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Use of Transposon Screening for Salicylic Acid-Assisted Desiccation Killing in Salmonella

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Use of Transposon Screening for Salicylic Acid-Assisted Desiccation Killing in *Salmonella*

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by

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ABSTRACT

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Salmonella enterica serovar Typhimurium is one of the most prevalent food-borne pathogens, affecting millions around the world every year, making it a threat to global health. *Salmonella* possesses the ability to survive the normally lethal condition of desiccation, however, discovery of the genes and mechanisms behind this phenomenon are still ongoing. Using a transposon mutagenesis approach to construct a broad transposon library, this study aimed to uncover genes that may be contributing to changes in *Salmonella*'s survivability under desiccation, particularly when exposed to the antimicrobial molecule salicylic acid. Building on previous findings showing salicylic acid can alter cell viability through differential gene regulation, transposon mutants were exposed to salicylic acid and subsequently desiccated to screen for mutants that displayed an alteration in survival phenotypes. This work identified a transposon mutant with an interruption of the porin-coding gene *ompC* that displayed an augmented survivability phenotype under these conditions, leading to further exploration into the origin of this phenomenon.

DEDICATION

I dedicate this thesis to Keldan. Every moment leading up to its completion and every moment after belongs to you just as much as it does to me. I share every victory and success with you. My darling, you have been my inspiration for every second, every risk, every decision, every aspiration, and every breath I have taken from the moment you entered this earth. I hope that you will follow your dreams and walk your own path, staying true to the beautiful, brave, kind, passionate, and caring soul that I have watched you become. Guiding you through the years has been the principal honor and highest accomplishment of my life. Don't be afraid to be different. Don't be afraid to take the road less traveled. I will stand beside you, support you, and love you until the day I return to the earth. You will never be alone, love.

A snail with grace and charm unending
Mantle of ivory sparkling with dew
Trails upon flowers she lovingly leaves
With haste and devotion, I chose to pursue.

In the meadows and forests did I search
For a sign of her glistening light
But she did hide among the moss
A pearl within the fading night.

In the pouring rain I caught a glimpse
And clambered to her side
For in her embrace, I am whole
I hold her close with beaming pride.

All that is gold does not glitter,
Not all those who wander are lost;
The old that is strong does not wither,
Deep roots are not reached by the frost.

-The Riddle of Strider
The Lord of the Rings: The Fellowship of the Ring by J.R.R. Tolkien

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CHAPTER 1. INTRODUCTION

Bacterial Disease in Global Health

Humans undoubtedly can attribute their recent evolutionary history and worldwide expansion to their unique relationship with animals in an agricultural context. While the domestication of animals has given many benefits, it has also come with disadvantages. Repeated close contact between humans and animals has given potential pathogens an opportunity to cross the species barrier, giving rise to disease (zoonoses). Zoonoses are thought to comprise 60% of emerging infectious diseases, and classified by etiology, bacterial pathogens are estimated to account for an average 40% of known zoonoses [1–3]. Domestic agricultural practices appear to be enabling the cross-species transmission of enteric bacteria via frequent exposure to environments contaminated by animal feces or through contaminated food, water, and non-food surfaces [4].

There are many bacterial zoonoses that are currently contributing to global mortality. Estimates place global zoonotic infections at one billion annually, with millions of deaths per year [2]. For children ages 5 and under, the second leading cause of death globally in children is diarrheal disease, with a disproportionate burden located in developing nations [5]. One such bacterial zoonosis causing significant diarrheal illness and subsequent mortality is the genus *Salmonella*, which is one of the most common food-borne bacterial infections globally [6].

Salmonella enterica

Genus *Salmonella* are a group of Gram-negative, non-sporulating, facultative anaerobic, flagellated bacilli bacteria belonging to the family Enterobacteriaceae. Ubiquitously found within the intestinal tract of both humans and a wide range of animals, Salmonellae are zoonotic pathogens that can be passed between species to cause disease [7]. Though *Salmonella* only has

two species within the genus, the species *Salmonella enterica* has diversified into over 2600 different serovars, many with the capability of causing disease in humans with varying degrees of severity depending on the serovar [8]. The serovars can be further divided into typhoidal and non-typhoidal varieties, meaning capable of causing Typhoid fever or Salmonellosis, respectively. Despite different disease outcomes, both spread via contaminated food and water [9]. Non-Typhoidal *Salmonella* (NTS) are of an endemic nature globally, which usually presents as a self-limiting gastroenteritis that causes diarrhea, abdominal cramps, fever, nausea, and vomiting [7,9,10]. An estimated 5% of infections disseminate to other organs post-bacteremia onset that can be followed by septicemia, resulting in hospitalization or death [9,11,12]. NTS has numerous animal reservoirs, which makes eradication and prevention more challenging [10,13,14].

Though there are thousands of serovars of NTS, there are a select few that have proven to be immensely more problematic than others. One such serovar, *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium) is responsible for 11% of food-borne infections globally and a primary causative agent of food-borne illness [6]. *S*. Typhimurium causes an estimated 94 million cases globally per year resulting in a subsequent 155,000 deaths, with a disproportionate burden being placed on children 5 and younger, elderly, and immunocompromised individuals in developing nations. Developed nations are not exempt, as the United States alone sees 1.35 million infections and 420 deaths per year [7,9,12].

Chickens as Primary *Salmonella* Reservoirs

Animals have long been primary reservoirs of *Salmonella,* as it can infect a wide range of domestic and agricultural animals. One such animal has earned a reputation for being a prevalent reservoir of *S*. Typhimurium and other serovars: the chicken [14]. Chickens have been found to be the primary cause of animal-product related foodborne illness in the United States. Though

widespread poultry vaccination campaigns have been enacted since the 1990s, it has been insufficient in eliminating the instances of Salmonellosis in humans from their products [15].

Chickens possess a biology that allows for harboring and transmission of *Salmonella* bacteria. Adult chickens are colonized in the intestinal distal ileum and cecum via oral inoculum, and the ovary and oviduct are subsequently colonized in hens [16]. While generally asymptomatic, colonization causes subclinical systemic infection that allows colonized birds to transmit bacteria without intervention [16–18]. While chicken meat can be contaminated during butchering processes, contamination of the chicken egg is also of concern. Prior to egg laying, bacteria can infiltrate the egg interior such as the yolk, albumin, or shell membranes, when calcification of the shell has not yet been completed [19]. During egg laying, bacteria-laden feces are again deposited on eggshells as the egg passes through the oviduct, or laid eggs can become infected horizontally from a contaminated environment [16,18,19]. Contamination by *S.* Typhimurium is of great consequence, as widespread incidence of *Salmonella*-infected eggs within the global market is of chief concern.

Salmonella and Desiccation

Biologically, eggshells possess multifactorial components that form a generally antibacterial environment, making them a poor surface for microbial growth. The eggshell exterior is composed of a thick layer of mostly hydrophobic $CaCO_3$, constituting a dry and harsh barrier that is generally prohibitive for bacterial survival. Beneath the calciferous shell lies a series of membranes that contain numerous chemical barriers such as lysozyme, ovotransferrin, and proteases [19–21]. While microscopic pores line the exterior shell and generally serve as a method of water and gas exchange for the growing embryo, these pores can harbor bacteria that can survive the harsh conditions of the shell, forming entry points into the inner layers of the egg

[19,22]. *S.* Typhimurium is a key pathogen that can subvert the defense mechanisms the egg possesses due to its ability to survive desiccation on the surface of the eggshell and inside the egg proper [19,23]. These mechanisms have yet to be fully elucidated, so more research is needed to understand how *Salmonella* survives desiccation.

It has long been accepted that *S.* Typhimurium is able to survive drying and desiccation for extended periods, with previous studies showing it able to survive extreme heat and still retain a reasonable population of viable cells after dehydration [23]. One well-studied mechanism for survival of desiccation is formation of biofilms, which form as a result of generally circulating planktonic cells in a contaminated environment attaching to a surface in response to environmental stress [24]. Biofilms are complex communities of bacteria that aggregate on surfaces encased in a matrix of extracellular polymeric substances, which are polymers of proteins, lipids, nucleic acids, and exopolysaccharides. Contributing to bacterial persistence, biofilms have been shown to offer protection against many stressors including ultraviolet radiation, desiccation, and antibiotics [24–26].

Salicylic Acid Reduces *Salmonella* Desiccation Survival Through an Unknown Mechanism

Due to *Salmonella*'s persistence on desiccated surfaces and subsequent human exposure, there is a push to identify the mechanisms that permit persistence post-desiccation and chemicals that can inhibit *Salmonella* desiccation survival. Preliminary data from our lab suggests that addition of salicylic acid prior to desiccation can have adverse effects on *Salmonella* survival [27,28]. Salicylic acid is an organic compound that is known to have antimicrobial properties in addition to being utilized by bacteria, plants, and fungi for many cellular processes. Well studied in plants, it is used as a cell signaling molecule to mediate host defenses against microbial pathogens [29]. Salicylic acid has also been shown to impact a broad array of bacterial families

causing a diverse range of functional alterations spanning from antibiotic resistance to modulation of biofilm formation [30–32]. In *Salmonella* and other closely related *Enterobacteriaceae*, it has been observed to alter transcriptional and translational regulation, resulting in changes to protein expression. Most notably, it induces *marRAB*, a global regulatory operon controlling many survival and virulence genes that allows for multiple antibiotic resistance and expression of outer membrane porins and efflux pumps [33–35]. Though its effects on desiccation have not been studied to date, salicylic acid has been shown to affect expression and mechanisms of common bacterial virulence factors, such as biofilm formation and quorum sensing [30–32,36]. Given salicylic acid's penchant for interfering with proper function of numerous virulence factors, it is an interesting avenue for exploration of potential effects on *Salmonella* desiccation survival with much yet to uncover.

Discovering the mechanisms behind *S.* Typhimurium's ability to survive desiccation is crucial to the reduction of food-borne illness, making it of significant importance to global health. Building on previous findings from our lab related to the relationship between salicylic acid and desiccation survival, this study aimed to uncover potential genes that are responsible for *S.* Typhimurium persistence post-desiccation with an emphasis on survival post-salicylic acid exposure, utilizing a transposon mutagenesis approach [27]. To this end, a library of over 50,000 *S.* Typhimurium transposon (Tn) mutants was constructed and subjected to desiccation assays and growth studies with various concentrations of salicylic acid to discover and isolate mutants with pronounced survival phenotypes, yielding valuable insight about roles and functions of genes related to desiccation survival and salicylic acid–mediated killing.

CHAPTER 2. MATERIALS AND METHODS

Strains, Primers, & Recombinant DNA

Salmonella enterica serovar Typhimurium strain 14028S (CS093) was used as the wild-type reference strain for all experiments unless otherwise noted (Table 1). *S.* Typhimurium was typically cultivated on Luria Bertani (LB) agar or in LB broth. If needed, media was supplemented by antibiotics at the following concentrations: ampicillin 100 ug/ml, chloramphenicol 34 ug/ml, gentamicin 30 ug/ml, and kanamycin 100 ug/ml unless otherwise listed (Table 2). All *Escherichia coli* strains were propagated in LB broth, with antibiotics when appropriate, at 37°C for 24 hours shaking unless otherwise specified. Freezer stocks were prepared by mixing 670 µL 40% glycerol with 670 µL overnight culture and stored at –80°C.

Table 1 Bacterial Strains

Strain Name	**Genotype**	**Source, PMID**
CS093	*Salmonella enterica* serovar Typhimurium strain 14028S	ATCC [28] PMID: 26060330
EPST138	CS093 *sseG*::Cam	This study
EPST137	CS093 with spontaneous streptomycin resistance	This study
SEST001	EPST138 Tn7::pGRG37-ara-Gent	This study
SEST002	CS093 Tn7::pGRG37-ara-Gent	This study
MC1061	*Escherichia coli araD139, Δ(araA-leu), Δ(lac)X74, galK16, galE15(GalS), lambda-, e14-, mcrA0, relA1, rpsL150(strR), spoT1, mcrB1, hsdR2*	ATCC [37] PMID: 6697493
SM10 λ*pir*	*Escherichia coli thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km λ*pir*	Invitrogen [38]
S17-1 λ*pir*	*Escherichia coli thi, pro, hsdR, recA, endA* [RP4-2 Tc::Mu Km::Tn7 (Tp Sm)], λ*pir*	Invitrogen [39] PMID:11160899

SM10-pBT30	SM10 lambda *pir*, pBT30	This study
pir+ DH5α	*Escherichia coli fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17 λ pir*	Invitrogen [40] PMID: 1943786
SETn library	EPST137 with integrated Mariner Tn from pBT30	This study
SEST003	EPST138 Tn7::pBAD	This study
EM369	CS093, pKD46	ATCC [28] PMID: 26060330
SEST004	EM369 LRompCTn::Cam	This study
SEST005	EM369 LRompCFL::Cam	This study
SEST006	EM369 LRompCFL::Kan	This study
SEST007	CS093 ompCTn::Cam	This study
SEST008	CS093 ompCFL::Cam	This study
SEST009	CS093 ompCFL::Kan	This study
SEST010	SEST007 Tn7::Gent-ompC Comp	This study
SEST011	CS093 Tn7::Gent-empty	This study
SEST012	SEST007 Tn7::Gent-empty	This study
SEST013	SEST008 Tn7::Gent-empty	This study

Table 2 Recombinant DNA, Bacteriophage, and Plasmid Vectors

Name	**Description**
pRK2013	Helper plasmid with R2K transfer genes, KanR [41] PMID: 377280
pTNS1	Tn7 transposase expression vector, AmpR, RSF Ori [42] PMID: 15908923
pGRG37-ara-Gent	Tn7 gateway cloning vector for *Enterobacteriaceae*, AmpR/GentR, araBAD promoter
P22HT*int*	*S.* Typhimurium bacteriophage with high transduction and integrase deletion mutations [43] PMID: 1250221

pKD3	Bacterial vector commonly used for CamR fragments, CamR/AmpR, R6K [44] PMID: 10829079
pKD4	Bacterial vector commonly used for KanR fragments, KanR/AmpR, R6K [44] PMID: 10829079
pBT20	Himar1 Mariner Transposon, GentR/AmpR, R6K [45] PMID: 15659157
pBT30	Himar1 Mariner Transposon, GentR/AmpR, R6K [45] PMID: 15659157
pKeldSE01	Himar1 Mariner Transposon in SM10, CamR/AmpR, R6Kγ
pKeldSE02	Himar1 Mariner Transposon in SM10, CamR/AmpR, R6Kγ
pKeldSE03	Himar1 Mariner Transposon in S17-1, KanR/AmpR, R6Kγ
pKeldSE04	Himar1 Mariner Transposon in SM10, KanR/AmpR, R6Kγ
pKeldSE05	Himar1 Mariner Transposon in S17-1, KanR/AmpR, R6Kγ
pKD46	Temperature sensitive assembly vector possessing arabinose-inducible Lambda RED machinery, AmpR [44] PMID: 10829079
pTn7Gent-empty	Suicide vector with Tn7 transposon, GentR, oriT
pTn7Gent-OmpC-$_p$ompC	Suicide vector with Tn7 transposon, GentR, oriT, native *ompC* gene & promoter

Table 3 Primers

Name	**Sequence**
Gent NEW Int 5' Out#2	GCCCATACTTGAGCCACC
pBAD MCS GRvs	GGTGAATTCCTCCTGCTAGCCC
R6kgamma-GFwd	GGGCTTCTCAGTGCGTTAC
R6kgamma-GRvs	CAACAAGCCAGGGATGTAACG
Cam-pBT30-GFwd	CGATGTTTGATGTTATGGAGCAACGATGGAGAAAAAAATCACTGGATATACCACCG
Cam-pBT0-GRvs	GAAGCCGATCTCGGCTTGAACGAATTGTCATCGCAGTACTGTTGTAATC
Kan-pBT30-GFwd	CGATGTTTGATGTTATGGAGCAGCAACGATGATTGAACAAGATGGATTGCACGC
Kan-pBT30-GRvs	GAAGCCGATCTCGGCTTGAACGAATTGTCAGAGAACTCGTCAAGAAGGC

EM411	GCACTGCATAATTCTCTTACTGTCATGCCATCC
EM410	GGATGGCATGACAGTAAGAGAATTATGCAGTGC
pBT30-marker-GFwd	CATCGTTGCTGCTCCATAACATC
pBT30-marker-GRvs	CAATTCGTTCAAGCCGAG ATG
Kan Int 5' Out	CCTCTCCACCCAAGCG
TDR1 Term-Cam	GGATGGCCTTTTTGCGTTTCTACAAACTCTTCATCGCAGTA CTGTTGTATTCATTAAGCA
KanFwd-PciI[1]	ATATTA*ACATGT*CGGATGAATGTCAGCTACTGG
KanRvs-PciI[1]	ATATTA*ACATGT*CGATTCCGAAGCCCAACC
CamFwd-PciI[1]	ATATTA*ACATGT*CCCTGCCACTCATCGC
CamRvs-PciI[1]	ATATTA*ACATGT*GCCTACCTGTGACGGAAG
Gm-up2	GATGTTACGCAGCAGGGCAGTC
Gm-down2	GTGCATCACTTCTTCCCGTATGCC
Rnd1-ARB1-Pa[2]	*GGCCACGCGTCGACTAGTAC*NNNNNNNNNNAG
Rnd1-TnM30	CACCGCTGCGTTCGGTCAAGGTTC
Rnd2-ARB	GGCCACGCGTCGACTAGTAC
Rnd2-TnM30	CGAACCGAACAGGCTTATGTCAATTC
BTK30TnMSeq	TGGTGCTGACCCCGGATGAAG
Kan Int 3' Out	CGCCTTCTATCGCCTTCTTG
Kan Int 5' Out	CCTCTCCACCCAAGCG
pKD3 Cam 5'	GTATCAACAGGGACACCAGG
Cam Int 3' Out	GCGATGAGTGGCAGGG
GentGFwd	GGATCGTCACCGTAATCTGC
OmpC_Full_LRKO_Fwd[3]	GGTTAATAACATGAAAGTTAAAGTACTGTCCCTCCTGGTA *GTGTAGGCTGGAGCTGCTTC*
OmpC_Full_LRKO_Rvs[3]	CTTTTGCTGATTAGAACTGGTAAACCAGACCCAGCGCTAC *CATATGAATATCCTCCTTAG*
OmpC_Tn_LRKO_Fwd[3]	TTACTACTTCAACAAAAACATGTCCACCTATGTTGATTAA *GTGTAGGCTGGAGCTGCTTC*

OmpC_Tn_LRKO_Rvs[3]	AAAAAGGGCCCGCAGGCCCTTTAGCAACATCTTTTGCTGA *CATATGAATATCCTCCTTAG*
pTn7Gent_GRvs	CATGCATGATCGAATTAGCTTCAAAAGCGC
OmpC_Comp_Tn7_Fwd[4]	*GCGCTTTTGAAGCTAATTCGATCATGCATG*CTGTATTTTTGCG GAGAATGGAC
OmpC_Comp_Tn7_Rvs[4]	*GAAAATCTTCTCTCATCCGCCAAAACAGCC*CAG GCCCTTTAGCAACATC
rrnB Term Gfwd	GGCTGTTTTGGCGGATGAGAGAAG
oriT Gfwd	CGGGACTACGGGCTTGAGC
oriT GRvs	CGAACGACATGGAGCGGCAC
SseG LRKO Fwd	TAGCCCAAATGCTCAGGTAGGA
SseG LRKO Rvs	CACGTTGTTCTGGCGTTACCTGA

[1] Italicized nucleotides indicate the *PciI* site

[2] Italicized nucleotides indicate the region of complementarity for the Arb-Rnd2 primer, N represents a random addition of A, C, G, or T nucleotides

[3] Italicized nucleotides indicate the region of complementarity for the antibiotic resistance marker, while the 5' sequence indicates the region of homology with the *Salmonella* genome

[4] Italicized regions indicate the region of homology with the generated vector PCR fragments to be used for Gibson Assembly

Table 4 Enzymes

Reagent	**Source**
PciI	New England Biolabs
TSAP	Promega
DpnI	ThermoFisher Scientific
T4 DNA Ligase	New England Biolabs

Table 5 Colony PCR Reaction

Reagent	**Volume**
2x Go*Taq* DNA Master Mix	5.0 µL
Primer 1	0.5 µL (12.5 pmol)

Primer 2	0.5 µL (12.5 pmol)
Lysed Culture gDNA	1.0 µL
H_2O	3.0 µL
Total	10.0 µL

Table 6 Phusion PCR Reaction

Reagent	**Volume**
Primer 1	1.0 µL (25 pmol)
Primer 2	1.0 µL (25 pmol)
Template	0.5 µL
DNase free H_2O	16.25 µL
Buffer	5.0 µL
Phusion	0.25 µL
dNTP	1.0 µL
Total	25.0 µL

Table 7 Arbitrarily Primed PCR 1 Reaction

Reagent	**Volume**
2xGo*Taq* Master Mix	12.5 µL
Genomic DNA	1.0 µL
Rnd1-ARB	2.0 µL
Rnd1-TnM30	0.8 µL
DNAse Free H_2O	8.7 µL
Total	25.0 µL

Table 8 Arb1 Reaction Thermal Cycler Protocol

Step	**Temperature**	**Time**
Step 1	94°C	3 minutes
Step 2	94°C	30 seconds
Step 3	49°C, Reducing temp by 1°C for each subsequent round	30 seconds
Step 4	72°C	3 minutes
Step 5	Go to Step 2: 15 Rounds	

Step 6	94°C	30 seconds
Step 7	60°C	30 seconds
Step 8	72°C	3 minutes
Step 9	Go to Step 6: 20 Rounds	
Step 10	12°C	Infinite Hold

Table 9 Arbitrarily Primed PCR 2 Reaction

Reagent	**Volume**
2xGo*Taq* Master Mix	25.0 µL
Rnd1 PCR Product (Template)	2.0 µL
Rnd2-ARB	0.8 µL
Rnd2-TnM30	0.8 µL
DNAse Free H_2O	21.4 µL
Total	50.0 µL

Table 10 Arb2 Reaction Thermal Cycler Protocol

Step	**Temperature**	**Time**
Step 1	94°C	3 minutes
Step 2	94°C	30 seconds
Step 3	60°C	30 seconds
Step 4	72°C	2 minutes
Step 5	Go to Step 2: 30 Rounds	
Step 6	72°C	2 minutes
Step 10	12°C	Infinite Hold

Table 11 Lambda RED Recombineering Reaction

Reagent	**Volume**
LR Primer 1	0.5 µL (25 pmol)
LR Primer 2	0.5 µL (25 pmol)
Template (pKD3/4)	0.5 µL
DNase free H_2O	11.0 µL
2x Go*Taq* Master Mix	12.5 µL

Total	25.0 µL

Table 12 Lambda RED Recombineering Thermal Cycler Protocol

Step	**Temperature**	**Time/Cycles**
Step 1	95°C	3 minutes
Step 2	95°C	30 seconds
Step 3	45°C	30 seconds
Step 4	72°C	2 minutes
Step 5	Go to Step 2: 5 Rounds	
Step 6	95°C	30 seconds
Step 7	72°C	30 seconds
Step 8	72°C	2 minutes
Step 9	Go to Step 6: 30 Rounds	
Step 10	72°C	5 minutes
Step 11	12°C	Infinite Hold

Table 13 10x M63 Salts (1L)

Reagent	**Quantity**	**Molar Mass**	**0.5x Solution Final Concentration**
K_2HPO_4 (dibasic)	75.0 g	174.2 g/mol	21.0 mM
KH_2PO_4 (mono)	30.0 g	136.0 g/mol	11.0 mM
NH_4SO_4	10.0 g	114.1 g/mol	4.4 mM
Dissolve in 800 mL dH_2O Adjust pH to 7.4 Volumize to 1L and Autoclave			

Table 14 Complete M63 Defined Media (10 mL)

Reagent	**Quantity**
10x M63 Salt Solution (Table 13)	500.0 µL
50x Essential Amino Acids	200.0 µL
100x Non-essential Amino Acids	100.0 µL
40% Glycerol	57.5 µL
$MgCl_2$	10.0 µL

$FeCl_2$	1.0 µL
NaCl	2.0 µL
H_2O	9.1 mL

Table 15 “Green” Plates for Phage Selection (1L)

Reagent (dry)	**Quantity**
Dextrose	7.5 g
Yeast Extract	1.0 g
Bacto-Tryptone	8.0 g
NaCl	5.0 g
Bacto-Agar	15.0 g
Methyl Blue	65.0 mg
Alizarin Yellow	600.0 mg
Distilled H_2O	Volumize to 1 L

Conjugative Tn7 Integration into *S.* Typhimurium

Quadparental mating was utilized several times during this study to integrate a Tn7 vector into a single site within the *Salmonella* genome. All quadparental mating series shared a common protocol using the conjugative helper plasmid pRK2013, the Tn7 transposase containing vector pTNS1, a recipient *Salmonella* strain, and a donor Tn7 vector. In all cases except the noted complementation strains, the recipient *Salmonella* strain was *sseG::Cam S.* Typhimurium EPST138. An antibiotic-resistant *Salmonella* recipient strain was required to isolate *Salmonella* from the *E. coli* donor containing the Tn7 vector. Construction of the strain SEST001 to be paired with the created Mariner Transposons used donor Tn7 vector pGRG37-ara-Gent to establish a gentamicin-resistant strain for transposon mutagenesis. Future complementation studies involved inserting either an empty Tn7 transposon (pTn7Gent-empty) or the Tn7 transposon containing the *ompC* coding region and native promoter (pTn7Gent-

OmpC-$_p$ompC) either into the *sseG*::*Cam* strain for transfer to WT or into *Salmonella* deletion strains directly.

For quadparental mating events, all strains and plasmids were cultured in LB broth with their respective antibiotics at 37°C overnight with shaking. The entire 3 mL of each culture was pelleted for 2 minutes, washed in 750 µL LB broth to remove the antibiotics, pelleted again, decanted, and resuspended in 800 µL LB broth. 250 µL of each culture were then added to 1.5 mL tubes to form conjugation samples, combined in a manner that created an experimental sample (recipient *Salmonella*, the Tn7 vector, the pRK2013 helper, and pTNS1 transposase), a *Salmonella* control lacking a Tn7 vector, and a Tn7 control lacking a *Salmonella* recipient. These samples were pelleted and resuspended in 50 µL LB broth, then spotted on an LB agar plate to incubate at 37°C overnight. The following day, a small swath of each conjugation was streaked onto selective plates with the appropriate antibiotics and incubated overnight at 37°C. After checking the control plates for lack of growth, good colonies from experimental samples were cultured in 3 mL LB broth with antibiotics overnight at 37°C with shaking to be frozen for preservation and for colony PCR (Table 5) confirmation if needed.

P22 Phage Transductions

Transduction events were conducted using a mutant bacteriophage P22HT*int* to transfer genes or mutations, such as deletions or Tn7 integrations, from one *Salmonella* strain to another [46]. This integrase-defective phage permits incorporation of random genomic DNA into the phage particles, limiting the rate of lysogenic phage formation. P22HT*int* is maintained in wild-type *Salmonella* until it is introduced to a donor mutant *Salmonella* strain (Fig. 1).

The first reaction transduced the gentamicin-resistant Tn7 from donor strain SEST001 *sseG::Cam* Tn7::pGRG37-ara-Gent out of the *sseG::Cam* background into wild type CS093 to yield strain SEST002. The second set of reactions moved lambda RED-mediated deletions from donor strains SEST004, SEST005, SEST006 out of the pKD46-containing *Salmonella* EM369 background. Similarly to the first reaction, the third transduction moved *sseG::Cam* background Tn7 donor strain SEST003 into CS093 to form strain SEST011.

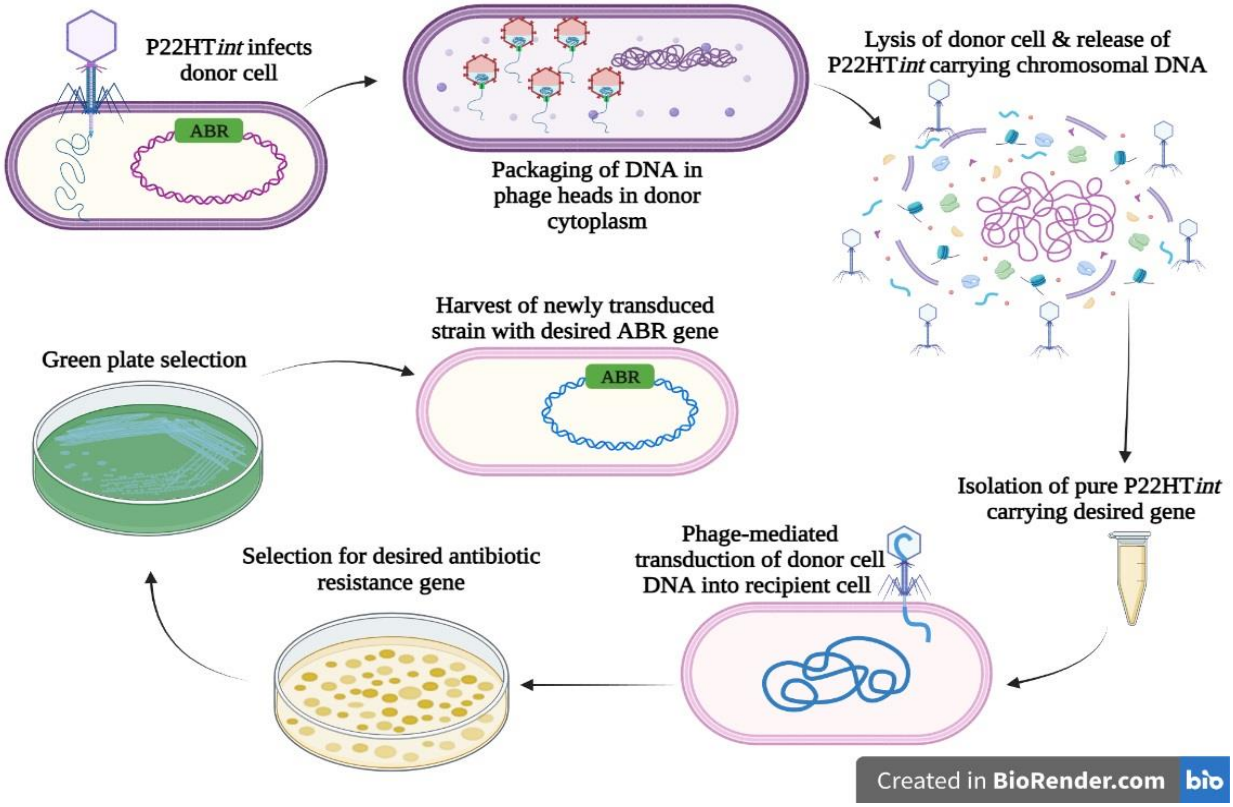

Fig. 1 P22 phage transduction protocol. P22HT*int* bacteriophage was mixed with a donor *Salmonella* strain to transfer antibiotic resistance gene (ABR)-marked deletions or insertions from one strain of *Salmonella* to another. After phage is produced, some of which contains the ABR fragment from the donor bacteria, the phage are purified and allowed to infect the recipient *Salmonella* strain. Selection by antibiotic resistance and clearance of remaining replicative phage generates the desired mutant.

Salmonella donor strains were cultivated in 2 mL overnight LB broth cultures overnight at 37°C, after which 500 µL of overnight donor strain culture were combined with 1.5 µL P22HT*int* and 1 mL LB broth to create the phage reactions. These were incubated overnight at 37°C with shaking, as was 2 mL LB broth cultures of recipient strain cultures (i.e., CS093 *S.* Typhimurium 14028S). P22HT*int* phage was prepped from phage reactions by pelleting 1 mL of the overnight phage culture and decanting 900 µL supernatant into a new 1.5 mL tube, then adding 100 µL chloroform to kill any remaining bacteria. This mixture was vortexed, allowed to sit for 5 minutes, vortexed again, and then centrifuged for 2 minutes. The top layer of aqueous supernatant containing pure Tn7 carrying P22HT*int* phage was removed and diluted for transduction by mixing 2µL phage and 1 mL LB broth. 150 µL of recipient *S.* Typhimurium overnight culture and 150 µL diluted P22HT*int* phage were combined in a new tube to a final phage dilution of 1:1000 and incubated for 2 hours at 37°C with shaking. Two negative controls were prepped by mixing 150 µL of either recipient *S.* Typhimurium or diluted phage with 150 µL LB. The phage reaction and controls were plated onto selective LB agar containing the appropriate antibiotics and incubated at 37°C overnight, whereafter a resistant colony was streaked onto green plates (Table 15). These green plates identify phage-infected colonies via the presence of pH reactive dyes in the media that are acidified following phage-mediated lysis of bacteria. Phage transductants were purified on green plates twice in succession, selecting only large white colonies for re-streaking. A resulting white colony following double green plate selection was transplanted to 2 mL LB broth with appropriate antibiotics and grown overnight at 37°C with shaking to serve as template for colony PCR confirmation (Table 5), then prepared into freezer stocks.

Construction of Mariner Transposon Vectors

To expand the possible range of antibiotic resistance marker options within potential Tn vectors, antibiotic resistance cassettes for chloramphenicol and kanamycin were cloned from the vectors pKD3 and pKD4 respectively using Phusion PCR (Table 6) with primers KanFwd-PciI, KanRvs-PciI for pKD4 and CamFwd-PciI, CamRvs-PciI (Table 3) for pKD3 [44]. PCR products were treated with 0.5 µL *DpnI* (Table 4) at 37°C for 4 hours to remove residual vector DNA, subjected to 1% agarose gel electrophoresis, and positive amplicons gel extracted with an IBI PCR Cleanup/Gel Extraction kit. 30 µL antibiotic resistance fragments were digested with 0.5 µL *PciI* in 3.5 µL 10x r3.1 Buffer and 1 µL DNase-free H_2O for 4 hours at 37°C followed by heat inactivation at 80°C for 20 minutes (Table 4) and purification with an IBI gel extraction kit. Vectors pBT20 and pBT30 were linearized by digestion with *PciI* at a site within the gentamicin resistance cassette and purified in an identical manner, with the addition of 0.5 µL thermosensitive alkaline phosphatase (TSAP) (Table 4).

Ligations were divided into reactions of pBT20-Cam, pBT30-Cam, pBT20-Kan, and pBT30-Kan conducted using T4 DNA Ligase (Table 4) at 16°C for 18 hours, then chemically transformed into *pir+* competent MC1061 *E. coli* using 25 µL of ligation reaction and 25 µL cells. Samples were heat-shocked for 30 seconds and recovered in 250 µL SOC for 37°C for 2 hours with shaking, after which 150 µL of each transformation reaction were plated onto either LB Cam34/Amp100 or LB Kan100/Amp100 agar plates. Positive transformants were picked and subjected to colony PCR (Table 5) for confirmation using primers Gm-up2 and Gm-down2 (Table 3) to confirm presence of pBT20/30 plasmid, whereafter confirmed colonies were propagated in 2 mL LB broth cultures with antibiotics for freezer stock preservation and plasmid extraction using an IBI plasmid mini prep kit.

Purified plasmids were then moved to *pir+* conjugation *E. coli* strains SM10 and S17-1 using transformation via electroporation. 1.5 mL of SM10 and S17-1 were washed 3 times with cold, sterile MilliQ H_2O. 5 µL of purified vector plasmid was added to 95 µL of washed *E. coli* and chilled on ice for 5 minutes, then 100 µL of each sample was added to a chilled cuvette and electroporated. Following transformation, the samples recovered in 250 µL SOC at 37°C for 2 hours with shaking, after which 150 µL of each sample were plated on LB Cam34/Amp100 or LB Kan100/Amp100 agar plates for overnight incubation at 37°C. Positive transformants were confirmed by colony PCR (Table 5) for the presence of pBT20/30 using the primers Gm-down2 and R6Kgamma GRvs (Table 3). Transformants with the vector present were frozen for future use. New vectors were named pKeldSE01-05. Further testing was conducted to confirm efficacy via conjugation trials with strain SEST002.

Mariner Transposon Mutagenesis Pre-testing and Tn Library Construction

A transposon mutagenesis protocol was prepared that could be used for both initial testing of the available plasmids, as well as in subsequent construction of the transposon library itself (Fig. 2). 3 mL cultures of the transposon strain (pKeldSE01-pKeldSE05 or SM10-pBT20/30) and the recipient *Salmonella* strains (SEST002, EPST137) were grown in broth containing their respective antibiotics overnight at 37°C shaking. 150 µL of each broth culture was then spread onto LB agar with the appropriate antibiotics and incubated overnight at 37°C. The recipient *Salmonella* strain plates were moved to a 42°C incubator for 2 hours prior to initiation of the conjugation experiment to shut down native restriction enzyme systems and increase transposition efficiency.

All plates were scraped into tubes with 1000 µL LB broth to form a concentrated culture, vortexed, and a portion diluted 1:100 to determine the OD600 of the concentrated sample. The

transposon and recipient strains were then back-diluted to a 40-OD600:20-OD600 transposon donor: recipient ratio. 300 µL of each concentrated culture was then combined and plated as 50 µL spots onto LB agar plates for a 2-hour conjugation at 37°C. Following conjugation, each spot was scraped into an individual tube containing 800 µL LB broth and lightly vortexed. 150 µL of each suspended spot was spread on LB agar with appropriate antibiotics and incubated for 24 hours at 37°C to select for successful transposon mutants. An estimate of colonies per plate was recorded to determine the relative efficacy of each transposon into the indicated recipient *Salmonella* strain. Selective control plates were made of all individual transposon and recipient strains and plated on the same media under the same conditions to test for background colony formation.

For Mariner Transposon library pre-testing, a random colony from each plate was streaked on LB Amp100 agar and grown overnight at 37°C to show there was no chromosomal integration of the vector into the recipient genome. During initial testing, resultant colonies from combined conjugations served as evidence the vectors worked appropriately as transposon delivery suicide vectors. The vector + recipient combination with the highest efficiency, lowest background growth, and fastest colony growth, SM10-pBT30 and EPST137, was chosen for Tn library construction. During Tn library construction, each plate of transformants was counted, scraped into 670 µL LB broth, vortexed, and prepared into glycerol freezer stocks for storage. transformants was counted, scraped into 670 µL LB broth, vortexed, and prepared into glycerol freezer stocks for storage.

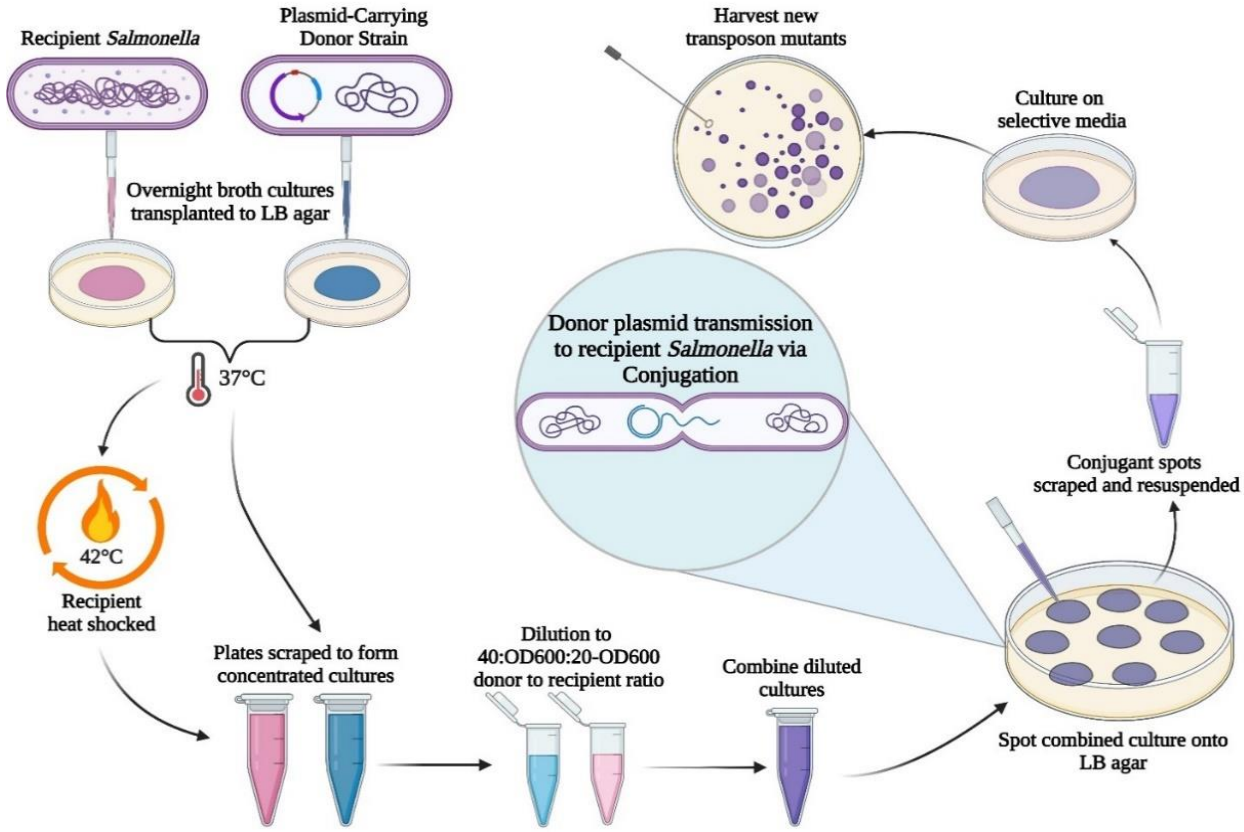

Fig. 2 Mariner Transposon pre-testing & Tn library construction via conjugation. Recipient *Salmonella* and conjugative donor *E. coli* containing the Mariner Tn vector were cultured in broth, then streaked to an agar plate. Following plate incubation, the recipient *Salmonella* was heat-shocked at 42°C to inactivate the native restriction systems. Both recipient and Tn donor strains were scraped, resuspended at a ratio of 40-OD600 Tn: 20-OD600 recipient, then spotted to an LB agar plate. After 2 hours, conjugation spots were resuspended and plated to selection plates for Tn mutant isolation. Resulting colonies were counted and frozen for further use.

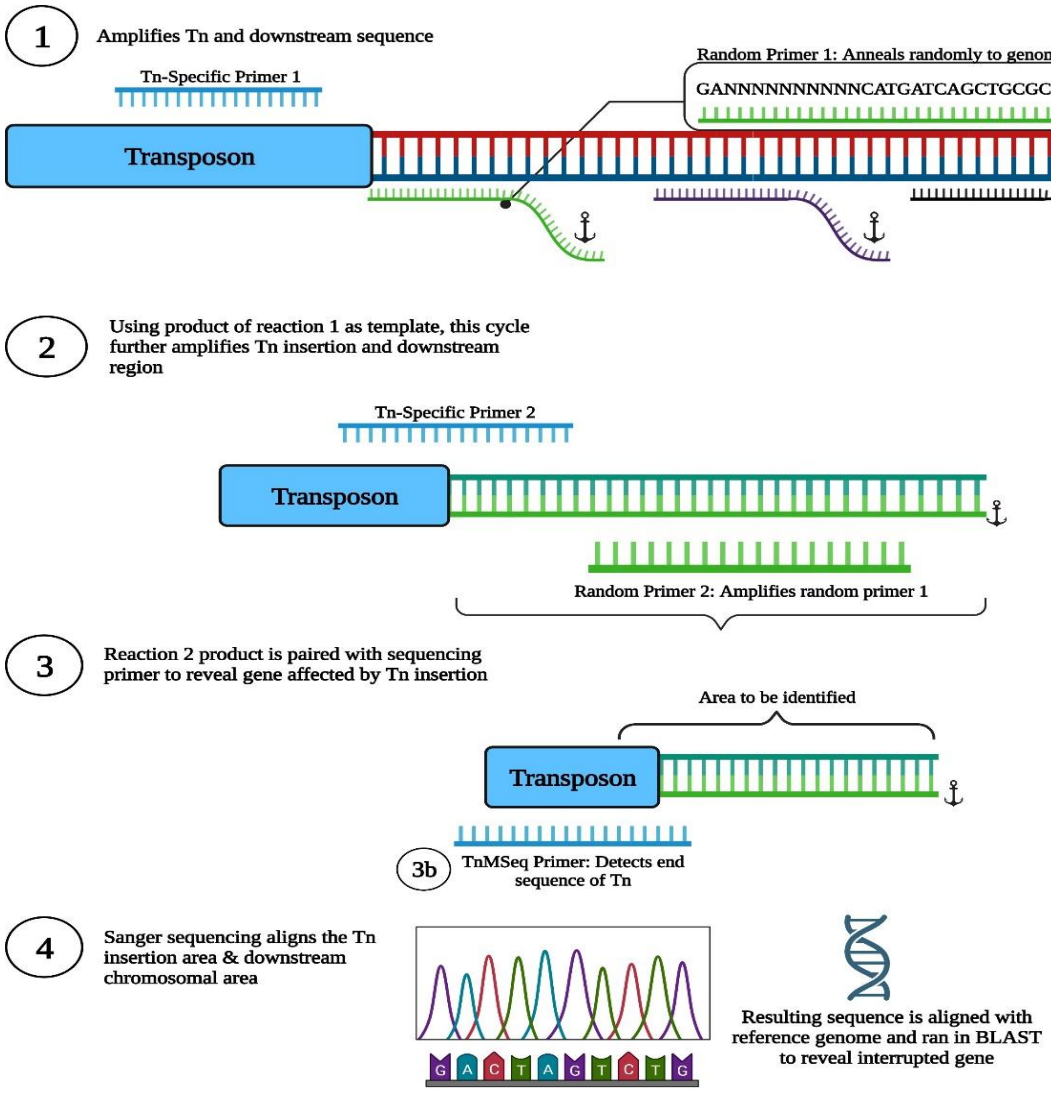

Fig. 3 Arbitrarily Primed PCR to identify chromosomal transposon insertion. In the first PCR round, Random Primer 1 is in possession of an identifiable anchor and random sequence that will arbitrarily anneal to the genome, which is then paired with Tn-specific Primer 1 to amplify the Tn and downstream region. This product is amplified in a second reaction template using Tn-specific primer 2 paired with Random Primer 2, which detects the first random primer and its annealed sequence. Primer BTK30TnMSeq is used to amplify the sequence annealed to the random primers and the flank of the Tn, which is identified by Sanger sequencing.

Transposon Insertion Sequencing via Arbitrarily Primed PCR

To identify the insertion site of an integrated transposon, a two-step PCR protocol was used that incorporates a primer stock with a random assortment of nucleotides (N) to enable varied annealing to the neighboring genomic DNA sequence (Fig. 3). Lysates from selected individual transposon colonies were created by resuspending either a single colony or a pellet from 50 µL of overnight culture in 50 µL of DNase-free H_2O. This bacterial suspension was then boiled in a thermal cycler at 98°C for 15 minutes to generate lysate, spun down for 5 minutes, and the supernatant used as DNA template. Arbitrary primer Rnd1-ARB1-Pa and transposon primer Rnd1-TnM30 (Table 3) were used in arbitrary reaction 1, the resultant product being used as template for arbitrary reaction 2 (Tables 7-8). Arbitrary reaction 2 utilized nested primer Rnd2-ARB and the transposon specific primer Rnd2-TnM30 (Tables 3, 9-10). The PCR product was cleaned up using an IBI gel extraction/PCR cleanup kit and eluted in 20 µL warmed DNase-free H_2O. Samples were submitted for Sanger sequencing by Genewiz using the primer BTK30TnMSeq (Table 3), and the resulting sequence was aligned to the *S.* Typhimurium genome to identify the transposon insertion site (*Supplementary Table 1*).

Tn Library Testing for Salicylic Acid Desiccation Resistance

For testing the Tn mutant library under desiccation conditions paired with exposure to varying concentrations of salicylic acid (SA), I used an adapted plastic desiccation assay (Fig. 4). 3 mL cultures of the combined SETn library were grown in M63 defined media (Tables 13-14) at 37°C overnight with shaking to match previous conditions. The next day, overnight cultures were triple washed with sterile MilliQ H_2O. After the final pelleting, they were resuspended in 1 mL MilliQ H_2O and diluted to 2.0 OD600. The cultures were split into four separate samples by adding 100 µL of culture into four 1.5 mL microfuge tubes, representative of the four different

SA concentrations used in this assay. 50 µL of 2x SA stocks were added to each sample to generate final SA concentrations of 125 µM, 250 µM, 500 µM, and 1000 µM, respectively, yielding a final bacterial OD600 of 1.0.

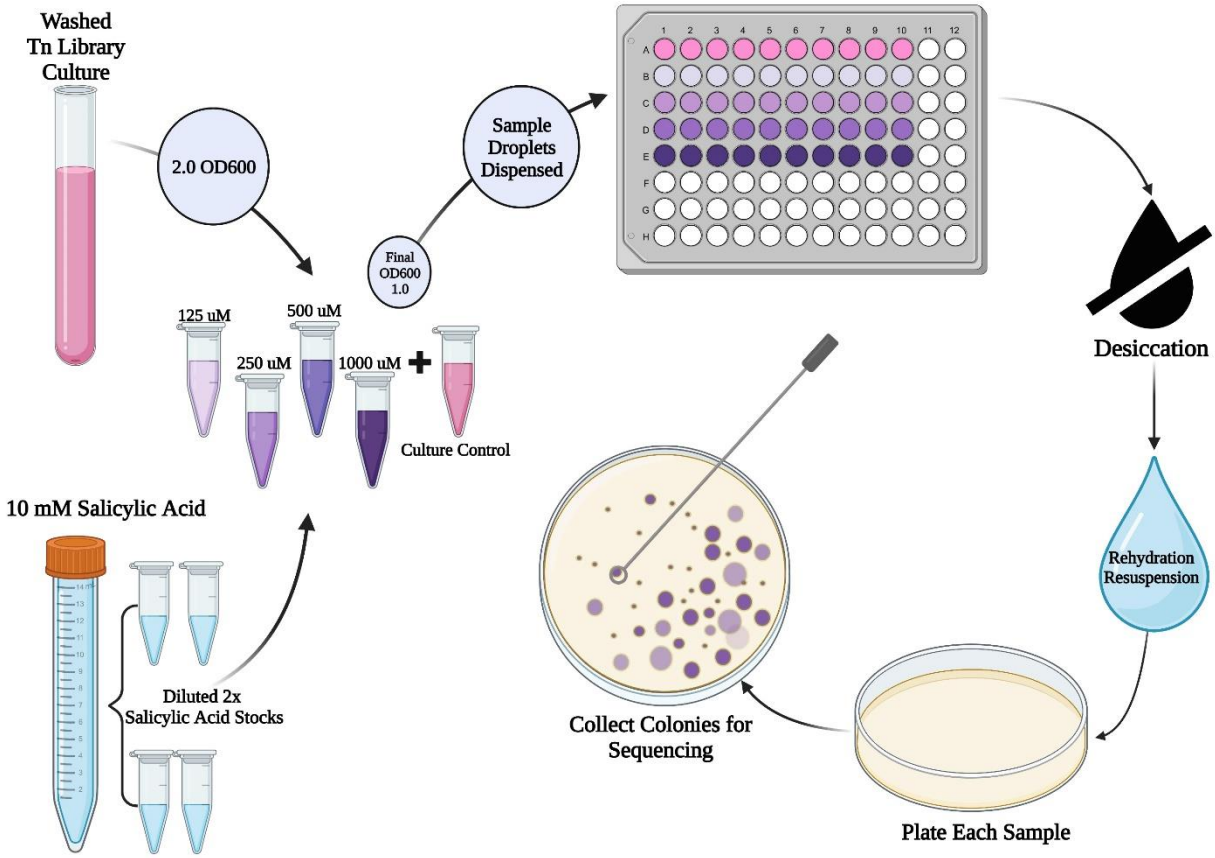

Fig. 4 Overview of transposon library screening for salicylic acid desiccation resistance. An overnight culture of the SETn library was washed, adjusted to 2.0 OD600, and combined with 2x salicylic acid stocks according to experimental concentrations, yielding a final OD600 of 1.0. Samples were dropped into a 96-well plate, desiccated, and allowed to incubate overnight. After rehydration and resuspension, the contents of each well were transferred to selective plates. Surviving mutants were collected, prepared via Arbitrarily Primed PCR, and subjected to Sanger sequencing.

In a sterile 96-well plate, a control row consisting of unexposed culture, as well as four experimental rows containing the salicylic acid exposed samples were dispensed by placing 10 µL of each culture in the wells. The samples in the 96-well plate were allowed to desiccate for 4 hours until completely dry, after which they were incubated at 25°C overnight. The following day, the desiccated samples were rehydrated with 200 µL of 1x PBS and resuspended via shaking at 1000 rpm for 20 minutes. 200 µL of each well was dispensed and spread on a dry, warmed LB agar plate and allowed to incubate at 37°C for 24-48 hours depending on growth needs. Resulting mutants displaying tolerance for desiccation under salicylic acid exposure were moved to an LB agar master grid plate, of which there were 21 in total over the triplicate study. To ensure the colonies were not contaminants, they were transferred to 2 mL LB Strep100/Gent30 broth to undergo selection and allowed to incubate overnight at 37°C with shaking and frozen as glycerol stocks thereafter. Individual cultures were sequenced as described above (*Supplementary Table 2*).

Lambda RED-Mediated Deletion Mutant Construction

Deletion mutants in *S.* Typhimurium were generated through lambda RED-mediated recombination (Fig. 5) [44]. Primers were designed to create PCR fragments that included chloramphenicol or kanamycin resistance markers from template vectors pKD3/4, respectively, wherein homologous primer overhangs correspond to the regions of desired deletions (Table 3) and generated through lambda RED PCR reactions (Tables 11-12). Fragments were confirmed via 1% agarose gel electrophoresis and subsequently gel extracted using an IBI gel extraction kit in preparation for lambda red mutagenesis. For transformation into recipient *S.* Typhimurium containing the lambda RED recombination machinery plasmid pKD46 (EM369), a 5 mL culture was grown in LB Amp100 broth overnight at 30°C with shaking to maintain the temperature-

sensitive pKD46. The next day, this overnight culture was diluted at a 1:30 ratio in 5 mL fresh LB Amp100 broth and incubated at 30°C with shaking for approximately 1.5 hours until the OD600 was between 0.5 and 1.0. The lambda RED machinery was induced by adding 0.2% arabinose and further incubated for 1 hour at 30°C, whereafter the culture was pelleted for 2 minutes at 2°C, triple washed with cold MilliQ H_2O, and resuspended. 95 µL of washed EM369

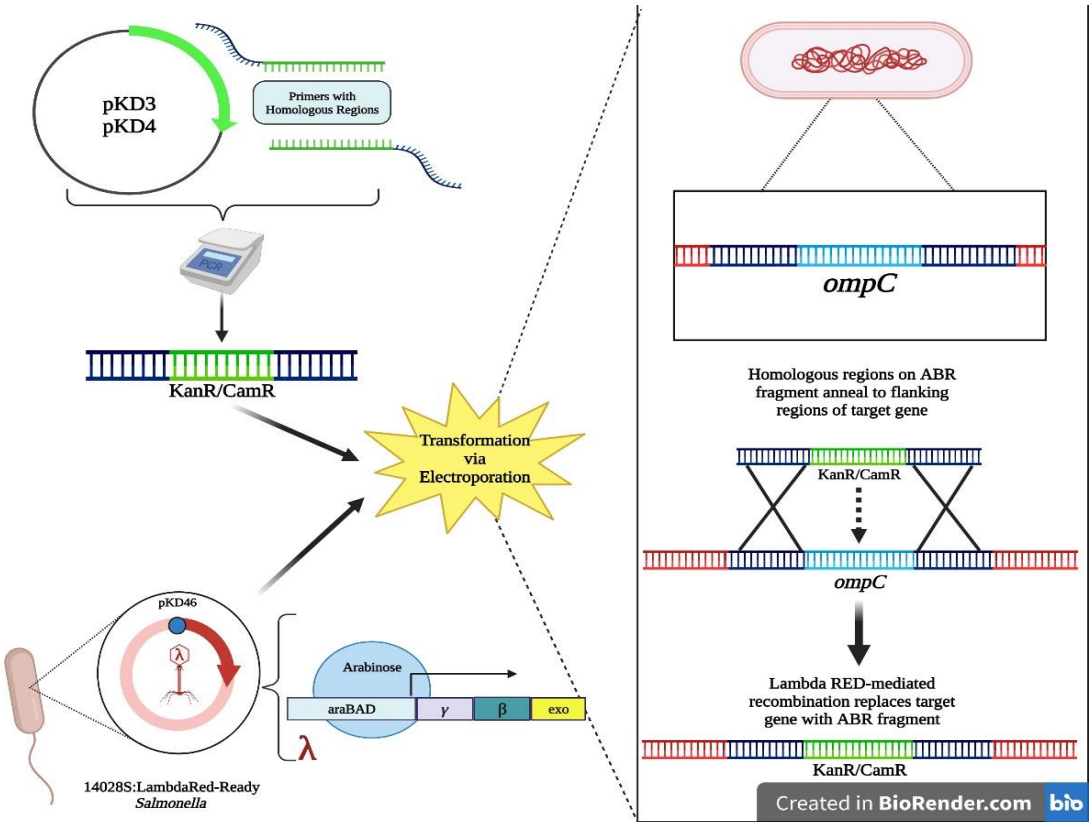

Fig. 5 Deletion construct generation via Lambda RED Recombineering. Antibiotic resistance fragments containing homologous sequence to the flanking regions of the target gene were generated via Lambda RED reaction using template plasmid and specialized primers. This fragment was electroporated into induced Lambda RED *Salmonella.* Recombination of flanking sequences occurs upon transformation, where generated ABR fragments with homologous overhangs, paired with lambda RED machinery will remove the target gene (not to scale).

and 5 µL of purified PCR fragment were combined, transformed via electroporation, and recovered in 250 µL SOC for 2 hours at 37°C with shaking. 125 µL of each transformation was plated on selective LB Kan100 or Cam34 agar plates, along with a negative control of non-transformed EM369, and grown for 24-48 hours at 37°C. Resultant transformation colonies were moved to 3 mL LB Kan100 or Cam34 broth and incubated overnight at 37°C with shaking to ensure colonies were not the result of spontaneous antibiotic resistance, then screened via PCR (Table 5) to confirm the presence of antibiotic resistance markers and appropriate size at the indicated genetic locus (strains SEST004, SEST005, and SEST006). Positive colonies were made into freezer stocks. While pKD46 is temperature sensitive and should be lost at 37°C, the culture was used for phage generation in P22 phage transduction to move the deletion into a clean wild-type *Salmonella* strain using the protocol as described above.

Salicylic Acid Desiccation Assay of *S.* Typhimurium Strains

To test whether isolated strains could survive salicylic acid exposure during desiccation, washed *S.* Typhimurium strains were prepared as described for the SETn library assay. Testing salicylic acid desiccation survival was conducted in two different ways. For the first, a gradient series of SA concentrations was assembled wherein every sample would be subjected to the final SA concentrations of 1000 µM, 500 µM, 250 µM, 125 µM, 62.5 µM, 31.25 µM, and an H_2O desiccation control. 50 µL of each 2x SA concentration were added to 1.5 mL tubes, which were mixed well with the 2.0 OD600 cultures, yielding a final OD600 of 1.0 for each sample/concentration. 10 µL of each sample/concentration were spotted in triplicate in a 96-well plate and allowed to desiccate in the cell culture hood for 4 hours until completely dry, then incubated at 25°C overnight. After overnight desiccation, 200 µL of LB was added to each well and surviving bacteria was quantified by allowing each well to replicate within a Synergy HTX

plate reader wherein the OD600 was measured every 5 minutes for 22-24 hours with orbital shaking at 1000 rpm at 37°C. The compiled OD600 readings were exported and analyzed to determine the point of time when an OD600 of 0.5 was reached.

The second method exposed washed *Salmonella* to salicylic acid before plating to determine the number of surviving colony forming units through manual counting (CFUs). The desiccated samples and the remainder of the 2.0 OD600 cultures for each strain were plated for colonies using the 6x6 drop plate method for the inoculum CFU count and experimental CFU count, respectively. The inoculums were serially diluted by placing 160 µL 1x PBS into rows A-D on a new 96-well plate, adding 40 µL of the 2.0 OD600 cultures to column 1 wells, mixing via pipette, and pulling 40 µL of column 1 contents into column 2. This process was repeated out to column 12. Contents of columns 7-12 were plated in 7 µL droplets in 6 replicates on dry LB agar using a multichannel pipette to create a 6x6 format. For desiccation sample CFU determinations, 10 µL drops of each experimental sample were placed into columns 1 and 7 of a 96-well plate. After desiccation overnight at 25°C, cells were rehydrated by adding 200 µL 1x PBS to the desiccated samples and resuspended with shaking 1000 rpm for 20 mins, after which 160 µL 1x PBS was added to all remaining wells in columns 2-6 and 8-12. Five-fold serial dilutions were created in the same manner as the inoculums and similarly plated in the 6x6 plating format (Fig. 6).

After plating both the inoculum and desiccated sample plates, the drops were allowed to absorb fully into the agar before incubating overnight at 25°C, agar side up. The following day, plates were briefly incubated at 37°C until colonies were visible and well-defined. Colonies were

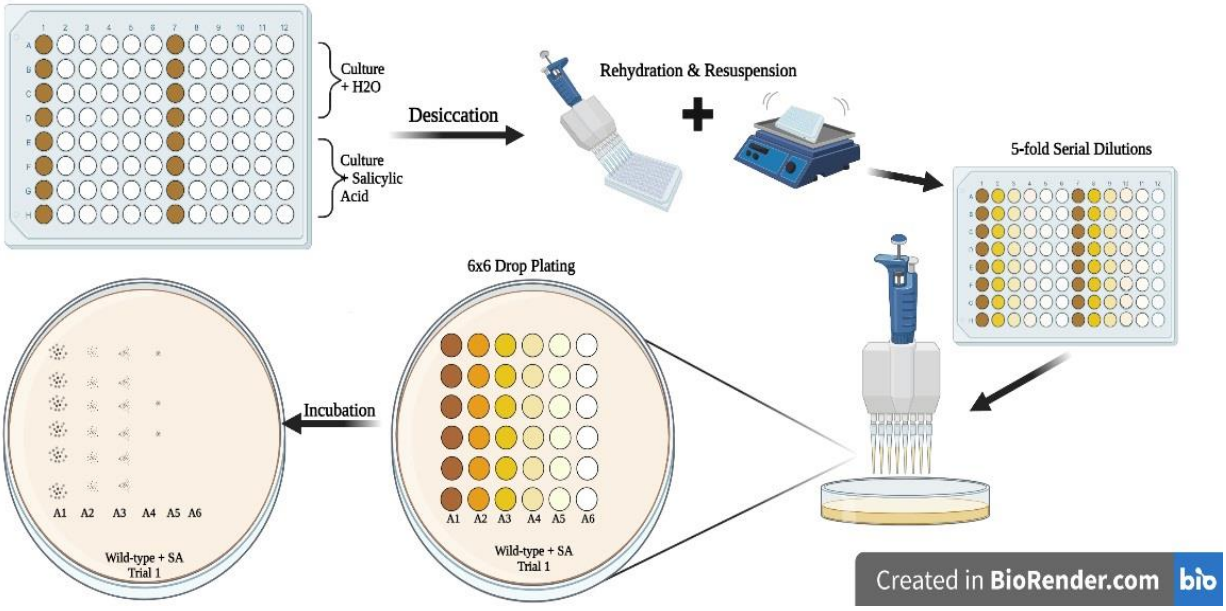

Fig. 6 Desiccation assay setup for CFU quantification. Washed bacteria were combined with H_2O or salicylic acid and desiccated overnight in columns 1 and 7 of a 96-well plate. After desiccation, surviving bacteria were resuspended, serially diluted in columns 2-6 and 8-12 of the 96-well plate, and plated to an LB plate for colony forming unit (CFU quantification).

counted in each row of a single dilution and recorded, with the sum of the 6 wells in a single row being used to calculate the average CFUs/mL. Relative survival percentages compared the number of surviving desiccated compared to H_2O desiccated samples. This assay was repeated in the same manner for individual concentration testing of 125 µM, 500 µM, and 1000 µM.

Complementation Vector pTn7Gent-OmpC-$_p$ompC Construction

To ensure proper expression of complementation constructs for the *ompC* deletions, I decided to integrate the *ompC* gene and its native promoter within the *S.* Typhimurium genome using the Tn7 transposon and its single integration site [47]. A PCR fragment consisting of the *ompC* locus was inserted into the pTn7Gent-pBAD vector using Gibson assembly [48]. Fragment generation consisted of three PCR reactions, wherein reaction 1 used the primers

OmpC_Comp_Tn7_Fwd and OmpC_Comp_Tn7_Rvs, which amplify the *ompC* locus and anneal to the pTn7 fragments. Reactions 2 and 3 used the primers pTn7Gent-GRvs/oriT Gfwd and primers oriT GRvs/rrnB Term Gfwd (Table 3), respectively, to amplify the pTn7Gent-pBAD vector and concurrently remove the pBAD promoter. PCR reactions were conducted using Phusion PCR (Table 6) to generate their products, all of which utilized an annealing Tm of 55°C and adjusted extension times according to size. As reactions 2 and 3 used pTn7 plasmid as template, template DNA was removed by digesting the finished PCR reaction with 0.5 µL *DpnI* (Table 4) for 4 hours at 37°C followed by heat inactivation at 80°C for 20 minutes.

Each reaction was visualized on a 1% agarose gel via electrophoresis and subsequently gel extracted using an IBI Gel Extraction/PCR Cleanup Kit. DNA concentrations were measured using the Take3 protocol on a Synergy HTX plate reader and used to calculate the fragment quantities needed to create 5 µL of fragment in appropriate proportion for cloning. The 5 µL of the proportionately combined fragments were added to 15 µL pre-assembled 2x Gibson Master Mix and Gibson Assembly conducted at 50°C for 60 minutes (Fig. 7) [48]. For transformation, chemically competent *pir*+ DH5*α E. coli* (Table 1) were thawed and mixed with 2.5 µL of each Gibson reaction, then left to sit on ice for 5 minutes. The DH5α cell/Gibson mixture was heat-shocked at 42°C for 30 seconds, rescued in 125 µL complete SOC media, then allowed to incubate at 37°C with shaking for 2 hours. As a control, 25 µL of non-transformed DH5α cells were added to 125 µL SOC media and incubated identically. 150 µL of both the control and transformation were plated on LB Gent30 agar and incubated at 37°C for 24-48 hours, depending on growth speed. Transformants were transferred to 2 mL LB Gent30 broth and incubated at 37°C overnight with shaking. Colony PCRs (Table 5) were assembled using the primers OmpC_Comp_Tn7_Fwd and OmpC_Comp_Tn7_Rvs (Table 3) to detect the presence of the

insertion into the vector. The vector was then purified using an IBI Miniprep kit to be submitted for sequencing to confirm the integrity of the *ompC* complementation fragment (Genewiz). The overnight cultures of positive transformants, now termed pTn7Gent-OmpC-$_p$ompC, were frozen into glycerol freezer stocks.

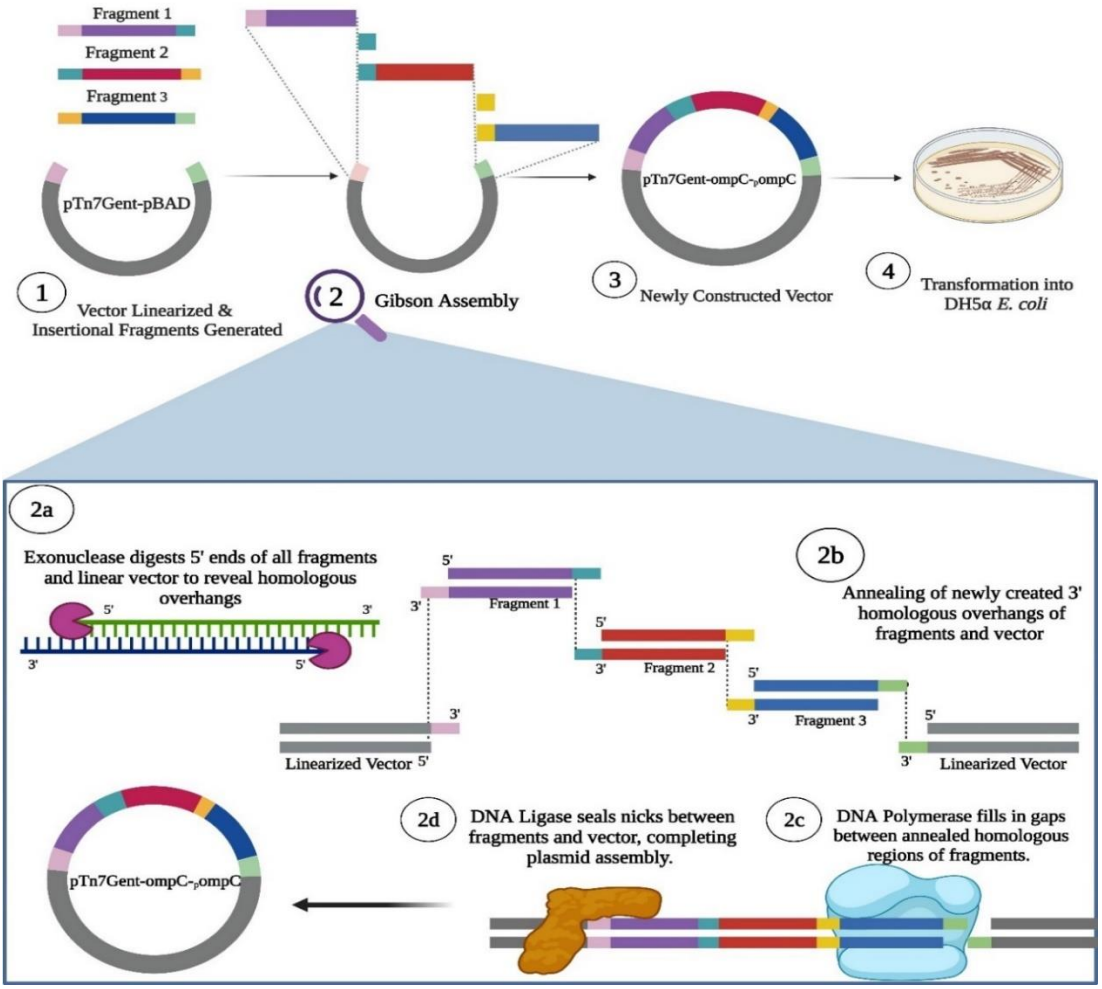

Fig. 7 Detailed overview of 3-part Gibson Assembly for construction of complementation vector pTn7Gent-OmpC-$_p$ompC. Three fragments were generated via PCR, engineered with homologous ends to the pTn7 vector, *ompC* encoding and promoter locus, and the other fragments. The fragments and linear vector were combined in an enzymatic mixture wherein a DNA exonuclease, polymerase, and ligase perform Gibson Assembly, linking the regions of complementarity between the fragments to re-circularize the vector.

CHAPTER 3. RESULTS

Construction of Mariner Transposon Library

Previous research has determined that salicylic acid significantly reduces survival of *S.* Typhimurium during desiccation [27]. This study was conducted with the intention of elucidating the mechanisms and genes involved in desiccation survival of *Salmonella enterica* serovar Typhimurium during exposure to salicylic acid. To this end, I constructed a transposon library of over 50,000 mutants by arbitrarily interrupting genes within the genome of *S.* Typhimurium. The rationale behind the 50,000 mutant goal is the *Salmonella* genome is roughly 5 million base pairs constituting approximately 5,000 genes. 50,000 transposon insertions would hypothetically yield an insertion every 100 base pairs, thus interrupting every gene in the genome. This would also lay a solid foundation to identify novel genes and pathways required for desiccation survival, or any other survival study undertaken in the future. While our lab and collaborators had also been constructing larger mutant libraries for this purpose, an issue had been discovered wherein the *Salmonella* strains were developing spontaneous resistance to the antibiotics used for selection. This has resulted in unwanted background growth of pseudo-mutants, adding difficulty to construction of the library.

To address the issue of spontaneous resistance, it was decided to create new combinations of vectors and strains. The ideal vectors to use for library construction were Himar1 Mariner transposons pBT20 and pBT30, which create random genetic insertions to cover a wide breadth of genes within the recipient genome [28,49]. Both vectors possess an ampicillin resistant backbone for cultivation, a gentamicin resistance cassette within a Tn5 transposon, and a transposase for genetic insertion. The primary difference between the two transposons is pBT30's possession of a transcriptional terminator to block outward transcription from the transposon [49]. To maximize

my chances of successfully generating the Tn library, I endeavored to replace the gentamicin resistance of vectors pBT20 and pBT30 with either chloramphenicol or kanamycin resistance cassettes. pBT20/30 share a *PciI* restriction enzyme site within the first half of the *aacC1* gentamicin resistance gene, allowing antibiotic resistant fragments to be inserted into the transposon. These vectors would be tested for efficiency and low background pairings that would accommodate optimal Tn library construction.

As conjugation was the preferred method of introducing transposon mutagenesis to our parental CS093 *Salmonella* Typhimurium 14028S strain (Table 1) due to its high efficiency, it was required to produce strains of *S.* Typhimurium that possessed resistance to an antibiotic cassette that was different from those of the constructed vectors. To construct this strain, I sought to integrate a gentamicin-resistance marker into the Tn7 site within the *S.* Typhimurium genome to be paired with my chloramphenicol and kanamycin-resistant transposons. Because this integration also required the use of a *S.* Typhimurium strain containing an antibiotic resistance cassette, I utilized a chloramphenicol-resistant mutant already present within the lab, *sseG::Cam S.* Typhimurium EPST138, as the parent strain. The *sseG::Cam* strain was conjugated with the pTn7 vector pGRG37-ara-Gent, along with helper plasmid pRK2013 and Tn7 transposase plasmid pTNS1. In this conjugation, pRK2013 allows for transmission of plasmids that are otherwise not self-transmissible through the RK2 transfer genes while pTNS1 lends a Tn7 transposase that will grant pGRG37-ara-Gent autonomous movement into the recipient genome [42,50]. The integrated Tn7 cassette was then transferred back to the wild-type 14028S parent *S.* Typhimurium strain using a mutant variant of the P22 *Salmonella* phage P22HT*int* [46]. For the gentamicin-resistant parental pBT20 and pBT30 vectors, a spontaneously streptomycin-resistant strain of *S.* Typhimurium was used as the recipient strain.

Transposon Mutant Library Pre-Testing and Construction

To determine the best combination of transposon and recipient strain for Tn library construction, I needed to test the variants for efficiency. Thus far, I had gentamicin and streptomycin resistant *Salmonella* strains and transposons that were resistant to gentamicin, chloramphenicol, and kanamycin to create an efficient and high output combination for library construction. These strains and vectors were conjugated using the same protocol, and their efficiency was determined (as measured by colonies counted after incubation) to compare differences between vectors. Conjugation reactions between the parental pBT30 paired with streptomycin-resistant *Salmonella* yielded the greatest quantity of colonies, averaging ~500 colonies per plate over a 24-hour period. In addition, the kanamycin resistant vectors required reducing selective antibiotic concentrations by half to yield equal colonies, which could potentially allow the growth of spontaneously resistant pseudo-mutants. While it was found the period of conjugation could be extended to increase colony counts in the chloramphenicol and kanamycin resistant vectors, this could have instead simply been replication of already-generated conjugants. This outcome would not be ideal for this transposon library, as it required the greatest possible number of unique insertions. Therefore, initial testing of Tn integration determined that the highest efficiency and lowest background colony production occurred by use of pBT30-Gent transposon and the streptomycin-resistant *Salmonella* recipient strain.

Given the results of the pre-testing, the full transposon library was constructed via conjugating SM10-pBT30-Gent transposon and the streptomycin-resistant *Salmonella* recipient strain using the same conjugation method as used for testing. The resulting colonies were counted, scraped from the plates, and stored as freezer stocks until an approximate colony count of 50,000 was reached. Upon isolating the full library, I made a freezer stock by culturing large

scrapings from each freezer stock and combining them in a 30 mL culture that was grown overnight and aliquoted into several small stocks to constitute a representation of the entire Tn mutant library for future study. To confirm that these were unique *Salmonella* insertions with minimal background, spontaneously resistant growth, and little to no vector integrations, a sampling of eight randomly selected colonies spanning different trials were isolated for Sanger sequencing, being prepared via Arbitrarily Primed PCR (Fig. 3). These results showed insertions in diverse *S.* Typhimurium genes, with no vector sequence in the sample (*Supplementary Table 1*), suggesting that my library would be suitable for further use.

Tn Library Exposure to Salicylic Acid During Desiccation

As previous work in our laboratory suggests salicylic acid could have a detrimental effect on desiccation survival, I sought to determine the mechanism of salicylic acid killing using this transposon library [27]. To test if any of the Tn mutants in the library can withstand the antimicrobial effects of salicylic acid during desiccation stress, I used an adapted plastic desiccation assay which our lab regularly uses (Fig. 4). Samples of Tn library from various aliquots plus controls of wild-type *S.* Typhimurium strain CS093 were exposed to increasing concentrations of salicylic acid, ranging from 125 µM to 1000 µM, with 10 samples per concentration and subsequently desiccated. After rehydration, they were plated on LB agar and monitored for colony growth. The presence of salicylic acid has previously been shown to increase desiccation-mediated killing of *Salmonella*, so surviving mutants would display interruptions in genes whose normal presence would promote cell death under those conditions. Three desiccation trials were conducted, yielding a total of 21 viable transposon colonies harvested from all exposure concentrations tested in this assay. These colonies were prepared for sequencing using Arbitrarily Primed PCR (Fig. 3) and sent to Genewiz for Sanger sequencing.

Interestingly, all 21 colonies appeared to be the result of a single mutant within the library that contained a Tn insertion 1,041 base pairs into the gene *ompC (Supplementary Table 2)*. Results of this assay suggest that this particular mutation in *ompC* is giving a competitive advantage for survival when desiccated in the presence of salicylic acid. In response to these results, I decided to further delve into *ompC* and its interactions with salicylic acid and desiccation.

OmpC Protein Structural Analysis & Bioinformatics

As these 21 colonies seemed to have an advantage in terms of desiccation survival under salicylic acid exposure, I wanted to explore the protein product of the *ompC* gene and ascertain exactly what segments were being affected by the transposon insertion. OmpC is a conserved protein found in many bacteria that encodes for an outer membrane porin that functions as a monoatomic ion transporter for hydrophilic molecules across the outer membrane and is an important component of outer membrane structural integrity [51–53]. DNA and protein sequence analysis shows *ompC* is 1134 base pairs, encoding for 378 amino acids. The protein structure consists of a trimer of beta-barrels, composed of numerous beta sheets with interspersed structural alpha helices to form a functional porin, with a signal peptide at the terminal end (Fig. 8a & 8b) [54]. The insertion disrupted the last 32 amino acids (96 base pairs), interrupting formation of the final segments of each beta barrel, indicating perturbation of these terminal amino acids was enough to compromise the integrity of the entire OmpC porin (Fig. 8c) [53].While there have been no studies to date specifically examining the role of *ompC* in salicylic acid aided desiccation killing, there have been investigations into *ompC* and related genes, but the exact mechanisms still need further study.

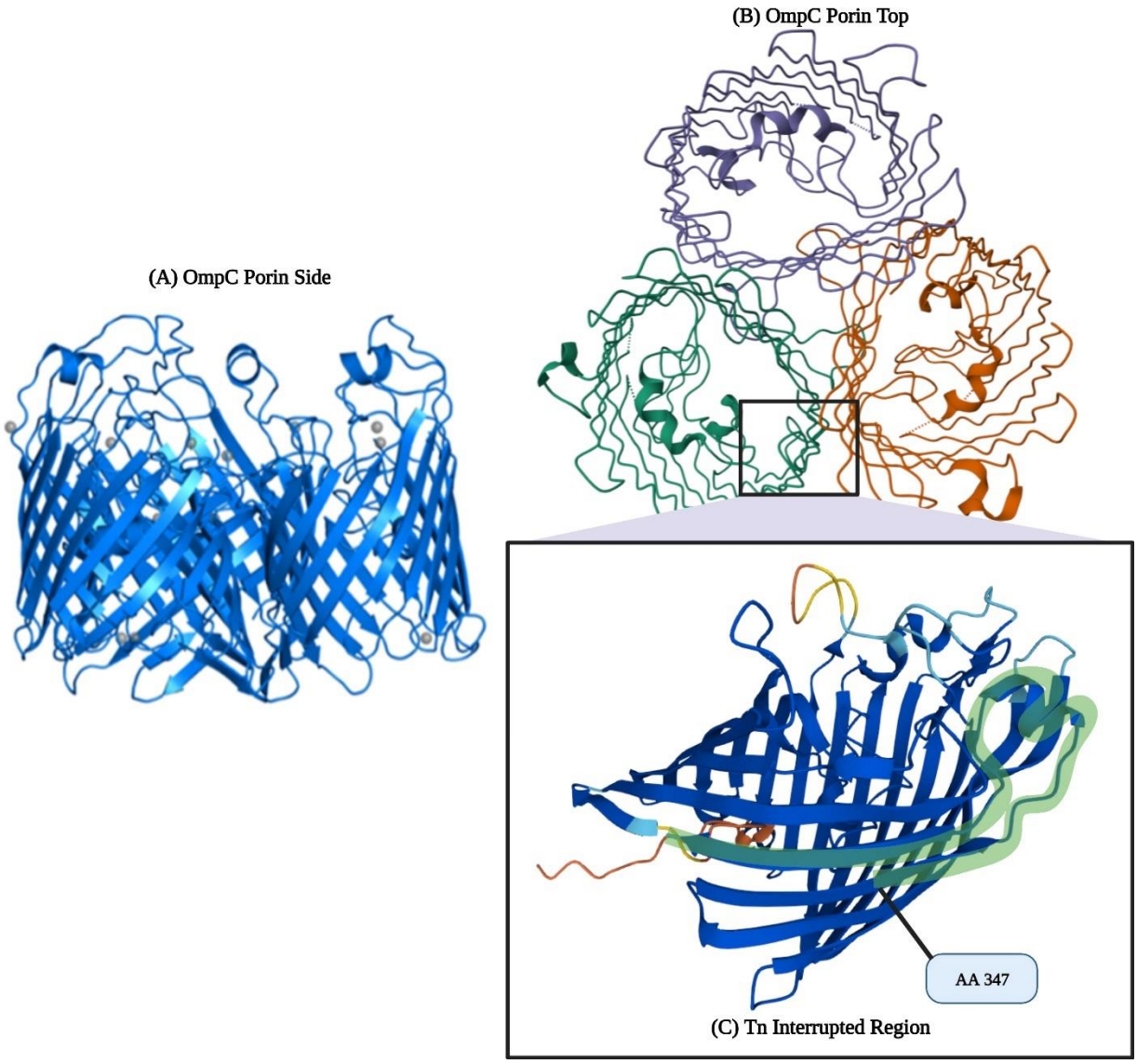

Fig. 8a-8c Visualization of Salmonella OmpC protein structure. The OmpC porin consists of a trimer of beta barrels shown from the Top [54] (a) Side [55] (b). The Tn insertion interrupted amino acid 347 and the following 32 amino acids, affecting a segment of all three beta barrels, highlighted in green (c).

ompC Mutation Desiccation Survival under Salicylic Acid Exposure

As the sequencing data suggested an interruption in *ompC* was affecting *Salmonella*'s survivability under desiccation, I endeavored to uncover more about this phenomenon. The

location of the insertion posed the question of whether the interruption of the terminal 32 amino acids was sufficient to cause the salicylic acid desiccation resistance phenotype, or if the interruption of the entire *ompC* gene was responsible (Fig. 8c). Using Lambda RED Recombineering, I undertook construction of both a full-deletion mutant and a partial deletion mimicking the Tn insertion (Fig. 5) (Tables 11-12).

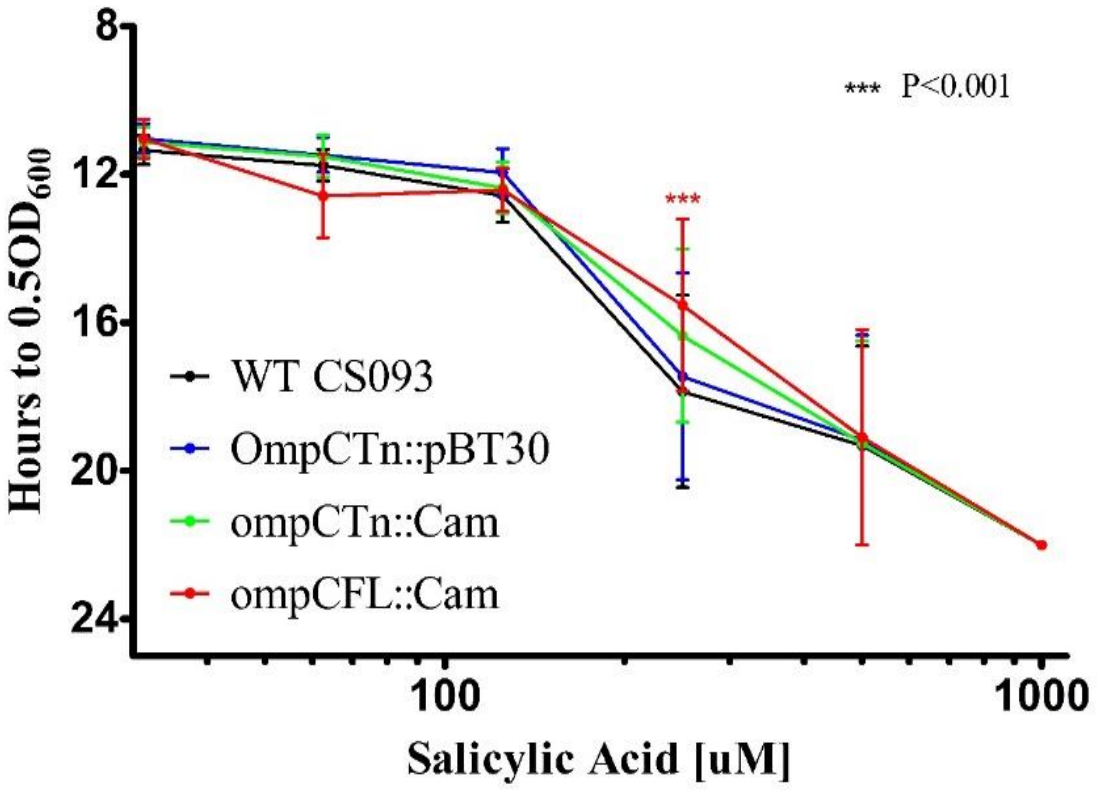

Fig. 9 *ompC* mutant growth analysis post-salicylic acid desiccation. *ompC* full and partial deletion mutants and wild-type *Salmonella* were desiccated post-salicylic acid exposure with concentrations ranging from 1000 µM to 31.25 µM (with H_2O culture desiccation reference) and subjected to growth studies. Growth of samples were monitored for the time point at which they reached 0.5 OD600, signifying the starting density of cells that survived each strain condition, and analyzed using 2-way ANOVA with n=4.

After generating these deletion mutants, I wanted to examine if there was phenotypic variation between the full deletion, partial deletion, and the Tn insertion mutant that I isolated in previous assays (termed OmpCTn::pBT30 for simplicity). I began by doing manual desiccation assays with a 1000 µM salicylic acid concentration, but this proved to be too high, causing death to all strains. Thereafter, testing entailed introducing each strain to a gradient of increasing salicylic acid concentrations from 31.25 µM to 1000 µM (plus H_2O control) and desiccated in a 96-well plate. Surviving bacteria were subjected to growth curve analysis by resuspension in LB media and incubating the 96-well plate within a plate reader for kinetic OD600 measurements.

The experiment which measured higher bacterial cell density, thus higher survival consisted of 4 trials containing a triplicate of each, wherein the full *ompC* deletion mutant showed a higher rate of resistance to salicylic acid desiccation at 250 µM with significance to the wild-type (Fig. 9). From these tests, it was found the ideal concentration range of salicylic acid for addition assays was between 125 µM and 500 µM. Using this concentration range, I shifted back to the manual plating assay that would reveal the average CFU/mL for each strain, showing the actual quantity of viable cells within a dilution that are able to proliferate, which could be more valuable than the time point to mid-log phase. The manual assay was prepared in nearly the same manner as the previous assay, except the samples are serially diluted and plated for CFUs rather than subjected to a growth analysis. After plating, the colonies are counted within a single dilution per strain condition and averaged across 6 replicates, the CFUs/mL of each strain with and without salicylic acid were compared. Initially, 1000 µM and 500 µM concentrations were tested, but was found to be too high as no bacteria from any strain were detected by plating after salicylic acid exposure. A triplicate of 125 µM salicylic acid desiccation assays were conducted

and compared to strains desiccated in only H_2O, which found no significant difference between any of the strains (Fig. 10).

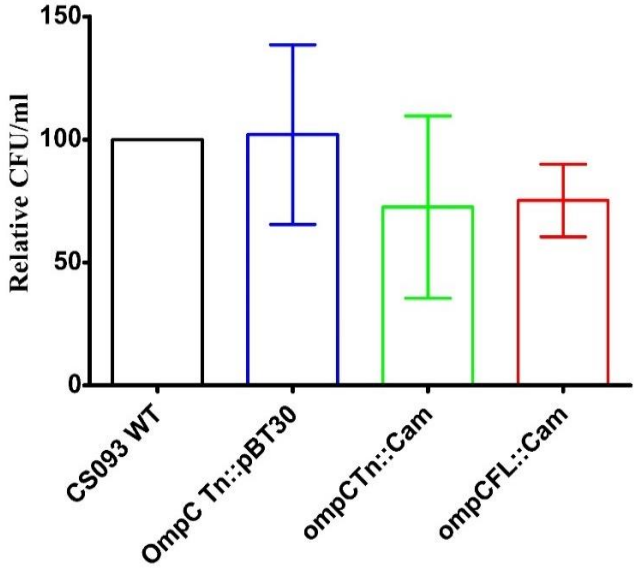

Fig. 10 125 µM salicylic acid desiccation assay of *ompC* mutants. *ompC* mutants and wild-type *Salmonella* were desiccated post-salicylic acid exposure with a 125 µM concentration and plated. Relative CFUs/mL calculated by comparing the survival rates with salicylic acid and without exposure using H_2O. Analysis was conducted via 1-way ANOVA with n=3. None of the samples were statistically significant at this salicylic acid concentration.

Complementation of the *ompC* Deletion Mutants

Concurrently with the assays with the *ompC* mutant variants, I began construction on complementation vectors and a *S.* Typhimurium strain which would allow for the restoration of wild-type phenotypes back to the *ompC* full and partial deletion mutants under control of their native promoter. Because we were unsure how successful complementation from a plasmid-based induction system would work during desiccation assays, I elected to complement the *ompC* gene and its native promoter through insertion into the Tn7 genomic site. After construction of the new Tn7 vector, I inserted this construct into both *ompC* deletion mutants

through a series of conjugative events through the quadparental mating protocol used in this study. I also generated an empty Tn7 variant that contained the parental pTn7Gent-empty transposon without the *ompC* fragment inserted into both a wild-type strain and the mutant strains. As the salicylic acid desiccation assays did not yield any significant results and the original phenotype of the *ompC* transposon mutants could not be replicated, complementation trials could not take place. However, these strains will be available for future research.

CHAPTER 4. DISCUSSION

The zoonotic enteric bacterium *Salmonella enterica* serovar Typhimurium remains a global health threat due to its pervasive nature, widespread and numerous animal reservoirs, and survivability in harsh environments that would be lethal for many bacteria. As a result, it has become a threat to the global food supply, particularly in the poultry product sector, due to its innate ability to survive desiccation on the surface of eggshells. While there have been attempts to elucidate the genes and mechanisms behind desiccation survival, definitive answers are still scant and there is much yet to be uncovered. In an effort to identify chemicals that reduce survival of *S.* Typhimurium during desiccation, our lab previously identified salicylic acid as able to significantly and specifically reduce desiccation survival [27]. Additionally, salicylic acid has been shown to affect cellular processes involved in desiccation survival, but the genes responsible and subsequent interactions with this compound are still unknown [28]. In this study, a transposon mutant library representative of the *S.* Typhimurium genome was constructed with the goal of identifying genes responsible for desiccation survival upon salicylic acid exposure.

An approximate 50,000 Tn mutants were screened under desiccation conditions with varying salicylic acid concentrations and screened for growth during such time a unique desiccation resistant phenotype entailing a transposon insertion at the terminal end of *ompC* was discovered, which initially displayed resistance to varying salicylic acid concentrations. Full gene and mock-Tn partial *ompC* deletions were constructed and tested against several salicylic acid concentrations followed by desiccation. The phenotype was partially recreated by the full *ompC* deletion mutant at the 250 μM salicylic acid concentration in the growth studies, however this phenotype was not evident in later testing.

Published reports indicate an interaction between OmpC and salicylic acid entailing an upregulation and changes in *ompC* expression upon salicylic acid induction [34,51,56]. Paired with its cognate porin-encoding gene *ompF*, *ompC* was originally thought to be solely regulated by the EnvZ-OmpR two-component system acting in response to environmental osmolarity [51–53]. Recent discoveries have found *ompC/ompF* to be under control of additional regulators such as MarA, the global regulator of the *marRAB* operon within the major virulence regulon *mar-sox-rob* [33,34,52]. *marRAB* is generally attributed to multidrug resistant phenotypes by through mechanisms like overexpression of efflux pumps and modulating outer membrane permeability [35,56–58]. Salicylic acid is a known inducer of *marA*, whose downregulation of OmpF leads to decreased porin size and quantity to prohibit antimicrobial passage into the cell [35,56,57]. However, it is unclear whether *marA*'s induction is solely responsible for the upregulation seen in *ompC*, as additional studies suggest restriction of MarA's cognate repressor MarR to be culpable, some even proposing the existence of *mar*-independent pathways for salicylic acid induction [35,59].

Some studies in related enterics also suggest the capability of *ompC* eliciting a salicylic acid response independently of MarA, but a consensus has not yet been reached about the mechanisms of this phenomenon [34,60,61]. This has also been presented in studies of related Omp porins that influence OmpF/OmpC and are likewise under regulation of MarA [62]. It is also possible that interruption of *ompC* could have compromised a regulatory or mechanical pathway that is not part of the canonical EnvZ/OmpF system, which might have more relevance to this study's conditions. Taken together, the workings of an alternative unknown mechanism or regulatory system could be influencing the salicylic acid desiccation survival phenotype seen in this study.

Another possibility was an unknown secondary mutation event either through insertional polar effects to downstream genes, multiple transposon insertions, or another random mutation elsewhere in the genome. Regarding polar effects, directly downstream of *ompC* is the gene *apbE*, an FAD binding protein thought to contribute to outer membrane stabilization and periplasmic transport that could potentially have a role in desiccation survival or porin function [63]. However, the intergenic region following *ompC* showed a high probability for promoter presence suggesting the two are not part of an operon, making polar effects unlikely [64]. A hypothetical secondary mutation event could be responsible for the salicylic acid desiccation resistance phenotype being exhibited, rather than an interruption of *ompC* itself. However, single-strain experiments with the originally isolated transposon mutant suggest this is not likely the case, as the transposon mutant would still be displaying the phenotypes of any such effects in subsequent assays, which was not seen. Regardless, this avenue remains open for future study, as the *ompC* transposon mutants were harvested over a triplicate of trials in various concentrations, suggesting a level of validity to this phenotype. Though the mechanisms behind this initial phenotype have not been elucidated, it is clear there are other factors influencing what was seen in this study.

Given the wide breadth of possibilities and potential for discovery, there are many future directions this study could take. The findings of this study suggest that further investigation into genes within and related to the *mar-sox-rob* regulon, including their interactions with the Omp porins and their respective genes, should be investigated in the context of survival post-salicylic acid exposure and desiccation. As our lab now possesses many confirmed single deletion mutant strains in this relative group in addition to the deletion variants of *ompC*, the opportunity for additional screenings and adaptations of assays exists as prospective work (*Supplementary Table*

4). Meanwhile, in conjunction with our collaborators, a large-scale TnSeq study is being undertaken on a separate Tn library to monitor changes of gene function throughout the *S.* Typhimurium genome under desiccation with the addition of compounds postulated to affect survival, including salicylic acid. In addition to offering an opportunity to again identify *ompC* mutants exhibiting increased survival, we hope to further uncover other genes responsible for this phenotype. Finally, a qRT-PCR study conducted by our collaborating group on *ompC*, in response to the observations of this study, is currently being anticipated. Together, these future efforts will help to piece together the mechanisms behind salicylic acid treatment of desiccated *S.* Typhimurium as we endeavor to determine inhibitory chemicals to limit human infection.

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APPENDICES

Appendix A. Supplemental Tables

Supplementary Table 1 Tn Library Sequencing Sample Results

Sample	**Gene with Tn Insertion**
1	14028S plasmid, uncharacterized gene
2	*ompR*
3	*STM14_3365* (hypothetical protein)
4	*STM14_5121* (putative inner membrane protein)
5	*fdhE*
6	*treB*
7	14028S plasmid, *traT*
8	*cysC*

Supplementary Table 2 Sequencing Results of Viable Tn Mutants Post-SA Desiccation

Date	**Concentration SA**	**Well of Isolation**	**Affected Gene**	**Location (bp)**
Tn1 3/27	1000 uM	E1	*ompC*	2417091-2417470
	1000 uM	E2	*ompC*	2417091-2417470
	1000 uM	E3	*ompC*	2417091-2417470
	1000 uM	E4	*ompC*	2417091-2417470
	1000 uM	E5	*ompC*	2417091-2417470
	1000 uM	E6	*ompC*	2417091-2417470
	1000 uM	E7	*ompC*	2417091-2417470
	1000 uM	E8	*ompC*	2417091-2417470
	1000 uM	E9	*ompC*	2417091-2417470
	1000 uM	E10	*ompC*	2417091-2417470
Tn1 4/2	125 uM	B1	*ompC*	2417091-2417470
	250 uM	C5 (1)	*ompC*	2417091-2417470
	250 uM	C5 (2)	*ompC*	2417091-2417470
	250 uM	C5 (3)	*ompC*	2417091-2417470
	500 uM	D6 (1)	*ompC*	2417091-2417470
	500 uM	D6 (2)	*ompC*	2417091-2417470

	1000 uM	E5	*ompC*	2417091-2417470
Tn2 4/2	125 uM	B2	*ompC*	2417091-2417470
	125 uM	B3 (1)	*ompC*	2417091-2417470
	125 uM	B3 (2)	*ompC*	2417091-2417470
	1000 uM	E5	*ompC*	2417091-2417470

Supplementary Table 3 Restriction Enzymes for Mutant Confirmation

Reagents	**Source**
EcoRI	Thermo Scientific
PstI	Thermo Scientific
NdeI	Thermo Scientific
BamHI	Thermo Scientific
SalI	Thermo Scientific

Supplementary Table 4 Single Deletion Mutants

Gene	**Locus**	**Annotation**	**Information**	**Source**
bcsA	*STM14_4358*	UDP-forming cellulose synthase catalytic subunit	SL14028 *bcsA::kan*	Mills et al., 2015
kdpB	*STM14_0824*	P-type ATPase, high-affinity potassium transport system	SL14028 *kdpB::kan*	Porwollik et al., 2015
uvrY	*STM14_2365*	Putative *luxR/uhpA* family response regulator	SL14028 *uvrY::kan*	Porwollik et al., 2015
acrB	*STM14_0559*	RND family, acridine efflux pump	SL14028 *acrB::kan*	Porwollik et al., 2015
marR	*STM_1838*	Multiple antibiotic resistance protein	SL14028 *marR::kan*	Porwollik et al., 2015
marA	*STM_1837*	*araC/xylS* family transcriptional activator of defense systems	SL14028 *marA::kan*	Porwollik et al., 2015
emrB	*STM14_3400*	Putative MFS superfamily multidrug transport protein	SL14028 *emrB::kan*	Porwollik et al., 2015
yhcN	*STM14_4055*	Putative outer membrane protein	SL14028 *yhcN::kan*	Porwollik et al., 2015
emrA	*STM_3399*	Multidrug resistance secretion protein	SL14028 *emrA::kan*	Porwollik et al., 2015

emrR	*STM_3397*	Transcriptional repressor of *emrAB* operon	SL14028 *emrR::kan*	Porwollik et al., 2015
tolC	*STM14_3859*	Outer membrane channel	SL14028 *tolC::kan*	Porwollik et al., 2015
hsdS	*STM14_5436*	Specificity determinant for *hsdM* and *hsdR*	SL14028 *hsdS::cam*	Porwollik et al., 2015

Appendix B. Single Deletion Mutant Confirmations

During the course of the study, our lab acquired 12 mutant *S.* Typhimurium strains containing single deletions in genes that might have associations with desiccation survival from our collaborators. Many of these genes are known or suspected to also have interactions with *ompC* or its regulatory genes, which presented a potential avenue of exploration following this study. In preparation for future studies, I tested and confirmed these mutants for contamination and correct mutation. Upon acquisition, the freezer stocks were tested for contamination by streaking on LB agar plates for colony isolates and ensuring appropriate colony morphology, whereafter a clean colony from each was transplanted for growth overnight at 37°C shaking into LB broth containing their respective antibiotics (either Cam34 or Kan100) to be preserved in freezer stocks. The mutants were then screened for possession of the appropriate deletion and antibiotic resistance markers via colony PCR (Table 5) against their parent *S.* Typhimurium 14028S strain. To amplify the deletion region, primers were made for each mutant that flanked the gene in question. Mutants and a control of wild-type 14028S were grown in 2 mL LB broth cultures overnight at 37°C with shaking from freezer stocks. Genomic DNA was isolated using an IBI genomic DNA extraction kit for each mutant to be used as template for confirmation colony PCRs (Table 5). Reactions were visualized using 1% agarose gel electrophoresis.

Mutants with expected amplicon sizes similar to their wild-type genes had their PCR products digested with restriction enzymes using cut sites within each respective gene, with comparative controls consisting of uncut mutant fragment with cut and uncut wild-type 14028S. Samples, plus controls of wild-type 14028S and pKD3/4, were digested with 0.5 µL enzyme (selected for single cutting within the desired gene) for 2 hours at 37°C, followed by heat inactivation at 80°C for 20 minutes (*Supplemental Table 3*). The contents were subjected to gel

electrophoresis and gel imagery to visualize the desired “band drop” and to confirm the presence or absence of the wild-type gene. Of the 12 mutants, SL14028 *bcsA::kan* and SL14028 *emrB::kan* were excluded due to lack of correct mutation, with the remaining confirmed mutants being cataloged for use in future studies.

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