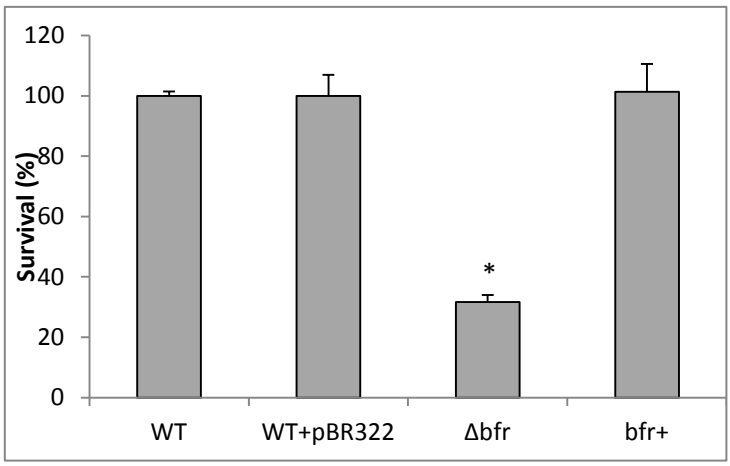


Figure 15. Survival of STm Δ*bfr* mutant and complementation strain following drying. STm cells were dried for 1.5 h and the relative survival was determined (WT survival is considered as 100%). The data represent the mean survival ±SD from three independent experiments, each performed in triplicate. Asterisks indicate significant difference ($p < 0.05$) in percentage of survival between the mutant and the WT strain.

3.2.5 Effect of anaerobic growth conditions on desiccation tolerance

Few of the differentially-expressed proteins identified in shaking cultures were related to antioxidative activity (see, Table 7). SodB is superoxide scavenging enzyme; Bfr (bacterioferritin) is involved in detoxification of iron and protection against O_2 and its radical products (Carrondo, 2003); and Gst (glutathione S-transferase) together with glutathione enable protection against oxidative stress (Vuilleumier and Pagni, 2002). Under static growth conditions, an oxygen gradient is formed: where the highest concentration is found at the meniscus of the culture and the inner volume has a lowered oxygen level (Wyckoff et al., 2002; Gaines et al., 2005). We therefore assumed that a relatively high oxygen level during growth in shaking- compared to static-culture, may have affected the expression of the anti-oxidative proteins, which might be responsible for the higher desiccation tolerance in shaking cultures of STm.

In order to examine if the reason for the higher desiccation tolerance is related to a previous adaptation to oxidative stress, STm cells were grown on agar plates under ambient conditions or under defined anaerobic conditions and the survival of bacteria was tested following desiccation of 1.5 h, or incubation for a similar time period in SDDW (Fig. 16). Indeed, STm grown on agar plates under defined anaerobic atmosphere has a lower survival compared to cells grown aerobiacally.

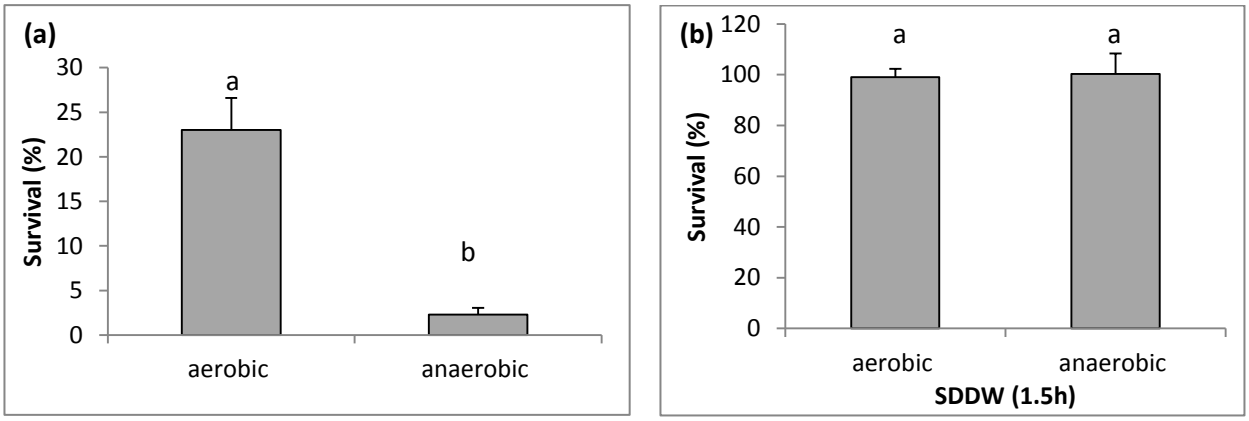


Figure 16. Survival of STm following 1.5 h drying (a) or incubation in SDDW (b). Plates were incubated at 37°C under ambient conditions (aerobic) or inside anaerobic jars (anaerobic conditions) and the bacteria were harvested, washed and tested for desiccation tolerance. The columns represent the average percentage of surviving cells ±SD in three independent experiments, each performed in triplicate. Means followed by different letters indicate significant differences between cultures survival ($p<0.01$).

3.3 Association between desiccation tolerance and oxidative stress

During desiccation the cell encounters oxidative stress due to the accumulation of hydrogen peroxide (H_2O_2) and superoxide anion (O^{2-}) (Billi and Pots, 2002; Contreras-Porcia et al., 2011). Therefore, it is possible that the ability of bacterial cells to withstand oxidative stress may also affect its tolerance to desiccation.

3.3.1 Aeration affects STm susceptibility to reactive oxygen species

Desiccation tolerance of STm grown under aerobic and anaerobic conditions was compared to their susceptibility to oxidative stress caused by hydrogen peroxide (H_2O_2) and superoxide anion (O^{2-}). STm grown aerobically or anaerobically was challenged with hydrogen peroxide, or paraquat (an intracellular superoxide anion generator) (Fig. 17).

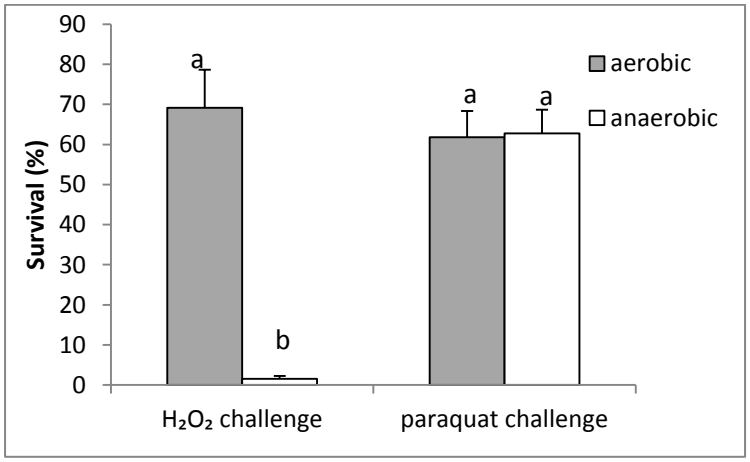


Figure 17. Survival of aerobic and anaerobic cultures of STm following oxidative stress. STm suspension in SDDW was xposed for 1.5 hour to H_2O_2 (60 mM), or paraquat (O^{2-}) (60 mM) and the number of CFU was determined. The columns represent the average percentage (compared to time zero) of surviving cells ±SD in three independent experiments, each performed in triplicate. For each treatment, means followed by different letters indicate significant differences between aerobic and anaerobic cells survival ($p < 0.01$).

STm grown aerobically was found to be more tolerant to H_2O_2 compared to bacteria grown under anaerobic conditions. The growth conditions, however, did not affect STm susceptibility to superoxide anions. Since, STm grown aerobically is also more resistant to desiccation (Fig. 16), it is likely that increased tolerance to desiccation stress may be related to increased tolerance to H_2O_2.

3.3.2 Effect of *bfr* mutation on tolerance to H_2O_2 stress

The *bfr* gene encodes a bacterioferritin, an iron (ferric) storage protein, whose secondary function is detoxification of iron and protection of cells against oxidative damage by O_2 and its radical products (Carrondo, 2003). Since desiccation induces an oxidative stress (Contreras-Porcia et al., 2011), it is possible that the reduced desiccation tolerance of the mutant is associated with decreased anti-oxidative activity. In order to examine this assumption, the *Δbfr* mutant was exposed to H_2O_2 stress and the survival of the WT and mutant strains was recorded (Fig. 18). The *Δbfr* mutant survived the H_2O_2 challenge, similar to the WT strain, suggesting that the reduced dehydration tolerance of the *Δbfr* mutant is not related to the anti-oxidative activity of Bfr.

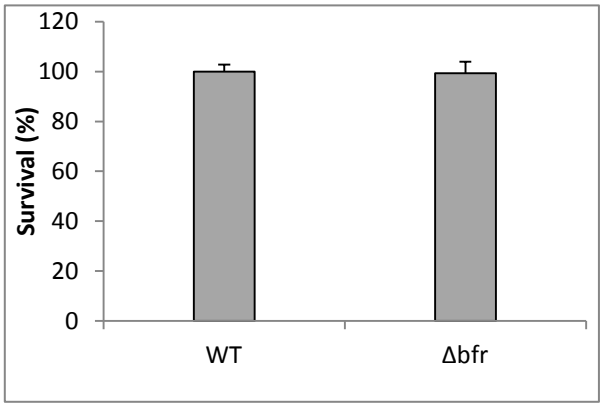


Figure 18. Survival of STm WT and Δ*bfr* mutant following H_2O_2 challenge (60 mM, 1.5 h). Survival is expressed as a percentage of the WT survival (considered as 100%). The data represent the mean survival ±SD from three independent experiments, each performed in triplicate.

3.3.3 Role of global oxidative stress response regulators, *oxy*R and *sox*R, in desiccation tolerance

If indeed, tolerance to H_2O_2 is associated with desiccation tolerance, then oxidative stress response regulators might also be involved in desiccation tolerance. Knockout mutations were generated in two major oxidative stress response regulators: *oxyR* (peroxide stress regulator) and *soxR* (superoxide stress regulator) and the mutants were examined for their susceptibility to H_2O_2, paraquat, and desiccation. We found that Δ*oxyR*, but not Δ*soxR* mutant was compromised in both H_2O_2 and desiccation tolerance, demonstrating ~ 5% survival compared to the WT strain (Fig. 19). No effect was observed following exposure to paraquat. These results further support a linkage between the ability of STm to withstand H_2O_2 challenge and desiccation tolerance.

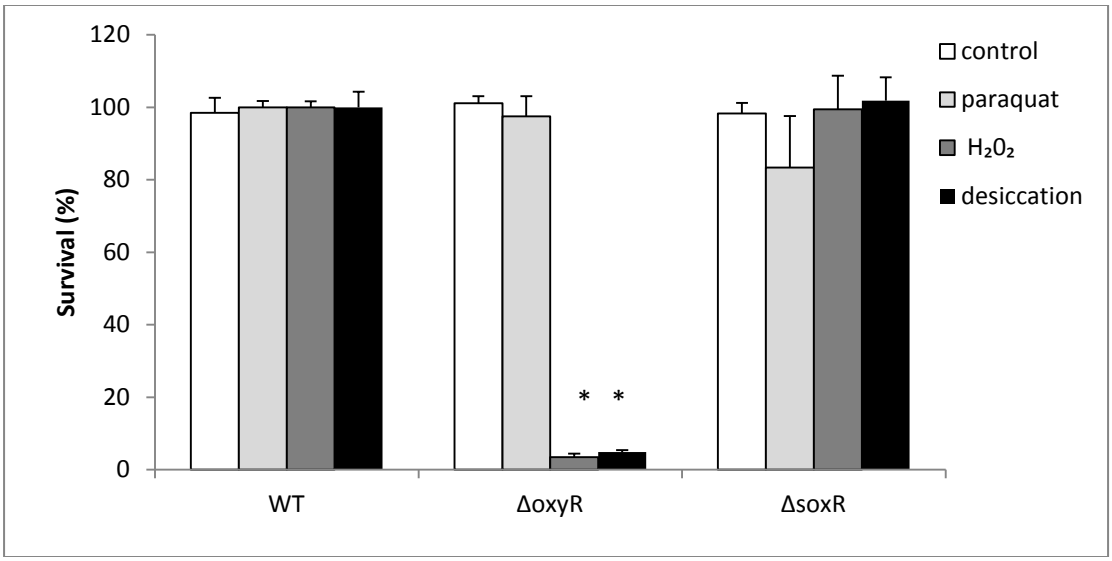


Figure 19. Survival of Δ*oxyR* and Δ*soxR* mutants following exposure to oxidative stress and dehydration. WT and mutants' cells suspended in SDDW were exposed to H_2O_2 (60 mM), paraquat (O^{2-}) (60 mM) or dehydrated for 1.5 h. WT and mutants incubated in SDDW for the same period of time served as control. Survival is expressed as a percentage of viable bacteria (CFU) relative to the WT strain (considered as 100%). The data represent the mean survival ±SD from three independent experiments, each performed in triplicate. Asterisk indicates significant difference ($p < 0.05$) in percentage of survival between the mutant and the WT strain.

In order to confirm that single mutation in *oxyR* is indeed responsible for the reduced desiccation tolerance, genetic complementation was performed in the Δ*oxyR* mutant with a copy of the WT gene. Indeed, *oxyR* complementation, but not the vector alone, restored the desiccation tolerance of mutant back to the WT's level (Fig. 20). These results support the involvement of *oxyR* gene in desiccation tolerance of STm.

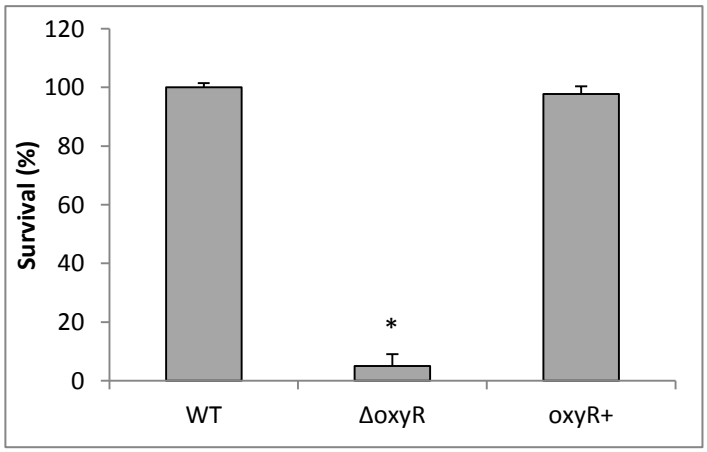


Figure 20. Survival of STm WT and Δ*oxyR* mutant strains following H_2O_2 challenge (60 mM, 1.5 h). Survival is expressed as a percentage of the WT survival (considered as 100%). The data represent the mean survival ±SD from three independent experiments, each performed in triplicate.

3.3.4 Effect of *de-novo* protein synthesis on STm tolerance to desiccation and H_2O_2 stresses

STm cells grown overnight under aerobic or anaerobic conditions were pretreated with chloramphenicol (Cm) and tested for desiccation and H_2O_2 tolerance (Fig. 21).

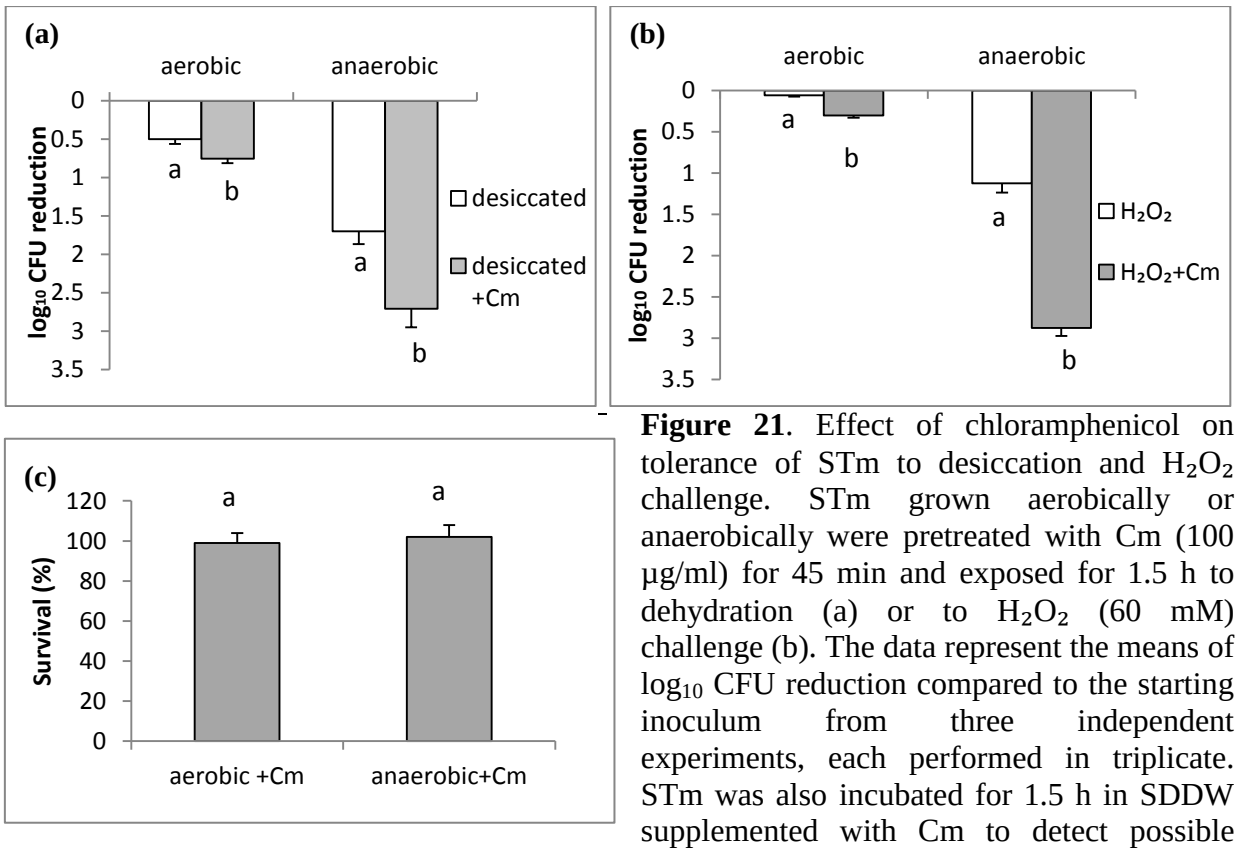


Figure 21. Effect of chloramphenicol on tolerance of STm to desiccation and H_2O_2 challenge. STm grown aerobically or anaerobically were pretreated with Cm (100 µg/ml) for 45 min and exposed for 1.5 h to dehydration (a) or to H_2O_2 (60 mM) challenge (b). The data represent the means of $\log_{10}$ CFU reduction compared to the starting inoculum from three independent experiments, each performed in triplicate. STm was also incubated for 1.5 h in SDDW supplemented with Cm to detect possible effect of the antibiotic on bacterial survival, regardless of the stress imposed (c). The data represent the percentage of surviving bacteria from three independent experiments, each performed in triplicate. Error bars denote standard deviations of the means (n=9). Different letters indicate significant difference ($p < 0.05$) between the treatments (Cm) and control (no Cm).

Pretreatment with Cm decreased the survival of STm following drying, regardless of the growth conditions. Yet, the effect was higher in cells grown anaerobically compared to aerobically grown cells (2.8 log reduction, versus 0.7, respectively) (Fig. 21a). Similarly, *de-novo* protein synthesis is needed to withstand H_2O_2 challenge (Fig.21b). To negate the possibility that Cm itself affects STm viability, the fate of STm following 1.5 h incubation in SDDW supplemented with Cm was also examined (Fig.21c). The results indicate that *de novo* protein synthesis is needed for maximal survival of STm following both H_2O_2 and desiccation stresses.

3.3.5 Total oxidative scavenging capacity (TOSC) of STm cells

According to our results (see, Fig. 17) and previous studies (Imlay and Linn, 1986), anaerobically grown cells are more susceptible to oxidative stress than aerobically grown cells. This has been attributed to their reduced ability to scavenge reactive-oxygen species (Imlay, 2002). In order to examine this hypothesis, total oxidative scavenging capacity (TOSC) of STm was determined in aerobically and anaerobically grown cells (Fig. 22).

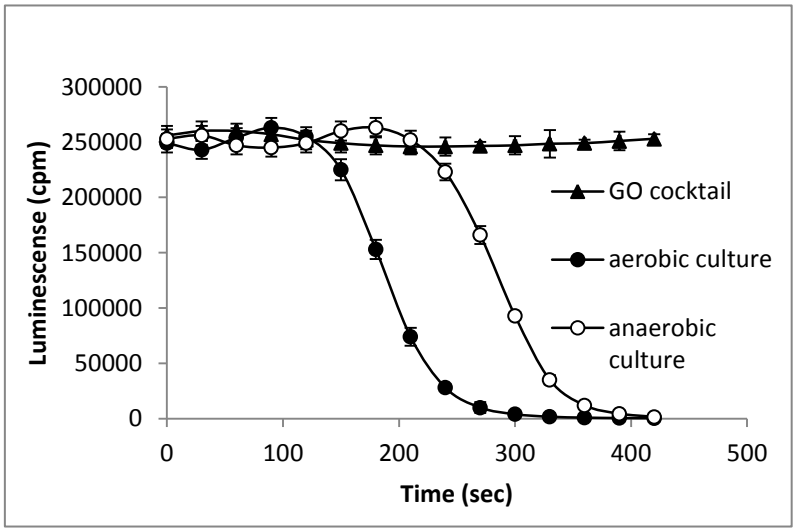


Figure 22. Quantification of total oxidative scavenging capacity of STm cells. STm cultures were grown aerobically or anaerobically to a density of (~$2x10^9$ CFU/ml), and the TOSC was measured using the LDCL assay, which assesses the ability of cells to quench luminescence induced by reactive oxygen species by the 'GO' cocktail. Quenching of light, expressed as counts per minute (cpm), was monitored for several minutes. Data are expressed as the average of 3 independent experiments, each performed in duplicate.

Aerobically grown cells quench the luminescence much faster than anaerobically grown cells. These data suggest that anaerobically grown cells have reduced ability to scavenge ROS compared to aerobically grown cells.

TOSC of Δ*oxyR* mutant

OxyR up-regulates *katG and ahpCF* encoding two scavengers of hydrogen peroxide, catalase and alkyl hydroperoxide reductase, respectively (Imlay, 2008). Consequently, the *oxyR* mutant strain is expected to be compromised in TOSC. Indeed, the Δ*oxyR* mutant had much lower TOSC compared to the WT strain (Fig. 23).

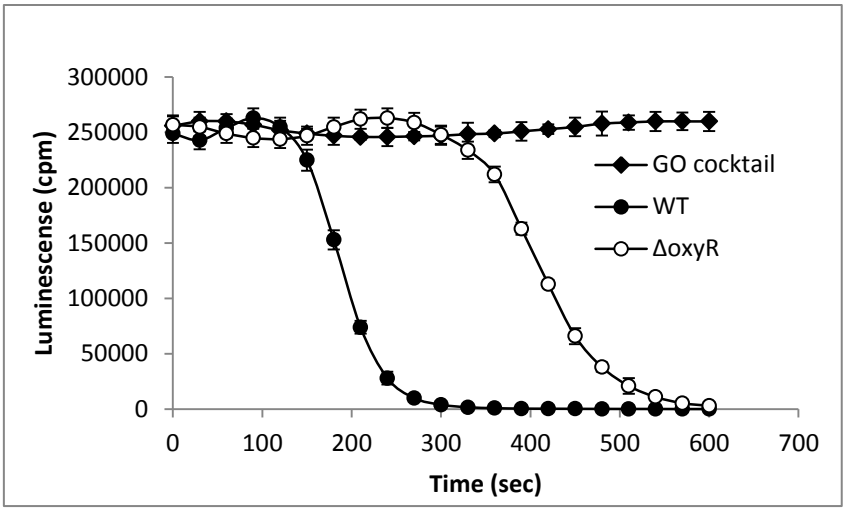


Figure 23. TOSC determination in the WT and Δ*oxyR* mutant. Aerobically grown cells ($2x10^9$/ml) were mixed with 'GO' cocktail and the luminescence was measured up to 10 min. Data are presented as mean luminescence values ± SD of three independent experiments, each performed in duplicate.

To examine if the reduced total oxidative scavenging capacity of anaerobic cells is related to lower catalase activity, the effects of a catalase inhibitor (sodium azide; NaN_3) on TOSC was tested (Fig. 24). Pretreatment of both aerobic and anaerobic cultures of STm with sodium azide resulted in slower luminescence quenching, probably due to catalase inhibition. Anaerobic culture showed much slower luminescence quenching than aerobic culture (32 minutes compare to 13 minutes, respectively).

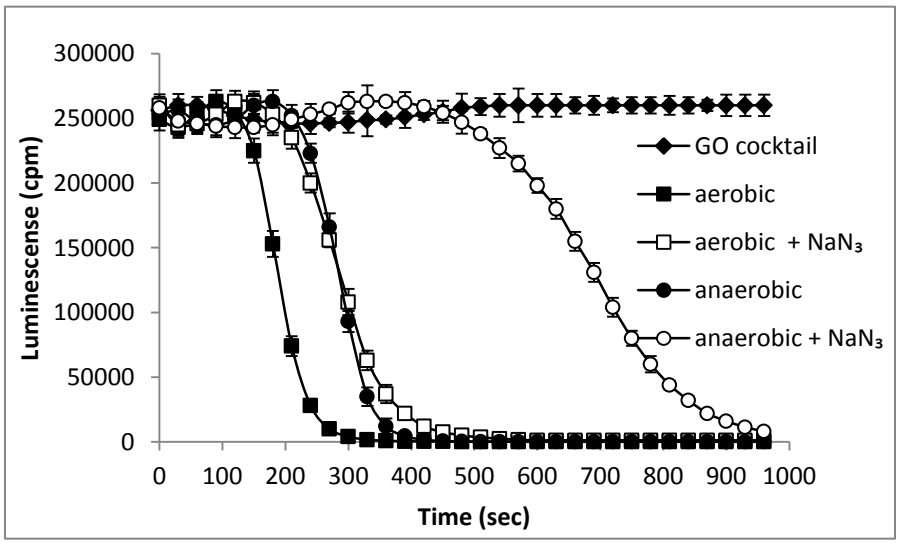


Figure 24. Effects of the catalase inhibitor NaN_3 on TOSC. STm cells ($2x10^9$/ml) were pretreated with sodium azide (1 mM) for 1 minute, mixed with the 'GO' cocktail and the luminescence was measured. The Data are presented as mean luminescence values ± SD of 3 independent experiments, each performed in duplicate.

3.3.6 Effect of sodium azide (NaN_3) on tolerance of STm to desiccation and H_2O_2 stresses

Since NaN_3 decreased bacterial TOSC, we assumed that pretreatment of cells with sodium azide will also decrease STm tolerance to H_2O_2 and to desiccation. Indeed, sodium azide pretreatment reduced the survival of STm challenged with H_2O_2 and desiccation. These results suggest that catalase is involved in the development of tolerance to desiccation stress.

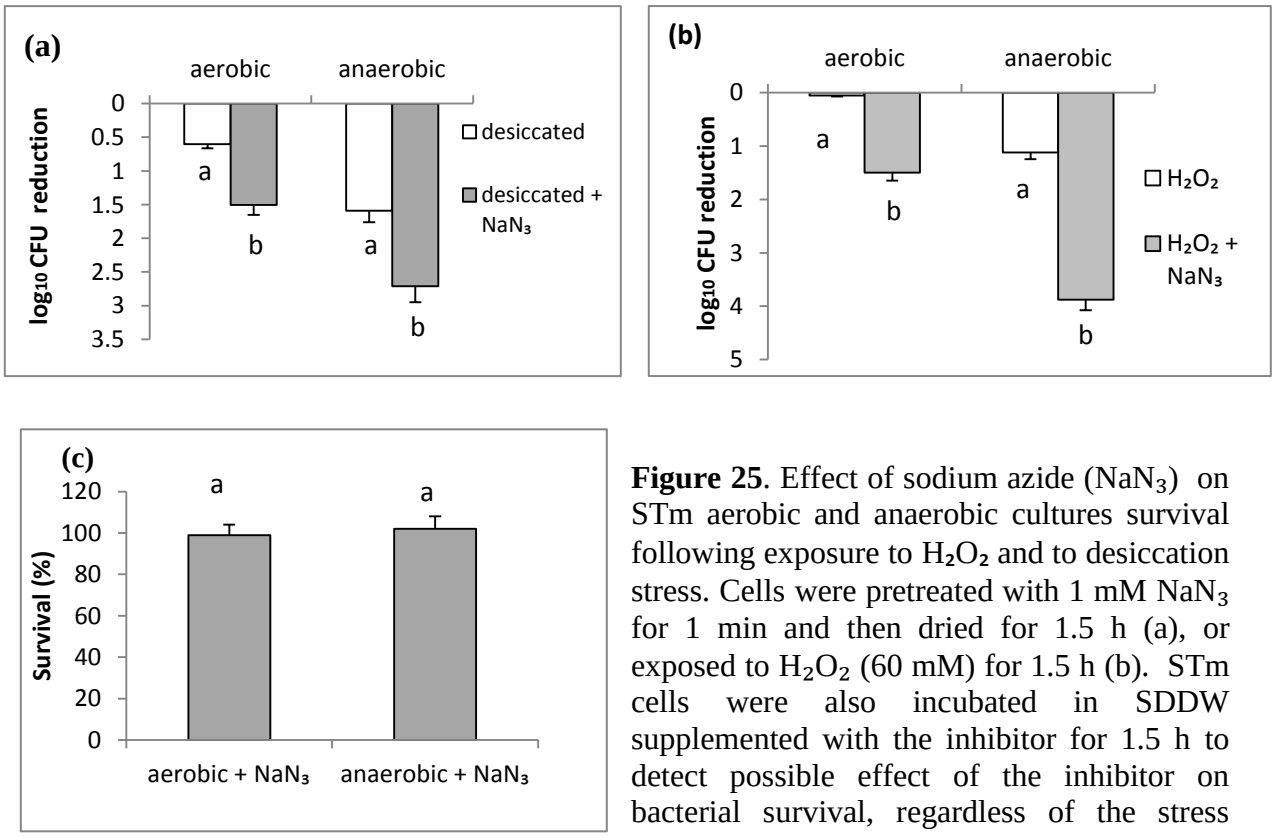


Figure 25. Effect of sodium azide (NaN_3) on STm aerobic and anaerobic cultures survival following exposure to H_2O_2 and to desiccation stress. Cells were pretreated with 1 mM NaN_3 for 1 min and then dried for 1.5 h (a), or exposed to H_2O_2 (60 mM) for 1.5 h (b). STm cells were also incubated in SDDW supplemented with the inhibitor for 1.5 h to detect possible effect of the inhibitor on bacterial survival, regardless of the stress imposed and their viability was determined (c). The data represent the mean log_{10} CFU reduction compared to the initial inoculum (panels a and b) or the percentage of surviving bacteria (c). All the data were derived from three independent experiments, each performed in triplicate. Error bars denote standard deviations of the means (n=9). Different letters indicate significant difference ($p < 0.05$) between the treatment (NaN_3) and the control (no NaN_3).

3.3.7 Involvement of hydrogen-peroxide scavengers in STm tolerance to desiccation stress

Our data thus far indicate a role for hydrogen-peroxide scavengers in desiccation tolerance. STm possesses multiple H_2O_2 scavengers including three catalases (KatE, KatG, and KatN) and two alkyl hydroperoxide reductases (AhpCF and TsaA) (Hebrard et al., 2009). The *katE* and *katN* genes are induced by sigma factor *rpoS*, while the *ahpCF* and *katG* genes are regulated by

oxyR (Robbe-Saule et al., 2001, Imlay, 2008). Previous studies reported that *katE* mutant as well as the *ahpCF*/*katG* double mutant are highly susceptible to hydrogen-peroxide compared to the WT strain (Seaver and Imlay, 2001). In contrast, neither *katN* nor *tsaA* mutant were more susceptible to hydrogen peroxide (Hebrard et al., 2009; Robbe-Saule et al., 2001). Consequently, we decided to tests the involvement of the *katE*, *katG* and *ahpCF* genes in desiccation tolerance of STm. Knockout mutants in these genes were generated and tested for desiccation tolerance. Since absence of *ahpCF* can be compensated by overexpression of *katG* (Seaver and Imlay, 2001), an *ahpCF/katG* double mutant was also generated. To verify that the mutants are susceptible to H_2O_2, they were exposed to H_2O_2 stress, as described in section 2.2.4.

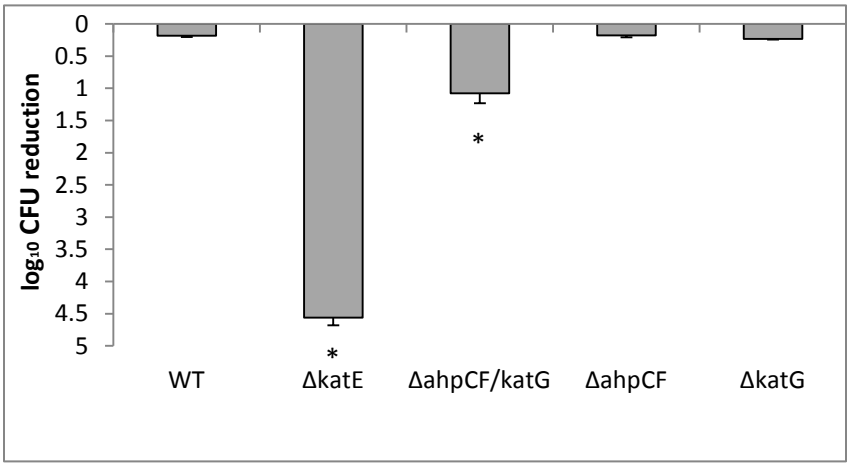


Figure 26. Survival of STm mutants following H_2O_2 challenge (60 mM, 1.5 h). The data represent the mean $\log_{10}$ CFU reduction (compared to time 0 before the challenge) ±SD of three independent experiments, each performed in triplicate. Asterisks indicate significant difference ($p < 0.05$) in survival between the mutant and the WT strain.

The *Δ*katE* mutant displayed a greater susceptibility to H_2O_2 compared to the WT strain (4.3 versus 0.1 log CFU reduction), while the *ΔahpCF/katG* double mutant showed a moderate susceptibility (1.1 log CFU reduction). The survival of the other mutants was comparable to that of WT.

The mutants were then tested for survival following desiccation stress (Fig. 26). Interestingly, the Δ*ahpCF/katG* double mutant, but not the *Δ*katE* mutant was found to be compromised in desiccation tolerance, demonstrating ~ 10% survival compared to the WT strain. The survival

of the other mutants Δ*katE*, Δ*ahpCF*, and Δ*katG* was comparable to that of the WT strain. All the mutants survived similarly (100%) following incubation in SDDW.

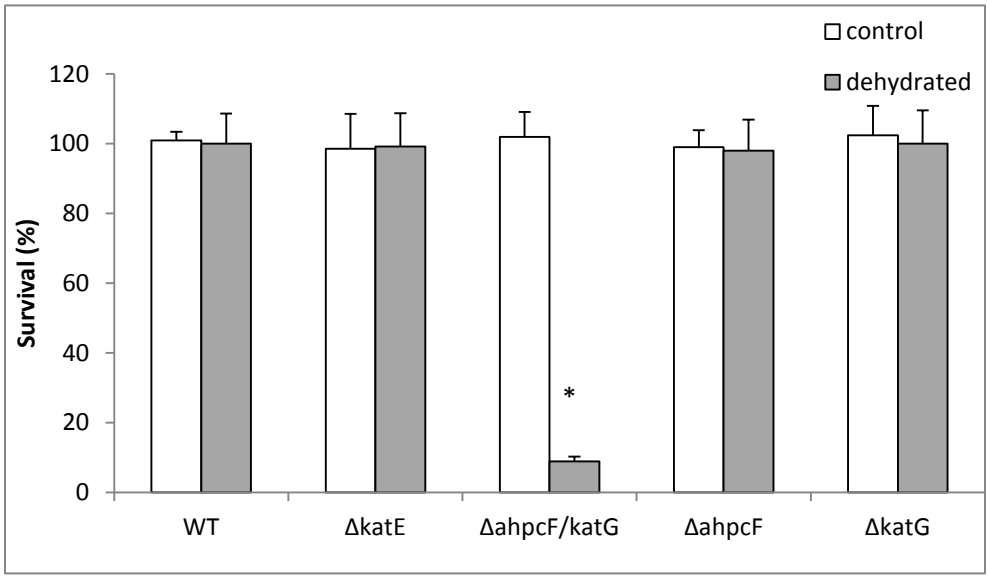


Fig. 27.Survival of STm mutants following dehydration. STm WT and mutant strains grown aerobically were dried for 1.5 h or remained suspended in SDDW (control) for the same period of time. Survival is expressed as a percentage of surviving bacteria relative to the WT strain (considered as 100%). The data represent the mean percentage of survival ±SD from three independent experiments, each performed in triplicate. Asterisk indicates significant difference ($p < 0.05$) in survival between a mutant and the WT strain.

4. Discussion

Although bacteria cannot replicate in food with low water-activity, during the last decade, *Salmonella* has been involved in several national and international outbreaks related to consumption of low-water-activity foods such as snacks, almonds, peanut butter, chocolate, and paprika (Isaacs et al., 2005; MMWR, 2007; Anonymous, 2009). *Salmonella* has been occasionally detected in granola, pistachio, and corn flakes (Weber et al., 2005; Anonymous, 2010a; Anonymous, 2010b). The ability of the pathogen to survive on dry surfaces in food processing operations, including restaurants, enhance the risk of cross-contamination and re-entry of the pathogen into the food supply chain (de Cesare et al., 2003).

4.1 Role of intrinsic and extrinsic factors on Salmonella tolerance to desiccation

One important factor that affects desiccation tolerance of bacteria is the rate of dehydration (Antheunisse and Arkesteijn-Dijksman, 1979; Mary et al., 1985). But, when testing the effect of dehydration-rate on the desiccation tolerance of STm cells ,no difference in the survival was observed immediately following rapid or slow drying (1.5 and 22 h, respectively). However, during 1-month of storage at 25°C under 40% RH, the number of viable cells was much higher after slow drying as compared to rapid drying (Fig. 4). These results are in agreement with the report of Antheunisse and Arkesteijn-Dijksman (1979), who found that bacterial cells from different genera, including *Escherichia* and *Pseudomonas* have similar survival rates immediately after rapid (20 min) or slow (32 h) drying and decreased survival during prolonged storage (35 days) after rapid- as compared to slow-drying. The authors proposed that rapid drying damages the slime capsule (the capsule is torn), while less damage occurs by slow drying, enabling the cells to keep their water content longer and thus to survive during prolonged storage.

Another hypothesis that may explain these results is that slow drying enables STm to adapt to the dry environment, possibly by synthesizing the necessary proteins and compounds required to retain intracellular water content, and limit cellular damages caused by the osmotic stress and the reduced water content. This adaptation is subsequently advantageous during prolonged storage in dry environment.

If indeed bacteria may manipulate their physiological status to withstand desiccation, then other intrinsic and extrinsic factors may affect this adaptation, as well. Since, we utilized stationary-

phase cells for the earlier studies; in the next step we investigated the effect of growth phase on desiccation tolerance. Others have reported that during stationary phase bacteria achieve a multi-resistant state, becoming more thermo-tolerant and more resistant to oxidative-, acid- and osmotic-stresses than at logarithmic phase (Ishihama, 1997). It was suggested that in stationary-phase the metabolism of the cell is optimized for survival, whereas in the log-phase bacteria do not express the general stress response factor (RpoS) and their metabolism is optimized for growth (Ward et al., 2010). Accordingly, we have assumed that STm at logarithmic-phase of growth will be less tolerant to desiccation stress than stationary-phase cells. Indeed, we found that logarithmic-phase cells were significantly more susceptible to rapid drying than stationary phase cells (Fig.5); however after slow drying, no difference in desiccation tolerance between log- and stationary-phase cells was found. Since, logarithmic phase cells are intrinsically less adapted to withstand stresses, as mentioned above, during slow- but not during rapid- drying (22 h), bacteria have the necessary time to adapt to the reduced water content and synthesize the necessary compound required to withstand the stress.

Previous studies found that STm SJW1103 mutants lacking O-polysaccharide element (OPS) of LPS are sensitive to desiccation (Garmiri et al., 2008). In order to examine if desiccation tolerance in STm strain 14208 also requires an intact LPS we tested 3 mutants lacking the O-polysaccharide element. The Δ*waaL* mutant cannot ligate the OPS to outer core of LPS, the Δ*wba*P mutant cannot catalyze the first step in the OPS subunit biosynthesis, while the Δ*waaP* mutant cannot synthesize LPS inner core. We found that Δ*wbaP* and Δ*waaL* mutants are less tolerant to desiccation compare to the WT, while the Δ*waaP* did not display significant difference compare to the WT (Fig.6). These results differ from those of Garmiri et al., 2008, who found that all the 3 were more sensitive to desiccation.

The phenotype of the *ΔwbaP* and *ΔwaaL* mutants was presented only after rapid drying, while during slow drying all the mutants survived similar to the WT strain. These results suggest that OPS provide defense against rapid drying, and it seems to be less important during slow drying. This finding supports the notion that slow drying is less severe to the cell than rapid drying because the cells have enough time to adapt and activate other defense mechanisms.

In order to examine if the growth conditions (static versus shaking) affect STm desiccation tolerance, we exposed to dehydration both static and shaking cultures (Fig. 7). We found that static cultures were more susceptible to desiccation only following rapid drying, but not under

slow drying. Since, differences in cells' survival were observed after rapid- but not after slow-drying, we decided to continue studying STm tolerance to rapid drying. We further investigated the effect of dehydration on the survival of STm grown in shaking and static cultures. STm survival was dramatically decreased between 1-1.5 h of drying when the water activity dropped to its lowest value (Fig. 8). These results support previous findings suggesting that loss of water is directly linked to cell death (Billi & Potts 2002).

In order to examine if reduced tolerance to desiccation of static culture is unique to the tested STm strain (14208), we have tested desiccation tolerance of another STm strain SL1344, as well as two other serotypes of *S. enterica: S.* Newport, and *S.* Enteritidis. We found that reduced tolerance to desiccation of static culture is common in all three tested *Salmonella* serotypes (Fig.9-11).

4.2 Comparison between shaking and static cultures

To gain knowledge regarding the mechanisms responsible for the differences in desiccation tolerance between shaking and static cultures, we first compared the growth curves to make sure that both cultures were found to be at the same growth phase when harvested and tested for desiccation tolerance. Indeed, both cultures were already at early stationary phase at 8 h (Fig. 12), indicating that the observed difference between static- and shaking-cultures was not attributed to the growth phase.

Shaking and static cultures differ in their physiologic state due to the different level of aeration. In order to identify specific proteins that participate in the adaptation of STm to the two growth conditions, we have compared total intracellular proteins derived from shaking and static culture by SDS-PAGE and mass spectroscopy. Several proteins were identified which were differentially expressed in shaking culture: SbmC, YbgI, SodB, Gst, and Bfr (Table 7). SbmC is a DNA gyrase inhibitor that belongs to the SOS regulon of *E. coli*. The SOS response is a global regulatory network activated as a response to DNA damages (Oh et al., 2001). YbgI is a hydrolase-oxidase**,** metal binding protein of unknown function. SodB is an iron-binding superoxide dismutase, which catalyzes the conversion of superoxide anion into hydrogen peroxide, thus protecting cells from ROS damages (Benov, 2001). Gst is a glutathione S-transferase, which protects the cell against oxidative-stress by catalyzing the addition of glutathione to endogenous or xenobiotic, often toxic electrophilic chemicals (Vuilleumier and Pagni, 2002); Bfr is a bacterioferritin, a member of a broad superfamily of iron storage

proteins, named ferritins. The primary function of bacterioferritins is likely to be detoxification of iron and protection against oxidative damages induced by O_2 and its radical products (Carrondo, 2003). Knockout mutants of the genes encoding for these proteins were prepared and tested for dehydration tolerance. Only the Δ*bfr* mutant was compromised in desiccation tolerance, demonstrating ~ 30% survival compared to the WT strain (Fig.14). The involvement of the *bfr* gene in tolerance to desiccation stress was confirmed by genetic complementation (Fig.15). The fact that *sbmC, gst, ybgI, sodB* mutations didn't affect desiccation tolerance doesn't necessary mean that these genes are not involved in the cellular response to drying. *Salmonella* is known to have three superoxide dismutases genes (*sodA, sodB, sodC*), and nine glutathione S-transferases (Fang et al., 1999; Vuilleumier and Pagni , 2002), therefore knockout in one of the genes may not be sufficient to affect cellular survival upon desiccation stress. It is important to note that 1D SDS-PAGE did not reveal all existing differences between the proteomes of shaking and static cultures. For a more comprehensive proteomic analysis, other methodologies must be used, such as two-dimensional gel electrophoresis or quantitative mass spectrometry.

Bacterioferritin (Bfr) is an iron storage protein that also plays a role in protecting cells during aerobic conditions, when bioavailable form of iron, ferrous ion Fe(II) may be cytotoxic, by reacting with oxygen to generate toxic free radicals. Bacterioferritins sequester the iron inside a cavity, away from dioxygen, hydrogen peroxide and superoxide (Carrondo, 2003). The *bfr* gene is regulated by the *fur* (ferric uptake) global regulator. *fur* acts as a repressor of about 30 genes, which are involved in environmental iron uptake (Asad et al., 2004).

It is possible that the sensitivity of the Δ*bfr* mutant to desiccation may be a result of enhanced formation of hydroxyl radical. Absence of Bfr protein may result in high levels of available Fe(II), which may react with H_2O_2 (that is generated during desiccation) to produce OH^- in a Fenton reaction (see below). ROS produced during this reaction are harmful to DNA and other biomolecules (Imlay et al., 1988).

Fenton reaction (Benov, 2001):

$$(1)\ Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\cdot + OH^-$$

$$(2)\ Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + OOH\cdot + H^+$$

To examine this assumption, we have challenged the Δ*bfr* mutant to H_2O_2 stress, expecting that the mutant, having higher levels of Fe(II), will be more sensitive to H_2O_2 compared to the WT. Nevertheless, the Δ*bfr* mutant withstood H_2O_2 stress similar to the WT strain. These results are in agreement with a study of Touati et al. (1995) who found in *E. coli* that Bfr did not affect cellular H_2O_2 sensitivity, while ferritin iron storage protein (Ftn), another cellular iron storage protein, did. The major difference between Ftn and Bfr is the presence of heme moieties in the Bfr complex, which reduces the core iron for its export into the cytoplasm (Yasmin et al., 2011).

Thought it was suggested that the function of *bfr* in *Salmonella* and *E. coli* is to protect cells against oxidative stress (Carrondo, 2003), neither we nor Touati et al. (1995) did observe *bfr*-related antioxidative properties. Further investigation is required in order to understand the function of the *Salmonella* bacterioferrtitin (Bfr) in desiccation tolerance.

MS analysis revealed three proteins with anti-oxidative functions (Bfr, SodB and Gst) that were highly expressed in shaking culture compared to static culture. We assumed that the difference in protein expression between shaking and static cultures is related to differences in the level of oxygen during bacterial growth. This is supported by previous studies, demonstrating that under static conditions bacterial culture has a lowered oxygen level (Wyckoff et al., 2002; Gaines et al., 2005). Lowered oxygen level during growth may down-regulates the expression of oxidative-stress response genes. The cellular capacity to respond and protect the cells from oxidative stress seems to be an important component of desiccation tolerance (Kranner et al., 2005; Fredrickson et al., 2008; Contreras-Porcia et al., 2011). This notion may explain why static STm cultures are more susceptible to desiccation stress than shaking cultures.

4.3 Anaerobic culture susceptibility to desiccation and oxidative stresses

In order to provide evidences that the higher susceptibility of static culture to desiccation stress is indeed related to lowered oxygen access during growth, STm cells were grown under controlled anaerobic conditions and its survival following dehydration was investigated. Anaerobically-grown cells were found to be more susceptible to desiccation stress than aerobically grown cells (Fig.16).

In order to further investigate the hypothesis that anaerobically grown cells are more sensitive to desiccation because of their reduced ability to respond to oxidative stress, we challenged aerobically- and anaerobically-grown cells to hydrogen peroxide and paraquat (an intracellular

superoxide anion generator). Anaerobic culture was found to be more susceptible to desiccation and H_2O_2 stresses than aerobic culture, while both cultures had comparable tolerance to paraquat (Fig.17). These results suggest that susceptibility of anaerobic culture to desiccation may be related to the increased susceptibility to H_2O_2.

Imlay, (2008) hypothesize that anaerobic *E. coli* is more tolerant to paraquat than to H_2O_2 because even under anaerobic conditions bacteria express SODs at a level sufficient to scavenge superoxide anion, and because superoxide anions are considered less toxic to cell compared to H_2O_2 .

During aerobic growth microorganisms continuously produce ROS as a side effect of metabolic pathways, mainly during oxidative phosphorylation. In order to protect themselves from ROS, bacteria activate mechanisms to scavenge free radicals, and repair damaged molecules, such as DNA and proteins. It has been shown that aerobically grown *E. coli* and *S.* Thypimurium express a number of such enzymes, including superoxide dismutases, which scavenge superoxide anion; catalases, which reduces hydrogen peroxide, and alkyl-hydroperoxidases, which reduces organic peroxides (Far et al., 1991; van der Straaten et al., 2004). It is likely that the presence of these or some of these enzymes, in aerobically grown dehydrating cells enable STm to better cope with the oxidative stress occurring during desiccation, compared to anaerobically-grown cells.

4.4 Involvement of soxR and oxyR in desiccation tolerance

In order to confirm that the cellular oxidative-stress response affects STm survival during dehydration, desiccation tolerance was examined in mutants deficient in central oxidative stress response regulators, *soxR* (superoxide stress sensor) and *oxyR* (peroxide stress sensor). SoxR activator is a member of two-component signal transduction pathways responsible for the dismutation of superoxide anion to molecular oxygen and hydrogen peroxide (*soxRS*). Superoxide is sensed by SoxR, by oxidizing its iron cluster $[2Fe\text{-}2S]^{+}$ and then the activated SoxR up-regulates expression of SoxS, which is a transcription regulator of the *soxRS* regulon (Lushchak, 2010). The *soxRS* regulon includes 20 genes, some of them have ROS protective activities, such as *sodA*, encoding a scavenger of O^{2-} anions, *nfo* encoding an endonuclease IV involved in repairing DNA damages, and *fur*, encoding a global regulator that represses iron uptake, which reduces available iron for Fenton reaction (Imlay, 2008). Other enzymes induced by SoxRS are involved in cellular metabolism, including oxidant-resistant dehydratase

isozymes, such as *fumC* encoding a fumarase C, and *acnA,* encoding aconitase A. A few other enzymes that are regulated by SoxRS have an iron sulfur cluster repair activity or stabilization properties: Zwf is a Glucose-6-phosphate dehydrogenase; FldA is a flavodoxin A, and FldB is a flavodoxin B; other group of genes regulated by SoxRS regulon are related to efflux of antibiotics and organic solvents, including *acrAB,* a drug efflux pump, *tolC,* an OMP component of drug efflux pumps, *micF* an *ompF* antisense sRNA, *marAB*- encoding a multiple antibiotic resistance operon, *nfnB*- nitroreductase, and *rimK,* -encoding ribosome modification enzyme (responsible for post translational modification of ribosomal protein S6) (Hidalgo et al., 1998; Imlay et al., 2008).

OxyR is a one component signal transduction system consisting of OxyR protein, which is a peroxide sensor and a transcription regulator of *oxyR* regulon which is responsible for the decomposition of hydrogen peroxide to water and molecular oxygen (Martinez et al., 1997).

OxyR protein belongs to the LysR family of transcription activators and exists in both oxidized and reduced forms. OxyR is activated after oxidation by hydrogen peroxide. Activated OxyR regulates the expression of about 20 genes, including *katG* and *ahpCF* encoding two hydrogen peroxide scavengers; *dps*–encoding a DNA protective protein (Halsey, et al., 2004); *grxA*-encoding glutoredoxin I that catalyzes the reduction of disulfides using reduced glutathione; *trxC* encoding a thioredoxin able to reduce disulfides; *hemH* encoding ferrochelatase that catalyzes the last step of protoheme biosynthesis (heme is an essential cofactor for both catalases *katG* and *katE,* increased heme levels associated with increased hydroperoxidase production); *gor* encoding a glutathione reductase; *fur* (see above), *suf* operon whose exact function is not clear; and few other genes with unknown function (*yaaA*, *yaiA*, *ybjM*, *yljA*) (Zeng et al., 2001; Imlay 2002).

Interestingly, the survival of the Δ*soxR* mutant, following desiccation, H_2O_2 and paraquat challenges, was comparable to that of the WT strain (Fig. 19). This finding is in contrast to previous studies, which reported that an *E. coli soxR* deletion mutant was highly sensitive to paraquat (Wu and Weiss**,** 1992; Membrillo-Hernández et al., 1997). However, these studies used cells in exponential growth phase while in our study, stationary phase cells were used. It is important to note that there is no available data on the sensitivity of *Salmonella soxR* mutant to paraquat, while a *Salmonella soxS* deletion mutant was reported to be sensitive to paraquat during exponential growth (Fang et al., 1997). Further investigation is required regarding

potential difference in *Salmonella* sensitivity to paraquat at exponential and stationary phase of growth in both the WT and the *soxR* deletion mutant.

Previous study found that STm *oxyR* deletion mutant is sensitive to H_2O_2 and shows a high spontaneous mutation rate (Cristman et al., 1989). We found that the Δ*oxy*R mutant was compromised in both H_2O_2- and desiccation-stress tolerance, demonstrating ~ 5% survival compared to the WT strain (Fig. 19). The involvement of the *oxyR* gene in tolerance of STm to desiccation stress was confirmed by genetic complementation (Fig. 20). These results further support the notion that there may be connection between the cellular H_2O_2 response and desiccation tolerance.

4.5 Desiccation tolerance requires de-novo protein synthesis and H_2O_2 detoxification

Cm treatment only slightly reduced desiccation- and H_2O_2 -tolerances in aerobic culture, while a significant decrease (2-log reduction) in cell viability occurred upon exposure of anaerobic culture to the two stresses (Fig. 21). *De-novo* protein synthesis in anaerobic culture is required in order to adapt to the two stresses. These results further support our assumption that during anaerobic growth the expression of anti-oxidative stress genes is inhibited, so in order to withstand oxidative stress, *de novo* protein synthesis is needed. On the other hand, aerobically grown cells express high basal levels of anti-oxidative stress proteins, which can act immediately upon exposure to oxidative stress during dehydration.

To further support this notion, total oxidative scavenging capacity (TOSC) of STm was measured (Fig. 22). Our results suggest that anaerobically-grown cells have reduced TOSC compare to aerobically-grown cells. Similarly, the TOSC of the *ΔoxyR* mutant was decreased compared to that of the WT strain (Fig. 23), perhaps due to reduced expression of the two *oxyR*-regulated scavengers of hydrogen peroxide, the KatG catalase and the AhpCF alkyl hydroperoxide reductase (Imlay, 2008).

In order to further investigate the role of H_2O_2-detoxification in desiccation tolerance, both aerobic and anaerobic cultures were pretreated with the catalase inhibitor, sodium azide (NaN_3), and TOSC, desiccation- and H_2O_2-stress tolerance were examined. TOSC of both cultures was reduced probably due to inhibition of catalases. Anaerobic cultures showed much lower TOSC than aerobic culture, indicating the presence of less ROS scavengers (Fig. 24). Sodium azide significantly reduced STm tolerance to both H_2O_2- and desiccation-stress (Fig. 25), supporting the idea that catalase inhibition reduces the ability of cells to scavenge H_2O_2

and leads to increased susceptibility of STm to both stresses. The greater effect of the catalase inhibitor was on anaerobic culture, in either TOSC analysis or H_2O_2 and desiccation challenges, further supports the notion that anaerobic culture may initially have lower catalases level compared to aerobic cultures.

STm possesses multiple H_2O_2 scavengers including three catalases (KatE, KatG, and KatN) and two alkyl hydroperoxide reductases (AhpCF and TsaA) (Hebrard et al., 2009). *katE* and *katN* genes are induced by sigma factor *rpoS*, while *ahpCF* and *katG* genes are up-regulated by *oxyR* (Farr and Kogoma, 1991; Zeng et al., 2001; Robbe-Saule et al., 2001, Imlay, 2008). Previous studies found that *katE* mutant as well as *ahpCF* and *katG* double mutant are more susceptible to hydrogen peroxide challenge than the WT strain (Seaver and Imlay, 2001). Neither *katN* nor *tsaA* mutant were found to be susceptible to hydrogen peroxide (Robbe-Saule et al., 2001; Hebrard et al., 2009). We therefore decided to tests the involvement of the three genes *katE*, *katG* and *ahpCF* in desiccation tolerance of STm. Knockout mutants in each of these genes were generated and tested for desiccation tolerance. Since absence of the *ahpCF* gene can be compensated by overexpression of *katG* (Seaver and Imlay, 2001), a double mutant containing both *ahpCF* and *katG* mutations was also generated.

We found that *ΔkatE* and *ΔahpCF/katG* mutants were more susceptible to H_2O_2 than the WT strain, while survival of the single mutant, Δ*ahpCF*, or Δ*katG* was comparable to that of the WT strain (Fig. 26). These results are in agreement with previous findings in *E. coli* (Seaver, et al., 2001; Imlay, 2002 and 2001-above). The Δ*ahpCF/katG* double mutant (but not the others) was compromised also in desiccation tolerance, demonstrating ~ 10% survival compared to the WT strain (Fig. 27). These results demonstrate that both Δ*ahpCF* and *katG* genes are required for cellular tolerance to desiccation and support the role of H_2O_2 scavenging in desiccation tolerance. It was expected that the *ΔkatE* mutant, which was also more sensitive to H_2O_2, will be less tolerant to desiccation, however, its tolerance to desiccation was comparable to that of the WT strain (Fig. 27), suggesting that the KatE catalase is not involved in STm desiccation tolerance. Previous studies found that low concentrations of H_2O_2 are scavenged by both and AhpCF and KatG (Seaver et al., 2001; Imlay, 2002), while KatE is activated only in the presence of high H_2O_2 concentrations (Mulvey et al., 1990; Far et al., 1991; Imlay, 2008). It is possible that during desiccation, relatively low concentrations of H_2O_2 are generated, which do not result in *katE* induction.

4.6 Summary

In this research we report for the first time that anaerobically-grown *Salmonella* are more susceptible to desiccation than aerobically-grown bacteria. We showed the involvement of the *bfr* gene, which encodes bacterioferitin in desiccation tolerance of STm cells. In addition we showed that the oxidative stress global regulator, *oxyR* and both alkylperoxide reductase and catalase, encoded by the *ahpCF* and the *katG* genes are required for desiccation tolerance. Desiccation tolerance was shown to be related to bacterial TOSC, supporting previous suggestion on the role of oxidative stress response in desiccation tolerance (Kranner et al., 2005; Fredrickson et al., 2008; Contreras-Porcia et al., 2011).

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תקציר

בשנים האחרונות עלה בעולם מספר הדיווחים על התפרצויות של מחלת הסלמנלוזיס כתוצאה מאכילת מוצרי מזון עם תכולת מים נמוכה, כגון שוקולד, חטיפים שונים, חמאת בוטנים, תבלינים ואבקת חלב. נמצא כי חיידקי *Salmonella* הגורמים למחלה יכולים לשרוד במצב יבש על משטחים שונים, בקרקע ובמוצרי מזון לאורך שנים. הישרדות בתנאי יובש, בחומר הגלם ועל פני משטחים במפעלי מזון, מעלה את פוטנציאל הזיהום של הפתוגן ומדגישה את הצורך לחקור ולהבין את מנגנוני ההישרדות של החיידק בתנאי יובש. כיום, יש מידע מועט בלבד על הגורמים המאפשרים הישרדות של *Salmonella* ביובש. מטרת עבודה זו היא להרחיב את הידע לגבי הגורמים המשפיעים על עמידות החיידק ליובש, לזהות גנים שמעורבים בעמידות ליובש ולנסות להבין את מנגנון פעילותם.

במחקר זה הראנו כי ישנם גורמים חיצוניים שמשפיעים על הישרדות של *Salmonella* ביובש. מצאנו שקצב ייבוש מהיר פוגע ביכולת של החיידק לשרוד במצב יבש לאורך זמן. כמו כן, מצאנו שחיידקים שנמצאים בשלב גידול לוגריטמי יותר רגישים ליובש לעומת חיידקים בשלב הגידול הסטציונרי. מצאנו לראשונה כי חיידקים שגדלו בתרבית עם טילטול עמידים יותר בתנאי יובש לעומת אלה שגדלו בתנאים ללא טילטול. מכאן ניתן להניח כי חשיפה מוקדמת לריכוזי חמצן שונים משפיעה על ההישרדות בעקת יובש. בכדי למצוא חלבונים המעורבים בעמידות ליובש, בוצעה אנליזת חלבונים של תרבית עם ובלי טילטול. נמצא שחיידקים שגדלו עם טילטול בטאו מספר חלבונים, שלא נמצאו בתרבית שגדלה ללא טלטול. החלבונים שזוהו הם: SodB- superoxide dismutase, Gst- glutathione-S-transferase, Bfr- bacterioferritin, SmbC- DNA gyrase inhibitor, YbgI- hydrolase oxidase. לצורך בדיקת מעורבות של גנים אלה בהישרדות של החיידק ביובש, יצרנו מוטנטים איזוגניים בכל אחד מהם (Δ*bfr,* Δ*sodB,* Δ*gst,* Δ*smbC,* Δ*ybgI*) ובדקנו את עמידותם ליובש. מצאנו שרק מוטציה בגן *bfr* שמקודד ל-bacterioferitin, המעורב בפעילות אנטיאוקסידטיבית בתא, פוגעת ביכולת של החיידק לשרוד ביובש.

כיוון שבהעדר טילטול נוצרים בתרבית תנאים של מחסור בחמצן, בדקנו גם הישרדות של חיידקים שגדלו בתנאים אנארוביים מבוקרים על מצע אגר ומצאנו שגידול בתנאים אנארוביים אכן פוגע ביכולת החיידק לשרוד ביובש. הראנו כי תנאי הגידול משפיעים על יכולתו הכללית של החיידק לנטרל צורוני חמצן חופשיים(TOSC) וכי בתנאים אנארוביים לחיידקים יכולת ניטרול נמוכה של צורוני חמצן. כמו כן, הראנו לראשונה שעיכוב פעילות הקטאלזות על ידי סודיום אזיד, גרם לירידה בעמידות ה- *Salmonella* ליובש, ממצא המחזק את חשיבות היכולת האנטי-חימצונית של החיידק בעמידות ליובש. עם זאת, מוטציה ב-*katE* המקודד לאחד מהקטלאזות, אשר גורמת לרגישות למי חמצן, לא השפיעה על עמידות החיידק ליובש. מאחר ש-KatE מבוטא בתא רק בחשיפה לריכוזים גבוהים של H_2O_2, יתכן כי במהלך ההתייבשות נוצרים בחיידק ריכוזים נמוכים יחסית של H_2O_2 אשר אינם גורמים לביטוי האנזים ועל כן אנזים זה אינו מעורב בניטרול של H_2O_2. גם מוטציות ב-*katG* אשר מקודד לקטלאז אחר וב-*ahpCF* אשר מקודד ל- alkylperoxidase לא השפיעו על עמידות החיידקים ל-H_2O_2 וליובש. אולם, מוטציה כפולה *ahpCF/katG* פגעה בעמידות של החיידקים גם למי חמצן וגם לעקת יובש. ממצא זה תואם את הידוע בספרות כי מוטציה בכל אחד מהגנים הללו גורמת לביטוי יתר של הגן השני ונדרשת מוטציה כפולה בשני הגנים האלה כדי לפגוע ביכולת הניטרול

של H_2O_2. לחיזוק הקשר בין עמידות לעקה חימצונית לעמידות לעקת יובש, הראנו לראשונה כי מוטציה ב-*oxyR* המקודד לחלבון בקרה מרכזי של עקה חימצונית גרמה לירידה בהישרדות החיידקים בחשיפה לשתי העקות.

תוצאות מחקר זה תומכות בממצאים קודמים המצביעים על קשר אפשרי בין תגובה של חיידקים לעקת חימצון לבין עמידותם ליובש ומציעות תפקיד מרכזי ליכולתו של התא לנטרל צורוני חמצן חופשיים בהישרדות החיידקים ביובש. במחקר זה הראנו לראשונה מעורבות של קטלזות וחשיבות ה-TOSC בעמידות של החיידקים ליובש. על מנת להבין את מנגנון הפעילות המשותף לשתי העקות יש צורך במחקרים נוספים; חשוב למדוד את רמות צורוני החמצן שנוצרים כתוצאה מהתייבשות החיידק בכדי לבנות מודל מחקרי מדוייק יותר. כמו כן, חשוב לחקור את המעורבות של גנים נוספים המעורבים בתגובה לעקה חימצונית בעמידות ליובש, כגון, גנים שקשורים לתיקון נזקי חימצון. נושא נוסף שמצריך מחקר, הוא השפעת אנטיאוקסידנטים על הישרדות החיידקים ביובש, לאור העובדה שהם נמצאים בשימוש נרחב כתוספים בתעשיית המזון.

זיהוי גנים המעורבים בעמידות של חיידק הסלמונלה ליובש

עבודת-גמר

מוגשת לפקולטה לחקלאות, מזון וסביבה על שם רוברט ה. סמית

של האוניברסיטה העברית בירושלים

לשם קבלת תואר 'מוסמך למדעי החקלאות'

על ידי

תמי חזין

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