Norwegian University of Life Sciences
Faculty of Veterinary Medicine and Biosciences
Department of Chemistry, Biotechnology and
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An Investigation of Type I Toxin-Antitoxin Sytems from *E.coli*

Nazia Koser Zaman

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Abbreviations

Amp^R cassette	Ampicillin resistant cassette
AR	Amplex Red
Bp	Base pair
CFU	Colony forming units
DNA	Deoxyribonucleic acid
DSB	double-strand DNA breaks
DSE	double-strand ends
DSMO	dimethyl sulfoxide
dNTP	Deoxynucleotide phosphate
EDTA	Ethylenediaminetetraacetic acid
E.coli	*Escherichia coli*
FAD	Flavin adenine dinucleotide
FMN	Flavin adenine mononucleotide
HRP	Horseradish peroxidase
Kan^R cassette	Kanamycin resistant cassette
Kb	kilobase
LB	Luria-Bertani
M	Molar
nt	nucleotide
NER	Nucleotide excision repair
OD	Optical density
ONC	Overnight culture

PBS	Phosphate buffered saline
PCR	polymerase chain reaction
PFU	Plaque forming units
RCF	Relative centrifugal force
RPM	Revolutions per minute
RNA	Ribonucleic acid
SOC	Superoptimal broth with catabolite repression
ssDNA	Single stranded DNA
TAE	Tris acetate buffer
TE	Tris-HCl and Ethylenediaminetetraacetic acid
Tris	Tris(hydroxymethyl)aminomethane
TLS	Translesion synthesis
UV	Ultraviolet

Sammendrag

Bakterier utvikler seg til å bli motstandsdyktige mot flere antibiotika. Dette er muligens på grunn av seleksjon av eksisterende resistensgener og mer moderne evolusjon gjennom mutagenesis. Å forstå prosessen som bidrar til motstandsdyktighet er viktig for å utvikle alternativer til nye antibiotika. *Escherichia coli* er en organisme som er ganske godt studert men fortsatt er funksjonen til mange små proteiner ukjent. Noen av de små proteinene er SOS regulerte og viktige i celle-funksjoner, slik som regulering, signalisering og bekjempelse av andre bakterier. Toxin-antitoxin loci i bakterier består av to gener, hvorav det ene genet koder for et lite protein som er potensielt giftig. DinQ er et toxin som veldig giftig ved moderat overproduksjon og DinQ har blitt foreslått til å være en kandidat for anti-celle-envelope antibiotika mot *E.coli* infeksjoner og muligens mot andre gram-negative bakterier. Overproduksjon av TisB danner persister celler som er uvirksomme celler, som har sterk toleranse mot antibiotika. Det er bekymrende at DNA-skadende antibiotika induserer celler som er tolerante mot flere ulike antibiotika.

Basert på TA-systemet *dinQ-agrB,* som ble karakterisert nylig, ble funksjonen til TA-systemene *tisB-istR, shoB-ohsC* and *ldrD-rdlD* undersøkt. Mutantene *tisB, istR, shoB, ohsC, ldrD, rdlD, dinQ* og *agrB* ble stresset på flere ulike måter og den biofysiske responsen til bakteriene ble observert ved deres evne til å replikere og danne kolonier. Ulike genetiske bakgrunner ble kombinert for å finne gener som kan påvirke disse systemene.

Resultatene i disse studiene viser at *ohsC* mutanten er 9000 ganger mer sensitiv mot basisk stress i forhold til villtype (MG1655). Under kronisk oksidativ stress viste *shoB* og *ohsC* mutantene motsatt resultater når de ble stresset med hydrogen peroksid og superoksid, som gir indikasjon på at ShoB har en spesifikk rolle under oksidativ stress. Mulighetene for at ShoB øker oksidativ skade via Fe^{2+}-opptaksproteiner ble undersøkt. Fjerning av både *shoB* og *iscA* indikerer at det er en sterk genetisk interaksjon mellom *shoB* og *iscA,* siden det inhiberer mutantene i å replikere.

Abstract

Bacteria are evolving to be multidrug resistant, this is probably a combination effect of selection for previously existing resistance genes and more modern evolution through mutagenesis. Understanding the processes contributing to resistance development is important in an attempt to produce novel antibiotics. *Escherichia coli* is a well-studied organism but function of many small proteins are still unknown. Some of the small proteins are SOS regulated and are important in cellular processes such as regulation, signaling and antibacterial action. Toxin-antitoxin (TA) loci in bacteria consist of two genes, of which one of the genes encodes a small protein which is often highly toxic upon moderate overexpression. The toxin DinQ has shown to be highly lethal upon modest overexpression and DinQ has been suggested to be a candidate for anti-cell-envelope antibiotic against *E.coli* infections and possibly infections by other gram-negative bacteria. Overproduction of the toxin TisB has shown to form persister cells, which are dormant cells that are highly tolerant to antibiotics, and it is concerning that DNA-damaging antibiotics induce multidrug tolerant cells.

Based on the recently characterized TA-system, *dinQ-agrB*, the function of the TA-systems *tisB-istR, shoB-ohsC* and *ldrD-rdlD* was investigated. The mutants *tisB, istR, shoB, ohsC, ldrD, rdlD, dinQ* and *agrB* were stressed in several ways and biophysical responses of the bacteria was observed by their ability to replicate and form colonies. Various genetic backgrounds were combined to define the epistasis groups of these systems.

The results obtained show that *ohsC* mutant had dramatically reduced survivors by almost 9000-fold compared to wild type (MG1655) under 1 hour alkaline challenge. Under chronic oxidative stress, *shoB* and *ohsC* mutants showed opposite result when stressed with hydrogen peroxide and superoxide, indicating a specific role of ShoB under oxidative stress. Possibility of ShoB increasing oxidative stress through Fe^{2+} uptake proteins was investigated. Removing both *shoB* and *iscA* is indicating there is a strong genetic interaction between *shoB* and *IscA* as replication is strongly inhibited.

1 Introduction

For the past 70 years, different classes of small molecule drugs have provided considerable defense against bacterial infections. However, as bacteria are evolving to be multidrug resistant, understanding the processes contributing to resistance development is important in an attempt to creating alternatives to antibiotics, as they gradually lose their effectiveness. The function of several small proteins, here defined as proteins of 50 amino acids or fewer, in *Escherichia coli* are still unknown. Some of the proteins which are well-characterized participate in diverse cellular functions ranging from morphogenesis and cell division to transport, enzymatic activities, regulatory networks, and stress response (Storz et al. 2014). Some of the small proteins are SOS regulated proteins which are important in cellular processes such as regulation, signaling and antibacterial action. In *E.coli* more than 50 small proteins, which are encoded chromosomally, have been identified with a validated expression of less than 50 amino acids.

Among those small proteins are small hydrophobic toxins located in the inner membrane which are a part of a toxin-antitoxin system (TA). TA loci in bacteria consist of two genes. One of the genes encodes a protein which is potentially toxic, and the second gene encodes an antitoxin to repress the first gene's function or expression. The *dinQ-agr* locus has recently been characterized and *dinQ* produces five transcripts of which only one is actively translated. The actively translated transcript translates into a hydrophobe toxic transmembrane peptide localized in the inner membrane, and is 27 amino acids long. *dinQ* RNA is regulated by *agrB* RNA interference to neutralize toxicity by DinQ. *dinQ-agrB* locus belongs to the type I toxin-antitoxin (TA) system and DinQ has been hypothesized to be a transmembrane peptide that modulates membrane-dependent activities such as nucleoid compaction and recombination. Modest overexpression of DinQ leads to highly increased sensitivity to DNA damage. Ectopic expression has also shown to be highly lethal (Weel-Sneve et al. 2013). DinQ has been suggested to be a candidate for anti-cell-envelope antibiotic against *E.coli* infections and possibly infections by other gram-negative bacteria (Booth et al. 2015). The TA- systems *tisB-istR* and *shoB-ohsC* are similar to *dinQ-agrB,* and also produce a hydrophobic peptide of similar length and have a reasonable amount of sequence similarity.

2 Theoretical background

2.1 Toxin-Antitoxin systems

Toxin-antitoxin (TA) loci in bacteria consist of two genes. One of the genes encodes a protein which is potentially toxic, and the second gene encodes an antitoxin to repress the first gene's function or expression. The classification of the TA systems depends on the mode of action of antitoxins. While toxins are always proteins, the antitoxin can either be a protein or RNA. There are in total five TA systems. Type I TA system rely on RNA antitoxin that bind toxin mRNA in an anti-sense manner. This leads to inhibition of translation initiation and degradation of RNA duplex. Some type I TA systems are found in several phyla while some are only found in a limited number of bacteria. *Escherichia coli* O157:H7 Sakai strain is predicted to have up to 26 type I loci. While type II and type III systems spread by horizontal gene transfer, type I loci arise by duplication in specific lineages and are inherited vertically. In general, type I toxins are inner membrane proteins that disrupt the proton motive force upon modest overexpression (Goeders & Van Melderen 2014).

Type I TA systems have been found in both gram-negative and gram-positive bacteria. The chromosomally encoded systems are often present in multiple copies. The toxins are small hydrophobic proteins (except SymE) which are less than 50 amino acids long and induce pores into the cell membrane. As a consequence, replication, transcription and translation may be inhibited and lead to cell death. In most cases, only overproduction of the toxin protein shows a toxic effect. Many of the toxins interfere with phage propagation and modulate the cell membrane or prevent mature particle formation (Brantl 2012).

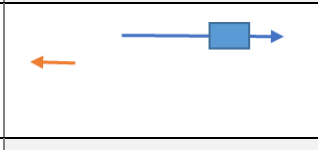
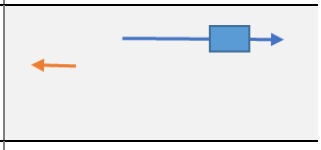
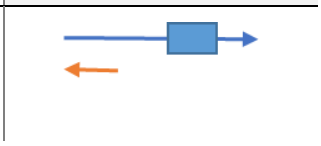
The *Hok-Sok* system was the first and best characterized TA system that is involved in inheritance of plasmids (Alix & Blanc-Potard 2009). The earliest described examples of TA systems came from studies examining the control of plasmid replication. TA loci were later identified on plasmids. The toxin gene products are highly stable while antitoxin gene products are unstable. If a daughter cell did not inherit the plasmid upon cell division, the unstable antitoxin would be degraded. The stable toxin would then exert its toxicity and the plasmid-less daughter cell would be killed. Homologs to the different TA systems described on plasmids have been recently found on the chromosomes of bacteria. Some recently identified loci with no apparent homology to the mobile genetic elements have also been found. The biological function

of the chromosomal loci may, therefore, be different from the loci found on plasmids and needs to be investigated (Wen & Fozo 2014).

The type I TA systems can be arranged as overlapping, convergently transcribed gene pairs directly antisense to the toxin gene and not in some other chromosomal or plasmid location, or as divergently transcribed gene pairs located apart. The antitoxin in the first case is a *cis*-encoded sRNA, and in the second case, it is a *trans*-encoded sRNA (Brantl & Jahn 2015). The majority of antitoxins are *cis*-encoded (Wen & Fozo 2014). The regulatory RNAs are referred to as small RNAs or sRNAs because they are between 50 and 200 nucleotides in length (Fozo, Elizabeth M et al. 2008). They often have limited complementarity to their targets because they are not encoded directly antisense. Their complementarity is therefore limited to 6-12 nucleotides. TisB-istR pair was the first of the divergent systems to be identified, and shortly after ShoB-ohsC and Zor-OrZ gene pairs were discovered (Fozo 2012).

While many of the sRNAs in *E.coli* require the protein Hfq to stabilize their interactions with their target mRNA, type I antitoxin do not require this protein. The primary mode of action of the antitoxin appears to be through inhibition of mRNA translation, although most antitoxins may stimulate RNA degradation upon formation of RNA duplex by bacterial endoribonuclease (RNase III) (Wen & Fozo 2014). Many of the toxins are induced by the SOS response, also known as DNA damage response. These TA systems regulate cell growth and death under various growth and stress conditions (Yamaguchi et al. 2014).

Table 1: Genetic orientation of the different TA-systems.

Locus	*Genetic orientation*
DinQ/AgrB	
TisB/IstR1	
ShoB/OhsC	
Ldr/rdl	

2.1.1 The TA-system tisAB-istR-1

The *tisAB-istR-1* locus is a part of SOS response to DNA damage. *tisAB* (toxicity induced by SOS) gene encodes a peptide localized in the inner membrane, TisB, which is toxic on overexpression. While *istR-1*(inhibitor of SOS-induced toxicity by RNA) encodes a small RNA which acts as an antitoxin. IstR-1 has a LexA-independent promoter and controls the production of TisB. TisB is a 29 amino acid long protein and conserved in enterobacteria (Vogel et al. 2004).

tisA is an untranslated open reading frame that contains antisense RNA binding site and ribosome binding site for *tisB. istR-1* is transcribed throughout growth but *tisAB* transcription is regulated by DNA damage response (Dörr et al. 2010). The toxicity of *tisAB* is inhibited by *istR-1* base-pairing to a small region in the mRNA of *tisAB.* The mRNA is inactivated for translation by this antisense interaction. The inactivation involves RNase III-dependent cleavage. SOS induction leads to depletion of IstR-1, which normally is present in high concentrations over its target, which leads to accumulation of *tisAB* mRNA. Under these conditions, cell growth will be slowed down by TisB exerting its toxic effect. *istR-1* possesses 21 nucleotides that are complementary to the 5‘ UTR of *tisB* mRNA. For *tisB* mRNA to be transcribed or interact with *istR-1*, it must be cleaved at the 5‘end. The cleaved mRNA gives *tisB* an altered structure that possesses a single stranded stretch where *istR* can bind. Ribosomes also bind to this stretch, known as a standby site,

and move to the true binding site and start translation. The *tisB-istR-1* genes are encoded divergent from each other. A deletion of *istR-1* locus gives an increase in *tisB* mRNA levels (Wen & Fozo 2014).

Overexpression of Tis B leads to decrease of membrane potential which results in reduced intracellular ATP levels. This again leads to shutdown of macromolecular synthesis, which includes synthesis of DNA, RNA and protein. Consequently cell death will occur in a fraction of the population (Brantl & Jahn 2015). A study by (Steinbrecher et al. 2012), showed that synthetic TisB monomers bound rapidly to membranes and antiparallel dimers were postulated to assemble via a ladder of salt bridges. The electrostatic charge zipper could then enable protons to pass across the hydrophobic membrane. Overexpression of TisB also induces many genes, including genes involved in regulation of superoxide stress response such as *soxS* (Fozo, Elizabeth M. et al. 2008).

Expression of TisB is not toxic under some stress conditions but has shown to increase survival by forming persisters. Persisters are dormant cells that are highly tolerant to antibiotics. A study by Dörr et al., 2010, showed increased levels of persister cells when TisB production was induced 1000-fold by SOS response caused by DNA-damaging antibiotic. Cells that produced TisB toxin were tolerant to multiple antibiotics that caused DNA damage. Two different strategies of survival are linked upon induction of persisters by TisB toxin. The strategies involve repairing the damage and forming persisters. When DNA-damaging agents are present, it is optimal to use both strategies. Ciprofloxacin is a broad-spectrum antibiotic that is widely used and kills cells by damaging the DNA. This antibiotic is dependent on ATP. TisB is a hydrophobic peptide that binds to the membrane and disrupts the proton motive force, leading to a drop in levels of ATP. Drop in ATP levels prevent DNA damage caused by antibiotics. Strain deleted for *IstR-1* gave 10-a to 100-fold increase in level of persisters while deletion of the entire *tisB/istR-1* locus lead to decrease in persisters tolerant to ciprofloxacin. It is concerning that DNA-damaging antibiotics induce multidrug tolerant cells (Dörr et al. 2010).

2.1.2 The TA-system shoB-ohsC

The toxin ShoB (short hydrophobic ORF) is a short hydrophobic peptide which is 26 amino acids long and hypothesized to be localized in the inner membrane. The *shoB* and *ohsC (*oppression of hydrophobic ORF by sRNA) genes are like *tisB-istR1* encoded divergently from each other and the

TA system share a 19-nucleotide region of complementarity. ShoB and *ohsC* were previously referred to as RyfB and RyfC (Fozo, Elizabeth M. et al. 2008). *ohsC* RNA regulates levels of ShoB in the cell by base pairing to *shoB* mRNA in the 5' UTR. Upon binding *ohsC* may prevent translation of an internal open reading frame. ShoB-OhsC were discovered in a cloning based strategy to identify sRNAs in *E.coli* (Fozo 2012).

To examine if high levels of ShoB depolarizes the cells, Fozo et al., did an experiment to test the ability of cells to take up the dye $DiBAC_4$. The dye enters the cells upon membrane depolarization and gives an increase in fluorescence signal which can be analyzed with flow cytometry. Overexpression of ShoB lead to reduction in membrane potential and also induction of several genes. Among those genes is *soxS* which is a transcriptional regulator of the superoxide stress response. The deletion of *ohsC* gene has not shown to affect levels of *shoB* mRNA. (Fozo, Elizabeth M. et al. 2008).

2.1.3 The TA-system ldrD-rdlD

E.coli has four copies of long repetitive elements called long direct repeat (LDR) sequences. One of these sequences is *ldrD* which is 450 bp in length and encodes a 35-amino acid peptide. The antitoxin of this peptide is RdlD (regulator in LDR) RNA encoded by the gene *rdl.* Unlike *tisB-istR, shoB-ohsC* and *dinQ-agrB, rdl* is located in the promoter region of the *ldr* gene on the opposite strand. Overexpression of LdrD causes rapid growth inhibition but the mechanism is not known, and it also causes nucleoid condensation but this might be a secondary effect. Genes encoding proteins in the membrane are affected by overexpression of LdrD(Yamaguchi et al. 2014). Overproduction of LdrD also leads to induction of *soxS.* The toxins TisB, ShoB and LdrD induce a common set of genes but a subset of these toxins repress or induce an additional set of genes indicating that small toxins do not act in an identical fashion (Fozo, Elizabeth M. et al. 2008) .

2.2 SOS response

A multitude of DNA damaging agents exist in the environment ranging from ultraviolet (UV) light to fungal metabolites and other DNA-damaging agents such as reactive oxygen species which can be produced by the cell itself in metabolic pathways as by-products and intermediates (Smith & Walker 1998). To maintain the integrity of the genome all species require DNA repair pathways. The SOS response is an inducible repair system which allows the bacteria to survive

sudden increases in DNA damage(Michel 2005). The SOS response system in many bacterial species regulates repair of the DNA and genes involved in damage tolerance. Two key proteins control the SOS response: The repressor a LexA dimer and the inducer a RecA filament bound to ssDNA. RecA is the main recombinase. A LexA dimer binds to SOS boxes, which are a consensus palindromic DNA sequence of 20 base pair, in the absence of DNA damage. Basal-level expression of *lexA* ensures downregulation of the system in the absence of DNA damage. The binding of LexA represses transcription of a regulon which regulates more than 50 genes, including *lexA* and *recA*. (Zgur-Bertok 2013)

The ultimate trigger of the SOS response is formation of ssDNA. ssDNA regions accumulate at arrested replication forks. Single-stranded DNA binding proteins (SSB) immediately coat the ssDNA and are subsequently replaced by RecA. The activated form of RecA facilitates autocleavage of LexA bound to the operator region. Cleavage of LexA leads to derepression of more than 40 SOS genes including *recA, umuDC, dinB* and *sulA* (Tan et al. 2015). The complexes RecBCD or RecFOR recruits RecA to ssDNA. Double-strand DNA breaks (DSB) or double-strand ends (DSE) are recognized by RecBCD. Its nuclease and helicase activities result in formation of an ssDNA, which again is a substrate for RecA. DNA nicks and gaps are recognized by RecFOR, and recruits RecA to the ssDNA patch. Three main DNA repair pathways are induced by SOS: homologous recombination, nucleotide excision repair, and translesion synthesis. The formation of RecA nucleofilaments are central in all these three pathways for the induction of the SOS response. RecA is also recruited by other homologous recombination proteins such as RecBCD and RecFOR in the homologous recombination pathway. In the homologous recombination pathway single-stranded lesions are repaired (Baharoglu & Mazel 2014).

Nucleotide excision repair (NER) is driven by UvrABC and lesions are repaired where the DNA is double stranded. Recombinase and translesion DNA polymerase decrease the speed of replication fork progression during the DNA damage response in Escherichia coli cells. The first genes induced by the SOS are the *uvr* genes (Zgur-Bertok 2013). Lesions are recognized by UvrABC endonuclease and nicks the DNA. UvrD helicase removes the DNA patch which carries the lesion. The gap is filled by DNA polymerase Pol I (Baharoglu & Mazel 2014).

Translesion synthesis (TLS) is the third pathway which can be performed by different specific DNA polymerases, PolV, PolII, and PolIV encoded by *umuCD, polB,* and *dinB* respectively. UmuD is activated by the RecA nucleofilament which catalyzes proteolytic cleavage of UmuD. It is the active form of UmuD, UmuD' that forms the translesion synthesis DNA polymerase PolV in complex with UmuC (UmuD'(2)C. PolV and other translesion synthesis polymerases allow the replication of damaged DNA in a mutagenic manner. The TLS polymerases lack a proofreading activity and incorporates any base across from the DNA lesion that the proofreading polymerase PolIII cannot replicate (Baharoglu & Mazel 2014).

2.3 Oxidative stress in *E. coli*

Under aerobic conditions bacteria experience oxidative stress through formation of reactive oxygen species (ROS). ROS can damage several cellular sites, which include iron-sulfur clusters, mononuclear iron proteins, cysteine and methionine residues of proteins and DNA. Oxidative stress also cause mismetallation of enzymes with zinc, which is not as catalytically efficient as iron in the enzymes. To maintain activity of enzymes under oxidative stress the iron atom is replaced with manganese atom which is resistant to hydrogen peroxide (Imlay 2014). Numerous DNA lesions are induced upon oxidative stress. Both sugar and base moieties of DNA are vulnerable and the attack on bases produces 8-hydroxyguanine, hydroxymethyl urea, urea, thymine glycol, thymine, and adenine ring-opened and ring-saturated products (Farr & Kogoma 1991).

The most consequential impact of oxidative stress is mutagenesis but neither H_2O_2 nor O_2^- can damage DNA directly. By reacting with the intracellular pool of unincorporated iron, some of which is associated with DNA, H_2O_2 produces hydroxyl radicals that can oxidize both base and ribose moieties of the DNA. Guanine is not necessary the initial site for hydroxyl-radical attack, but because of its lower reduction potential, its electrons hop to electron holes in nearby oxidized base radicals. For example if a nearby Adenine is damaged a neighboring guanine lose its electron which is transferred to adenine, leading to a lesion on the guanine. 8-hydroxyguanine is a common product which is highly mutagenic due to its ability to base pair with adenine, in a way that escapes the essential mispair detection system of DNA polymerase. Oxidation of thymine produces lesions that are most likely non coding and lethal rather than mutagenic because polymerase progression is blocked. Oxidation of ribose moieties generates single-strand breaks

with 3’ glycolate residues 5’ to the break which also blocks the polymerase (Imlay 2013). The lipids and the membrane proteins of the membrane can also be damaged through oxidation.

ROS are by-products of oxygen exposure and utilization. Common reactive species in bacteria are superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and $HO^{\bullet}$ (Chiang & Shellhorn 2012). Molecular oxygen is small and non-polar, and it can therefore diffuse quickly across biological membranes. Hydrogen peroxide is a small and uncharged molecule, and can therefore cross membranes at a moderate efficiency that is similar to water (Imlay 2013). *E. coli* generates 10-15 μM per second of intracellular H_2O_2 when it grows on conventional substrates through accidental autoxidation of redox enzymes (Ravindra Kumar & Imlay 2013).

2.3.1 Sources of intracellular O_2^- and H_2O_2

O_2 can take electrons from metal centers, flavins and respiratory quinones. Flavins are organic cofactors that bind to redox enzymes in the form of Flavin adenine dinucleotide (FAD) or Flavin mononucleotide (FMN). Such cofactors are electron carriers in the respiratory chain. O_2^- and H_2O_2 are primarily produced by the accidental autoxidation of non-respiratory flavoproteins. Flavoproteins are found throughout metabolism and a wide variety of them release ROS *in vitro*. This includes glutathione reductase (Imlay 2013). To protect bacteria from O_2^- and H_2O_2 that are formed by enzyme autoxidation, basal oxidative defense mechanisms are sufficient, but additional responses are induced upon elevated levels of O_2^- and H_2O_2 stress that is artificially imposed in the laboratory. In nature plants and microbes excrete redox-cycling compounds that diffuse into nearby bacteria to generate ROS and induce the extra defenses in bacteria. Redox-cycling compounds are typically viologens, phenazines or quinones (Imlay 2008).

2.3.2 Enzymes that scavenge O_2^- and H_2O_2

To maintain low intracellular concentrations of O_2^- bacteria synthesize three superoxide dismutases (SOD): iron- and manganese-cofactored enzymes (Fe SOD and Mn SOD) in the cytoplasm and copper-zinc cofactored (Cu-Zn) in the periplasm. The spontaneous dismutation of O_2^- is not sufficient to maintain low intracellular concentrations. Mn-SOD is encoded by *sodA* and Fe-SOD is encoded by *sodB* and the third isozyme, Cu-Zn SOD is encoded by *sodC* (Tovmasyan et al. 2014). The enzymes must be located within the cellular compartment they are intended to protect because O_2^- cannot cross membranes. During exponential growth O_2^- is

produced in the periplasm but it is not harmful enough to warrant SOD synthesis in the periplasm. Cu-Zn is only synthesized when cells enter stationary phase (Imlay 2013).

Peroxidases and catalases are used in most organisms to scavenge hydrogen peroxide. Alkyl hydroperoxide reductase (Ahp), catalase G and catalase E are the three important enzymes that scavenge H_2O_2. In *E. coli* the two component NADH peroxidase, AhpCF is the primary scavenger of H_2O_2 with very high activity. The activity of Ahp is so high that even though H_2O_2 is produced endogenously at a rate of about 15 µM/s, the steady-state concentration of H_2O_2 does not exceed 20 nM. When Ahp is saturated by high levels of H_2O_2 OxyR is activated and catalase is strongly induced and becomes the primary scavenging enzyme (Imlay 2008).

Figure below shows the standard reduction potential oxygen and other reactive oxygen species. It also shows enzymes involved in reducing them to H_2O.

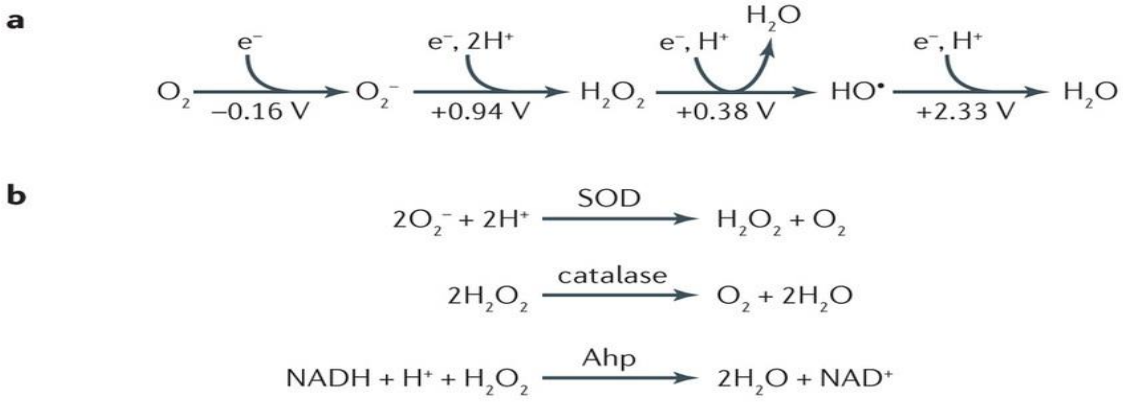


Figure 1: (A) The reduction series of oxygen and standard reduction potentials (pH 7) of molecular oxygen (O_2), superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (HO·). (B) Enzymes involved in scavenging hydrogen peroxide and superoxide.

2.3.3 Responses that are induced by ROS

The superoxide stress response and the hydrogen peroxide stress response protects bacteria against sudden increase in oxidative stress. The SoxRS regulon is activated by redox-cycling drugs, and not by superoxide itself. Redox-cycling drugs generate superoxide and because superoxide dismutase (SOD) is a member of the SoxRS regulon, superoxide was initially thought to be the activator of SoxR (Gu & Imlay 2011). When *E.coli* is exposed to redox-cycling drugs such as menadione or paraquat (methyl virologen), the [2Fe-2S] clusters in SoxR undergoes a reversible oxidation, which can activate transcription of *soxS*. SoxS is a transcriptional activator that activates more than one hundred genes. *fur* encodes an iron uptake regulatory protein and is

among the genes which are induced by the SoxRS regulon (Imlay 2008). Among the enzymes that are induced by SoxRS are proteins which work to exclude redox-cycling compounds from the cytoplasm by actively pumping them back to the cell envelope. Import of the redox-cycling compounds can also be slowed down by modification of the charge and porin content of the cell envelope, or by chemical modification. There is a disagreement in the literature whether O_2^- can oxidize SoxR directly, since it is a relatively ineffective activator. One argument against is that redox-cycling compounds are toxic to cells even under anoxic conditions where O_2^- cannot be made. SoxRS needs to sense the threat even when O_2^- is absent. (Imlay 2013). When oxidative stress is reduced SoxR is reduced by reducing systems encoded by *rseC* and *rsxABCDGE* and extant SoxS is degraded rapidly by proteolysis(Gu & Imlay 2011).

Hydrogen peroxide stress induces the OxyR H_2O_2-stress response. OxyR is a transcription factor that senses oxidative stress. The active site of the transcription factor contains a cysteine residue which reacts rapidly with H_2O_2. OxyR is inactive when intracellular levels of H_2O_2 is around 50 nM but is activated when intracellular reach around 200 nM which again promotes transcription of a dozen operons. 1 µM of extracellular H_2O_2 also activates the OxyR regulon. To drive the H_2O_2 concentrations back to innocuous levels, the OxyR induces synthesis of catalase G and Ahp. Other members of the H_2O_2 stress response is Dps which is involved in iron scavenging, and SufABCDE which is involved in FeS cluster assembly (Imlay 2013).

2.4 Iron homeostasis

Iron is an essential element to virtually all organisms but it also poses problems of toxicity and poor solubility. Iron is essential in many cellular processes, which includes DNA synthesis tricarboxylic acid (TCA) cycle and respiration.

2.4.1 Assembly of Fe-S clusters

Many proteins depend on iron as a cofactor for their function. Fe-S clusters are the oldest and most versatile inorganic cofactors which can participate in electron transfer, catalysis and regulatory processes. The rhombic [2Fe-2S] and the cubic [4Fe-4S] types are the chemically simplest Fe-S clusters, which contain $Fe^{2+/3+}$ and S^{2-}. Cysteine or histidine residues usually coordinate the iron ions to integrate Fe-S clusters into proteins. Electron transfer is the most common function of Fe-S clusters and is based on irons ability to switch between oxidative states +2 and +3. Fe-S clusters can adopt redox potential from -500 mV to +300 mV within a given

proteinaceous surrounding, which makes the clusters excellent electron acceptors and donors in biological reactions. Sensing environmental or intracellular conditions to regulate gene expression is the third general role of Fe-S clusters. Examples are the transcription factors IscR and SoxR which sense Fe-S clusters and superoxide respectively. (Lill 2009).

Regulation of expression of Fe-S clusters biogenesis under changes in Fe-S cluster demand is not well understood (Giel et al. 2013). Formation of Fe-S clusters can be achieved spontaneously *in vitro* with inorganic iron and sulfur sources, but *in vivo* Fe-S biogenesis systems are required. In bacteria three systems have been identified, nitrogen fixation (NIF), Iron Sulfur Cluster (ISC) and Sulfur assimilation (SUF) systems. *E.coli* only has the two latter systems. The maturation of all Fe-S proteins are permitted by the ISC and SUF systems. The systems involve a cysteine desulferase which produces free sulfide from L-cysteine and a scaffold provides a molecular platform where iron and sulfur are allowed to meet and form a cluster. A carrier then delivers the cluster to the terminal apotarget. All three systems contain members of the A-type carrier family of Fe-S biosynthesis proteins ($IscA^{NIF}$, IscA and SufA), which all have three conserved cysteine residues which are involved in Fe-S cluster coordination (Chahal et al. 2009).

The ISC system is a five-protein complex which assembles Fe-S clusters through controlled protein-protein interactions and associated conformational changes that take place. IscU acts as both iron and sulfur acceptor and a scaffold. IscU interacts with two chaperones (HscA and HscB)) to release the Fe-S clusters. (Roche et al. 2013). IscS catalyzes the production of sulfur from L-cysteine (Schwartz et al. 2000).

The SUF system mediates Fe-S cluster assembly under oxidative stress and iron limitation conditions in *E. coli*. *E. coli* carries the *sufABCDE* operon that is required for Fe-S cluster assembly (Chahal et al. 2009). Two sub-complexes, SufBCD and SufSE proteins, are required for the assembly. A [4Fe-4S] cluster can be transferred to an apoprotein by SufBCD. SufB is the scaffold in the SufBCD complex since it binds a [4Fe-4S] cluster. SufB interacts with SufD and SufC. SufD is a paralog to SufB and SufC is a soluble ATPase. For the Fe-S cluster assembly the SufSE heterodimeric complex serves as the sulfur donor. SufS is the cysteine desulferase, which mobilizes the sulfur from L-cysteine. The activity of the desulferase is greatly enhanced by the SufE protein interacting with SufS (Roche et al. 2013).

2.4.2 Iron transport and storage

Although iron is essential for most organisms, it can be extremely toxic under aerobic conditions (Seo et al. 2014). Under physiological conditions, iron exists as the reduced Fe^{2+} ferrous form or the oxidized Fe^{3+} ferric form. Various mechanisms have been evolved in bacteria to allow them to achieve effective iron homeostasis under a range of iron availability. Iron can be transported to the cytoplasm from the environment actively through specific iron-binding transporters in the bacterial outer membrane. Under iron-restricted conditions, highly efficient iron acquisition systems are used to transport iron from the environment. Extracellular ferric chelators called siderophores are produced in *E.coli* cells under conditions of iron limitation, and transport Fe^{3+} into the cells through the outer membrane. Enterobactin is a common siderophore in *E.coli.* While binding to the receptor proteins on the surface is energy independent, the energy-transducing proteins TonB, ExbB, and ExbD, also called TonB complex, drives the transport of the iron-siderophore complexes through the outer membrane. Iron is deposited into Fe-S proteins, heme or iron storage proteins when it is inside the cell. Three types of iron storage proteins are recognized in bacteria called ferritin and bacterioferritin encoded by *ftnA* and *bfr* respectively, and the smaller Dps (DNA-binding proteins from starved cells) proteins, which are only found in prokaryotes(Andrews et al. 2003).

Dps can provide protection to cells during exposure to oxidative stress, nutritional deprivation and other severe environmental assaults. Dps has three properties that provide protection: DNA binding, iron sequestration and ferroxidase activity. Through these properties Dps is extremely important in iron and hydrogen peroxide detoxification and acid resistance (Calhoun & Kwon 2011). Three common iron transporters encoded by *fhuB, fhuC* and *fhuD* transport the iron through the cytoplasmic membrane (Burkhardt & Braun 1987). Figure below shows proteins involved in iron transport.

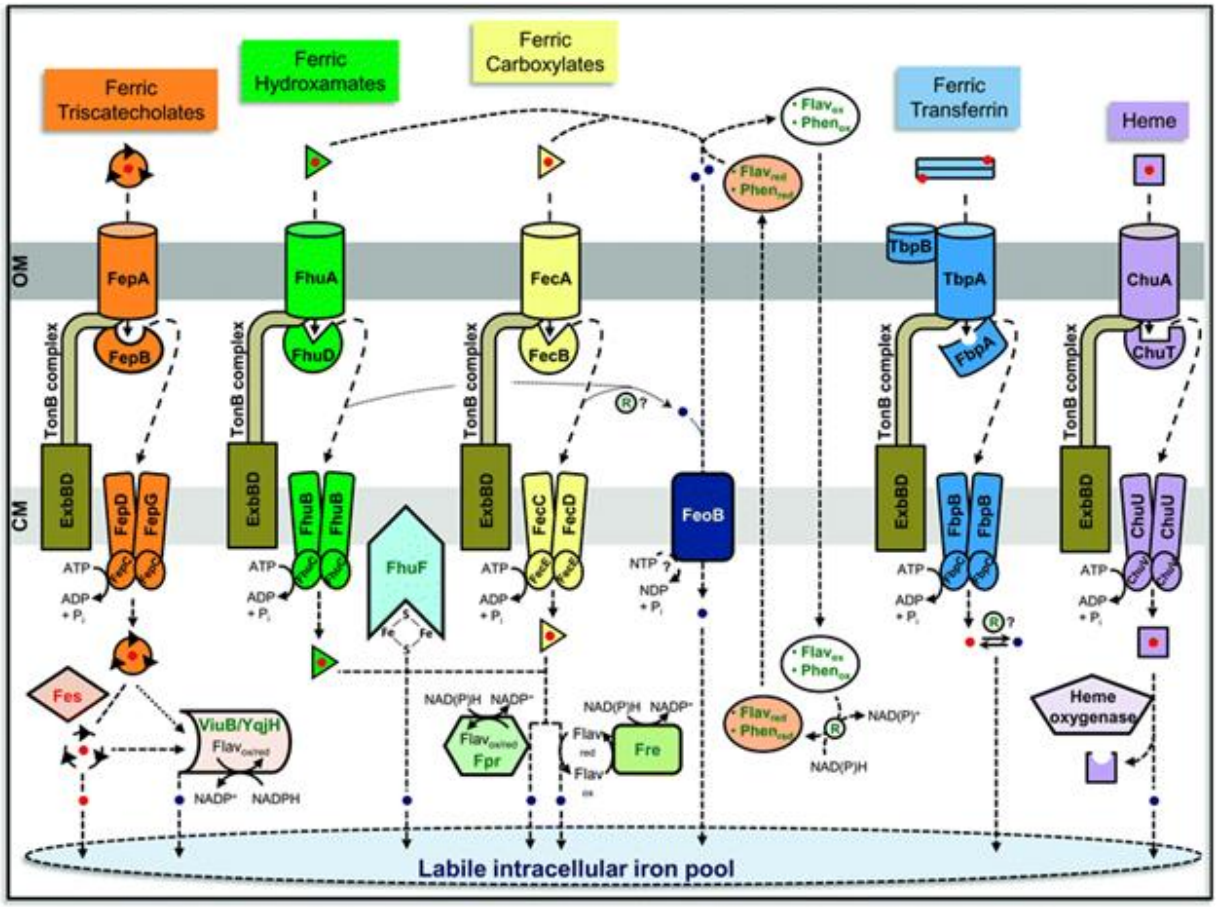


Figure 2: Iron transport. The Figure shows enzymes involved in transport of iron from outer membrane to a labile intracellular iron pool (Miethke 2013)

When the intracellular iron concentration is sufficient, synthesis of siderophores and transport proteins is shut off by the Fur (ferric uptake regulator) protein (Braun & Braun 2002). Through the binding of Fe^{2+} as a cofactor, Fur inhibits transcription of iron uptake genes by binding in the promoter region. A total of 81 genes are directly regulated by Fur under iron-replete (77 genes) or iron starvation (4 genes) conditions (Seo et al. 2014) Fur also repress a small RNA, named RhyB, which promotes degradation of the mRNAs encoding for Fe-using proteins. (Semsey et al. 2006).

RhyB down-regulates a set of iron-storage proteins when iron concentrations are limited. Levels of RhyB RNA are inversely correlated with mRNA levels of *sdhCDAB* operon, which encodes succinate dehydrogenase. Five other genes are also positively regulated by Fur which includes *acnA, fumA, ftnA bfr* and *sodB. acnA* and *fumA* encodes iron-binding enzymes in the TCA cycle. *sodB* encodes a Fe-superoxide dismutase. RhyB needs the RNA binding protein, Hfq, for activity and stability. *Fur* does not function as a repressor under conditions of iron limitation (Massé &

Gottesman 2002). Under aerobic conditions iron can interact with superoxide and hydrogen peroxide and produce highly reactive and damaging hydroxyl radical species by Fenton or Harber-Weiss reactions. The key reactions are listed below.

Iron reduction: (1) $O_2^- + Fe^{3+} \rightarrow Fe^{2+} + O_2$

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

Harber-Weiss reaction: (1) + (2):

$$O_2^- + H_2O_2 \xrightarrow{\text{Fe catalysis}} OH + {}^{\cdot}OH^- + O_2$$

It is therefore important that intracellular iron is maintained in non-toxic form. Cellular iron must not interact with reactive species in an unrestricted manner (Andrews et al. 2003; Keyer & Imlay 1996).

2.5 pH homeostasis

E.coli can tolerate or grow at external pH values that are outside the cytoplasmic pH range through mechanisms for pH sensing and cytoplasmic pH homeostasis. *E. coli* cells are crucially dependent on pH homeostasis because most proteins have distinct ranges of pH within which they can function. *E. coli*, which is a neutralophilic bacteria, can grow at external pH values of 5.5-9.0 but maintain cytoplasmic pH values in a narrow range of 7.5-7.7 (Padan et al. 2005). The proton concentration is involved in cellular bioenergetics. In bacteria, the proton motive force (PMF) is an electrochemical gradient of protons (H^+) across the bacterial cell membrane. The PMF of bacteria consists of two components. First component is a transmembrane pH gradient (ΔpH) and the second component is a transmembrane electrical potential ($\Delta\psi$). Typically, the ΔpH is more alkaline inside the cell relative to outside and $\Delta\psi$ is more negative inside the cell relative to outside. In bacteria the primary proton pumps generate the PMF which includes respiratory or other redox potential-driven pumps (for example respiratory chain pumps) or bond energy-driven pumps (for example proton-pumping ATPases). Under pH stress *E. coli* exhibit "reversal" of the orientation of one of the PMF components. As the pH gradient decreases the electrical potential increases to maintain a cytoplasmic pH value between 7.5-7.7 when external pH is between 5-9, as seen in the figure below (Krulwich et al. 2011).

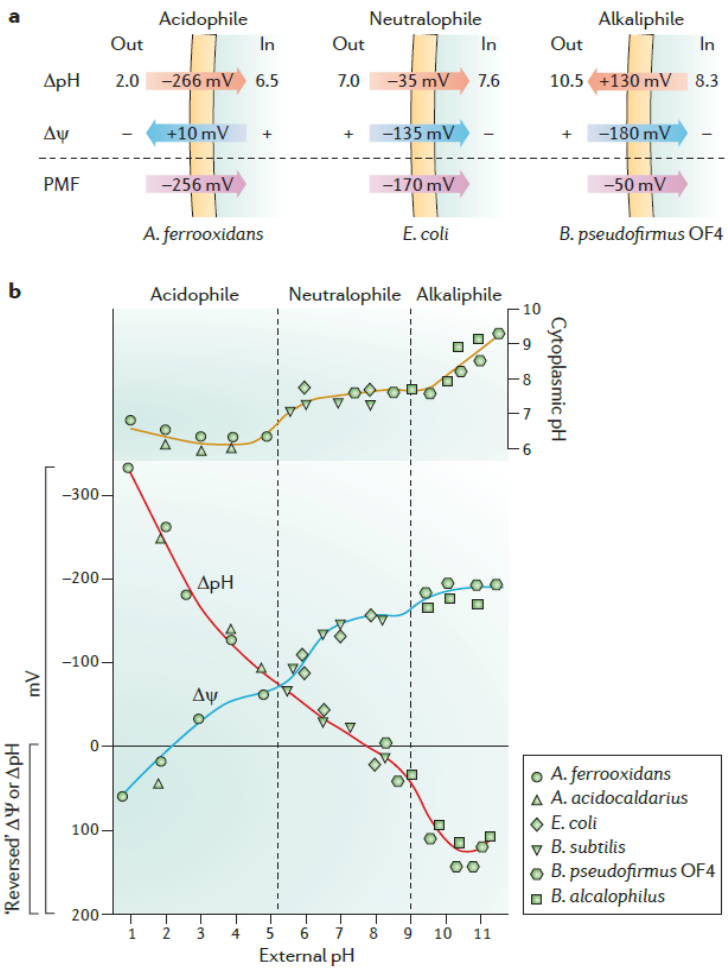


Figure 3: (A) The proton motive force and its pattern in diverse bacteria. (B)Measurement of cytoplasmic pH, ΔpH and Δψ at different external pH values in diverse bacteria. (Krulwich et al. 2011)

Transporters are used for active uptake or efflux of protons. The transporters include primary proton pumps and secondary active transporters, such as cation-proton antiporters. The cation-proton antiporters use PMF generated by respiration or ATPases to take up protons in exchange for cytoplasmic cations such as Na^+ or K^+. The expression of respiratory chain complexes that pump protons out of the cell is increased under conditions of acid stress and expression of the ATP synthase which brings protons into the cell is decreased. Under alkaline stress, protons are actively transported inward, which involves activation and transcriptional upregulation of key cation-proton antiporters. Expression of non-proton-pumping cytochrome *bd* is increased and expression of proton-pumping respiratory chain complexes is decreased to minimize loss of protons from the cytoplasm during PMF generation. Increased expression of F_1F_0-ATP synthase further enhances proton capture (Krulwich et al. 2011).

There are three known Na^+/H^+- antiporters in *E.coli:* NhaB, NhaA and ChaA. NhaA and NhaB are antiporters that exchange Na^+ or Li^+ for H^+, while ChaA exchanges H^+ for $Ca^+$and K^+ in addition to Na^+. The antiporters are found in the cytoplasmic membranes of almost all cells (Krulwich et al. 2011). NhaA has 12 transmembrane segments that form a cytoplasmic funnel and a periplasmic funnel with a barrier separating them (Williams 2000). NhaA primarily functions under adaptation to high salinity, protects against Li^+ toxicity, adaptation to alkaline pH (in the presence of Na^+), and is a prominent antiporter. The stoichiometry is $2H^+/Na^+$ for NhaA and NhaA is dramatically dependent on pH. The activity of NhaA is changed when changes in pH are detected by NhaA's "pH sensor" which is a cluster of ionizable residues. When the residues are mutated the pH sensor change the pH profile but not the Na^+/H^+ antiport capacity of the protein. Most of the residues are located at the opening of the cytoplasmic funnel while the active site is at the bottom of the cytoplasmic funnel (Krulwich et al. 2011). NhaA expels Na^+ from the cytoplasm by using the proton electrochemical gradient (Padan et al. 2004). The activity is increased 2000-fold between pH 6.5 and 8.5 due to conformational change in the protein (Rothman et al. 1997).

In contrast to NhaA, NhaB shows little or no pH dependency (Pinner et al. 1992). The stoichiometry is $2H^+/3Na^+$ for NhaB (Pinner et al. 1994). *nhaB* mutants are Na^+/H^+ antiporter negative up to pH 8.0 where ChaA and NhaA become active, and knocking out *nhaB* makes the strain unable to grow in medium with pH higher than 8.0 and the intracellular pH is not regulated between an external pH of 7.9 and 9.1 in the *nhaB* mutant. Wild type cells can maintain an intracellular pH at about 7.6 at extracellular pH range from 7.6 to 8.5, which means NhaB is essential for the regulation of intracellular pH under alkaline conditions (Shimamoto et al. 1994; Thelen et al. 1991). NhaA and NhaB have similar putative secondary structure but they do not share detectable sequence similarity (Pinner et al. 1993)

2.6 Aim of this Study

The overall aim of this project is to further understand the function of the toxin-antitoxin systems *tisB-istR, shoB-ohsC* and *ldrD-rdlD* based on the recently characterized toxin-antitoxin system, *dinQ-agrB.* How their expression is regulated is much better known than their biological function. The TA-system *dinQ-agrB* is a LexA sensitive TA-system. Better understanding of these TA-systems might contribute to development of novel antibiotics and a better understanding of existing classes of antimicrobial agents. This is of great importance, as number of infections by multidrug resistant bacteria are increasing

The mutants *tisB, istR, shoB, ohsC, ldrD, rdlD, dinQ* and *agrB* were stressed in several ways, by irradiation and by use of various chemicals, and biophysical responses of the bacteria were observed by their ability to replicate and form colonies. Genetic techniques such as general transduction was used to combine various genetic backgrounds to define the epistasis groups of these systems. The resultant bacteria were also stress tested. Flow cytometry was used to measure cell size of *tisB, istR, shoB, ohsC, ldrD* and *rdlD* mutants.

3 Materials and methods

3.1 Materials

3.1.1 Molecular marker

Standard	Manufacturer
GeneRuler™ DNA Ladder Mix	Thermo Scientific

3.1.2 Chemicals, solutions and reagents[1]

Chemicals/Reagents	Purity/Concentration	Manufacturer
Agar	-	Formedium™
Ammonium chloride (NH_4Cl) (s)	-	Merck
Amino acids	-	Sigma Aldrich
Ampicillin	50 mg/mL	Sigma Aldrich
Bacto-Tryptone	-	Difco Laboratorium
Calsium chloride ($CaCl_2$) (s)	-	Sigma Aldrich
Chloramphenicol	-	Sigma Aldrich
Amplex® Red reagent	-	Life technologies
Chloroform	>99.8%	Sigma Aldrich
Copper Sulphate monohydrate ($CuSO_4 \cdot H_2O$) (s)	-	Sigma Aldrich
Cobalt nitrate ($CoN_2O_6 \cdot 6H_2O$)	-	Kebo Lab
Catalase	-	Sigma Aldrich
Dimethyl sulfoxide (DMSO)	>99.5%	Sigma Aldrich
Diammonium sulphate ($(NH_4)_2SO_4$) (s)	-	Sigma Aldrich
Dipotassium hydrogen phosphate (K_2HPO4) (s)	-	J.T Baker
Disodium hydrogen phosphate (Na_2HPO_4) (s)	-	Sigma Aldrich
Dithiothreitol (DTT) (s)	-	Sigma Aldrich
Horse radish peroxide	-	Sigma Aldrich
Hydrogen peroxide	30%	Life technologies
Iron sulphate ($FeSO_4 \cdot 7H_2O$) (s)	-	Sigma Aldrich
Difco Luria Bertani (LB)-Broth	-	Miller
Ethanol	-	Kemetyl
Ethylenediaminetetraacetic acid (EDTA)	>99.8%	Sigma Aldrich
Ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid) (EGTA) (s)	>97%	Sigma Aldrich
Glucose	20%	Ullevål Universitetssykehus
Glycerol	60%	Ullevål Universitetssykehus
Hydrogen chloride (HCl)	38%	Sigma Aldrich
Kanamycin	50 mg/mL	Sigma Aldrich

Menadione Sodium bisulfite	>95%	Sigma Aldrich
Magnesium chloride (MgCl2) (s)	>98%	Sigma Aldrich
Magnesium sulphate ($MgSO_4$) (s)	-	Sigma Aldrich
Manganese dichloride heptahydrate ($MnCl_2 \cdot 4H_2O$) (s)	>97%	Sigma Aldrich
2-(*N*-morpholino)ethanesulfonic acid (MES)	>99,5%	Ullevål Universitetssykehus
3-(N-morpholino) propanesulfonic acid (MOPS) buffer	-	Ullevål Universitetssykehus
M9 salts x5	-	Ullevål Universitetssykehus
Potassium chloride (KCL) (s)	>99%	Merck
Phosphate buffered saline (PBS ×10)	-	Ullevål Universitetssykehus
Potassium dihydrogen phosphate (KH_2PO_4) (s)	-	Prolab®
Primers	-	Eurofins Genomics
SOC medium	-	Ullevål Universitetssykehus
Sodium chloride (NaCl) (s)	>99%	Sigma Aldrich
Sodium citrate dehydrate	-	Sigma Aldrich
Sodium hydroxide (NaOH) (s)	>99%	Merck
SYBR safe DNA gel stain	-	Applied Biosystems
UltraPure™ Agarose	-	Invitrogen
TAE-Buffer	-	Ullevål Universitetssykehus
Thiamine (s)	-	Sigma Aldrich
Yeast extract micro granulated	-	Formedium™
2,2′-Dipyridyl	-	Sigma Aldrich

[1] s=solid

3.1.3 Equipment and instruments

	Manufacturer
Centrifuges:	
Allegra ™ X-22R Centrifuge	Beckman Coultier
Spectrafuge maxi	Hitachi
Biofuge pico	Heraeus
Eppendorf Centrifuge	Eppendorf AG
Spectrophotometer:	
NanoDrop ND-1000	Saveen Werner
Gel electrophoresis :	
Electrophoresis power supply EPS	Amersham pharmacia biotech
PCR:	
Thermal cycler 2720	Applied biosystems

Diverse:	
Multiple well plate, OptiPlate ™ 96 F	Perkin Elmer
Multilabel Counter	Wallac Victor^2
Flow cytometer	Accurie C6
Safe Imager™ Transilluminator	Invitrogen
MicroPulser	BIO-RAD
Micro test plate 96 well	Sarstedt
Micro test plate 24 well	Sarstedt
Tubes, 30 mL	Sarstedt
Tubes, 50 mL	Sarstedt
Tubes, 15 mL	Sarstedt

3.2 Sequencing of tisB, istR, shoB, ohsC, ldrD and rdlD mutants

Recipes for all solutions used in this thesis are given in Appendix A. To confirm that the mutants had been correctly constructed, *tisB, istR, shoB, ohsC, ldrD* and *rdlD* mutants were sequenced. Colony PCR was performed to prepare the DNA template from each mutant. 50 µL reaction was prepared in a 0.5 mL PCR tube on ice. Components needed for 1 reaction are listed below.

Accuprime	Buffer 1×
Forward primer	0.3 µM
Reverse primer	0.3 µM
Accuprime Pfx DNA polymerase	1 unit/ 50 µL
DMSO	2%
Template bacteria*	1 µL
Nuclease-free water	to 50 µL

* 1 colony of template bacteria was grown in LB-medium for 3-4 hours and 1 µL was added to the reaction mix.

Primers used for the reaction are listed below. To see primer sequence see Appendix B.

Table 2: Primers ID for the PCR reaction

Region	Primers ID
tisB/istR	17781-17782
shoB/ohsC	17791-17794
ldrD/rdlD	17799-17800

Cycling conditions for PCR are listed below.

Cycle step	TEMP	TIME	CYCLES
Initial denaturation	95°C	3 minutes	1
Denaturation	95°C	15 seconds	25
Annealing	60° C	30 seconds	
Extension	68° C	60 seconds	
Final extension	68°C	10 minutes	1
Hold	4°C	∞	

50 µL of PCR product was run on 1% agarose gel with 1X TAE running buffer. 6 µL 6× loading dye was used. The gel migrated for 40 minutes with 90V current. GeneRuler^{TM} 1 kb DNA ladder was used and SYBR® Safe DNA gel stain was used to visualize the DNA. The fragments were visualized with blue light on a Safe Image^{TM} Transilluminator. UV-light was avoided to avoid damage on the DNA fragments. DNA bands were cut out from the gel and the DNA was extracted using "QIAEX II gel extraction kit", protocol for agarose gel. Total amount of DNA extracted was measured with NanoDrop. Purified PCR product, approximately 300 ng was premixed with 2.5 µL primer and the total volume was adjusted to 12 µL with H_2O. The DNA was sequenced by "LIGHTRUN". Primers used for sequencing are listed in the table below.

Table 3: Primers ID for sequencing.

Region	Primers ID
tisB/istR	17781-17782
shoB	17792-17791
ohsC	17793-17794
ldrD/rdlD	17799-17800

3.3 Screening for new interactions in the toxin-antitoxin systems

Wild type strain and mutants deleted for the genes *tisB, istR, shoB, ohsC ldrD* and *rdlD* were initially used to find acute and chronic toxicity levels for various chemical agents. Different chemical agents were used to cause a possible response in the wild type and the mutants to observe an effect in the mutants. Different salts, oxidative agents, acid, base and ion starvation were tested and spot survival assays were performed to look for a difference in survival between the wild type strain and the mutants. If an effect was seen for any of the chemicals, different genetic backgrounds were combined to find the epistasis group.

In all experiments wild type strain MG1655 and mutants made from this strain was used. All strains that were tested were grown in LB-medium in a 30 ml-tube and incubated at 37 ºC in shaker if not mentioned otherwise. Strains containing kanamycin cassette were grown with 50 µg/mL of kanamycin and strains containing a plasmid with ampicillin resistance were grown with 100 µg/ml ampicillin. Negative controls were included to make sure there was no contamination. The bacteria were grown to logarithmic phase (OD_{600} 0.4-0.8) before being tested. The bacteria needed to be in the logarithmic phase and not stationary phase to avoid mutagenesis due to nutrition depletion and other factors. The cells are generally more sensitive to stress in the exponential phase due to rapid growth.

3.3.1 Survival assay

Survival assays were performed to find toxicity levels of the chemical agents tested. For both chronic and acute testing the bacteria suspension was serial diluted in 1×PBS on a 96-well plate after reaching OD_{600} 0.4-0.8. Undiluted bacteria suspension was added in the first well and in the five other wells the bacteria was serial diluted 10^{-1}-10^{-5} in 1×PBS. The bacteria were spotted on LB-agar plate for acute testing and for chronic testing the bacteria were spotted on LB-agar plates with different chemicals and LB-agar plates with no chemicals as control. The plates were incubated at 37 ºC and inspected the next day.

For each chemical agent it was optimal to find a toxicity level that killed 90-99 % of the bacteria so that stress response could be initiated, and the biophysical response of the bacteria was observed by their ability to replicate and form colonies. This responds to the three first spots on the assay. If any of the TA-system showed less or more survival compared to the wild type, the survival assays were repeated on a 24 well-plate in three replicates. 1 ml melted LB-agar with different chemicals was added in each well for chronic testing. 10 µl bacteria suspension with the respective serial dilutions in 1×PBS was spotted in each well. As control the bacteria was also spotted on LB-agar without chemicals added. For acute testing, the strains were only spotted on LB-agar. The 24-well plates were incubated at 37 ºC and inspected the next day. Colonies were counted for each strain where it was between 10-100 colonies. CFU/ml was calculated and normalized to CFU/ml on the control.

Equation 1: $$Suvivors\ (\%) = \frac{\frac{CFU}{ml}(survivors)}{\frac{CFU}{ml}(Control)} \times 100$$

Equation 2: $\text{Survivors} = \text{Survivors}\ (\%)_{\text{mutant}} : \text{Survivors}\ (\%)_{\text{MG1655}}$

3.3.2 Chronic testing

See Appendix D to see all the concentrations tested in a stepwise manner.

3.3.2.1 Chronic oxidative stress caused by H_2O_2

H_2O_2 is a peroxide that leads to oxidative stress in *E.coli* and has been used by researchers as a source of ROS. ROS can damage several cellular sites, which include iron-sulfur clusters, cysteine and methionine residues of proteins, and DNA. Liquid H_2O_2 was used for testing and the stock solution was ≈ 10 M. Concentrations between 0.005 to 500 mM were tested.

3.3.2.2 Chronic oxidative stress caused by menadione sodium bisulfite

Menadione sodium bisulfite is a water soluble form of menadione, and belongs to the Vitamin K class of compounds. Like H_2O_2 menadione sodium bisulfite leads to ROS formation but by a different mechanism. Menadione sodium bisulfite causes formation of superoxide. 100 mg/mL of menadione sodium bisulfate was solved in H_2O before adding to melted agar. Concentrations between 20 nM to 8 mM were tested.

3.3.2.3 Chronic iron stress

The salt $FeSO_4 \cdot 7H_2O$ was used for iron stress. 250 mg/ml was dissolved in H_2O before adding to the melted LB-agar. The pH was measured and since it dropped to 6.4 it was adjusted to 7.40 with 5 M NaOH in the initial steps. Since the agar solidified so quickly while adjusting the pH, it was not adjusted in the later steps when it was tested in triplicate. Concentrations between 1.2 to 3 mg were tested.

Less growth was observed in the first spot (undiluted bacteria) than 10^{-1} dilution, which is diluted in PBS, for 1.4, 1.6 and 1.8 mg/mL. Therefore an alternative protocol was needed. The strains that were tested were grown as previously mentioned and 0.5 ml of bacteria culture for each strain was resuspended in 0.5 ml LB-medium and serial diluted 10^{-1} to 10^{-5} in LB-medium. The strains were also tested without resuspending in LB-medium but only serial diluting in LB-medium. 0.5 ml of the bacteria culture was also resuspended in 0.5 ml PBS and serial diluted 10^{-1} to 10^{-5} in PBS before spotting on the iron plates. For further experiments, bacterial culture was resuspended in LB-medium and serial diluted in LB-medium before spotting on iron plates.

3.3.2.4 Chronic iron stress + acute UV stress

To see if a combination of different types of stress could have an effect, the bacteria were stressed with chronic iron and acute UV. The iron plates were made and spotted as describes in "3.2.2.3 Chronic iron stress" before the bacteria was irradiated with 0, 2, 5, 10, 20 and 30 J.

3.3.2.5 Chronic iron removal stress

2,2′-Dipyridyl was used to remove iron in the cells. It can possibly remove other ions as well. 100 mg/mL of colorless solid 2,2′-Dipyridyl was solved in ethanol before adding to melted LB-agar. The concentrations 1, 1.5 and 2 mM were tested.

3.3.2.6 Chronic manganese stress

Manganese is one of the primary divalent transition metals in the *E.coli* cytoplasm (Imlay 2014).100 mg/ml $MnCl_2 \cdot 4H_2O$ was solved in H_2O before adding to melted LB-agar. Concentrations between 2.5 to 20 mM were tested.

3.3.2.7 Chronic Ca^{2+} removal

EGTA(ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N′*,*N′*-tetraacetic acid) was used to remove Ca^{2+}ions from the cells. 38 mg of solid EGTA was solved in 1 mL of 1 M NaOH. 1 ml of the EGTA solution was added to 21 ml LB agar. pH was 9.4 and was downregulated to 7.3 with 38% HCl. The agar was then serial diluted 10^{-1}-10^{-4} which gave concentrations between 5 mM-5 µM.

3.3.2.8 Chronic Copper stress

Solid $CuSO_4 \cdot H_2O$ was solved in H_2O to 300 mg/mL before adding to melted LB-agar. pH was adjusted to 7.0. Concentrations between 0.1 to 11 mM were tested.

3.3.2.9 Chronic Cobalt stress

Solid $CoN_2O_6 \cdot 6H_2O$ was solved directly in melted LB-agar. pH was 4.87 after adding the cobalt salt, but the pH was not adjusted as adding 5M NaOH gave precipitation. Concentration between 1 µM to 100 mM were tested.

3.3.2.10 Chronic alkaline and acidic stress

To test chronic acidic and alkaline stress, LB-agar plates were made with adjusted pH. Approximately 20 ml of melted agar was needed (included buffer) to make each plate. pH 5, 6, 7 and 8 was tested and the buffers MES and MOPS were used. The stock solution of MES was 1 M and pH 6. The stock solution of MOPS was 0.5 M and pH 7.5. 100 mM of each buffer was needed. To make the agar plates at pH 5 and 6, MES was used. For pH 7 and 8 MOPS was used. pH was adjusted to 5.0 and 7.0 with 38% HCl and to pH 6.0 and 8 with 5 M NaOH.

3.3.3 Acute testing

For the acute testing MG1655 was initially used to find the toxicity level. When toxicity levels were found for the different chemical agents the mutants were also tested. The bacteria strains were grown as mentioned above and then 1 ml bacteria culture was transferred to a 1.5 ml-Eppendorf tube and centrifuged at 21500g for 20-30 seconds in a “CT15RE” centrifuge at 4℃ or 20000g for 20-30 seconds in a “Centrifuge 5417R” centrifuge at 22℃. The bacteria pellet was

resuspended in 1 ml LB-medium with different chemical agent and without chemical agent as a negative control. The strains were again incubated at 37 ºC and grown for one hour. After one hour the bacteria was washed by resuspending in 1 ml LB-medium before they were spotted on the plates.

3.3.3.1 Acute oxidative stress caused by H_2O_2

Liquid H_2O_2 was used for testing and the stock solution was ≈ 10 M. Concentrations between 10 nM to 800 mM were tested.

The protocol did not seem stable because when clones of *tisB, istR, shoB, ohsC, ldrD,* rdlD mutants and MG16555 were tested several times, the level of survivors varied. MG1655 was grown for 9 generations to $OD_{600} \sim 0.6$ and each generation was stressed with 10 mM H_2O_2 for one hour, in an attempt to remove any "old" cells from the culture in case they were more or less sensitive to oxidative agents. To make sure that all bacteria are in logarithmic phase *tisB, istR, shoB, ohsC, ldrD,* rdlD , *dinQ, agrB* mutants and MG1655 were grown for 9 generations to $OD_{600} \sim 0.8$ and new glycerol stock was made immediately and frozen down. For further testing these freezer stocks were used. A survival curve was made for MG1655 to see how sensitive it is to different concentrations of hydrogen peroxide.

Six clones of MG1655, *tisB-istR, shoB-ohsC, ldrD-rdlD, dinQ-agrB* were tested at 8 mM but it was observed that some of the clones for a strain survived while other died. Possibilities of this being a genetic effect were investigated by testing 8 clones at 10 mM acute for 1 hour. The same colonies were restreaked and tested again the next day to see if the effect variated each time they were tested. This was repeated for four days. In the next step it was investigated whether it was because of technical differences, because centrifuge at both 4°C and 25°C had been used, depending on availability. The fourth day the samples were centrifuged at 4°C and 25°C to see if this has an effect on survival. To remove residual hydrogen peroxide, the reaction was stopped with catalase, to see if this has an effect on survival. Catalase was solved in potassium phosphate buffer (50 mM) and added to 10 µg/mL bacterial culture. The potassium phosphate buffer contains KH_2PO_4 (stock solution 1M) and K_2HPO_4 (stock solution 1 M). As control the reaction was stopped by resuspending in LB-medium.

3.3.3.2 Acute acidic and alkaline stress

Different pH levels were tested. The pH was down regulated with HCl (38%) and up regulated with 5 M NaOH. pH was measured with an electronic pH meter. pH between 1.70-4.50 was tested for acidic stress and between 9.35-11.80 for alkaline stress.

3.4 Genetic analysis

To define the epistasis groups of the TA systems various genetic backgrounds were combined and tested for the different types of stress. Genes were knocked out in MG1655 by general transduction by the bacteriophage T4GT7 (T4 bacteriophage, generalized transducer number 7). Under transduction by a bacteriophage the phage infects the donor bacterial cell and phage DNA and proteins are made inside the donor cell. The bacterial chromosome is broken into pieces and those pieces are occasionally packed into phage capsid. Phage particles with bacterial DNA are released upon lysis of the donor cell. New host cells can be infected by the phage carrying bacterial DNA (Tortora et al. 2010).

3.4.1 Lysate preparation from donor strain

Lysate was prepared from donor strains which already had the desired gene knocked out, to knock out genes in MG1655 by general transduction. The desired gene had been replaced with a kanamycin cassette in the donor strain.

ONC of the donor strains were made and T4GT7 lysate was serial diluted 1:10 in 100 µl T4 buffer in 5 Eppendorf tubes to make 10^{-1}-10^{-5} dilutions. 200 µl of donor strain and 1.2 µl tryptophane was added to each tube and incubated at room temperature for 25 minutes. Five 5 mL-tubes were prepared with soft agar to stop the reaction. The tubes were preheated to 60°C to avoid premature solidification of the soft agar. The reaction was stopped by adding the solution to 4 mL soft agar, and the soft agar was poured out on LB-plates and incubated at 37°C overnight.

3.4.2 Harvesting lysate

After the lysate had been prepared it was harvested the next day. Plates that contained plaques that were touching each other were harvested. Clear single plaques become identifiable usually at the 10^{-4}-10^{-5} plate.

Glass pipette was made to a scraper and the soft agar was scraped together into a 15 mL tube.The plate was washed 2 times with 1 mL T4 buffer. 0.2 mL $CHCl_3$ was added to the tube and mixed. The tubes were centrifuged at 4500 RPM for 5 minutes in "Allegra X-22R Centrifuge". The supernatant was transferred to a clean 2 mL tube which had 10 µl $CHCl_3$ added at the bottom of the tube. The lysate was stored at 4°C.

3.4.3 Transduction of MG1655

MG1655 was transduced with the lysates prepared in an attempt to remove the desired gene and replace it with kanamycin cassette.

ONC of MG1655 was made and T4GT7 lysate was serial diluted 1:10 in 100 µL T4 buffer in 4 Eppendorf tubes. This gave 10^{-1}-10^{-4} dilutions. 500 µL of ONC was centrifuged for 20 seconds on 14000 RPM and resuspended in 1 mL T4 buffer. 100 µL of bacteria was added to each tube and incubated at room temperature for 25 minutes. As a negative control 100 µL of bacteria was added to 100 µL of T4 buffer and included in the experiment. By including a negative control, one can make sure MG1655 is not already kanamycin resistant. Five 5 mL-tubes were prepared with soft agar to stop the reaction. The tubes were preheated to 60°C to avoid premature solidification of the soft agar.The reaction was stopped by adding the solution to 4 mL soft agar, and the soft agar was poured out on kanamycin plates and incubated at 37°C overnight. If transformants were observed, they were restreaked minimum two times to make sure no bacteriophage was left. To transduce *nhaA* mutant with istR::kan, ldrD::kan and rdlD::kan lysates, ONC was made in low salt LB medium which was also used in the soft agar prepared for the transduction. Low salt LB plates were used after the reaction was stopped in soft agar.

3.4.4 Colony PCR and gel electrophoresis to verify deletion

Colony PCR was performed to verify that the correct gene in MG1655 had been deleted and replaced with kanamycin. Forward or reverse primer of the selected gene was used and forward or reverse primer for kanamycin cassette was used to verify deletion. See Appendix B to see primer sequence. 12 µL reaction was prepared in a 0.5 mL PCR tube on ice. Components needed for 1 reaction are listed below.

Buffer	1×
Forward primer	0.5 µM
Revers primer	0.5 µM
dNTPs	1000 µM
Taq DNA polymerase	0.06 µL/ rxn
DMSO	2%
Template bacteria*	1 µL
$MgCl_2$	2 mM
Nuclease-free water	to 12 µL

* 1 colony of template bacteria was resuspended in 10 µL dH_2O and 1 µL was added to the reaction mix.

Cycling conditions for PCR:

Cycle step	**TEMP**	**TIME**	**CYCLES**
Initial denaturation	95°C	5 minutes	1
Denaturation	95 °C	30 seconds	25
Annealing	55° C	30 seconds	
Extension	72° C	45 seconds	
Final extension	72°C	10 minutes	1
Hold	4°C	∞	

Verification of the PCR products were carried out on 1% agarose gel with 2 µL of 6× loading dye. 12 µL was applied to the gel and ran for 35 minutes with 90V current.

3.4.5 Electrocompetent cells

Electrocompetent cells were generated from the strains that needed kanamycin cassette removal. Tubes and solutions were pre-chilled in ice-water and the host strain with the gene replaced by the kanamycin cassette was grown in 5 mL LB with kanamycin at 37 °C to OD_{600}~0.6. After reaching OD_{600}~0.6, the cells were chilled in ice-water bath. The cells were then transferred to a 15 mL tube and centrifuged for 10 minutes at 2500 RCF at 4°C. The cells were resuspended in 5 ml dH_2O. 2 ml of a solution of 1.5% mannitol + 20% glycerol was added to the tube under the water phase. The cells were again centrifuged for 10 minutes at 2500 RCF at 4°C. The supernatant was aspirated and each cell pellet was resuspended in 20 % glycerol + 1.5 % mannitol and H_2O so the final concentration of glycerol was 10 % and cell density was adjusted to OD_{600}=50. The cells were kept on ice until electroporation.

3.4.6 Removing antibiotic resistance with pCP20

pCP20 was electroporated in the host cells to remove Kan^R casette. pCP20 is a temperature sensitive plasmid which contains a Flp recombinase gene from yeast and an ampicillin resistant gene. The Flp enzyme is a site-specific recombinase which promotes recombination within a 65-nt sequence at a specific site termed Flp recombination target (FRT). The plasmid is 9.4 kb and is inherited stably at 30°C but at temperatures above 37°C it is inherited poorly. It is important to remove pCP20 upon a new transduction as it would flip out the kanamycin cassette upon a new transduction. It would then not be possible to select colonies with kanamycin. Free DNA can enter the cells though microscopic pores in the membrane created by an electrical pulse in the electroporation process (Tortora et al. 2010).

The cuvettes used for electroporation were pre-chilled to 4°C and the electroporator was set to 2.5 kV. 50 µl of electrocompetent cells were electroporated with 39 ng DNA (pCP20). 1 ml of SOC was added immediately to the cuvette, resuspended and transferred to a 15 mL tube. As a negative control, cells were electroporated without DNA added. The cells were grown at 30°C in shaker for 2 hours and 200 µl (and 20 µl in case the plates with 200 µl had colonies too close to each other) of cells were plated out on ampicillin plates. The cells were grown at 30°C for 16-24 hrs. Few transformants were colony purified non-selectively at 42°C by growing them in LB-medium for 3-4 hours. After 3-4 hours 1 µl from each bacterial culture was transferred to a tube

with fresh LB-medium and grown again for 3-4 hours. The bacteria was then streaked out on LB plates and incubated at 42°C overnight. The next day 3 colonies from each strain were grown in 3 mL LB-medium for 4-5 hours at 37°C and 100 µL was plated out on kanamycin and ampicillin plates to make sure that the antibiotic resistance is removed. Kanamycin plates were used to test for the presence/absence of kanamycin resistant gene in the genome of the host strain, while ampicillin plates were used to test for the presence/absence of ampicillin resistant from the pCP20 plasmid.

3.4.7 Genotypying

Forward and reverse primer of the selected genes were used to verify that kanamycin cassette has been removed by pCP20. The expected bands should be around 1000 base pairs if it has been removed. Same procedure as described in "**3.3.4 Colony PCR to verify deletion"** was used.

3.4.8 Making mutants with different genetic background

After making the single mutants of MG1655, the strains were transduced with tisB::kan, istR::kan, shoB::kan, ohsC::kan, ldrD::kan or rdlD::kan lysates to make double mutants. To transduce with these lysates, procedure described in "3.3.3 Transduction of MG1655" was followed. To again verify that the gene of interest has been replaced with kanamycin cassette, colony PCR was performed as described in "3.3.4 Colony PCR and gel electrophoresis to verify deletion". The process was repeated to make triple or quadruple mutants. The table below shows strains made.

Table 4: Mutants made. Δ indicates it is a deletion mutant and:: indicates its an insertion mutant.

Single mutants	**Mutants in combination**
ΔnhaA	*ΔnhaA tisB::kan* *ΔnhaA istR::kan* *ΔnhaA shoB::kan* *ΔnhaA ohsC::kan* *ΔnhaA ldrD::kan* *ΔnhaA rdlD::kan*

ΔnhaB	*ΔnhaB tisB::kan* *ΔnhaB istR::kan* *ΔnhaB shoB::kan* *ΔnhaB ohsC::kan* *ΔnhaB ldrD::kan* *ΔnhaB rdlD::kan*
ΔtonB	*ΔtonB tisB::kan* *ΔtonB istR::kan* *ΔtonB shoB::kan* *ΔtonB ohsC::kan* *ΔtonB ldrD::kan* *ΔtonB rdlD::kan*
Δdps	*Δdps tisB::kan* *Δdps istR::kan* *Δdps shoB::kan* *Δdps ohsC::kan* *Δdps ldrD::kan* *Δdps rdlD::kan*
Δftn	*Δftn tisB::kan* *Δftn istR::kan* *Δftn shoB::kan* *Δftn ohsC::kan*
Δfur	*Δfur tisB::kan* *Δfur istR::kan* *Δfur shoB::kan* *Δfur ohsC::kan*
ΔfeoB	*ΔfeoB tisB::kan* *ΔfeoB istR::kan* *ΔfeoB shoB::kan* *ΔfeoB ohsC::kan*
ΔfecC	*ΔfecC shoB::kan*

	ΔfecC ohsC::kan
ΔfecD	*ΔfecD shoB::kan* *ΔfecD ohsC::kan*
ΔfepD	*ΔfepD shoB::kan* *ΔfepD ohsC::kan*
ΔsodA	*ΔsodA shoB::kan* *ΔsodA ohsC::kan* *ΔsodA iscA::kan* *ΔsodA fhuB::kan* *ΔsodA ΔiscA shoB::kan* *ΔsodA ΔiscA ohsC::kan* *ΔsodA ΔfhuB shoB::kan* *ΔsodA ΔfhuB ohsC::kan*
ΔsodB	*ΔsodB shoB::kan* *ΔsodB ohsC::kan*
ΔsodC	*ΔsodC shoB::kan* *ΔsodC ohsC::kan*
ΔsufA	*ΔsufA shoB::kan* *ΔsufA ohsC::kan*
ΔsufE	*ΔsufE shoB::kan* *ΔsufE ohsC::kan*
ΔiscA	*ΔiscA shoB::kan* *ΔiscA ohsC::kan*
ΔfhuB	*ΔfhuB shoB::kan* *ΔfhuB ohsC::kan*
ΔkatG	*ΔkatG shoB::kan* *ΔkatG ohsC::kan* *ΔkatG ΔkatE* *ΔkatG ΔkatE ΔahpC* *ΔkatG ΔkatE ΔahpC shoB::kan* *ΔkatG ΔkatE ΔahpC ohsC::kan*

ΔkatE	*ΔkatE shoB::kan* *ΔkatE ohsC::kan*
ΔahpC	ΔahpC shoB::kan ΔahpC ohsC::kan

3.4.9 Making glycerol stocks

Glycerol stocks were made of all new strains for future screening and to avoid contamination. The strains were grown in LB-medium with or without antibiotic overnight or 5-6 hrs. 1200 µL bacteria culture was mixed with 600 µL of 60% glycerol and kept at -80°C. The final concentration of glycerol was 20 %.

3.5 Complementation plasmid

Complementation plasmids were electroporated into *tisB, istR, shoB, ohsC, ldrD* and *rdlD* mutants and tested again to see if the effect seen is because of the peptide/sRNA or because of interference in the genomic area. Low-copy-number plasmid (pMW119) was used so that the ectopic expression of the gene itself is not toxic. High-copy number plasmid was also used (pUC57) for *tisB, istR, ohsC, ldrD* and rdlD. The high-copy-number plasmid expresses the toxin/antitoxin in 500-700 copies and the low-copy-number plasmid expresses the toxin/antitoxin in 15-20 copies (Cohrt 2015). The high-copy-number plasmid with ShoB was too toxic and could not be transformed into the *shoB* mutant. Both plasmids were selected on ampicillin plates. Procedure described in "3.3.6 Rremoving antibiotic resistance with pCP20 " was used to transform the plasmid into the strains.

3.6 Flow Cytometry

Flow cytometry was performed to measure size of the cells. In this method a moving fluid containing bacteria is forced through a small opening and bacteria can be detected through detection of difference in electrical conductivity between cells and the surrounding medium. A laser is used to illuminate the fluid passing through the opening which scatters light that provides information about cell size, shape, density and surface. The data is analyzed by a computer. Fluorescence can be used to detect cells that are tagged with fluorescent dyes (Tortora et al. 2010). Side-scattered light (SSC) is proportional to cell granularity or internal complexity and Forward-scattered light (FSC) is proportional to cell-surface area or size (Tzur et al. 2011).

3.6.1 Measuring cell size

pH of 1×PBS and LBK medium was adjusted to pH 5.5 and 8.0. MES buffer was used for pH 5.5 and MOPS buffer was used for pH 8.0. In PBS the buffers were added to a final concentration of 20 mM, and in LBK the buffers were added to a final concentration of 20 mM. On a 96 well plate 100 µl of PBS with pH adjusted to 5.5 and 8.0 was added in as many wells as needed. The mutants *tisB, istR, shoB, ohsC, ldrD, rdlD* and wild type were grown in LBK medium adjusted to pH 5.5 and 8 for 1-1.5 hour before OD_{600} and forward scatter light measurements were made. Measurements were made with 30-minute intervals in between. Flow cytometer was used to measure cell size.1-10 µL of bacteria culture was added in each well to keep events/sec below 2500.

3.7 Endogenous H_2O_2 Detection

To continuously monitor the formation of intracellular H_2O_2, the extracellular H_2O_2 levels were measured. In this experiment an ultrasensitive Amplex red/horseradish peroxidase (AR/HRP)-based fluorescence assay was used. AmplexRed(10-acetyl-3,7-dihidroxyphenoxazine) is a colorless substrate that reacts with H_2O_2 with a 1:1 stoichiometry in the presence of HRP and produces red-fluorescent resorufin with excitation maxima at 570 nm and emission maxima at 585 nm (ThermoFisher)

Method described by (Seaver, L. C. & Imlay, J. A. 2001) was used for H_2O_2 detection and H_2O_2 scavenging by whole cells with some modifications. Instead of using 50 mM potassium phosphate (KPi, pH 7.8), 50 mM potassium phosphate buffer at pH 7.80 was used. Samples were loaded in Black opaque 96-well polystyrene plate and measured on a walllac Victor^2 1420 multilabel counter at wavelength settings of 570 nm (excitation) and 590 (emission). For each measurement 50 µL of sample was mixed with 25 µL AR solution and 25 µL of HRP solution. MQ filtered water was autoclaved with platinum plate to remove trace H_2O_2 and all solutions were prepared with this water. A standard curve was obtained by diluting H_2O_2 to 10 mM in water. This dilution was further diluted to 10 µM in potassium phosphate buffer (50 mM, pH 7.8). Serial two-fold dilutions in 50 mM potassium phosphate buffer at pH 7.80, were used to prepare calibration standards which gave the concentrations 0, 0.078, 0.156, 0.3125, 0.625, 1.25, 5 and 10 µM. Sample without H_2O_2 was used as blank. Fluorescence of resorufin was measured immediately after adding all reagents.

To measure H_2O_2 scavenging by whole cells method described by Seaver & Imlay (2001) was used with modifications. Instead of taking 0.45-mL aliquots, 50 µL aliquots were taken and assayed immediately for H_2O_2 content by the AR/HRP method. This assay was done for the mutant *shoB* and wild type (MG1655). To monitor H_2O_2 production rates the intracellular formation of H_2O_2 was measured by measuring extracellular H_2O_2 levels. Cells from plates were grown in M9 medium with 0.2% glucose medium containing 0.5 mM each of the 20 amino acids. MG1655 and *shoB* were grown aerobically to an OD of ≈0.1; however, *katG katE ahpC* mutants were grown only to an OD of ≈0.05 in order to measure H_2O_2 production before growth was significantly inhibited. Cells were then washed in fresh medium containing only 0.02% glucose and 0.05 mM amino acids, resuspended at an OD of 0.1 in the same medium, and incubated with shaking at 37°C. Glucose was added to the M9 medium immediately before use. Aliquots were removed at intervals, and their H_2O_2 content was measured. This assay was performed for MG1655 and *shoB* mutant and MG1655, *katG katE ahpC, katG katE ahpC shoB::kan* and *katG katE ahpC, katG katE ahpC ohsC::kan* mutants. All solutions were shielded from light after preparation to avoid chemical production of hydrogen peroxide.

4 Results

The mutants *dinQ, agrB, tisB, istR, shoB, ohsC, ldrD* and *rdlD* were stressed by irradiation and different chemicals. The biophysical response of the bacteria to the stress was observed by their ability to replicate and form colonies. To define the epistasis group of the TA-systems, various genetic backgrounds were combined. The response of *shoB* and *ohsC* mutants to superoxide was opposite of hydrogen peroxide stress, so the main focus after the screening was to investigate the role of ShoB. All figures presented are from experiments done in triplicate if not mentioned otherwise. Mean and standard deviation was calculated from the triplicates. *tisB, istR, shoB, ohsC, ldrD* and *rdlD* mutants were complemented with a high-copy-number plasmid (pUC57) and a low-copy-number plasmid (pMW119) to see if the effect seen is caused by the peptide (toxin)/ sRNA (antitoxin) or because of interference in the genomic area. A high-copy-number could give less survival since overexpression of toxin is toxic. *shoB* mutant could not be complemented with a high-copy-number plasmid as it was too toxic for the cells. An inducible system is a possible alternative.

Different genetic backgrounds were combined to define the epistasis group of the TA systems. To verify the new genotype of the strains, PCR was performed with respective primers. Expected size of the bands is shown in Appendix C. Genes encoding superoxide dimutases (*sodA, sodB* and *sodC),* iron-sulphur synthesis proteins (*sufA* and *sufE),* iron-sulphur cluster assembly protein (*iscA),* proteins involved in iron homeostasis (*fhuB, fecC, fecD fepD, ftn, fur, feoB, dps,* and *tonB)* and antiporters involved in pH homeostasis (*nhaA* and *nhaB)* were deleted and tested for different types of stress. These single mutants were combined with *tisB::kan, istR::kan, shoB::kan, ohsC::kan, ldrD::kan,* and *rdlD::kan*.

4.1 Chronic oxidative stress caused by H_2O_2

The strains were stressed chronically with hydrogen peroxide. Concentrations between 0.005-500 mM were tested for single colonies. The concentation 0.7 mM of H_2O_2 seemed to kill 99 % of the bacteria on the spot assay. It was repeatedly observed that MG1655 had a very dense growth in the first spot, which contained undiluted bacteria, while no growth was seen in the second spot, which contained 10^{-1} dilution of the bacteria in PBS. To investigate if MG1655 produced signal molecules that increased survival, the bacteria culture was resuspended in PBS before it was diluted in PBS. It was also resuspended in LB medium and then serial diluted in both PBS and

LB-medium. No difference in survival was observed (data not shown). Figure below shows strains stressed with hydrogen peroxide and their survival.

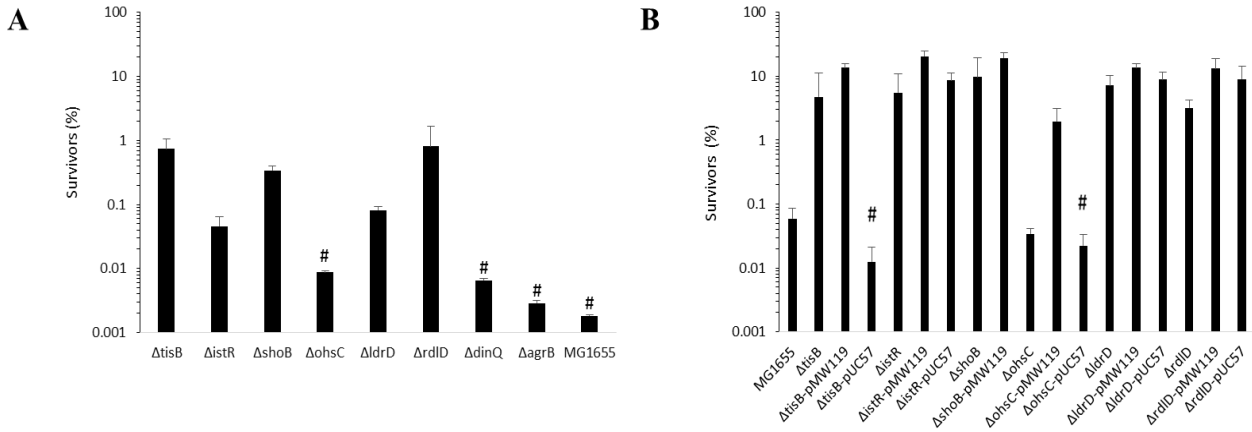


Figure 4: Response to chronic oxidative stress caused by H_2O_2 (0.7 mM) (A) Response of TA-systems *tisB-istR, shoB-ohsC, ldrD-rdlD dinQ-agrB* and wild type. (B) Response of TA-systems tisB-istR, shoB-ohsC, ldrD-rdlD and with low- and high-copy-number plasmid. # CFU/mL were set to 10 000 as the colonies were uncountable due to dense bacterial growth.

It was observed that wild type had 0.00018 ± 0.000097 % survivors while the mutants had 0.00028-0.74 % survivors, as seen in Figure 4A, which is up to 4400-fold increase in survivors among the mutants. The deletion of toxin or antitoxin gave increased oxidative stress response. In general, it was noticed that the deletion of toxin lead to a higher percentage of suvivors than deletion of antitoxin, compared to wild type, except for *ldrD* and *rdlD* deletion. The biggest difference in survival between toxin and antitoxin was noted between *shoB* and *ohsC* mutants. The deletion of *shoB* gave almost 400-fold increase in survivors compared to the deletion of *ohsC*. The deletion mutants were complemented with high-copy-number plasmid and low-copy-number plasmid. Their response against oxidative stress is shown in Figure 4B. Complementation of *tisB* mutant with high-copy-number plasmid brought the survival level back to the same level as wild type. Complementation of the *ohsC* mutant with high-copy-number plasmid reduced the sensitivity against oxidative stress.

ohsC mutant was tested with H_2O_2 at a concentration of 0.6 mM, where it showed almost 10-fold increased sensitivity compared to wild type (data not shown). Figure below shows mutants in combination with *shoB::kan* and *ohsC::kan.*

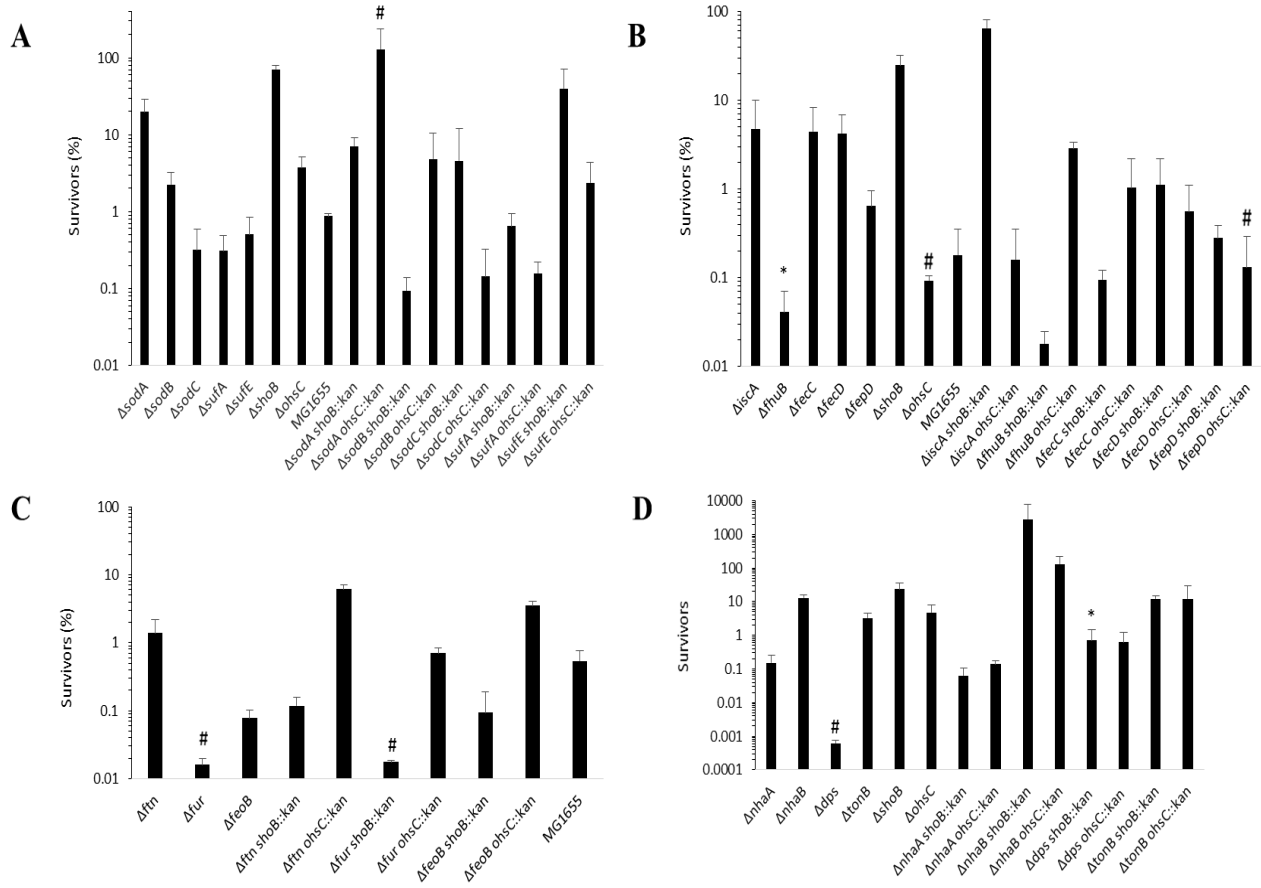


Figure 5: Response to chronic oxidative stress caused by H_2O_2 (0.7 mM). # CFU/mL were set to 10 000 as the colonies were uncountable due to dense growth. *One of the triplicates had less than 10 colonies in the well that was countable. (D) Survivors are calculated as described in Materials and methods (equation 2).

The mutants *sodC*, *sufA* and *sufE* showed the same level of sensitivity as wild type, while *sodA* and *sodB* mutants showed decreased level of sensitivity, as seen in Figure 5A. Upon examination, the *sodA* mutant also showed a 20-fold increase in survivors compared to wild type. Mutants in combination with *shoB::kan* and *ohsC::kan* showed similar survival levels as the single mutants, except *sodB shoB::kan* mutant, which showed increased sensitivity compared to *sodB* and *shoB* mutants. However, the *sodA ohsC::kan* mutant showed decreased sensitivity compared to the single mutants *ohsC* and *sodA.*

Figure 5B shows increased survival for the *iscA, fecC, fecD* and *fepD* single mutants compared to wild type, where *fhuB* mutant had almost the same survival level as wild type. *fhuB* in combination with *shoB::kan* showed increased sensitivity to oxidative stress. The effect of *fhuB* mutant

dominated over *shoB* mutant. *fhuB* in combination with *ohsC* had decreased sensitivity compared to *fhuB* and *ohsC* single mutants. Deleting the gene encoding the transcription factor Fur also gave a mutant which was very sensitive to oxidative stress, as seen in Figure 5C. This effect is seen dominant in the double mutant *fur shoB::kan* but not in *fur ohsC::kan.* In general, it was observed that *ohsC::kan* in combination with *ftn, fur* and *feoB* gave a decreased level of sensitivity compared to the single mutants. In Figure 5D the survivors are calculated with Equation 2, as described in Materials and methods. Values below 1 indicate strains that have less survivors than MG1655, while values above 1 indicate more survivors than MG1655. The *dps* mutant showed increased sensitivity to oxidative stress, while *nhaA, nhaB* and *tonB* mutants showed decreased sensitivity, compared to MG1655. Mutants in combination with *shoB::kan* and *ohsC::kan* showed similar survival levels as the single mutants, except *nhaB shoB::kan* and *nhaB ohsC:kan,* which had 100- and 2500-fold increase in survival compared to MG1655, respectively. Figure below shows mutants in combination with *tisB::kan, istR::kan, ldrD::kan* and *rdlD::kan.*

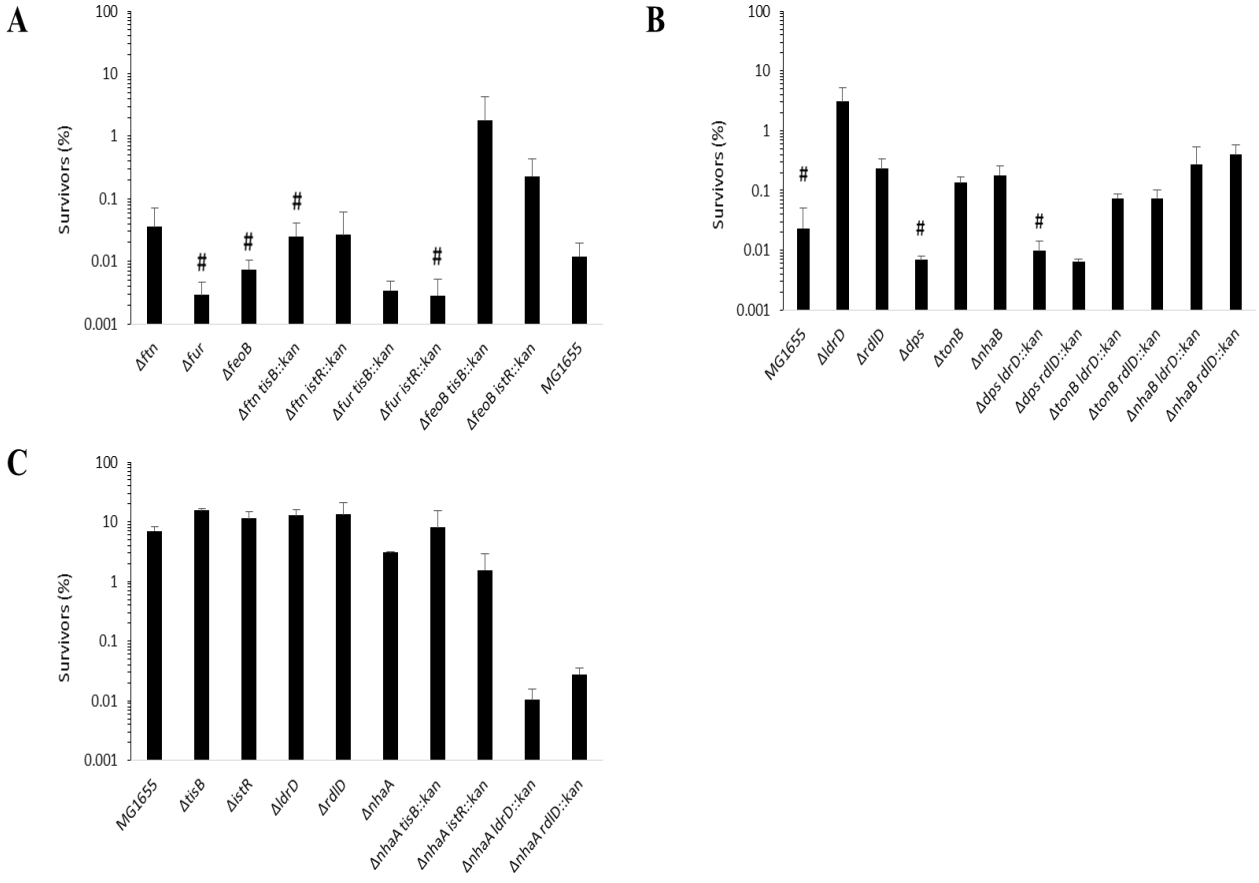


Figure 6: Response to chronic oxidative stress caused by H_2O_2 (0.7 mM). #CFU/ml were set to 10 000 as the colonies were uncountable due to dense growth.

Observations revealed that *fur* mutant is very sensitive to oxidative stress compared to MG1655 and this effect dominates in the *fur tisB::kan* and *fur istR::kan* mutants (Figure 6A). It was further seen that the *feoB* mutant is also sensitive to oxidative stress compared to MG1655, but in combination with *tisB::kan* and *istR::kan* the sensitivity is reduced by 30- to 230-fold. When *tisB::kan, istR::kan, ldrD::kan* and *rdlD::kan* were combined with *dps, tonB, nhaA* and *nhaB,* the percentage of survivors was brought back to the same levels as of the single mutants, as shown in Figure 6B and 6C, except for the *nhaA ldrD::kan* and *ldrD::kan* mutants, which had 10-fold less survivors.

4.2 Chronic oxidative stress caused by menadione sodium bisulfite

Menadione causes a different type of oxidative stress than hydrogen peroxide. Menadione gives production of superoxide, which can cause oxidative stress in bacteria. Concentrations between 2 µM -8 mM were tested for single colonies. Toxicity level which kills 99 % of wild type was not found but an effect was seen in some of the mutants. Figures below show strains tested and their survival.

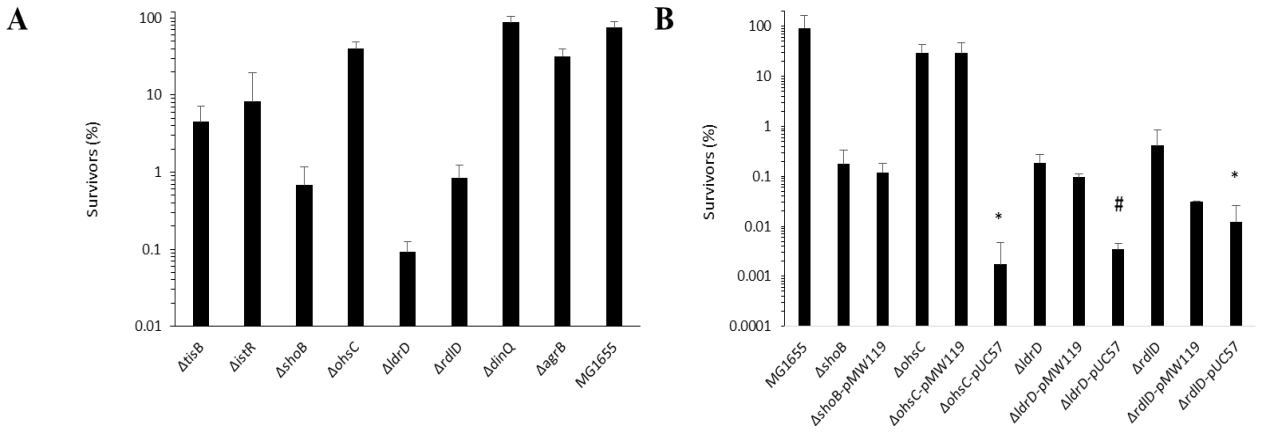


Figure 7: Response to chronic oxidative stress caused by menadione. (A) Response of TA-systems *tisB-istR, shoB-ohsC, ldrD-rdlD, dinQ-agrB* and wild type tested at 5 mM. (B) Response of TA-*systems shoB-ohsC, ldrD-rdlD* and with low- and high-copy-number plasmid tested at 7 mM. #CFU/mL were set to 10 000 as the colonies were uncountable due to dense growth. *One of the triplicates had less than 10 colonies in the well that was countable.

It was observed that MG1655 was not sensitive to oxidative stress caused by menadione sodium bisulfite at the concentrations tested. Concentrations above 8 mM could not be tested, as menadione sodium bisulfite gave precipitation. However, *shoB* and *ldrD* mutants were very sensitive to concentration of 7 mM, as seen in Figure 7A. These two mutants showed 100- and 800-fold decrease in survivors compared to MG1655, respectively. Deletion of the antitoxin

RdlD also showed a 100-fold decrease in survivors. *ldrD, rdlD* and *ohsC* mutants were tested with low- and high-copy-number plasmids, while *shoB* was tested with low-copy-number. The results are shown in Figure 7B. Complementation of the four mutants did not remove the effect seen. However, complementation of *ohsC* with high-copy-number plasmid reduced survival by more than 1000-fold.

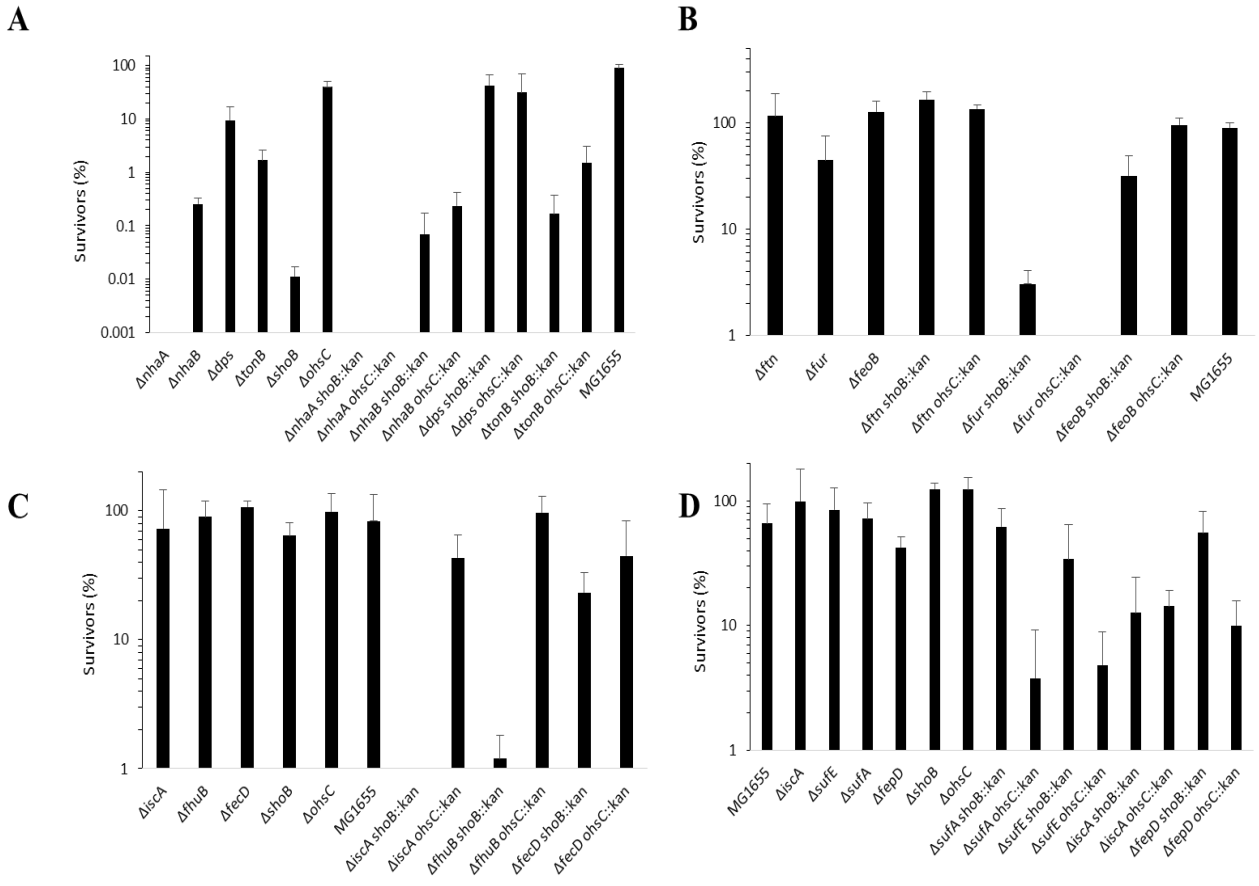


Figure 8: Response to chronic oxidative stress caused by menadione in combination with different genetic backgrounds tested at a concentration of 7 mM.

Mutant lacking the gene *nhaA*, which encodes the antiporter NhaA, was found to be very sensitive to oxidative stress caused by menadione. No survivors were observed at the concentration tested, as seen in Figure 8A. This antiporter primarily functions under adaptation to high salinity, and protects against Li^+ toxicity and adaptation to alkaline pH. Mutants in combination are also sensitive and had no survivors as well. The *nhaB* mutant was also sensitive to oxidative stress and had more than 300-fold increased sensitivity compared to MG1655. Single mutants in combination with *shoB::kan* or *ohsC::kan* brought the survival back to the same level

as the single mutants. It was further observed that deleting the genes *ftn*, *fur* or *feoB* gave the same percentage of survivors as MG1655. The same was observed when they were combined with *shoB::kan* and *ohsC::kan. fur* mutants in combination with *shoB::kan* and *ohsC::kan* did not follow the same trend, as shown in Figure 8B. *fur shoB::kan* mutant had almost 30-fold less survivors compared to MG1655 while *fur ohsC::kan* had no survivors. Figures 8C and 8D do not show the same level of sensitivity for *shoB* mutant, as seen in previous observations, where *shoB* had been stressed with menadione. *iscA shoB:kan* and *fhuB shoB::kan* mutants still showed a decrease in survival compared to wild type. No survivors of *iscA shoB:kan* were observed, while *fhuB shoB::kan* mutant showed almost 70-fold less survivors compared to MG1655.

The mutants lacking genes for the superoxide dismutates, encoded by *sodA*, *sodB* and *sodC*, showed different sensitivity against oxidative stress caused by menadione. A *sodA* mutant had no survivors at a concentration of 7 mM (data not shown), so the *sodA* mutants were tested at lower concentration. The results are shown in the figure below along with other mutants tested as well.

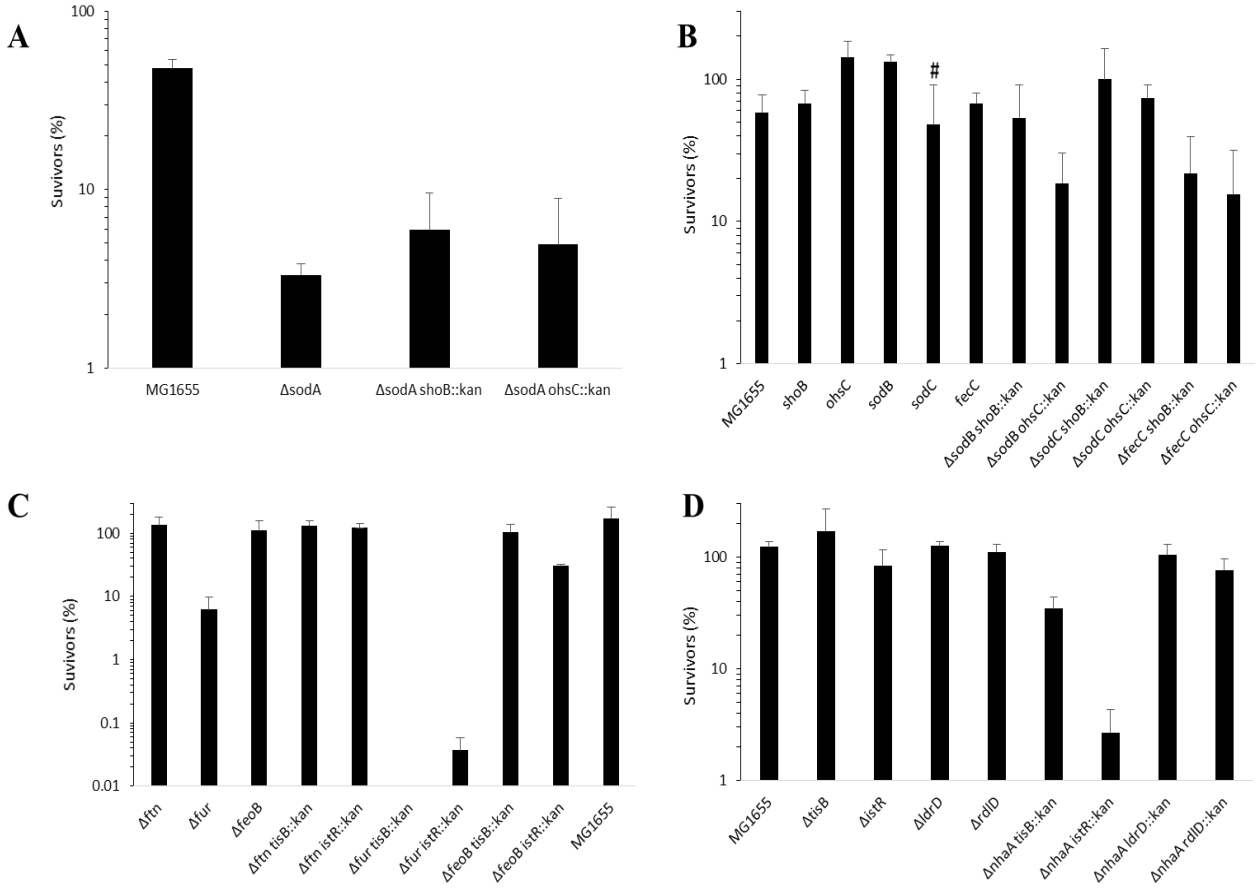


Figure 9: Response to chronic oxidative stress caused by menadione in combination with different genetic backgrounds. The *sodA* mutants were tested at a concentration of 4 mM and the other mutants were tested at 7 mM.

The *sodA* mutants were stressed with menadione at a concentration of 4 mM. No difference in survivors between the *sodA* mutant and the double mutants *sodA shoB::kan* and *sodA ohsC::kan* was observed (Figure 9A). The three single mutants *sodB, sodC, fecC* and the double mutants in combination with *shoB::kan* and *ohsC::kan,* showed the same level of survivors as MG1655, as seen in Figure 9B. Figures 9C and 9D show the response of mutants in combination with *tisB::kan, istR::kan, ldrD::kan* and *rdlD::kan.* Upon deletion of *fur* in combination with *tisB::kan* or *istR::kan,* an increase in sensitivity was observed, giving no survivors for *fur tisB::kan* and only 0.1 ± 0.02 % survivors for *fur istR::kan*. *nhaA istR::kan* also showed sensitivity to menadione with a 50-fold decrease in survivors compared to MG1655.

4.3 Chronic iron stress

The mutants *tisB, istR, shoB, ohsC, ldrD, rdlD,* and wild type were stressed chronically with iron. Concentrations between 4.3-10.8 mM were tested for single colonies and 5.7 mM seemed to kill 99 % on the spot assay. Figure below shows strains tested and their response to chronic iron stress.

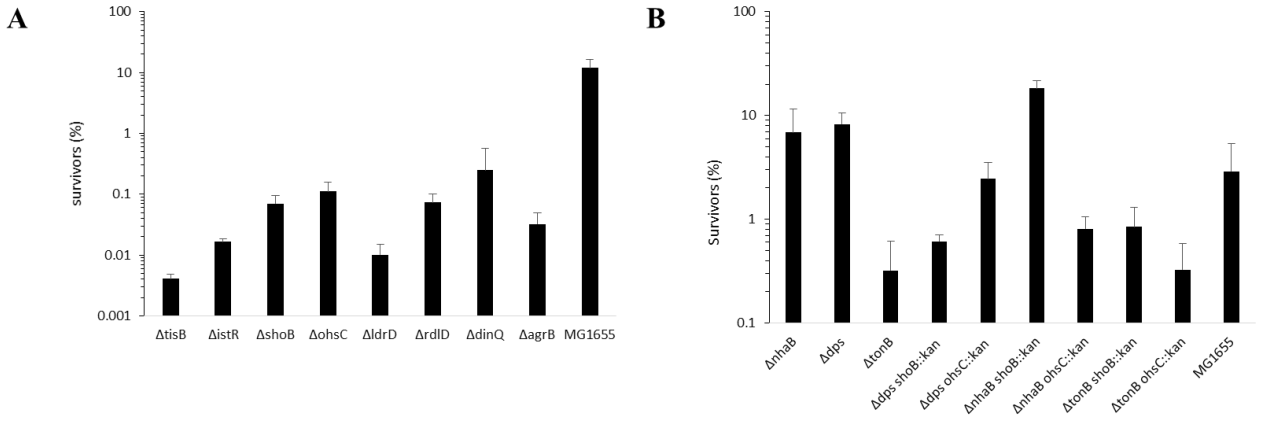


Figure 10: Response to chronic iron stress. Fe^{2+} was added to a final concentration of 5.7 mM and survival of the different strains was observed. (A) Response to iron stress by single mutants and wild type (MG1655). (B) Response of strains with different genetic backgrounds to chronic iron stress.

MG1655 had around 10 % survivors when it was stressed with Fe^{2+}. The single mutants, where toxin or antitoxin is deleted, showed an increase in sensitivity against iron stress. The sensitivity increased 50- to 3000-fold relative to MG1655 (Figure 10A). Deleting *tonB,* which encodes a protein that aids in the import of Fe^{2+} and other low concentration substrates, reduced survival by almost 10-fold, relative to MG1655 (Figure 10B). It was observed that double mutants in combination with *shoB::kan* or *ohsC::kan* brought the survival level back to the level of the single mutants.

4.4 Chronic stress by different chemicals and metals

The mutants *tisB, istR, shoB, ohsC, ldrD, rdlD* and wild type were stressed with the metals copper, manganese and cobalt at different concentrations, but a difference in ablity to replicate and form colonies was not observed. Stressing the strains with EGTA (ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N′*,*N′*-tetraacetic acid and 2,2′-Dipyridyl, which remove Ca^{2+}-ions and Fe^{2+}-ions respectively, did not show any difference in survival between wild type and mutants.

pH 5, 6, 7 and 8 were tested chronically but no effect was seen as well. To investigate if two types of stress combined has any effect, the mutants and wild type were stressed with iron and varying doses of UV radiation. Again, no effect was seen.

4.5 Acute oxidative stress caused by H_2O_2

Initially MG1655 was stressed with acute oxidative stress caused by H_2O_2 for one hour, as described in Materials and methods. Concentrations between 10 nM to 100 mM were tested. Concentration of 10 mM H_2O_2 seemed to kill 99 % of wild type. The mutants *tisB, istR, shoB, ohsC, ldrD* and *rdlD* were tested twice at this concentration but percentage of survivors varied (data not shown). The protocol seemed unstable as some strains showed 10 % survivors first time tested and no survivors second time tested. To find out if the effect seen was caused by different amount of internal ROS, MG1655 was grown for 9 generations and each generation was tested at OD_{600} 0.6 with 10 mM H_2O_2 for 1 hour. The results are shown in Figure 11A. As the old freezer stocks could contain both stationary phase and logarithmic phase bacteria, *tisB, istR, shoB, ohsC, ldrD, rdlD, dinQ, agrB* mutants and MG1655 were grown for 9 generation to OD_{600} 0.8 and new freezer stocks were made to make sure that all bacteria are in logarithmic phase. Stationary phase cultures are more resistant to ROS.

Five clones of MG1655 were grown for four generations and each generation was stressed with 12 mM H_2O_2 for one hour to see how many of the clones would survive in each generation. The purpose of the experiment was also to observe if the effect seen was genetic. Results are shown in Figure 11B. A survival curve was made for MG1655 when stressed with different concentrations of H_2O_2. The results are shown in Figure 11C. Six clones of *tisB, istR, shoB, ohsC, ldrD, rdlD, dinQ, agrB* mutants were tested at 8 mM to see if any of the mutants show the same effect as MG1655. MG1655 was included as control. Results are shown in Figure 11D.

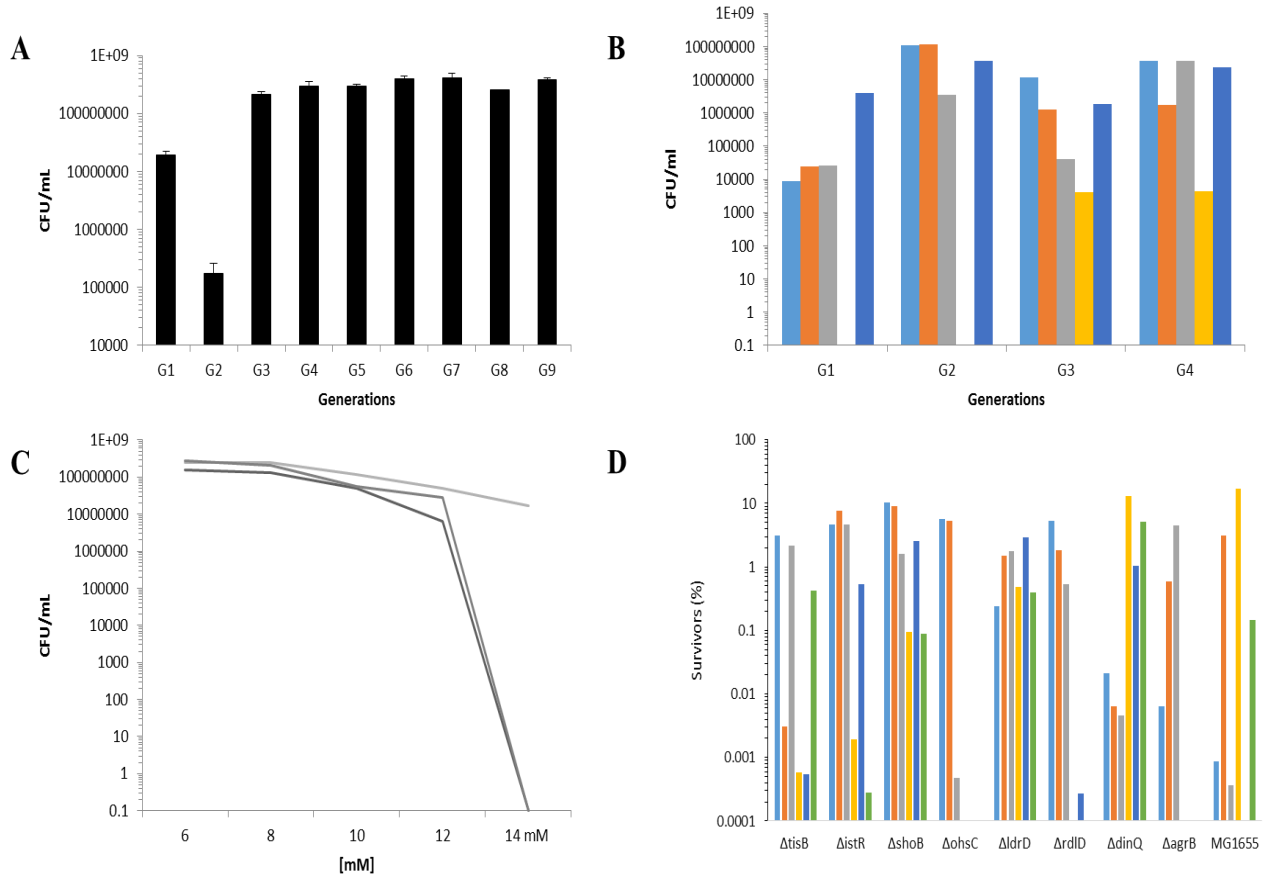


Figure 11: Response to chronic oxidative stress caused by H_2O_2. (A) Survival rate of one clone at different generations of MG1655 tested at 10 mM H_2O_2. (B) Survival rate of 5 clones of MG1655 at different generations tested at 12 mM H_2O_2. (C) Survival curve of three MG1655 clones tested at different concentrations of H_2O_2. (D) Response of six clones of the mutants *tisB, istR, shoB, ohsC, ldrD, rdlD, dinQ, agrB* and wild type to acute oxidative stress at a concentration of 8 mM H_2O_2.

When generation two was stressed with 10 mM H_2O_2, it showed poor survival. Generation two had 180 000 CFU/mL, while generation three to nine had more than 2.5 $\times 10^8$ CFU/ml, as seen in Figure 11A. Based on these observations, it seems that MG1655 can handle oxidative stress better in generations three to nine. Five clones were tested at 12 mM of H_2O_2. Clone number four had no survivors in generation one and two, and relatively low CFU/mL in generation three and four compared to the other clones, as seen in Figure 11B. In general, there were less CFU/mL in generation one compared to generations two, three and four.

Upon studying the survival curve, it is revealed that small changes in amount of H_2O_2 cause great differences in CFU/mL for MG1655, as seen in Figure 11C. Furthermore it can be seen that with an increase in concentration from 12 to 14 mM, the CFU/mL are reduced from an average of 2.81 $\times 10^7$ to 0. One of the clones survived at 14 mM but as seen in Figure 11B survival can vary from

clone to clone. The survival from clone to clone varies also for the *tisB, istR, shoB, ohsC, ldrD, rdlD, dinQ, agrB* mutants which were tested at 8 mM as shown in Figure 11D.

To investigate if the effect seen is genetic or because of technical differences, 8 clones were tested at 10 mM acute for 1 hour. The same colonies were restreaked and tested again the next day to see if there is a variation in, which of the clones die, and which survive. This was tested for four days. The fourth day the samples were also centrifuged at 4°C and 25°C to see if this has an effect on survival. To further investigate if residual H_2O_2 is causing the bacteria to die, H_2O_2 was removed with catalase, added to a concentration of 10 µg/mL bacterial culture. As control, the bacteria culture was resuspended in LB-medium to stop the oxidative stress. The results are shown in the figure below.

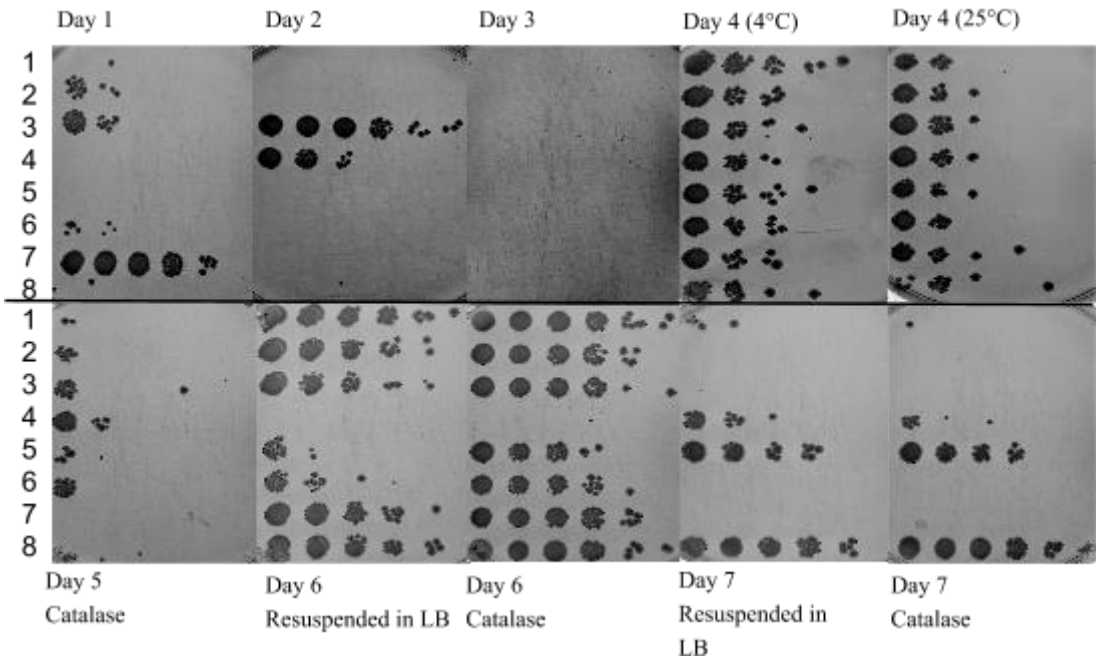


Figure 12: Response to oxidative stress caused by H_2O_2 (10 mM) for one hour. 8 clones of MG1655 were tested at different days. Different conditions for stopping the reaction were tested as described in Materials and methods.

Figure 12 shows a great difference in survival among the 8 clones that were tested. The clones that died or survived varied each day the experiment was conducted. Centrifuging the cells at different temperatures when resuspending the cells did not affect survival (Day 4). Stopping the reaction with catalase did not increase the survival either.

4.6 Acute acidic stress

The strains were stressed one hour, with acidic stress. pH between 1.70 and 4.50 was tested to find the pH that kills 90-99 % of *tisB, istR, shoB, ohsC, ldrD, rdlD* mutants and wild type. pH 3.70 seemed to kill 99 % of the cells. Figure below shows strains tested and their survival.

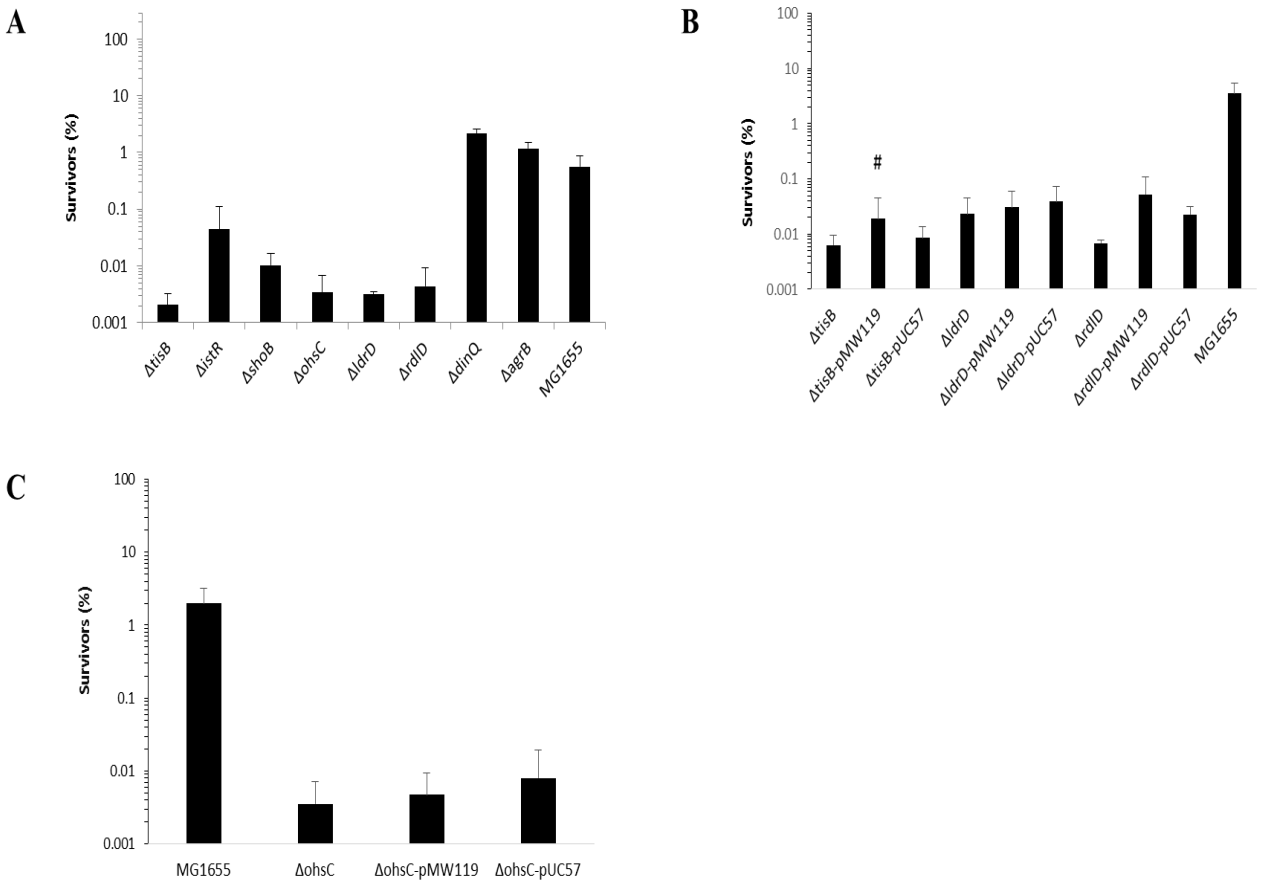


Figure 13: Acute acidic stress at pH 3.70 (A) Response of TA-systems *tisB-istR, shoB-ohsC, ldrD-rdlD dinQ-agrB* and wild type. (B) Response of *tisB, ldrD, rdlD* and with low- and high-copy-number plasmid. #One of the triplicates had less than 10 colonies. (C) Response of *ohsC* mutant without and with high- and low-copy-number plasmid.

Wild type had 1± 0.31 % survivors while *tisB, istR, shoB, ohsC, ldrD* and *rdlD* mutants had about 10- to 300- fold less survivors. Deletion of *dinQ* or *agrB* gave a slight increase in survivors. The four strains, which had the most decrease in survivors, *tisB, ohsC, ldrD* and *rdlD*, were tested with complementation plasmid, but percentage of survivors did not increase back to the same level as wild type.

4.7 Acute alkaline stress

The strains were stressed acute, 1 hour, with alkaline stress. pH between 9.35 and 11.80 was tested to find the pH that kills 90-99 % of wild type cells and *tisB, istR, shoB, ohsC, ldrD* and *rdlD* mutants. pH 9.70 seemed to kill 99 % of the cells. Figure below shows strains tested and their survival.

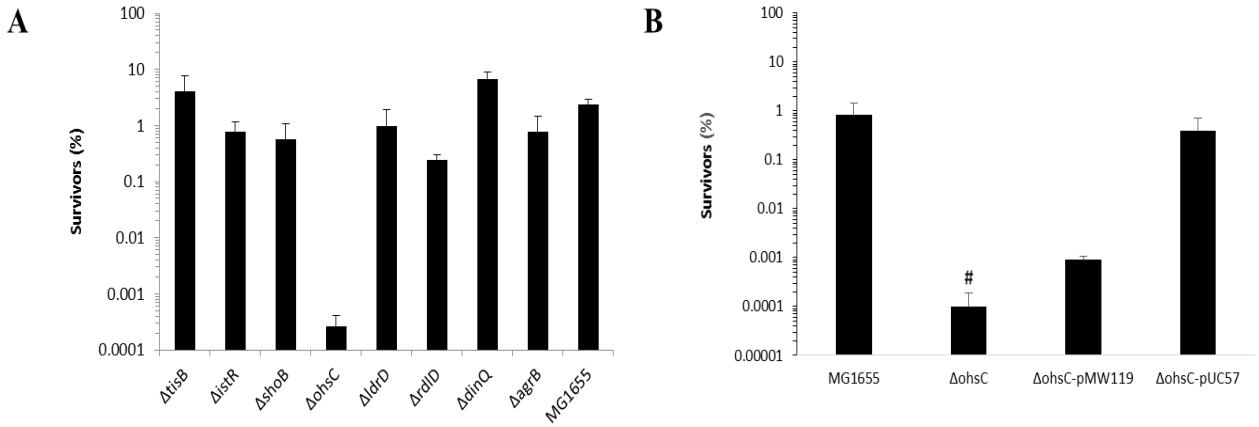


Figure 14: Acute alkaline stress at pH 9.70 (A) Response of TA-systems *tisB-istR, shoB-ohsC, ldrD-rdlD dinQ*-agrB and wild type. (B) Response of *ohsC* without and with high- and low-copy-number plasmid. #One of the triplicates had less than 10 colonies and one of the colonies had no survivors

For wild type, 2 ±0.6 % survivors were observed. Deletion of toxin or antitoxin gave a slight increase or decrease in percentage of survivors, except for the deletion of *ohsC*. For the *ohsC* mutant there was a 9000-fold reduction of survivors compared to MG1655, as seen in Figure 14A. Complementation with high-copy-number plasmid in the *ohsC* mutants brought the survival back to the same level as MG1655.

4.8 Scavenging of H_2O_2 by whole cells

To detect if there is a difference in amount of H_2O_2 produced or scavenged by the *shoB* mutant, an ultrasensitive Amplex Red/horseradish peroxidase assay was used. The amount of the fluorescent oxidation product resorufin was measured fluorometrically. The ability of *shoB* mutant to scavenge H_2O_2 when it is added to PBS and to remove trace H_2O_2 in M9 medium was measured. Results are shown below in Figure 15.

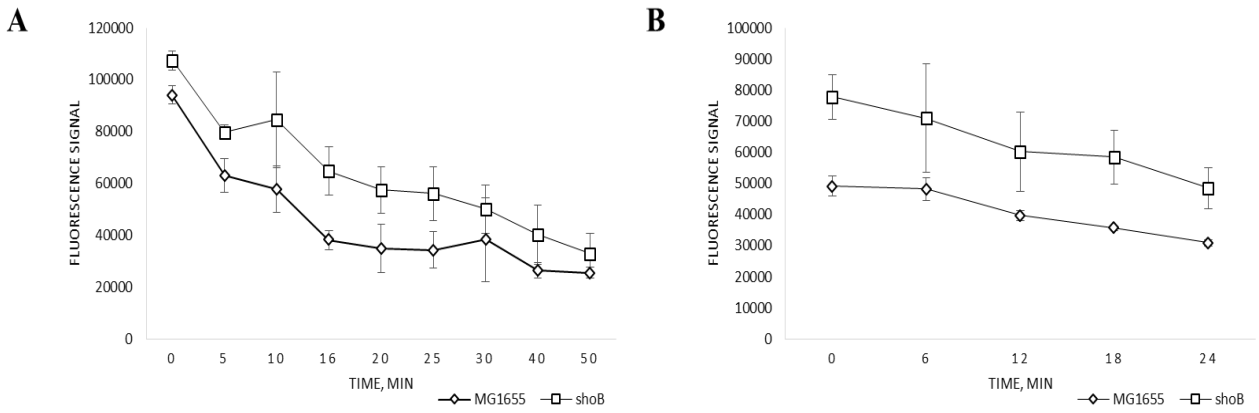


Figure 15: Measurement of H_2O_2 scavenging. (A) Scavenging of H_2O_2 by whole cells. Cultures of MG1655 (wild type) and *shoB* mutant were grown aerobically in LB medium and resuspended in PBS at an OD_{600} of 0.1. H_2O_2 was added to a final concentration of 2.0 µM and the H_2O_2 concentration was measured at various time points after addition as described in Materials and methods. (B) Cultures of MG1655 (wild type) and *shoB* mutant were grown aerobically in M9 medium with 0.2 % glucose and 0.5 mM of the 20 amino acids. It was then resuspended in M9 medium, with 0.02% glucose and 0.05 mM of each of the amino acids, at an OD_{600} of 0.1. The H_2O_2 concentration was measured at various time points after resuspension.

The statistical significance was determined by performing a statistical T-test, using a 95% confidence interval. A *p* value higher than 0.05 was considered statistically insignificant. Both figure 15A and 15B show that the *shoB* mutant had a significantly higher amount of H_2O_2 at time point 0. Also, at several other time points, the amount of H_2O_2 in the *shoB* mutant was significantly higher. The genes *katG*, *katE* and *ahpC* where deleted in combination with *shoB::kan* or *ohsC::kan* to observe differences in amount of H_2O_2 when the strains cannot remove endogenous H_2O_2. The results are shown in figure below.

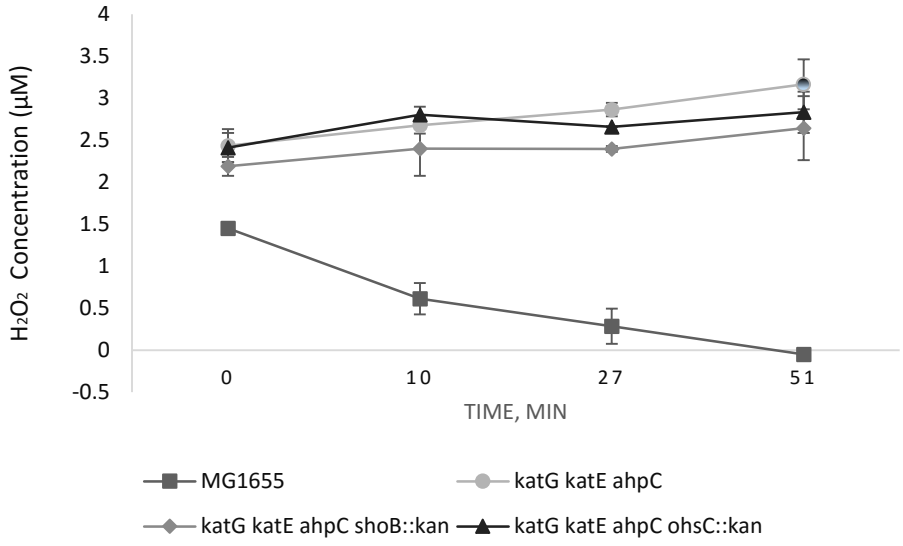


Figure 16: Cultures of MG1655 (wild type), *katG katE ahpC, katG katE ahpC shoB:.kan* and *katG katE ahpC ohsC::kan* mutants were grown aerobically in M9 medium with 0.2 % glucose and 0.5 mM of the 20 amino acids. It was then resuspended in M9 medium, with 0.02% glucose and 0.05 mM of each of the amino acids, at an OD_{600} of 0.1. The H_2O_2 concentration was measured at various time points after resuspension.

Substantial H_2O_2 accumulated in the medium of the *katG katE ahpC, katG katE ahpC shoB::kan katG katE ahpC ohsC::kan* mutants, as seen in Figure 16. This is expected because all the enzymes (*ahpC, katG* and *katE)* which have an important role in degrading H_2O_2 are deleted. Thus, MG1655 had 1.5 µM of H_2O_2 after resuspension while *katG katE ahpC* mutants had ~2.0 µM. The H_2O_2 level dropped below zero for MG1655 because this strain degrade the H_2O_2 that is present in the initial medium. No clear difference is seen between the *katG katE ahpC shoB::kan* and *katG katE ahpC ohsC::kan* mutant.

4.5 Measurement of cell size with Flow cytometry

The mutants *tisB, istR, shoB, ohsC, ldrD, rdlD* and wild type were grown in LBK medium adjusted to pH 5.5 and 8. OD_{600} and forward scatter light measurements were made with 30-minute intervals in between. Forward scattered light (FSC) is proportional to cell size. Three independent experiments were carried out with one colony from each strain. Figure below shows result for one of the colonies of *shoB, ohsC* mutants and wild type, grown at pH 5.5. The results of the other experiments at pH 5.5 and 8.0 are shown in Appendix H. The flow cytometry data was analyzed by James Booth.

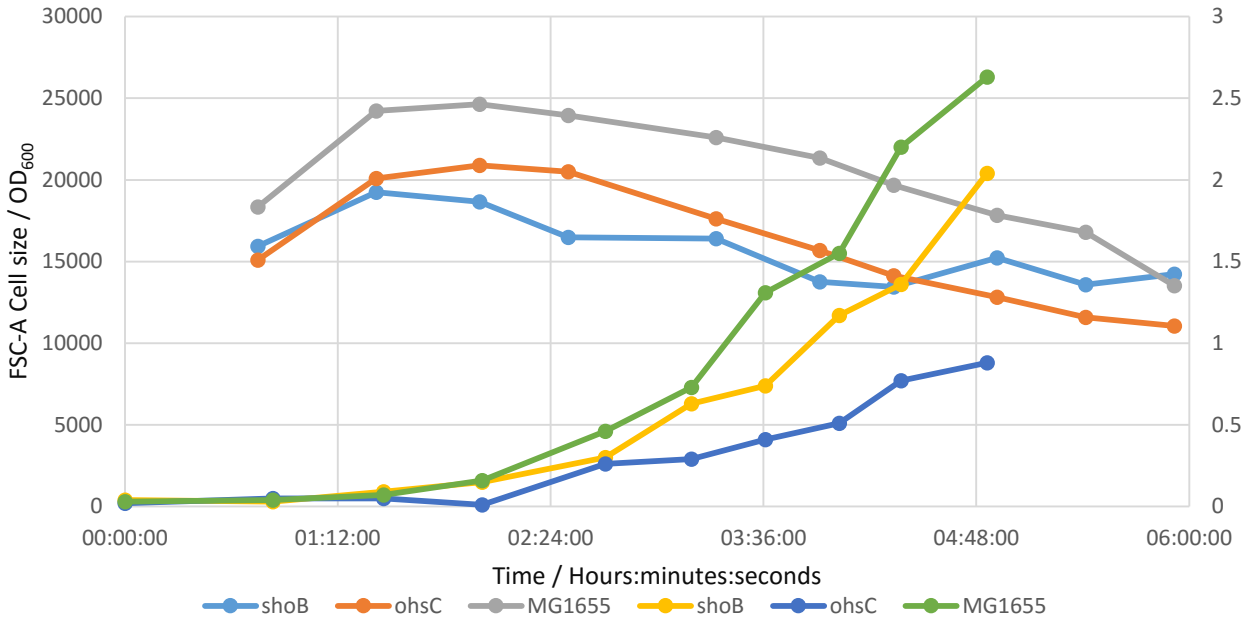


Figure 17: OD_{600} and forward scatter light measuremeants of *shoB, ohsC* mutants and MG1655. *shoB, ohsC* mutants and MG1655 were grown in LBK medium at pH 5.5 and OD_{600} and forward scatter light was measured at different time intervals. Y-axis to the left shows forward scatter and y-axis to the right shows OD_{600}.

Size reduction of *tisB, istR, shoB, ohsC, ldrD, rdlD* mutants and wild type cells along the growth curve was obsereved with flow cytometry at pH 5.5 and 8.0. The *shoB* mutant had an increase in FSC from OD_{600} ~ 0.6-0.7 at pH 5.5 (see Figure 17). Results from all three independent experiments show a similar trend at both pH 5.5 and 8.0 (see Appendix H). From the growth curves obtained, *ohsC* mutant showed slow growth rate at both pH 5.5 and pH 8.0.

5 Discussion

In the initial screening phenotypes that varied from the wild type was investigated by looking at responses to stress in order to direct further study into epistasis groups. In the TA-systems *tisB-istR, shoB-ohsC* and *ldrD-rdlD* revealed how *shoB* and *ohsC* mutants had opposite results when stressed with superoxide and hydrogen peroxide. *shoB* showed decreased sensitivity toward hydrogen peroxide but increased sensitivity toward superoxide stress. The *ohsC* was sensitive to hydrogen peroxide while not sensitive to superoxide stress. Based on these results, possible genetic partners with ShoB were investigated. It has been postulated that antiparallel dimers are assembled with TisB, which forms a amphiphilic α-helix in the inner membrane, but this study was *in vitro* (Steinbrecher et al. 2012*).* TisB has similar characters as ShoB, so it could also be forming a protein channel with other small proteins. ShoB does not necessarily have any interaction protein and could be an independent protein. A lot more data is available about on the TA-system *tisB-istR* than *shoB-ohsC* and *ldrD-rdlD.* Many of the chemicals tested did not show any effect.

5.1 ShoB possibly increase oxidative stress

Stressing the mutants *shoB* and *ohsC* with different chemicals to find a phenotype, shows that *shoB* mutant has 400-fold reduced sensitivity against H_2O_2 compared to *ohsC* mutant. This is indicating that ShoB compromise the effective treatment of oxidative damage in an *ohsC* mutant, which possibly has increased level of ShoB. By reinforcing the removal of ShoB resistance to oxidative stress is increased. ShoB could be inhibiting the removal of oxidized compounds in order to increase the mutations rate due to oxidative stress caused by H_2O_2. In an *ohsC* mutant the increased level of ShoB therefore increases the oxidative stress while deletion of *shoB* reduces the level of oxidative stress. This also reduces the possibility for evolutionary adaptation to the oxidative stress.

The H_2O_2 detection assay shows an increased amount of H_2O_2 in a *shoB* mutant compared to wild type, indicating a slower removal of H_2O_2 at initial time point, both when H_2O_2 is added and removal of residual H_2O_2, as seen in Figure 15A and B. This could be causing oxidative damage which leads to SOS response seen with flow cytometry at OD_{600} 0.6-0.7 at pH 5.5. Upon SOS induction, the cells keep replicating, whereas cell division is inhibited which results in

filamentation. *ohsC* mutant also showed slow growth at both pH 5.5 and 8.0, and is possibly dividing too slow in relation to rate of replication.

H_2O_2 produces hydroxyl radicals that can oxidize both base and ribose moieties which are both lethal and mutagenic. (Imlay 2013). Increased concentrations of H_2O_2 in the *shoB* mutant could also be a result of different amount of cells at the time of resuspension, as more cells would be able to scavenge H_2O_2 faster, but this was accounted for by measuring OD_{600} right before resuspending the cells. An experimental difficulty arose from the fact that glucose media can chemically generate H_2O_2. The *katG katE ahpC* mutants were difficult to grow in both minimal A medium and M9 medium as growth was most likely inhibited by H_2O_2. *E. coli* generates about 14 μM hydrogen peroxide per second when it grows exponentially in glucose medium, but H_2O_2 is also generated in the medium when it is exposed t visible light. Therefore, when cells are first inoculated into media, the primary oxidative damage is from H_2O_2 generated by the medium rather than H_2O_2 generated by metabolism (Seaver, Lauren Costa & Imlay, James A. 2001). Growing the strains anaerobically overnight before detecting rate of intracellular H_2O_2 did not increase the growth of the mutants. The assay must be improved as the results indicate that H_2O_2 is generated in high amount under preparation of growth medium.

Interestingly, stressing *shoB* and *ohsC* mutant with menadione gives opposite results than hydrogen peroxide. *ohsC* mutants are only slightly sensitive while *shoB* mutants are almost 60-fold more sensitive, compared to *ohsC* mutants, as seen in Figure 7A. Albeit superoxide and hydrogen peroxide both cause oxidative stress, it is known that two different mechanisms are involved in handling oxidative stress caused by hydrogen peroxide and superoxide in *E.coli.* Superoxide stress activates the SoxRS system while the OxyR system is activated by hydrogen peroxide. The SoxRS system also induces the ferric uptake regulator (Fur) which regulates amount of iron in the cells to reduce oxidative stress through the Fenton reaction. This mononuclear iron protein can itself lose activity during superoxide or hydrogen peroxide stress as Fe^{2+} can be oxidized to Fe^{3+}, giving increase in iron importers. Both iron-sulphur dependent dehydratases, such as Aconitase and Fumarase in the TCA cycle, Serine dehydratase, and mononuclear iron proteins are sensitive to oxidative stress. There is a possibility that ShoB influence Fe^{2+} levels via uptake proteins in the inner membrane leading to reduced or increased amount of free iron in the cell. Free iron in the cytoplasm is sequestered by Dps which is

upregulated under oxidative stress and strongly suppresses DNA damage, but if shoB influences uptake of iron in the inner membrane, a *ohsC* mutant might be less sensitive to superoxide since superoxide also induce uptake of iron to reduce oxidative stress, which reduce amount of free iron in cell to reduce damage on different cellular sites through the Fenton reaction.

The genes encoding superoxide dismutases (*sodA, sodB* and *sodC)* where deleted in combination with *shoB* or *ohsC* to investigate if an increase in superoxide in different compartments of the cell is the reason behind the effect seen with menadione stress. The superoxide dismutases are localized in different compartments of the cell because superoxide cannot cross membranes. Mn SOD and Fe SOD, encoded by *sodA* and *sodB* respectively, dismutate superoxide in the cytoplasm. Superoxide stress in the periplasm is removed by Cu-Zn SOD encoded by *sodC.* In general, mutants lacking *sodA* were more sensitive to mendione than *sodB* and *sodC* mutants.

The genes *fepD, fhuB, fecC, fecD, feoB, tonB,* which all encode proteins in the inner membrane involved in iron uptake, were also knocked out in combination with *shoB::kan* or *ohsC::kan* to find potential protein ShoB interacts with to influence levels of Fe^{2+}. The genes *dps* and *ftn* were knocked out to reduce the strains ability to store free iron in the cytoplasm. *fhuB ohsC::kan* mutant has reduced sensitivity against hydrogen peroxide stress compared to *ohsC* and *fhuB* mutants. This indicates there might be an interaction between ShoB and FhuB.

The mutants *IscA shoB::kan, fhuB shoB::kan, sufA ohsC::kan, sufE ohsC::kan* and *sufA ohsC::kan,* shows high sensitivity against superoxide stress, especially *iscA shoB::kan* which had no survivors at the concentration tested. IscA is a iron-sulphur cluster assembly protein which is localized in the cytoplasm and binds iron. Under oxidative stress, IscA can no longer bind iron because of oxidation of the thiol group in the protein, which possibly gives an increase of free iron in the cells (Imlay 2015). Removing both ShoB and IscA is indicating there is a strong interaction since it inhibits the mutant from replicating. There might be a more direct interaction between ShoB and IscA as deleting other iron-sulphur cluster assembly proteins (SufA and SufE) in combination with ShoB does not give increased sensitivity against superoxide stress. To further investigate a direct interaction between ShoB and IscA, the two proteins must be isolated together which is difficult because shoB is a hydrophobic membrane protein and membrane proteins are difficult to isolate. Thus, combining different genetic backgrounds is a good method for investigating interaction between membrane proteins.

The initial screening for new interactions among the TA-systems also indicated possible interaction with the antiporters NhaB or NhaA under acidic stress, as acidic stress has shown to induce the oxidative stress regulon which includes induction of *ahpC.* Alkaline stress has shown to repress oxidative stress genes (Maurer et al. 2005).

Both deletion of toxin (TisB, ShoB, LdrD) and antitoxin (IstR, OhsC, RdlD) gave increased sensitivity against acidic stress. Interestingly stressing an *ohsC* mutant with alkaline stress for one hour increases the sensitivity by almost 9000-fold and a plasmid with high ectopic expression of the antitoxin OhsC increased the survival level back to the same level as for wild type, as seen in Figure 14. This indicates that the effect seen is because of deleting the antitoxin, and not because of changes in the genomic area. Since the result is only seen for one of the mutants it gives a strong indication that ShoB has a very specific role under alkaline stress. This result strengthens the hypothesis about ShoB increasing the oxidative stress in the cells, as alkaline stress repress genes involved in response to oxidative stress.

Under alkaline stress the protons are actively transported into the cell and transcription of cation-proton antiporters is upregulated and expression of proton-pumping respiratory chain complexes is decreased to minimize proton loss from the cytoplasm during PMF generation (Krulwich et al. 2011). Overproduction of ShoB leads to a reduction in membrane potential, disrupting the PMF and levels of ATP. This could be causing the sensitivity seen in the *ohsC* mutant, as the strain cannot protect itself from the alkaline stress due to decreased levels of ATP, which again inhibits import of H^+ into the cell. However, overproduction of most small membrane proteins disrupts the PMF, so it must be a more direct interaction for the *ohsC* mutant. Stressing the mutants *nhaA shoB::kan, nhaA ohsC::kan, nhaB shoB::kan* and *nhaB ohsC::kan* does not reveal any interaction between nhaA, nhaB and ShoB under superoxide and hydrogen peroxide stress. It does not seem like ShoB has an interaction with the antiporters NhaA and NhaB or the iron storage protein Dps, from these survival assays. NhaA and NhaB are localized in the inner membrane while Dps is localized in the cytoplasm.

Complementing the *shoB* mutant with a plasmid which expresses ShoB in low-copy-number does not bring the survival level back to that of wild type under different type of stress that was tested. There could be several reasons behind this. ShoB might not be expressed in the same amount or same compartment as it would be endogenously. However, complementation of *ohsC* with high-

copy-number plasmid decreased survival more than 1000-fold. This could possibly be because higher level of antitoxin removed most or all of ShoB, which gives the same effect as in the *shoB* mutant. A plasmid with high-copy-number of *shoB* was also not transformable in a *shoB* mutant as it was lethal to the cells. The increased sensitivity against superoxide stress and reduced sensitivity against hydrogen peroxide stress, can be as a result of deleting the toxin ShoB, but it can also be due to possible elevated levels of antitoxin or because of changes in the genomic area.

5.2 No interaction apparent in *ldrD-rdlD* system under oxidative stress

ldrD and *rdlD* have 400- and 4000-fold increase in survivors when stressed with hydrogen peroxide, and 1000- and 100-fold decrease in survivors when stressed with superoxide compared to wild type. But for both types of stresses it is less than 10-fold change between *ldrD* and *rdlD.* Thus the *ldrD-rdlD* system appears not to interact with oxidative stress responses whether it is caused by hydrogen peroxide or superoxide. Complementation of these two mutants did not remove the effect seen indicating that the effect seen is because of changes in the genomic area around the deleted gene.

Unlike the other TA-systems tested *ldrD* and *rdlD* are encoded convergently, which makes it difficult to delete one gene without affecting the genetic area of the other. The resultant mutants might be showing this effect as a result of changes in the genomic area and not because of the specific role of the toxin or antitoxin. The double mutants *nhaA ldrD::kan* and *nhaA rdlD::kan* both were very sensitive to hydrogen peroxide stress, having ~100-fold less survivors compared to the single mutants *nhaA, ldrD, rdlD* and wild type. Since both double mutants showed this sensitivity against hydrogen peroxide any specific role of toxin or antitoxin in interaction with NhaA cannot be suggested. Deletion of toxin does not necessarily give higher expression of antitoxin, which means both deletion of *ldrD* and *rdlD* could give almost similar levels of *rdlD* and same effect is seen for both *nhaA ldrD::kan* and *nhaA rdlD.*

5.3 TisB and ShoB show similar stress response to hydrogen peroxide stress

Curiously both deletions of *tisB* and *istR* (potentially elevated TisB) lead to increased oxidative stress response when stressed with hydrogen peroxide. Effects similar to *shoB* and *ohsC* were seen for *tisB* and *ohsC* mutants, when they were stressed with hydrogen peroxide but not when stressed with superoxide. The survival level of *tisB* mutant complemented with plasmid with high ectopic expression of TisB, removes the effect seen when stressed with hydrogen peroxide,

indicating that the effect is because of the toxin. TisB could, like ShoB, inhibit removal of oxidized compounds to increase the mutations rate due to oxidative stress caused by H_2O_2. Many toxins have some general roles but also some specific.

5.4 Different survival rates in wild type against oxidative stress

Survival level of the mutants *tisB, istR, shoB, ohsC, ldrD, rdlD* and wild type variated from time to time when they were tested against chronic oxidative stress caused by H_2O_2 or menadione sodium bisulfite. This difference can be due to photochemical production of H_2O_2 in LB-medium when it is exposed to sun light, which gives slightly different concentrations of H_2O_2, and results in big difference in survival as the bacteria are very sensitive to small changes of oxidative stress. The LB-medium had been exposed to sunlight and room light for different amount of time which can give higher or lower concentration of H_2O_2 that was already in the the LB-medium prior to the experiments. Repeatedly, it was seen that MG1655 had very dense growth for bacterium culture which was undiluted before spotting on the plate and no colonies at all in the first dilution which was diluted in PBS. 10-fold less bacteria would be expected from one dilution to the second, but since this was not the case, possibilities of MG1655 producing signal molecules in the growth medium which protects against H_2O_2 was investigated, but it did not seem like this was the case.

MG1655 was stressed one hour at different concentrations of H_2O_2 to make a survival curve. Concentrations from 6 to 14 mM were tested. 12 mM gave ~ 1×10^8 CFU/ml while 14 mM gave no survivors for two of the clones. This shows how steep the survival curve is and how small changes in H_2O_2 concentration exerts a great difference in survival. The freezer stock might have contained bacteria that were in different growth phases before it was frozen down, giving some “old” and some “new” bacteria. Stationary phase bacteria would be more protected against ROS. The strains were grown over four generations in an attempt to remove any "old" cells. More stationary phase cells, or those that have been in stationary phase may be more resistant to oxidative agents. Each generation was also stressed with 10 mM hydrogen peroxide for one hour to investigate possibilities of difference in survival between the generations. Also six clones of *tisB, istR, shoB, ohsC, ldrD and rdlD* were tested with MG1655 to see if this effect is only in

MG1655 or mutants as well. The results are shown in figure 11, and the figure shows how all the strains tested had 0 to 10 % survival among the clones of each strain.

To investigate if the effect seen is genetic or because of experimental differences, 8 clones of wild type (MG1655) were tested at 10 mM acute for 1 hr. The same colonies were restreaked and tested again the next day to see if there is a variation in which clones that die and which survive. This was tested for four days. The results shows that there was a variation in which clone survived and which died when tested, indicating that the effect might be because of epigenetics and not a genetic effect. Different approaches were tried to investigative if the effect seen was because of technical differences, such as using centrifuge at 4°C or 25°C. Cold shock can lead to decrease in membrane fluidity and translational block. The translational block induces cold shock proteins and ATP generation is also regulated. This could have given a difference in survival but no difference was observed. Thus the results are indicating there are clonal differences in transcription that leads to the bacteria being sometimes sensitive and other times not, or the other possibility is that it was an experimental error due to ROS in the growth medium, LB-plates and solutions. As mentioned previously, ROS can be generated photochemically.

Although most antibiotics used today target cell-wall assembly, protein synthesis, or DNA replication, recent reports have raised the possibility that their lethal effects are because of ROS that damage bacterial DNA. Understanding the mechanism behind the clonal differences is important, because major classes of bactericidal antibiotics were shown to stimulate the production of deleterious hydroxyl radicals which ultimately lead to cell death (Kohanski et al. 2007). It is concerning if clonal differences can protect *E.coli* from ROS and possibly ROS generating antibiotics. There are contradicting theories, arguing no involvement of ROS under cell death by antibiotics (Liu & Imlay 2013).

5.5 Acidic stress and iron possibly cause oxidative stress

The mutants *tisB, istR, shoB, ohsC, ldrD, rdlD* all showed increased sensitivity against acid stress at pH 3.70 for one hour, while *dinQ* and *agrB* mutants were less sensitive compared to wild type. *E.coli* has three acid resistant systems which make it possible to survive acid stress. The systems are glutamate-dependent, arginine-dependent and oxidative systems (Castanie-Cornet et al. 2010). Albeit oxidative stress response genes are upregulated under acidic stress, catalase is partially dissociated at pH between 3.0-4.0 (Samejima et al. 1962). Acidic stress could therefore

be inhibiting removal of hydrogen peroxide causing oxidative damage at low pH. At lower pH iron is also more soluble compared to neutral and high pH (Deitrich & Silver 2007). So both acid and iron stress might be causing oxidative damage as increased amount of free iron in the cell increase oxidative stress through the Fenton reaction.

5.6 Cell size reduction at pH 5.5 and 8.0

Size reduction of *tisB, istR, shoB, ohsC, ldrD, rdlD* mutants and wiltype cells along the growth curve was obsereved with flow cytometry. This could be a result of problems with iron metabolism, which leads to problems with serine deaminases and pyruvate production. As a concequence cell size, replication initiation and growth rate is reduced. The initiation of DNA replication and cell division, have been implicated as important control points in regulation of cell size. DnaA is a ATP-dependent protein that binds cooperatively to sequence specific DnaA boxes within the chromosomal origin of replication (oriC). Upon binding, DnaA drives open complex formation, facilitating loading of the replication machinery. Significantly reducing DnaA expression delays initiation and increases cell size, while overexpressing DnaA leads to premature initiation and a reduction in cell size in E. Coli (Chien et al. 2012).

5.7 Conclusion

The function of the TA-systems *tisB-istR, shoB-ohsC* and *ldrD-rdlD* was investigated through different types of both chronic and acute stress. The toxin ShoB seems to be involved in oxidative stress, and it possibly increase oxidative stress inside the cell through iron uptake proteins. No clear genetic interaction was found between any iron uptake proteins in the inner membrane, but it seems like it is a strong interaction between ShoB and IscA. There is also a strong indication that ShoB has a specific role under alkaline stress as an *ohsC* mutant is extremely sensitive when challenged with alkaline stress for one hour. The sensitivity against alkaline stress can also be through increase in oxidative stress by ShoB in the *ohsC* mutant.

Many clones of wild type strain show different amount of survivors when challenged with hydrogen peroxide stress for one hour. A clonal difference in transcription might be the cause of this effect seen.

5.8 Future aspects

Although hydrogen peroxide, superoxide and alkaline stress showed an effect in survival in the TA-system *shoB-ohsC*, other types of stress can be tried to find other stress responses in the TA-systems, such as heat and cold shock.

The oxidative stress by hydrogen peroxide and superoxide indicates that ShoB has a specific role under oxidative stress. OhsC could be regulating levels of ShoB during oxidative shock, which can further be investigated by real time quantitative PCR (RT-qPCR), by analyzing samples taken at different time points after treating *shoB* and *ohsC* mutants with oxidative agents.

Most of the effects seen in the mutants *tisB, istR, shoB, ohsC, ldrD* were not removed with complementation plasmid with high/low ectopic expression of the toxin or antitoxin. There could be several reasons behind this. The toxin or antitoxin might not be expressed in the same amount or same compartment as it would be endogenously. For further work a His-tag can be used to verify the ectopic expression of the toxins. A His-tag would allow the protein to be purified and detected without any protein-specific antibody or probe. It should be noted that the His-tag itself can alternate the assembly state, conformation or activity of the protein, giving nonreproducible survival assays. Expression of antitoxin sRNA can be confirmed with RT-qPCR. A plasmid with high-copy-number of *shoB* was also not transformable in a *shoB* mutant as it was lethal to the cells. In future work an inducible system should be used for high ectopic expression of ShoB. ShoB is hypothesized to be located in the inner membrane, to confirm this hypothesis ShoB can be labeled with a FLAG-tag and immunofluorescence can be used to visualize localization of ShoB. Interactions between ShoB and IscA can be studied though knocking out other members of the Isc system, which is a five-protein complex (IscU, HscA, HscB and IscS)

To further study the clonal differences in transcription, which possibly cause the bacteria to be sensitive to ROS sometimes and other times not during hydrogen peroxide stress, RT-qPCR can be performed. RT-qPCR would reveal if genes involved in ROS removal are up- or downregulated to see if the clones have more or less internal ROS.

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Appendix A: Recipes for solutions and buffers

Water used for the solutions is filtered with 0.2 millimeter filter, deionized and autoclaved.

Table 3: Recipes of buffers and solutions

Solution	Components
Agarose gel	0.5 g ultrapure agarose 50 mL 1x TAE 1.5 µL SYBR safe DNA gel stain
Gene Ruler Mix (500 µL)	100 µL gene ruler 83 µL 6x Loading dye 317 µL UR-water
Glucose (20 %)	200 g D(+)-Glucose, $C_6H_{12}O_6$ 1 L UR
Glycerol (60 %)	600 mL Glycerol 99,9% 1L UR-water
6x DNA Loading Buffer	30 % Glycerol, 20 mM EDTA, 0.01 % Bromophenol Blue
LB medium (1 L)	25 g Difco™ LB broth, Miller 1 L MQ
LB agar (1 L)	25 g Difco™ LB broth, Miller 15 g agar 1 L MQ
LB agar low salt (1 L)	10 g Bacto-Tryptone 5 g NaCl 5 g yeast extract 15 g agar 1 L MQ
LB medium low salt (1 L)	10 g Bacto-Tryptone 5 g NaCl 5 g yeast extract 1 L MQ
LBK medium (1L)	10 g Bacto-Tryptone 7.46 g KCl 5 g yeast extract 1 L MQ
MES buffer	195,24 g MES hydrate, pH 6.0 1 L UR-water
MOPS buffer	50 mM MOPS pH 7.4, 1 mM EDTA, 100 mM KCl, 1 mM DTT
Minimal A medium 5X 1L	52.5 g K_2HPO4 22.5 g KH_2PO_4

	5 g $(NH_4)_2SO_4$ 2.5 g Sodium citrate $\cdot 2H_2O$
M9 salts 5X 1L	33.9 g Na_2HPO_4 15 g KH_2PO_4 5 g NH_4Cl 2.5 g NaCl
M9 medium 1 L	200 mL M9 salts (5X) 20 mL glucose (20%) 2 mL $MgSO_4$ (1 M) 0.1 mL $CaCl_2$ (1 M) 1 µg/mL Thiamine MQ to adjust volume to 1 L
Phoshate buffered saline (PBS ×10)	3.2 g $NaH_2PO_4 \cdot H_2O$ 13.7 g $Na_2HPO_4 \cdot 2H_2O$ 85 g NaCl 1 L UR-water
Potassium phosphate buffer 0.1 M (pH 7.0)	61.5 mL KH_2PO_4 (1M) 38.5 mL K_2HPO_4 (1M)
SOC medium (1 L)	20 g Bacto tryptone 5 g Bacto yeast extrakt 0.5 g NaCl 0.19 g KCl 2.03 g $MgCl_2$ 8.9 mL Glukose 45% 1L UR-water
Soft agar (30 mL)	10 mL 1.5% LB agar 20 mL LB medium 120 µL tryptophan (5 mg/ml)
TAE-buffer (1 L)	242 g Tris (tris(hydroxymethyl)aminomethane), $H_2NC(CH_2OH)_3$ 57.1 mL Glacial acetic acid 100 mL EDTA 0,5 M pH 8,0 ($C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$) 1000 mL UR-water
TE-buffer	1M Tris-HCl (pH approximately 8.0) 0.1M EDTA
Tryptone broth (1 L)	10 g tryptone 2.5 g NaCl 1 L MQ
T4-buffer (10 mL)	1 mL tryptone broth 9 mL MQ 40 µL tryptophan

Appendix B: Primers used for genotyping

Gene	Primer id[1]	Sequence 5'→3'
dps	17172_r	AAATGATTGCCCTCACCCGT
	17173_f	GCGCTAAAATTGTGCACTCA
tonB	17169_r	ACTGAAACGTGTTCATAGACTCCTG
	17176_f	ACTTTCTTACGGCCGGTTGC
nhaA	15840_r	TATCTCTTCATCGCAATTATTGACG
	15841_f	ACATTTTATCGGCATAGCGA
nhaB	15842_r	CGCCAGTGTTTCAAGGATAT
	15843_f	CAATCAGCGCAGCACCCAGA
katG	19763_r	TATCTAACTTTAAACAGGCG
	19764_f	ATCAGAATCGCGAAATATTG
katE	19767_r	ATACAGAATTTCAGGTCATG
	19768_f	TACGGTGAAGAGATCAGTGA
ahpC	19759_r	GATGGTGACGCCCATATCCTGATAC
	19760_f	TGGTGATCAGGAAAGACGGC
ftn	19681_r	GCAGCACTGTAACGCGTAAA
	19682_f	CGATGGACTCTATTCACGGT
fur	19683_r	AACCATCGTTGGTCACTGGC
	19684_f	AGCTTGTGCGCAATCAACCG
feoB	19679_r	ACCTTAATTAAACATTAGCCAGTCC
	19680_f	ATACCGGGCTTTCCAAAACA
tisB	17781_r	GGGGTTGATCACGTTTTGTA
	17782_f	CGGGAGAGGAATCTATCACATT
istR	17782_r	CGGGAGAGGAATCTATCACATT
	17781_f	GGGGTTGATCACGTTTTGTA
shoB	17792_r	TAAGGGCTAATCTGAATGGC
	17791_f	ACGAGATTAACAGCGATAAG
ohsC	17793_r	ATGATTTTGATGACCCGTTT
	17794_f	ATTCAATGATGAGCTCGGCA
ldrD	17800_r	CGCAACAGGTGGAGAAGTCG
	17799_f	GGTGGTTCGCGTTCTCGATG
rdlD	17799_r	GGTGGTTCGCGTTCTCGATG
	17800_f	CGCAACAGGTGGAGAAGTCG
iscA	19869_f	CTCATCATTAATCGGTATCG
	19870_r	AAAGAAAGCAAATATTCCGC
sodA	19775_f	GCCTGTTCTGGAAAGGTCTG
	19776_r	CCAGTTTATCGCCTTTCAGC
sodB	19859_f	CACAAAGCGAAGGTGCAAAA

	19861_r	CGCAGCTATTAGCATGGTGG
sodC	19863_f	TGGCGGACGCGTTCAAACAT
	19864_r	CTGGCTCGCCGTTGGTTATT
fecC	19850_f	ACCTACGCTGAAAATTTGCA
	19851_r	CGCGAGAGCATCAGATAATC
fecD	19852_f	AACAAACTGATCCTCGCGGG
	19853_r	CAGCTCCTGGACTGTGATCC
fepD	19854_f	ATCGCCCCGGCCTGCATTAA
	19855_r	TCCACGCCCCGGTGTTAAAG
sufA	19865_f	AGGTTCGATGACCTTCTTAATACT
	19866_r	TACTTAAAAAGGCGTTCGCG
sufE	19867_f	CCACCGTCAGCCTGAGTGAA
	19868_r	GTCGTACCAGGTTTTGGCAC
fhuB	19848_f	GCGCGTAAATCGCTGACGGAAAT
	19849_r	TTGCGATGGCAGCGGGTTTC
Kanamycin	K1	CAGTCATAGCCGAATAGCCT
cassette	K2	CGGTGCCCTGAATGAACTGC

[1]The primer ids are automatically generated by the common primer database at Rikshospitalet, Oslo University hospital.

Appendix C: Genotyping to see if desired gene has been knocked out

Expected length when gene is replaced with kanamycin cassette is ~1000 bp. For *shoB::kan* and *ohsC::kan* expected length of band is ~500 bp. Double mutants of *tisB::kan, istR::kan, shoB::kan, ohsC::kan, ldrD::kan and rdlD::kan* had expected bands as shown in Figure 1A. Forward primer of the gene was used with the revers primer of the kanamycin cassette or revers primer of the gene with forward primer of the kanamycin cassette. For all other genes that were also replaced with kanamycin cassette, the expected length was around 1000 bp. Figure 1B shows example of how expected bands should look like when genes have been replaced with kanamycin cassette and size of bands when kanamycin has been successfully/ not successfully removed by pCP20.

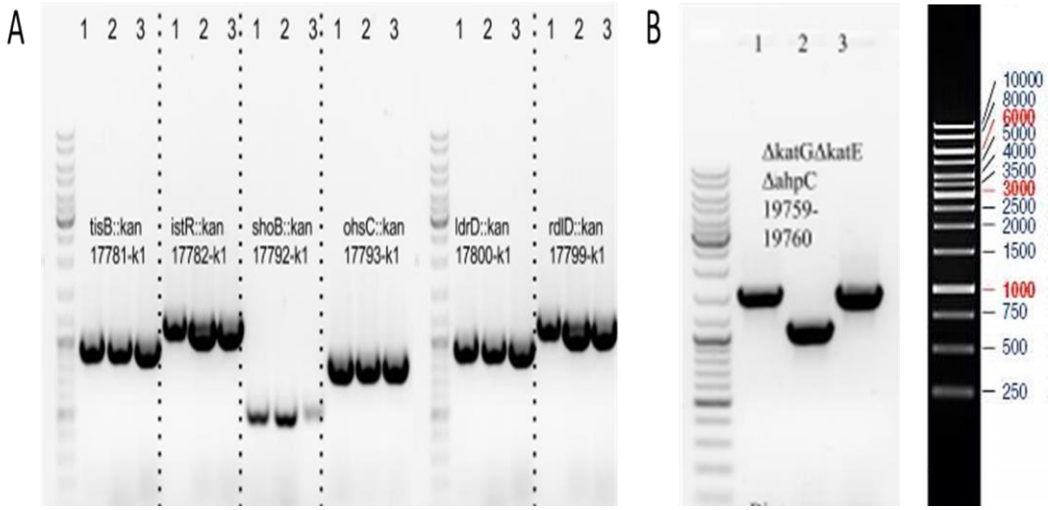


Figure 10: Figure A shows the size of expected bands when gene has been replaced with kanamycin cassette, Figure B shows size of expected bands when kanamycin cassette has been removed by pCP20 in lane 2, and size of bands when kanamycin cassette has not been removed successfully by pCP20 in lane 1 and 3. Figure to the right shows size of bands with GeneRuler^{TM} 1 kb DNA ladder.

Appendix D: Concentrations tested of different chemicals

The tables below show concentration of different chemicals tested in a stepwise manner to find toxicity level that kills almost 99 % of the bacteria.

Table 1: Chronic oxidative stress caused by H_2O_2

Step	**Concentration of H_2O_2 in mM**	**Strains tested**
1	500, 50, 5, 0.5, 0.05, 0.005	*MG1655, ΔtisB,ΔistR, ΔshoB, ΔohsC, ΔldrD,ΔrdlD*
2	0.5, 0.25, 0.05	*MG1655, ΔtisB,ΔistR, ΔshoB, ΔohsC, ΔldrD,ΔrdlD*
3	5, 2.5, 0.5	*MG1655, ΔtisB,ΔistR, ΔshoB, ΔohsC, ΔldrD,ΔrdlD*
4	0.25, 0.50, 1.0, 1.5	*MG1655, ΔtisB,ΔistR, ΔshoB, ΔohsC, ΔldrD,ΔrdlD*
5	0.3, 0.4, 0.5	*MG1655, ΔtisB,ΔistR, ΔshoB, ΔohsC, ΔldrD,ΔrdlD*
6	0.5, 0.6, 0.7	*MG1655, ΔtisB,ΔistR, ΔshoB, ΔohsC, ΔldrD,ΔrdlD*

Table 2: Chronic oxidative stress caused by menadione sodium bisulfite

Step	**Concentration of Menadione sodium bisulfite**	**Strains tested**
1	2 mM, 200 µM, 20 µM, 2 µM, 200 nM, 20 nM	*MG1655, ΔtisB,ΔistR, ΔshoB, ΔohsC, ΔldrD,ΔrdlD*
2	7 mM and 0.7 mM	*MG1655, ΔagrB::kan*
3	4, 5 and 6 mM	*MG1655, ΔtisB,ΔistR, ΔshoB, ΔohsC, ΔldrD,ΔrdlD*
4	2, 4, 6 and 8 mM	*Δ*sodA, MG1655

Table 3: Chronic iron stress ($FeSO_4 \cdot 7H_2O$)

Step	**Concentrations of ($FeSO_4 \cdot 7\ H_2O$)**	**Strains tested**
1	2.5 mg/ ml and 3mg/ml	*MG1655, ΔtisB,ΔistR, ΔshoB, ΔohsC, ΔldrD,ΔrdlD*
2	1, 2 and 3 mg/ml	*MG1655, ΔtisB, ΔistR, ΔshoB, ΔohsC, ΔldrD, ΔrdlD*

3	1.2, 1.4, 1.6 and 1.8 mg/ml	*MG1655, ΔtisB, ΔistR, ΔshoB, ΔohsC, ΔldrD, ΔrdlD*
4	1.2, 1.5 and 1.6 mg/ml	*MG1655, ΔtisB, ΔistR, ΔshoB, ΔohsC, ΔldrD, ΔrdlD*
5	1.4, 1.5 and 1.6 mg/ml	*ΔshoB, ΔohsC, Δdps shoB::kan, ΔtonB shoB::kan, ΔnhaB shoB::kan, Δdps ohsC::kan, ΔtonB ohsC::kan, ΔnhaB ohsC::kan*
6	1.4, 1.5 and 1.6 mg/ml	*ΔnhaB, ΔtonB, Δdps, ΔshoB, ΔohsC, MG1655*

Table 4: Chronic 2,2′-Dipyridyl stress (removal of Fe^{2+})

Step	Concentration of 2,2′-Dipyridyl	Strain tested
1	1, 1.5 and 2 mM	*MG1655, ΔtisB, ΔistR, ΔshoB, ΔohsC, ΔldrD, ΔrdlD*

Table 5: Chronic manganese stress ($MnCl_2 \cdot 4H_2O$)

Step	Concentration of Mn^{2+} in mM	Strain tested
1	2.5 and 7.7	*MG1655, ΔtisB, ΔistR, ΔshoB, ΔohsC, ΔldrD, ΔrdlD*
2	10, 12, and 14.	*MG1655, ΔtisB, ΔistR, ΔshoB, ΔohsC, ΔldrD, ΔrdlD*
3	16, 18, 20.	*MG1655, ΔtisB, ΔistR, ΔshoB, ΔohsC, ΔldrD, ΔrdlD*

Table 6: Chronic Copper stress ($CuSO_4\,5H_2O$)

Step	Concentration of $CuSO_4\,5H_2O$ in mM	Strain tested
1	10	MG1655 and *ΔtisB, ΔistR, ΔshoB, ΔohsC, ΔldrD, ΔrdlD*
2	0.1, 1, 5.	MG1655 and *ΔtisB, ΔistR, ΔshoB, ΔohsC, ΔldrD, ΔrdlD*
3	6 and 8	MG1655 and *ΔtisB, ΔistR, ΔshoB, ΔohsC, ΔldrD, ΔrdlD*

4	9, 10 and 11	MG1655 and *ΔtisB, ΔistR, ΔshoB, ΔohsC, ΔldrD, ΔrdlD*

Table 7: Chronic Cobalt stress ($CoN_2O_6.6H_2O$)

Step	**Concentration of ($CoN_2O_6.6H_2O$)**	**Strain tested**
1	100 mM, 10 mM, 1 mM, 100 µM, 10 µM	MG1655 and *ΔtisB, ΔistR, ΔshoB, ΔohsC, ΔldrD, ΔrdlD*
2	1 mM and 100 µM	MG1655 and *ΔtisB, ΔistR, ΔshoB, ΔohsC, ΔldrD, ΔrdlD*
3	0.2, 0.4, 0.6, 0.8 and 1 mM	MG1655 and *ΔtisB, ΔistR, ΔshoB, ΔohsC, ΔldrD, ΔrdlD*

Table 8: Acute oxidative stress caused by H_2O_2

Step	**Concentration of H_2O_2**	**Strain tested**
1	100 mM, 10 mM, 1mM, 100 µM, 10 µM, 1 µM, 100 nM, and 10 nM	MG1655
2	1 M	*MG1655, ΔtisB, ΔistR, ΔshoB, ΔohsC, ΔldrD, ΔrdlD*
3	0 , 200, 300 , 400, 500, 600, 700 and 800 mM	*MG1655*
4	0,100, 110, 120, 130, 140 and 150 mM	*MG1655*
5	0 , 1, 2, 3, 4, 6, 8 and 10 mM	*MG1655*
6	0, 10, 20, 40 , 60 and 80 mM	*MG1655*
7	0, 10, 20, 40, 60 and 80 mM	*MG1655*
8	0, 10, 12, 14, 16, 18 and 20 mM	*MG1655*
9	14 and 16 mM	*MG1655, ΔtisB, ΔistR, ΔshoB, ΔohsC, ΔldrD, ΔrdlD*
10	10 and 12 mM	*MG1655, ΔtisB, ΔistR, ΔshoB, ΔohsC, ΔldrD, ΔrdlD*
11	10 and 12 mM	*MG1655, ΔtisB, ΔistR, ΔshoB, ΔohsC, ΔldrD, ΔrdlD*
12	10 mM	*MG1655, ΔtisB, ΔistR, ΔshoB, ΔohsC, ΔldrD, ΔrdlD*

Table 9: Acute acidic and alkaline stress

Step	pH	Strains tested
1	1.7, 2.7, 3.9, 9.35, 10, 11.8	*MG1655*
2	3.1, 4.0, 9.4, 9.7	*MG1655*
3	3.6, 3.8 , 9.7 9.8, 10	*MG1655*
4	3.60 and 9.70	*MG1655, ΔtisB, ΔistR, ΔshoB, ΔohsC, ΔldrD, ΔrdlD*
5	4.50	*MG1655, ΔtisB, ΔistR, ΔshoB, ΔohsC, ΔldrD, ΔrdlD*
6	4.00	*MG1655, ΔtisB, ΔistR, ΔshoB, ΔohsC, ΔldrD, ΔrdlD*
7	3.60	*MG1655, ΔtisB, ΔistR, ΔshoB, ΔohsC, ΔldrD, ΔrdlD*
8	3.80	*MG1655, ΔtisB, ΔistR, ΔshoB, ΔohsC, ΔldrD, ΔrdlD*
9	3.70	*MG1655, ΔtisB, ΔistR, ΔshoB, ΔohsC, ΔldrD, ΔrdlD*

Appendix E: Raw data for acute and oxidative stress

# indicates too dense growth in well that was countable, CFU/mL was set to 10 000.

* Less than 10 colonies counted in the well that was countable.

Table 1: H_2O_2 0.7 mM chronic

	Tested			**Control**		
	No. of colonies	**Well**	**CFU/mL**	**No. of colonies**	**Well**	**CFU/mL**
ΔtisB	64	4	6400000	58	6	580000000
ΔtisB	31	4	3100000	48	6	480000000
ΔtisB	32	4	3200000	70	6	700000000
ΔistR	11	3	110000	36	6	360000000
ΔistR	11	3	110000	26	6	260000000
ΔistR	13	3	130000	20	6	200000000
ΔshoB	13	4	1300000	42	6	420000000
ΔshoB	16	4	1600000	53	6	530000000
ΔshoB	17	4	1700000	41	6	410000000
ΔohsC	100#	1	10000	13	6	130000000
ΔohsC	100#	1	10000	16	6	160000000
ΔohsC	100#	1	10000	81	5	81000000
ΔldrD	11	3	110000	16	6	160000000

ΔldrD	19	3	190000	21	6	210000000
ΔldrD	13	3	130000	15	6	150000000
ΔrdlD	11	4	1100000	41	6	410000000
ΔrdlD	11	4	1100000	30	6	300000000
ΔrdlD	100#	4	10000000	56	5	56000000
ΔdinQ	100#	1	10000	10	6	100000000
ΔdinQ	100#	1	10000	14	6	140000000
ΔdinQ	100#	1	10000	41	6	410000000
ΔagrB	100#	1	10000	75	6	750000000
ΔagrB	100#	1	10000	70	6	700000000
ΔagrB	100#	1	10000	17	6	170000000
MG1655	100#	1	10000	34	6	340000000
MG1655	100#	1	10000	73	6	730000000
MG1655	100#	1	10000	86	6	860000000

Table 2: H_2O_2 0.7 mM chronic

	Test			**Control**		
	No. of colonies	**Well**	**CFU/mL**	**No. of colonies**	**Well**	**CFU/mL**
MG1655	32	2	32000	38	5	38000000
MG1655	23	2	23000	38	5	38000000
MG1655	20	2	20000	63	5	63000000
ΔtisB	20	4	2000000	20	6	200000000
ΔtisB	27	4	2700000	22	6	220000000
ΔtisB	40	4	4000000	33	5	33000000
ΔtisB-pMW119	19	5	19000000	13	6	130000000
ΔtisB-pMW119	15	5	15000000	10	6	100000000
ΔtisB-pMW119	23	5	23000000	20	6	200000000
ΔtisB-pUC57	100#	1	10000	16	6	160000000
ΔtisB-pUC57	100#	1	10000	11	6	110000000
ΔtisB-pUC57	13	2	13000	59	5	59000000
ΔistR	20	4	2000000	12	6	120000000
ΔistR	29	4	2900000	25	5	25000000
ΔistR	38	4	3800000	11	6	110000000
ΔistR-pMW119	14	5	14000000	67	5	67000000
ΔistR-pMW119	19	5	19000000	12	6	120000000
ΔistR-pMW119	25	5	25000000	10	6	100000000
ΔistR-pUC57	31	5	31000000	38	6	380000000
ΔistR-pUC57	10	5	10000000	16	6	160000000
ΔistR-pUC57	17	5	17000000	15	6	150000000
ΔshoB	48	4	4800000	14	6	140000000
ΔshoB	55	4	5500000	26	5	26000000

ΔshoB	50	4	5000000	11	6	110000000
ΔshoB-pMW119	33	5	33000000	16	6	160000000
ΔshoB-pMW119	36	5	36000000	16	6	160000000
ΔshoB-pMW119	29	5	29000000	19	6	190000000
ΔohsC	102	2	102000	24	6	240000000
ΔohsC	60	2	60000	20	6	200000000
ΔohsC	93	2	93000	31	6	310000000
ΔohsC-pMW119	33	4	3300000	12	6	120000000
ΔohsC-pMW119	36	4	3600000	14	6	140000000
ΔohsC-pMW119	10	4	1000000	18	6	180000000
ΔohsC-pUC57	100#	1	10000	32	5	32000000
ΔohsC-pUC57	29	2	29000	11	6	110000000
ΔohsC-pUC57	44	2	44000	45	6	450000000
ΔldrD	19	5	19000000	19	6	190000000
ΔldrD	14	5	14000000	18	6	180000000
ΔldrD	10	5	10000000	27	6	270000000
ΔldrD-pMW119	27	5	27000000	21	6	210000000
ΔldrD-pMW119	20	5	20000000	17	6	170000000
ΔldrD-pMW119	26	5	26000000	16	6	160000000
ΔldrD-pUC57	23	5	23000000	33	6	330000000
ΔldrD-pUC57	20	5	20000000	26	6	260000000
ΔldrD-pUC57	17	5	17000000	14	6	140000000
ΔrdlD	58	4	5800000	16	6	160000000
ΔrdlD	35	4	3500000	18	6	180000000
ΔrdlD	44	4	4400000	11	6	110000000
ΔrdlD-pMW119	12	5	12000000	13	6	130000000
ΔrdlD-pMW119	20	5	20000000	10	6	100000000
ΔrdlD-pMW119	23	5	23000000	22	6	220000000
ΔrdlD-pUC57	10	5	10000000	22	6	220000000
ΔrdlD-pUC57	34	5	34000000	23	6	230000000
ΔrdlD-pUC57	19	5	19000000	25	6	250000000

Table 3: H_2O_2 0.7 mM chronic

	Test			**Control**		
	No. of colonies	**Well**	**CFU/mL**	**No. of colonies**	**Well**	**CFU/mL**
ΔsodA	46	5	46000000	17	6	170000000
ΔsodA	53	5	53000000	23	6	230000000
ΔsodA	34	5	34000000	36	6	360000000
ΔsodB	45	4	4500000	32	6	320000000
ΔsodB	45	4	4500000	23	6	230000000
ΔsodB	40	4	4000000	12	6	120000000

ΔsodC	27	4	2700000	44	6	440000000
ΔsodC	29	3	290000	31	6	310000000
ΔsodC	13	4	1300000	53	6	530000000
ΔsufA	40	4	4000000	78	6	780000000
ΔsufA	17	4	1700000	110	6	1100000000
ΔsufA	31	4	3100000	118	6	1180000000
ΔsufE	42	4	4200000	48	6	480000000
ΔsufE	20	4	2000000	45	6	450000000
ΔsufE	64	3	640000	33	6	330000000
ΔshoB	13	6	130000000	16	6	160000000
ΔshoB	26	6	260000000	38	6	380000000
ΔshoB	11	6	110000000	18	6	180000000
ΔohsC	80	4	8000000	15	6	150000000
ΔohsC	40	4	4000000	16	6	160000000
ΔohsC	53	4	5300000	16	6	160000000
MG1655	17	4	1700000	21	6	210000000
MG1655	20	4	2000000	23	6	230000000
MG1655	21	4	2100000	22	6	220000000
ΔsodA shoB::kan	34	5	34000000	39	6	390000000
ΔsodA shoB::kan	30	5	30000000	63	6	630000000
ΔsodA shoB::kan	19	5	19000000	24	6	240000000
ΔsodA ohsC::kan	7*	5	7000000	7	6	70000000
ΔsodA ohsC::kan	16	6	160000000	7	6	70000000
ΔsodA ohsC::kan	12	6	120000000	8	6	80000000
ΔsodB shoB::kan	26	3	260000	40	6	400000000
ΔsodB shoB::kan	35	*3	350000	50	6	500000000
ΔsodB shoB::kan	39	3	390000	27	6	270000000
ΔsodB ohsC::kan	80	5	80000000	70	6	700000000
ΔsodB ohsC::kan	62	4	6200000	32	6	320000000
ΔsodB ohsC::kan	50	4	5000000	48	6	480000000
ΔsodC shoB::kan	23	4	2300000	84	6	840000000
ΔsodC shoB::kan	15	4	1500000	98	6	980000000
ΔsodC shoB::kan	40	5	40000000	30	6	300000000
ΔsodC ohsC::kan	90	2	90000	37	6	370000000
ΔsodC ohsC::kan	67	3	670000	19	6	190000000
ΔsodC ohsC::kan	23	3	230000	45	6	450000000
ΔsufA shoB::kan	24	4	2400000	39	6	390000000
ΔsufA shoB::kan	35	4	3500000	37	6	370000000
ΔsufA shoB::kan	16	4	1600000	42	6	420000000
ΔsufA ohsC::kan	25	3	250000	20	6	200000000
ΔsufA ohsC::kan	49	3	490000	21	6	210000000
ΔsufA ohsC::kan	54	3	540000	46	6	460000000
ΔsufE shoB::kan	37	6	370000000	60	6	600000000

ΔsufE shoB::kan	20	5	20000000	53	6	530000000
ΔsufE shoB::kan	24	6	240000000	44	6	440000000
ΔsufE ohsC::kan	21	5	21000000	48	6	480000000
ΔsufE ohsC::kan	11	4	1100000	36	6	360000000
ΔsufE ohsC::kan	31	4	3100000	13	6	130000000

Table 4: H_2O_2 0.7 mM chronic

	Test			**Control**		
	No. of colonies	**Well**	**CFU/mL**	**No. of colonies**	**Well**	**CFU/mL**
ΔiscA	11	5	11000000	29	6	290000000
ΔiscA	16	5	16000000	38	6	380000000
ΔiscA	20	5	20000000	19	6	190000000
ΔfhuB	19	3	190000	39	6	390000000
ΔfhuB	42	3	420000	65	6	650000000
ΔfhuB	8*	3	80000	85	6	850000000
ΔfecC	42	4	4200000	27	6	270000000
ΔfecC	24	5	24000000	27	6	270000000
ΔfecC	10	5	10000000	35	6	350000000
ΔfecD	46	4	4600000	36	6	360000000
ΔfecD	15	5	15000000	24	6	240000000
ΔfecD	16	5	16000000	31	6	310000000
ΔfepD	48	4	4800000	53	6	530000000
ΔfepD	33	4	3300000	47	6	470000000
ΔfepD	10	4	1000000	33	6	330000000
ΔshoB	30	5	30000000	18	6	180000000
ΔshoB	27	5	27000000	10	6	100000000
ΔshoB	31	5	31000000	10	6	100000000
ΔohsC	100#	2	100000	10	6	100000000
ΔohsC	100#	2	100000	10	6	100000000
ΔohsC	100#	2	100000	13	6	130000000
MG1655	60	3	600000	16	6	160000000
MG1655	26	3	260000	28	6	280000000
MG1655	20	3	200000	28	6	280000000
ΔiscA shoB::kan	22	6	220000000	47	6	470000000
ΔiscA shoB::kan	35	6	350000000	47	6	470000000
ΔiscA shoB::kan	14	6	140000000	19	6	190000000
ΔiscA ohsC::kan	22	2	22000	13	6	130000000
ΔiscA ohsC::kan	30	3	300000	37	6	370000000
ΔiscA ohsC::kan	42	3	420000	11	6	110000000
ΔfhuB shoB::kan	23	2	23000	21	6	210000000
ΔfhuB shoB::kan	32	2	32000	13	6	130000000

ΔfhuB shoB::kan	41	2	41000	22	6	220000000
ΔfhuB ohsC::kan	47	4	4700000	14	6	140000000
ΔfhuB ohsC::kan	46	4	4600000	20	6	200000000
ΔfhuB ohsC::kan	29	4	2900000	10	6	100000000
ΔfecC shoB::kan	18	3	180000	16	6	160000000
ΔfecC shoB::kan	17	3	170000	27	6	270000000
ΔfecC shoB::kan	28	3	280000	26	6	260000000
ΔfecC ohsC::kan	32	3	320000	37	6	370000000
ΔfecC ohsC::kan	36	4	3600000	55	6	550000000
ΔfecC ohsC::kan	56	4	5600000	24	6	240000000
ΔfecD shoB::kan	17	4	1700000	43	6	430000000
ΔfecD shoB::kan	49	4	4900000	21	6	210000000
ΔfecD shoB::kan	20	4	2000000	32	6	320000000
ΔfecD ohsC::kan	43	3	430000	13	6	130000000
ΔfecD ohsC::kan	20	4	2000000	17	6	170000000
ΔfecD ohsC::kan	36	3	360000	22	6	220000000
ΔfepD shoB::kan	80	3	800000	20	6	200000000
ΔfepD shoB::kan	66	3	660000	34	6	340000000
ΔfepD shoB::kan	80	3	800000	33	6	330000000
ΔfepD ohsC::kan	100#	2	100000	20	6	200000000
ΔfepD ohsC::kan	44	3	440000	14	6	140000000
ΔfepD ohsC::kan	46	2	46000	18	6	180000000

Table 5: H_2O_2 0.7 mM chronic

	Test			**Control**		
	No. of colonies	**Well**	**CFU/mL**	**No. of colonies**	**Well**	**CFU/mL**
Δftn	34	4	3400000	17	6	170000000
Δftn	29	4	2900000	17	6	170000000
Δftn	12	4	1200000	27	6	270000000
Δfur	100#	1	10000	50	5	50000000
Δfur	100#	1	10000	70	5	70000000
Δfur	100#	1	10000	71	5	71000000
ΔfeoB	15	3	150000	30	6	300000000
ΔfeoB	30	2	30000	32	5	32000000
ΔfeoB	10	3	100000	11	6	110000000
Δftn shoB::kan	46	2	46000	54	5	54000000
Δftn shoB::kan	40	2	40000	25	5	25000000
Δftn shoB::kan	32	2	32000	30	5	30000000
Δftn ohsC::kan	38	4	3800000	55	5	55000000
Δftn ohsC::kan	30	4	3000000	59	5	59000000
Δftn ohsC::kan	33	4	3300000	52	5	52000000

Δfur shoB::kan	100#	1	10000	59	5	59000000
Δfur shoB::kan	100#	1	10000	53	5	53000000
Δfur shoB::kan	100#	1	10000	60	5	60000000
Δfur ohsC::kan	33	3	330000	57	5	57000000
Δfur ohsC::kan	34	3	340000	50	5	50000000
Δfur ohsC::kan	37	3	370000	43	5	43000000
ΔfeoB shoB::kan	60	2	60000	15	6	150000000
ΔfeoB shoB::kan	43	3	430000	21	6	210000000
ΔfeoB shoB::kan	66	2	66000	19	6	190000000
ΔfeoB ohsC::kan	60	4	6000000	15	6	150000000
ΔfeoB ohsC::kan	42	4	4200000	14	6	140000000
ΔfeoB ohsC::kan	108	4	10800000	30	6	300000000
MG1655	18	4	1800000	63	6	630000000
MG1655	21	4	2100000	29	6	290000000
MG1655	18	4	1800000	30	6	300000000

Table 6: H_2O_2 0.7 mM chronic

	Test			**Control**		
	No. of colonies	**Well**	**CFU/mL**	**No. of colonies**	**Well**	**CFU/mL**
ΔnhaA	26	3	260000	19	6	190000000
ΔnhaA	44	2	44000	14	6	140000000
ΔnhaA	41	3	410000	23	6	230000000
ΔnhaB	45	4	4500000	49	5	49000000
ΔnhaB	63	4	6300000	66	5	66000000
ΔnhaB	49	4	4900000	43	5	43000000
Δdps	100#	1	10000	23	6	230000000
Δdps	100#	1	10000	22	6	220000000
Δdps	100#	1	10000	19	6	190000000
ΔtonB	39	4	3900000	25	6	250000000
ΔtonB	67	4	6700000	22	6	220000000
ΔtonB	63	4	6300000	21	6	210000000
ΔshoB	53	5	53000000	19	6	190000000
ΔshoB	44	5	44000000	34	6	340000000
ΔshoB	60	5	60000000	43	6	430000000
ΔohsC	100#	1	10 000	13	6	130000000
ΔohsC	100#	1	10 000	16	6	160000000
ΔohsC	100#	1	10 000	81	5	81000000
ΔnhaA shoB::kan	10	3	100000	27	6	270000000
ΔnhaA shoB::kan	66	2	66000	23	6	230000000
ΔnhaA shoB::kan	23	3	230000	28	6	280000000
ΔnhaA ohsC::kan	38	3	380000	43	6	430000000

ΔnhaA ohsC::kan	36	3	360000	28	6	280000000
ΔnhaA ohsC::kan	12	3	120000	12	6	120000000
MG1655	24	3	240000	38	5	38000000
MG1655	29	3	290000	40	5	40000000
MG1655	32	3	320000	34	5	34000000
Δdps ohsC::kan	1*	1	100	16	6	160000000
Δdps ohsC::kan	10	1	1000	29	6	290000000
Δdps ohsC::kan	1*	1	100	48	5	48000000
ΔtonB ohsC::kan	10	1	1000	14	6	140000000
ΔtonB ohsC::kan	10	1	1000	73	5	73000000
ΔtonB ohsC::kan	10	1	1000	10	5	10000000
ΔnhaB ohsC::kan	112	2	112000	45	6	450000000
ΔnhaB ohsC::kan	27	2	27000	56	5	56000000
ΔnhaB ohsC::kan	22	2	22000	40	5	40000000
Δdps shoB::kan	1*	1	100	28	6	280000000
Δdps shoB::kan	5*	1	500	25	6	250000000
Δdps shoB::kan	10	1	1000	22	6	220000000
ΔtonB shoB::kan	21	2	21000	13	6	130000000
ΔtonB shoB::kan	21	2	21000	31	6	310000000
ΔtonB shoB::kan	22	2	22000	37	6	370000000
ΔnhaB shoB::kan	8	2	8000	58	6	580000000
ΔnhaB shoB::kan	30	2	30000	28	6	280000000
ΔnhaB shoB::kan	15	5	15000000	55	6	550000000
MG1655	100#	1	10000	18	6	180000000
MG1655	100#	1	10000	47	6	470000000
MG1655	100#	1	10000	47	6	470000000

Table 7: H_2O_2 0.7 mM chronic

	Test			**Control**		
	No. of colonies	**Well**	**CFU/mL**	**No. of colonies**	**Well**	**CFU/mL**
Δftn	80	3	800000	109	6	1090000000
Δftn	16	3	160000	54	6	540000000
Δftn	27	2	27000	60	6	600000000
Δfur	100#	1	10000	20	6	200000000
Δfur	100#	1	10000	61	6	610000000
Δfur	100#	1	10000	47	6	470000000
ΔfeoB	100#	1	10000	26	6	260000000
ΔfeoB	32	2	32000	36	6	360000000
ΔfeoB	106	2	106000	109	6	1090000000
Δftn tisB::kan	80	2	80000	80	6	800000000

Δftn tisB::kan	100#	2	100000	40	6	400000000
Δftn tisB::kan	41	2	41000	10	6	100000000
Δftn istR::kan	95	2	95000	14	6	140000000
Δftn istR::kan	13	2	13000	35	6	350000000
Δftn istR::kan	13	2	13000	17	6	170000000
Δfur tisB::kan	11	2	11000	52	6	520000000
Δfur tisB::kan	21	2	21000	42	6	420000000
Δfur tisB::kan	11	2	11000	35	6	350000000
Δfur istR::kan	15	1	1500	40	6	400000000
Δfur istR::kan	100#	1	10000	20	6	200000000
Δfur istR::kan	67	1	6700	21	6	210000000
ΔfeoB tisB::kan	31	4	3100000	66	5	66000000
ΔfeoB tisB::kan	85	3	850000	15	6	150000000
ΔfeoB tisB::kan	21	3	210000	27	6	270000000
ΔfeoB istR::kan	38	2	38000	22	6	220000000
ΔfeoB istR::kan	40	3	400000	10	6	100000000
ΔfeoB istR::kan	36	3	360000	13	6	130000000
MG1655	30	2	30000	42	6	420000000
MG1655	63	2	63000	80	6	800000000
MG1655	17	3	170000	81	6	810000000

Table 8: H_2O_2 0.7 mM chronic

	Test			**Control**		
	No. of colonies	**Well**	**CFU/mL**	**No. of colonies**	**Well**	**CFU/mL**
MG1655	100#	1	10000	12	6	120000000
MG1655	100#	1	10000	42	5	42000000
MG1655	100#	1	10000	46	5	46000000
ΔtisB	12	2	12000	30	6	300000000
ΔtisB	15	2	15000	15	6	150000000
ΔtisB	28	2	28000	11	6	110000000
ΔistR	10	2	10000	61	5	61000000
ΔistR	100#	1	10000	22	6	220000000
ΔistR	100#	1	10000	15	6	150000000
Δdps	100#	1	10000	20	6	200000000
Δdps	100#	1	10000	18	6	180000000
Δdps	100#	1	10000	16	6	160000000
ΔtonB	100#	1	10000	14	6	140000000
ΔtonB	100#	1	10000	14	6	140000000
ΔtonB	100#	1	10000	20	6	200000000
ΔnhaB	88	2	88000	14	6	140000000

ΔnhaB	93	2	93000	54	5	54000000
ΔnhaB	46	2	46000	11	6	110000000
Δdps tisB::kan	100#	1	10000	57	5	57000000
Δdps tisB::kan	100#	1	10000	22	6	220000000
Δdps tisB::kan	100#	1	10000	67	5	67000000
Δdps istR::kan	100#	1	10000	18	6	180000000
Δdps istR::kan	100#	1	10000	13	6	130000000
Δdps istR::kan	100#	1	10000	14	6	140000000
ΔtonB tisB::kan	24	2	24000	24	6	240000000
ΔtonB tisB::kan	100#	1	10000	41	6	410000000
ΔtonB tisB::kan	100#	1	10000	21	6	210000000
ΔtonB istR::kan	100#	1	10000	15	6	150000000
ΔtonB istR::kan	100#	1	10000	14	6	140000000
ΔtonB istR::kan	100#	1	10000	65	5	65000000
ΔnhaB tisB::kan	11	3	110000	21	6	210000000
ΔnhaB tisB::kan	14	3	140000	28	6	280000000
ΔnhaB tisB::kan	11	3	110000	18	6	180000000
ΔnhaB istR::kan	100#	2	100000	22	6	220000000
ΔnhaB istR::kan	100#	2	100000	33	6	330000000
ΔnhaB istR::kan	100#	2	100000	23	6	230000000

Table 9: H_2O_2 0.7 mM chronic

	Test			**Control**		
	No. of colonies	**Well**	**CFU/mL**	**No. of colonies**	**Well**	**CFU/mL**
MG1655	11	5	11000000	18	6	180000000
MG1655	13	5	13000000	20	6	200000000
MG1655	12	5	12000000	14	6	140000000
ΔtisB	36	5	36000000	24	6	240000000
ΔtisB	23	5	23000000	14	6	140000000
ΔtisB	29	5	29000000	19	6	190000000
ΔistR	16	5	16000000	11	6	110000000
ΔistR	12	5	12000000	16	6	160000000
ΔistR	12	5	12000000	10	6	100000000
ΔldrD	30	5	30000000	32	6	320000000
ΔldrD	26	5	26000000	20	6	200000000
ΔldrD	27	5	27000000	17	6	170000000
ΔrdlD	33	5	33000000	15	6	150000000
ΔrdlD	22	5	22000000	26	6	260000000
ΔrdlD	18	5	18000000	19	6	190000000
ΔnhaA	76	4	7600000	26	6	260000000
ΔnhaA	57	4	5700000	19	6	190000000

ΔnhaA	38	4	3800000	12	6	120000000
ΔnhaA tisB::kan	61	4	6100000	38	5	38000000
ΔnhaA tisB::kan	60	4	6000000	11	6	110000000
ΔnhaA tisB::kan	50	4	5000000	20	6	200000000
ΔnhaA istR::kan	70	4	7000000	23	6	230000000
ΔnhaA istR::kan	15	4	1500000	11	6	110000000
ΔnhaA istR::kan	32	3	320000	12	6	120000000
ΔnhaA ldrD::kan	11	2	11000	13	6	130000000
ΔnhaA ldrD::kan	18	2	18000	11	6	110000000
ΔnhaA ldrD::kan	10	2	10000	15	6	150000000
ΔnhaA rdlD::kan	27	2	27000	10	6	100000000
ΔnhaA rdlD::kan	23	2	23000	11	6	110000000
ΔnhaA rdlD::kan	40	2	40000	11	6	110000000

Table 10: H_2O_2 0.7 mM chronic

	Test			**Control**		
	No. of colonies	**Well**	**CFU/mL**	**No. of colonies**	**Well**	**CFU/mL**
MG1655	100#	1	10000	11	6	110000000
MG1655	100#	1	10000	18	6	180000000
MG1655	29	2	29000	53	5	53000000
ΔldrD	20	4	2000000	22	6	220000000
ΔldrD	28	4	2800000	54	5	54000000
ΔldrD	19	4	1900000	60	5	60000000
ΔrdlD	27	3	270000	16	6	160000000
ΔrdlD	54	3	540000	15	6	150000000
ΔrdlD	23	3	230000	14	6	140000000
Δdps	100#	1	10000	13	6	130000000
Δdps	100#	1	10000	17	6	170000000
Δdps	100#	1	10000	14	6	140000000
ΔtonB	19	3	190000	13	6	130000000
ΔtonB	21	3	210000	13	6	130000000
ΔtonB	17	3	170000	18	6	180000000
ΔnhaB	65	3	650000	26	6	260000000
ΔnhaB	14	3	140000	15	6	150000000
ΔnhaB	30	3	300000	15	6	150000000
Δdps ldrD::kan	100#	1	10000	69	5	69000000
Δdps ldrD::kan	100#	1	10000	18	6	180000000
Δdps ldrD::kan	100#	1	10000	11	6	110000000
Δdps rdlD::kan	100#	1	10000	16	6	160000000
Δdps rdlD::kan	100#	1	10000	14	6	140000000
Δdps rdlD::kan	100#	1	10000	16	6	160000000

ΔtonB ldrD::kan	13	3	130000	15	6	150000000
ΔtonB ldrD::kan	17	3	170000	23	6	230000000
ΔtonB ldrD::kan	93	2	93000	15	6	150000000
ΔtonB rdlD::kan	11	3	110000	11	6	110000000
ΔtonB rdlD::kan	71	2	71000	17	6	170000000
ΔtonB rdlD::kan	80	2	80000	10	6	100000000
ΔnhaB ldrD::kan	10	3	100000	14	6	140000000
ΔnhaB ldrD::kan	19	3	190000	11	6	110000000
ΔnhaB ldrD::kan	81	3	810000	14	6	140000000
ΔnhaB rdlD::kan	36	3	360000	11	6	110000000
ΔnhaB rdlD::kan	35	3	350000	13	6	130000000
ΔnhaB rdlD::kan	37	3	370000	61	5	61000000

Table 11: Menadione sodium bisulfate 5 mM chronic.

	Test			**Control**		
	No. of colonies	**Well**	**CFU/mL**	**No. of colonies**	**Well**	**CFU/mL**
ΔtisB	15	5	15000000	36	6	360000000
ΔtisB	48	4	4800000	24	6	240000000
ΔtisB	39	4	3900000	53	5	53000000
ΔistR	64	3	640000	57	5	57000000
ΔistR	55	4	5500000	24	6	240000000
ΔistR	14	5	14000000	66	5	66000000
ΔshoB	45	3	450000	37	5	37000000
ΔshoB	59	3	590000	11	6	110000000
ΔshoB	52	3	520000	18	6	180000000
ΔohsC	15	5	15000000	32	5	32000000
ΔohsC	48	5	48000000	11	6	110000000
ΔohsC	41	5	41000000	14	6	140000000
ΔldrD	11	3	110000	10	6	100000000
ΔldrD	11	3	110000	20	6	200000000
ΔldrD	10	3	100000	9	6	90000000
ΔrdlD	68	3	680000	63	5	63000000
ΔrdlD	73	3	730000	68	5	68000000
ΔrdlD	49	3	490000	13	6	130000000
ΔdinQ	72	5	72000000	67	5	67000000
ΔdinQ	45	5	45000000	60	5	60000000
ΔdinQ	47	5	47000000	57	5	57000000
ΔagrB	54	5	54000000	20	6	200000000
ΔagrB	28	5	28000000	10	6	100000000
ΔagrB	61	5	61000000	15	6	150000000

MG1655	66	5	66000000	10	6	100000000
MG1655	21	6	210000000	32	6	320000000
MG1655	14	6	140000000	15	6	150000000

Table 12: Menadione sodium bisulfate 7 mM chronic.

	Test			**Control**		
	No. of colonies	**Well**	**CFU/mL**	**No. of colonies**	**Well**	**CFU/mL**
MG1655	10	6	100000000	21	6	210000000
MG1655	18	6	180000000	10	6	100000000
MG1655	11	6	110000000	21	6	210000000
ΔshoB	19	2	19000	11	6	110000000
ΔshoB	44	3	440000	13	6	130000000
ΔshoB	30	3	300000	17	6	170000000
ΔshoB-pMW119	92	2	92000	10	6	100000000
ΔshoB-pMW119	93	2	93000	11	6	110000000
ΔshoB-pMW119	73	2	73000	39	5	39000000
ΔohsC	48	5	48000000	12	6	120000000
ΔohsC	41	5	41000000	12	6	120000000
ΔohsC	37	5	37000000	23	6	230000000
ΔohsC-pMW119	13	5	13000000	13	6	130000000
ΔohsC-pMW119	39	5	39000000	12	6	120000000
ΔohsC-pMW119	31	5	31000000	68	5	68000000
ΔohsC-pUC57	11	2	11000	21	6	210000000
ΔohsC-pUC57	0	1	0	10	6	100000000
ΔohsC-pUC57	0	1	0	15	6	150000000
ΔldrD	35	3	350000	21	6	210000000
ΔldrD	22	3	220000	20	6	200000000
ΔldrD	40	3	400000	14	6	140000000
ΔldrD-pMW119	81	2	81000	78	5	78000000
ΔldrD-pMW119	109	2	109000	10	6	100000000
ΔldrD-pMW119	76	2	76000	10	6	100000000
ΔldrD-pUC57	100#	1	10000	43	6	430000000
ΔldrD-pUC57	100#	1	10000	22	6	220000000
ΔldrD-pUC57	100#	1	10000	29	6	290000000
ΔrdlD	12	4	1200000	13	6	130000000
ΔrdlD	36	3	360000	17	6	170000000
ΔrdlD	22	3	220000	21	6	210000000
ΔrdlD-pMW119	78	2	78000	24	6	240000000
ΔrdlD-pMW119	58	2	58000	19	6	190000000
ΔrdlD-pMW119	75	2	75000	25	6	250000000
ΔrdlD-pUC57	59	2	59000	22	6	220000000

ΔrdlD-pUC57	35	2	35000	34	6	340000000
ΔrdlD-pUC57	4*	1	400	14	6	140000000

Table 13: Menadione sodium bisulfate 7 mM chronic.

	Test			**Control**		
	No. of colonies	**Well**	**CFU/mL**	**No. of colonies**	**Well**	**CFU/mL**
ΔnhaA	0	1	0	19	6	190000000
ΔnhaA	0	1	0	14	6	140000000
ΔnhaA	0	1	0	23	6	230000000
ΔnhaB	150	2	150000	49	5	49000000
ΔnhaB	118	2	118000	66	5	66000000
ΔnhaB	122	2	122000	43	5	43000000
Δdps	11	5	11000000	23	6	230000000
Δdps	12	5	12000000	22	6	220000000
Δdps	34	5	34000000	19	6	190000000
ΔtonB	17	4	1700000	25	6	250000000
ΔtonB	40	4	4000000	22	6	220000000
ΔtonB	53	4	5300000	21	6	210000000
ΔshoB	9	2	9000	19	6	190000000
ΔshoB	55	2	55000	34	6	340000000
ΔshoB	57	2	57000	43	6	430000000
ΔohsC	15	5	15000000	32	5	32000000
ΔohsC	48	5	48000000	11	6	110000000
ΔohsC	41	5	41000000	14	6	140000000
ΔnhaA shoB::kan	0	1	0	27	6	270000000
ΔnhaA shoB::kan	0	1	0	23	6	230000000
ΔnhaA shoB::kan	0	1	0	28	6	280000000
ΔnhaA ohsC::kan	0	1	0	43	6	430000000
ΔnhaA ohsC::kan	0	1	0	28	6	280000000
ΔnhaA ohsC::kan	0	1	0	12	6	120000000
MG1655	38	5	38000000	38	5	38000000
MG1655	29	5	29000000	40	5	40000000
MG1655	32	5	32000000	34	5	34000000
Δdps ohsC::kan	30	4	3000000	16	6	160000000
Δdps ohsC::kan	48	5	48000000	29	6	290000000
Δdps ohsC::kan	36	5	36000000	48	5	48000000
ΔtonB ohsC::kan	16	3	160000	14	6	140000000
ΔtonB ohsC::kan	93	3	930000	73	5	73000000
ΔtonB ohsC::kan	32	3	320000	10	5	10000000
ΔnhaB ohsC::kan	14	3	140000	45	6	450000000
ΔnhaB ohsC::kan	159	2	159000	56	5	56000000

ΔnhaB ohsC::kan	16	3	160000	40	5	40000000
Δdps shoB::kan	15	6	150000000	28	6	280000000
Δdps shoB::kan	15	6	150000000	25	6	250000000
Δdps shoB::kan	27	5	27000000	22	6	220000000
ΔtonB shoB::kan	53	3	530000	13	6	130000000
ΔtonB shoB::kan	28	3	280000	31	6	310000000
ΔtonB shoB::kan	26	2	26000	37	6	370000000
ΔnhaB shoB::kan	5	1	500	58	6	580000000
ΔnhaB shoB::kan	53	3	530000	28	6	280000000
ΔnhaB shoB::kan	12	3	120000	55	6	550000000
MG1655	15	6	150000000	18	6	180000000
MG1655	21	6	210000000	47	6	470000000
MG1655	35	6	350000000	47	6	470000000

Table 14: Menadione sodium bisulfate 7 mM chronic.

	Test			**Control**		
	No. of colonies	**Well**	**CFU/mL**	**No. of colonies**	**Well**	**CFU/mL**
Δftn	17	6	170000000	17	6	170000000
Δftn	33	6	330000000	17	6	170000000
Δftn	15	6	150000000	27	6	270000000
Δfur	40	5	40000000	50	5	50000000
Δfur	16	5	16000000	70	5	70000000
Δfur	22	5	22000000	71	5	71000000
ΔfeoB	40	6	400000000	30	6	300000000
ΔfeoB	50	5	50000000	32	5	32000000
ΔfeoB	10	6	100000000	11	6	110000000
Δftn shoB::kan	72	5	72000000	54	5	54000000
Δftn shoB::kan	43	5	43000000	25	5	25000000
Δftn shoB::kan	58	5	58000000	30	5	30000000
Δftn ohsC::kan	74	5	74000000	55	5	55000000
Δftn ohsC::kan	88	5	88000000	59	5	59000000
Δftn ohsC::kan	63	5	63000000	52	5	52000000
Δfur shoB::kan	17	4	1700000	59	5	59000000
Δfur shoB::kan	22	4	2200000	53	5	53000000
Δfur shoB::kan	13	4	1300000	60	5	60000000
Δfur ohsC::kan	0	1	0	57	5	57000000
Δfur ohsC::kan	0	1	0	50	5	50000000
Δfur ohsC::kan	0	1	0	43	5	43000000
ΔfeoB shoB::kan	66	5	66000000	15	6	150000000
ΔfeoB shoB::kan	83	5	83000000	21	6	210000000
ΔfeoB shoB::kan	24	5	24000000	19	6	190000000

ΔfeoB ohsC::kan	12	6	120000000	15	6	150000000
ΔfeoB ohsC::kan	13	6	130000000	14	6	140000000
ΔfeoB ohsC::kan	34	6	340000000	30	6	300000000
MG1655	60	6	600000000	63	6	630000000
MG1655	28	6	280000000	29	6	290000000
MG1655	24	6	240000000	30	6	300000000

Table 15: Menadione sodium bisulfate 8 mM chronic.

	Test			**Control**		
	No. of colonies	**Well**	**CFU/mL**	**No. of colonies**	**Well**	**CFU/mL**
ΔiscA	11	6	110000000	73	5	73000000
ΔiscA	14	5	14000000	30	6	300000000
ΔiscA	17	6	170000000	27	6	270000000
ΔfhuB	29	6	290000000	47	6	470000000
ΔfhuB	29	6	290000000	33	6	330000000
ΔfhuB	23	6	230000000	19	6	190000000
ΔfecD	24	6	240000000	25	6	250000000
ΔfecD	30	6	300000000	25	6	250000000
ΔfecD	21	6	210000000	20	6	200000000
ΔshoB	10	6	100000000	12	6	120000000
ΔshoB	20	6	200000000	37	6	370000000
ΔshoB	14	6	140000000	25	6	250000000
ΔohsC	11	6	110000000	12	6	120000000
ΔohsC	28	6	280000000	20	6	200000000
ΔohsC	15	6	150000000	23	6	230000000
MG1655	24	6	240000000	17	6	170000000
MG1655	26	6	260000000	40	6	400000000
MG1655	12	6	120000000	28	6	280000000
ΔiscA shoB::kan	0	1	0	15	6	150000000
ΔiscA shoB::kan	0	1	0	45	6	450000000
ΔiscA shoB::kan	0	1	0	13	6	130000000
ΔiscA ohsC::kan	10	6	100000000	26	6	260000000
ΔiscA ohsC::kan	16	6	160000000	24	6	240000000
ΔiscA ohsC::kan	11	6	110000000	45	6	450000000
ΔfhuB shoB::kan	19	4	1900000	10	6	100000000
ΔfhuB shoB::kan	22	4	2200000	32	6	320000000
ΔfhuB shoB::kan	16	4	1600000	16	6	160000000
ΔfhuB ohsC::kan	15	6	150000000	20	6	200000000
ΔfhuB ohsC::kan	27	6	270000000	20	6	200000000
ΔfhuB ohsC::kan	13	6	130000000	16	6	160000000
ΔfecD shoB::kan	48	5	48000000	14	6	140000000

ΔfecD shoB::kan	36	5	36000000	24	6	240000000
ΔfecD shoB::kan	37	5	37000000	18	6	180000000
ΔfecD ohsC::kan	51	5	51000000	30	6	300000000
ΔfecD ohsC::kan	39	5	39000000	14	6	140000000
ΔfecD ohsC::kan	51	5	51000000	57	5	57000000

Table 16: Menadione sodium bisulfate 8 mM chronic.

	Test			**Control**		
	No. of colonies	**Well**	**CFU/ml**	**No. of colonies**	**Well**	**CFU/mL**
MG1655	31	6	310000000	32	6	320000000
MG1655	21	6	210000000	35	6	350000000
MG1655	15	6	150000000	36	6	360000000
ΔiscA	11	6	110000000	39	6	390000000
ΔiscA	28	6	280000000	15	6	150000000
ΔiscA	15	6	150000000	18	6	180000000
ΔsufE	12	6	120000000	22	6	220000000
ΔsufE	31	6	310000000	47	6	470000000
ΔsufE	35	6	350000000	26	6	260000000
ΔsufA	14	6	140000000	22	6	220000000
ΔsufA	13	6	130000000	25	6	250000000
ΔsufA	22	6	220000000	22	6	220000000
ΔfepD	11	6	110000000	30	6	300000000
ΔfepD	15	6	150000000	40	6	400000000
ΔfepD	80	5	80000000	15	6	150000000
ΔshoB	13	6	130000000	12	6	120000000
ΔshoB	22	6	220000000	16	6	160000000
ΔshoB	14	6	140000000	11	6	110000000
ΔohsC	16	6	160000000	10	6	100000000
ΔohsC	12	6	120000000	12	6	120000000
ΔohsC	24	6	240000000	22	6	220000000
ΔsufA shoB::kan	15	6	150000000	24	6	240000000
ΔsufA shoB::kan	26	6	260000000	30	6	300000000
ΔsufA shoB::kan	11	6	110000000	30	6	300000000
ΔsufA ohsC::kan	37	3	370000	46	5	46000000
ΔsufA ohsC::kan	10	3	100000	19	5	19000000
ΔsufA ohsC::kan	21	5	21000000	21	6	210000000
ΔsufE shoB::kan	14	6	140000000	21	6	210000000
ΔsufE shoB::kan	11	5	11000000	15	6	150000000
ΔsufE shoB::kan	56	5	56000000	20	6	200000000
ΔsufE ohsC::kan	27	4	2700000	37	5	37000000
ΔsufE ohsC::kan	11	3	110000	12	6	120000000

ΔsufE ohsC::kan	38	4	3800000	54	5	54000000
ΔiscA shoB::kan	91	4	9100000	17	6	170000000
ΔiscA shoB::kan	78	4	7800000	12	6	120000000
ΔiscA shoB::kan	26	5	26000000	10	6	100000000
ΔiscA ohsC::kan	52	4	5200000	27	5	27000000
ΔiscA ohsC::kan	48	4	4800000	34	5	34000000
ΔiscA ohsC::kan	48	4	4800000	50	5	50000000
ΔfepD shoB::kan	33	5	33000000	40	5	40000000
ΔfepD shoB::kan	56	5	56000000	20	6	200000000
ΔfepD shoB::kan	30	5	30000000	53	5	53000000
ΔfepD ohsC::kan	18	5	18000000	11	6	110000000
ΔfepD ohsC::kan	10	5	10000000	19	6	190000000
ΔfepD ohsC::kan	10	5	10000000	12	6	120000000

Table 17: Menadione sodium bisulfate 4 mM chronic.

	Test			**Control**		
	No. of colonies	**Well**	**CFU/mL**	**No. of colonies**	**Well**	**CFU/mL**
MG1655	24	6	240000000	46	6	460000000
MG1655	15	6	150000000	30	6	300000000
MG1655	17	6	170000000	41	6	410000000
ΔsodA	75	4	7500000	25	6	250000000
ΔsodA	78	4	7800000	20	6	200000000
ΔsodA	67	4	6700000	22	6	220000000
ΔsodA shoB::kan	27	5	27000000	29	6	290000000
ΔsodA shoB::kan	24	5	24000000	37	6	370000000
ΔsodA shoB::kan	45	4	4500000	21	6	210000000
ΔsodA ohsC::kan	44	4	4400000	27	6	270000000
ΔsodA ohsC::kan	66	4	6600000	70	5	70000000
ΔsodA ohsC::kan	93	4	9300000	25	6	250000000

Table 18: Menadione sodium bisulfate 8 mM chronic.

	Test			**Control**		
	No. of colonies	**Well**	**CFU/mL**	**No. of colonies**	**Well**	**CFU/mL**
MG1655	20	6	200000000	46	6	460000000
MG1655	24	6	240000000	30	6	300000000
MG1655	21	6	210000000	41	6	410000000
ΔshoB	10	6	100000000	13	6	130000000
ΔshoB	11	6	110000000	23	6	230000000
ΔshoB	13	6	130000000	17	6	170000000
ΔohsC	13	6	130000000	13	6	130000000

ΔohsC	20	6	200000000	14	6	140000000
ΔohsC	66	5	66000000	36	5	36000000
ΔsodB	28	6	280000000	22	6	220000000
ΔsodB	20	6	200000000	17	6	170000000
ΔsodB	21	6	210000000	14	6	140000000
ΔsodC	100#	2	100000	33	6	330000000
ΔsodC	21	6	210000000	25	6	250000000
ΔsodC	19	6	190000000	32	6	320000000
ΔfecC	20	6	200000000	35	6	350000000
ΔfecC	18	6	180000000	28	6	280000000
ΔfecC	17	6	170000000	21	6	210000000
ΔsodB shoB::kan	14	6	140000000	80	6	800000000
ΔsodB shoB::kan	12	6	120000000	24	6	240000000
ΔsodB shoB::kan	14	6	140000000	15	6	150000000
ΔsodB ohsC::kan	54	5	54000000	20	6	200000000
ΔsodB ohsC::kan	45	5	45000000	19	6	190000000
ΔsodB ohsC::kan	12	5	12000000	23	6	230000000
ΔsodC shoB::kan	15	6	150000000	10	6	100000000
ΔsodC shoB::kan	28	6	280000000	23	6	230000000
ΔsodC shoB::kan	20	6	200000000	71	6	710000000
ΔsodC ohsC::kan	11	6	110000000	18	6	180000000
ΔsodC ohsC::kan	14	6	140000000	15	6	150000000
ΔsodC ohsC::kan	11	6	110000000	17	6	170000000
ΔfecC shoB::kan	46	5	46000000	12	6	120000000
ΔfecC shoB::kan	33	5	33000000	14	6	140000000
ΔfecC shoB::kan	39	4	3900000	11	6	110000000
ΔfecC ohsC::kan	25	4	2500000	11	6	110000000
ΔfecC ohsC::kan	16	5	16000000	48	5	48000000
ΔfecC ohsC::kan	15	5	15000000	14	6	140000000

Table 19: Menadione sodium bisulfate 7 mM chronic.

	Test			**Control**		
	No. of colonies	**Well**	**CFU/mL**	**No. of colonies**	**Well**	**CFU/mL**
Δftn	110	6	1100000000	109	6	1090000000
Δftn	65	6	650000000	54	6	540000000
Δftn	110	6	1100000000	60	6	600000000
Δfur	20	5	20000000	20	6	200000000
Δfur	21	5	21000000	61	6	610000000
Δfur	25	5	25000000	47	6	470000000
ΔfeoB	31	6	310000000	26	6	260000000
ΔfeoB	54	6	540000000	36	6	360000000

ΔfeoB	66	6	660000000	109	6	1090000000
Δftn tisB::kan	130	6	1300000000	80	6	800000000
Δftn tisB::kan	47	6	470000000	40	6	400000000
Δftn tisB::kan	12	6	120000000	10	6	100000000
Δftn istR	20	6	200000000	14	6	140000000
Δftn istR	34	6	340000000	35	6	350000000
Δftn istR	21	6	210000000	17	6	170000000
Δfur tisB::kan	0	1	0	52	6	520000000
Δfur tisB::kan	0	1	0	42	6	420000000
Δfur tisB::kan	0	1	0	35	6	350000000
Δfur istR	50	2	50000	40	6	400000000
Δfur istR	10	3	100000	20	6	200000000
Δfur istR	10	3	100000	21	6	210000000
ΔfeoB tisB::kan	67	5	67000000	66	5	66000000
ΔfeoB tisB::kan	100	5	100000000	15	6	150000000
ΔfeoB tisB::kan	38	6	380000000	27	6	270000000
ΔfeoB istR	70	5	70000000	22	6	220000000
ΔfeoB istR	31	5	31000000	10	6	100000000
ΔfeoB istR	36	5	36000000	13	6	130000000
MG1655	114	6	1140000000	42	6	420000000
MG1655	97	6	970000000	80	6	800000000
MG1655	99	6	990000000	81	6	810000000

Table 20: Menadione sodium bisulfate 7 mM chronic

	Test			**Control**		
	No. of colonies	**Well**	**CFU/mL**	**No. of colonies**	**Well**	**CFU/mL**
MG1655	65	6	650000000	53	6	530000000
MG1655	24	6	240000000	22	6	220000000
MG1655	113	6	1130000000	82	6	820000000
ΔtisB	24	6	240000000	17	6	170000000
ΔtisB	20	6	200000000	22	6	220000000
ΔtisB	28	6	280000000	10	6	100000000
ΔistR	16	6	160000000	20	6	200000000
ΔistR	20	6	200000000	17	6	170000000
ΔistR	11	6	110000000	20	6	200000000
ΔldrD	13	6	130000000	11	6	110000000
ΔldrD	14	6	140000000	11	6	110000000
ΔldrD	26	6	260000000	19	6	190000000
ΔrdlD	18	6	180000000	14	6	140000000
ΔrdlD	25	6	250000000	22	6	220000000
ΔrdlD	15	6	150000000	16	6	160000000

ΔnhaA tisB::kan	45	5	45000000	10	6	100000000
ΔnhaA tisB::kan	57	5	57000000	18	6	180000000
ΔnhaA tisB::kan	43	5	43000000	16	6	160000000
ΔnhaA istR::kan	82	4	8200000	18	6	180000000
ΔnhaA istR::kan	44	4	4400000	21	6	210000000
ΔnhaA istR::kan	31	4	3100000	22	6	220000000
ΔnhaA ldrD::kan	16	6	160000000	12	6	120000000
ΔnhaA ldrD::kan	18	6	180000000	19	6	190000000
ΔnhaA ldrD::kan	16	6	160000000	19	6	190000000
ΔnhaA rdlD::kan	12	6	120000000	20	6	200000000
ΔnhaA rdlD::kan	12	6	120000000	17	6	170000000
ΔnhaA rdlD::kan	109	5	109000000	11	6	110000000

Table 21: chronic iron stress

	Test			**Control**		
	No. of colonies	**Well**	**CFU/mL**	**No. of colonies**	**Well**	**CFU/mL**
ΔtisB	16	2	16000	38	6	380000000
ΔtisB	110	1	11000	23	6	230000000
ΔtisB	88	1	8800	27	6	270000000
ΔistR	56	2	56000	37	6	370000000
ΔistR	38	2	38000	20	6	200000000
ΔistR	42	2	42000	27	6	270000000
ΔshoB	21	3	210000	45	6	450000000
ΔshoB	80	3	800000	86	6	860000000
ΔshoB	43	3	430000	60	6	600000000
ΔohsC	48	3	480000	38	6	380000000
ΔohsC	59	3	590000	40	6	400000000
ΔohsC	28	3	280000	48	6	480000000
ΔldrD	98	2	98000	70	6	700000000
ΔldrD	40	2	40000	35	6	350000000
ΔldrD	30	2	30000	62	6	620000000
ΔrdlD	20	3	200000	43	6	430000000
ΔrdlD	46	3	460000	45	6	450000000
ΔrdlD	27	3	270000	38	6	380000000
ΔdinQ	52	3	520000	112	6	1120000000
ΔdinQ	70	3	700000	89	6	890000000
ΔdinQ	75	4	7500000	121	6	1210000000
ΔagrB	15	3	150000	88	6	880000000
ΔagrB	17	3	170000	59	6	590000000
ΔagrB	41	3	410000	80	6	800000000
MG1655	34	5	34000000	32	6	320000000

MG1655	29	5	29000000	17	6	170000000
MG1655	60	5	60000000	77	6	770000000

Table 22: Chronic iron stress

	Test			Control		
	No. of colonies	**Well**	**CFU/mL**	**No. of colonies**	**Well**	**CFU/mL**
ΔnhaB	27	4	2700000	18	6	180000000
ΔnhaB	25	5	25000000	26	6	260000000
ΔnhaB	20	5	20000000	21	6	210000000
Δdps	17	5	17000000	30	6	300000000
Δdps	42	5	42000000	40	6	400000000
Δdps	35	5	35000000	41	6	410000000
ΔtonB	54	4	5400000	86	6	860000000
ΔtonB	14	4	1400000	49	6	490000000
ΔtonB	20	3	200000	41	6	410000000
Δdps ohsC::kan	18	5	18000000	99	6	990000000
Δdps ohsC::kan	20	5	20000000	108	6	1080000000
Δdps ohsC::kan	41	5	41000000	112	6	1120000000
ΔtonB ohsC::kan	36	4	3600000	90	6	900000000
ΔtonB ohsC::kan	57	4	5700000	106	6	1060000000
ΔtonB ohsC::kan	21	3	210000	50	6	500000000
ΔnhaB ohsC::kan	25	4	2500000	36	6	360000000
ΔnhaB ohsC::kan	24	4	2400000	38	6	380000000
ΔnhaB ohsC::kan	23	4	2300000	21	6	210000000
Δdps shoB::kan	13	4	1300000	18	6	180000000
Δdps shoB::kan	10	4	1000000	18	6	180000000
Δdps shoB::kan	15	4	1500000	27	6	270000000
ΔtonB shoB::kan	23	4	2300000	50	6	500000000
ΔtonB shoB::kan	55	4	5500000	41	6	410000000
ΔtonB shoB::kan	33	4	3300000	45	6	450000000
ΔnhaB shoB::kan	23	6	230000000	107	6	1070000000
ΔnhaB shoB::kan	18	6	180000000	120	6	1200000000
ΔnhaB shoB::kan	11	6	110000000	60	6	600000000
MG1655	27	5	27000000	56	6	560000000
MG1655	20	5	20000000	53	6	530000000
MG1655	35	3	350000	27	6	270000000

Raw data for acute testing

Table 23: Acute oxidative stress caused by H_2O_2, 10 mM. 1 colony of MG1655 was grown for 9 generations and each generation was tested at 10 mM.

Generation	No. of colonies	Well	CFU/mL
1	22	5	22000000
1	19	5	19000000
1	16	5	16000000
2	27	3	270000
2	10	3	100000
2	15	3	150000
3	21	6	210000000
3	24	6	240000000
3	19	6	190000000
4	28	6	280000000
4	23	6	230000000
4	36	6	360000000
5	32	6	320000000
5	29	6	290000000
5	28	6	280000000
6	36	6	360000000
6	38	6	380000000
6	45	6	450000000
7	45	6	450000000
7	31	6	310000000
7	47	6	470000000
8	25	6	250000000
8	25	6	250000000
8	26	6	260000000
9	41	6	410000000
9	38	6	380000000
9	35	6	350000000

Table 24: Acute oxidative stress caused by H_2O_2, 12 mM. 5 colonies of MG1655 was grown for 4 generations and each generation was tested at 12 mM.

Generation	Number of colonies	well	CFU/mL
G1 1	86	1	8600
G1 2	25	2	25000

G1 3	26	2	26000
G1 4	0		0
G1 5	40	4	4000000
G2 1	11	6	110000000
G2 2	12	6	120000000
G2 3	34	4	3400000
G2 4	0		0
G2 5	36	5	36000000
G3 1	12	5	12000000
G3 2	13	4	1300000
G3 3	40	2	40000
G3 4	40	1	4000
G3 5	19	4	1900000
G4 1	38	5	38000000
G4 2	17	4	1700000
G4 3	37	5	37000000
G4 4	45	1	4500
G4 5	24	5	24000000

Table 25: Survival curve at different concentrations of H_2O_2. Three colonies of MG1655 were tested acute, 1 hour, with different concentrations of H_2O_2.

Concentration	No. of colonies	well	CFU/mL
6 mM	16	6	160000000
6 mM	24	6	240000000
6 mM	28	6	280000000
8 mM	13	6	130000000
8 mM	25	6	250000000
8 mM	21	6	210000000
10 mM	49	5	49000000
10 mM	12	6	120000000
10 mM	55	5	55000000
12mM	63	4	6300000
12mM	49	5	49000000
12mM	29	5	29000000
14 mM	0	1	0
14 mM	17	5	17000000
14 mM	0	1	0

Table 26: Acute oxidative stress caused by H_2O_2, 8 mM. 6 colonies of *tisB, istR, shoB, ohsC, ldrD, rdlD* mutants and MG1655 were tested at 8 mM.

	Test			Control		
	No. of colonies	Well	CFU/mL	No. of colonies	Well	CFU/mL
ΔtisB	32	5	32000000	102	6	1020000000
ΔtisB	22	2	22000	72	6	720000000
ΔtisB	16	5	16000000	74	6	740000000
ΔtisB	50	1	5000	87	6	870000000
ΔtisB	32	1	3200	59	6	590000000
ΔtisB	29	4	2900000	68	6	680000000
ΔistR	22	5	22000000	48	6	480000000
ΔistR	40	5	40000000	53	6	530000000
ΔistR	27	5	27000000	59	6	590000000
ΔistR	83	1	8300	43	6	430000000
ΔistR	28	4	2800000	52	6	520000000
ΔistR	16	1	1600	58	6	580000000
ΔshoB	26	5	26000000	25	6	250000000
ΔshoB	54	5	54000000	60	6	600000000
ΔshoB	10	5	10000000	62	6	620000000
ΔshoB	34	3	340000	36	6	360000000
ΔshoB	11	5	11000000	43	6	430000000
ΔshoB	33	3	330000	37	6	370000000
ΔohsC	41	5	41000000	73	6	730000000
ΔohsC	40	5	40000000	76	6	760000000
ΔohsC	38	1	3800	80	6	800000000
ΔohsC	0	1	0	58	6	580000000
ΔohsC	0	1	0	55	6	550000000
ΔohsC	0	1	0	41	6	410000000
ΔldrD	23	4	2300000	97	6	970000000
ΔldrD	16	5	16000000	106	6	1060000000
ΔldrD	18	5	18000000	102	6	1020000000
ΔldrD	36	4	3600000	74	6	740000000
ΔldrD	23	5	23000000	80	6	800000000
ΔldrD	20	4	2000000	50	6	500000000
ΔrdlD	51	5	51000000	95	6	950000000
ΔrdlD	17	5	17000000	93	6	930000000
ΔrdlD	53	4	5300000	99	6	990000000
ΔrdlD	0	1	0	43	6	430000000
ΔrdlD	15	1	1500	55	6	550000000
ΔrdlD	0	1	0	55	6	550000000
ΔdinQ	14	3	140000	67	6	670000000
ΔdinQ	38	2	38000	60	6	600000000

ΔdinQ	30	2	30000	65	6	650000000
ΔdinQ	47	5	47000000	36	6	360000000
ΔdinQ	45	4	4500000	43	6	430000000
ΔdinQ	28	5	28000000	54	6	540000000
ΔagrB	37	2	37000	57	6	570000000
ΔagrB	46	4	4600000	78	6	780000000
ΔagrB	29	5	29000000	64	6	640000000
ΔagrB	0	1	0	51	6	510000000
ΔagrB	0	1	0	36	6	360000000
ΔagrB	0	1	0	32	6	320000000
MG1655	31	1	3100	36	6	360000000
MG1655	16	5	16000000	52	6	520000000
MG1655	13	1	1300	35	6	350000000
MG1655	14	6	140000000	83	6	830000000
MG1655	0	1	0	38	6	380000000
MG1655	12	4	1200000	83	6	830000000

Appendix F: Raw date for H_2O_2 detextion assay

Table 1: Cultures of MG1655 (wild type) and shoB mutant were grown aerobically in M9 medium with 0.2 % glucose and 0.5 mM of the 20 amino acids, and was resuspended in M9 medium, with 0.02% glucose and 0.05 mM of each of the amino acids, at an OD_{600} of 0.1. The H_2O_2 concentration was measured at various time points after resuspension.

Time, minutes	Strain	Fluorescence signal
0	MG1655	49685
0	MG1655	45555
0	MG1655	51897
0	shoB	83127
0	shoB	80677
0	shoB	69517
6	MG1655	51438
6	MG1655	44379
6	MG1655	48735
6	shoB	84775
6	shoB	76700
6	shoB	51515
12	MG1655	40592
12	MG1655	40620
12	MG1655	37791
12	shoB	73962
12	shoB	58083
12	shoB	48671
18	MG1655	34756
18	MG1655	36031
18	MG1655	36003
18	shoB	67419
18	shoB	57717
18	shoB	50081
24	MG1655	31200
24	MG1655	31804
24	MG1655	29792
24	shoB	55992
24	shoB	45749
24	shoB	43832

Table 2: Cultures of MG1655 (wild type) and shoB mutant were grown aerobically in LB medium and resuspended in PBS at an OD_{600} of 0.1. H_2O_2 was added to a final concentration of 2.0 µM and the concentration was measured at various time points after addition as described in Materials and methods.

Time, min	Strain	Fluorescence signal
0	MG1655	90130
0	MG1655	94962
0	MG1655	97201
0	shoB	111272
0	shoB	106868
0	shoB	103937
5	MG1655	55809
5	MG1655	68913
5	MG1655	64381
5	shoB	77699
5	shoB	78408
5	shoB	82801
10	MG1655	47657
10	MG1655	64109
10	MG1655	61769
10	shoB	105979
10	shoB	72907
10	shoB	74821
16	MG1655	36110
16	MG1655	42419
16	MG1655	35983
16	shoB	75680
16	shoB	59134
16	shoB	59489
20	MG1655	27098
20	MG1655	45149
20	MG1655	32047
20	shoB	67159
20	shoB	49288
20	shoB	55994
25	MG1655	31273
25	MG1655	42373
25	MG1655	29339
25	shoB	67965
25	shoB	50767
25	shoB	49391
30	MG1655	22999
30	MG1655	36836

30	MG1655	55082
30	shoB	60790
30	shoB	43600
30	shoB	46036
40	MG1655	23507
40	MG1655	29345
40	MG1655	26421
40	shoB	52905
40	shoB	30949
40	shoB	36934
50	MG1655	25314
50	MG1655	23458
50	MG1655	27724
50	shoB	41793
50	shoB	28469
50	shoB	28259

Table 3: Cultures of MG1655 (wild type), ***katG katE ahpC, katG katE ahpC shoB::kan*** **and** ***katG katE ahpC ohsC::kan*** **mutant were grown aerobically in M9 medium with 0.2 % glucose and 0.5 mM of the 20 amino acids, and was resuspended in M9 medium, with 0.02% glucose and 0.05 mM of each of the amino acids, at an OD_{600} of 0.1. The H_2O_2 concentration was measured at various time points after resuspension.**

Time, minutes	**Strain**	**Fluorescence signal**
0	MG1655	90516
0	MG1655	94016
0	MG1655	93129
0	*katG katE ahpC*	118930
0	*katG katE ahpC*	130446
0	*katG katE ahpC*	131240
0	*katG katE ahpC shoB::kan*	117467
0	*katG katE ahpC shoB::kan*	122566
0	*katG katE ahpC shoB::kan*	114889
0	*katG katE ahpC ohsC::kan*	128337
0	*katG katE ahpC ohsC::kan*	119280
0	*katG katE ahpC ohsC::kan*	131129
10	MG1655	73478
10	MG1655	63488
10	MG1655	65562
10	*katG katE ahpC*	136823
10	*katG katE ahpC*	133043
10	*katG katE ahpC*	142003
10	*katG katE ahpC shoB::kan*	113287
10	*katG katE ahpC shoB::kan*	137448

10	*katG katE ahpC shoB::kan*	128235
10	*katG katE ahpC ohsC::kan*	147573
10	*katG katE ahpC ohsC::kan*	139108
10	*katG katE ahpC ohsC::kan*	142471
27	MG1655	62937
27	MG1655	42769
27	MG1655	44671
27	*katG katE ahpC*	149961
27	*katG katE ahpC*	142914
27	*katG katE ahpC*	144475
27	*katG katE ahpC shoB::kan*	123339
27	*katG katE ahpC shoB::kan*	126178
27	*katG katE ahpC shoB::kan*	123602
27	*katG katE ahpC ohsC::kan*	136129
27	*katG katE ahpC ohsC::kan*	136606
27	*katG katE ahpC ohsC::kan*	136453
51	MG1655	33229
51	MG1655	32456
51	MG1655	35961
51	*katG katE ahpC*	173515
51	*katG katE ahpC*	146368
51	*katG katE ahpC*	158518
51	*katG katE ahpC shoB::kan*	131415
51	*katG katE ahpC shoB::kan*	154851
51	*katG katE ahpC shoB::kan*	122858
51	*katG katE ahpC ohsC::kan*	149822
51	*katG katE ahpC ohsC::kan*	131476
51	*katG katE ahpC ohsC::kan*	151564

Appendix G: Raw data for standard curve made for hydrogen peroxide detection assay

Fluorescence signal was measured and converted to amount of H_2O_2.

	10 µM	**5 µM**	**2,5 µM**	**1,25 µM**	**0,625 µM**	**0,3125 µM**	**0,156 µM**	**0,078 µM**	**0**
	404224	247262	130654	78853	61886	55018	42459	36307	35075
	408120	239534	127286	85592	63335	47524	43222	39665	33680
	411319	242565	129373	92206	78348	52795	43479	39235	38226
Average	407888	243120	129104	85550	67856	51779	43053	38402	35660
SD	3553,201	3893,815	1699,998	6676,598	9114,889	3848,922	530,5057	1827,304	2328,839

Appendix H: OD measurements and flow cytometry data

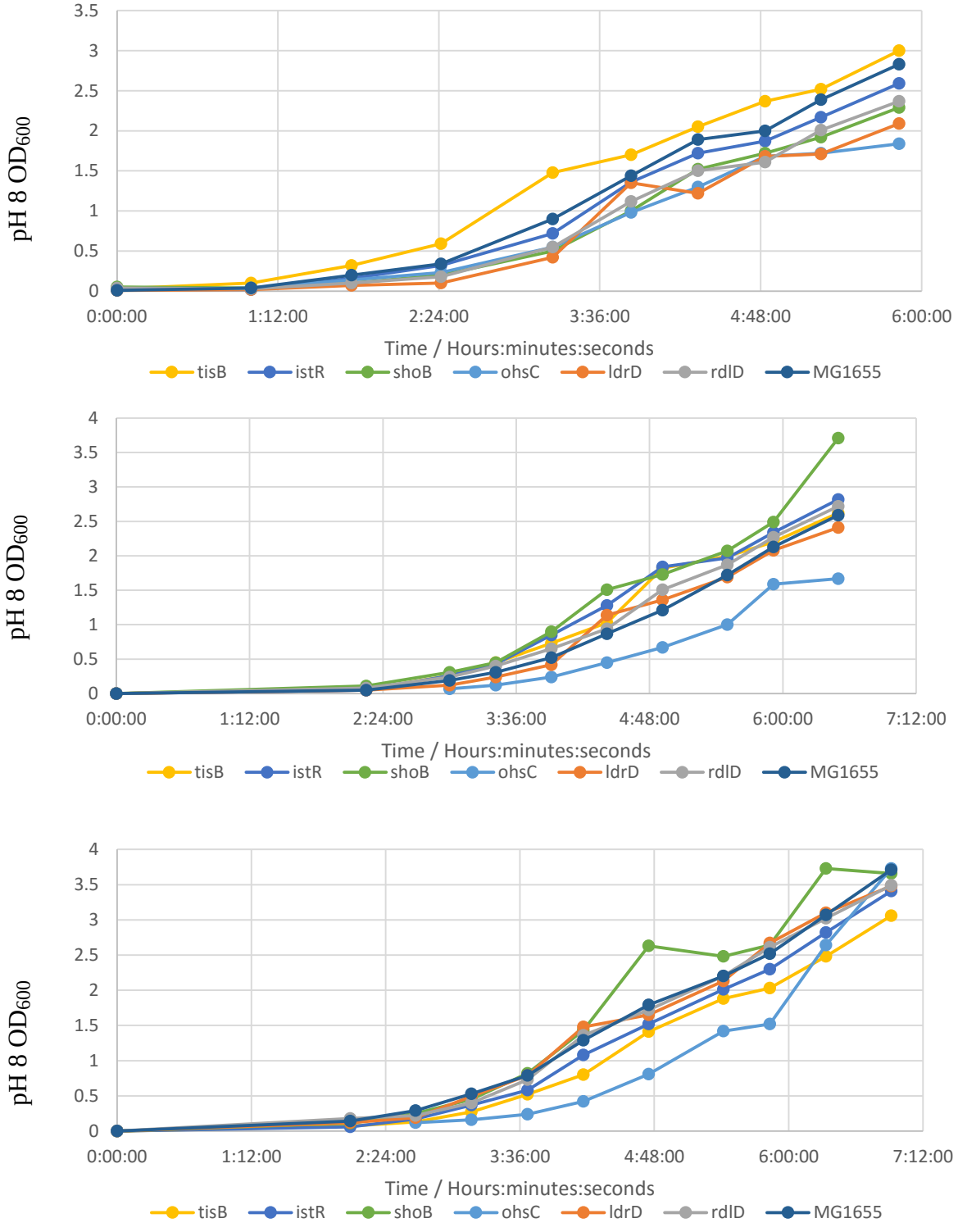


Figure 1: Three independent OD_{600} measurements of *tisB, istR, shoB, ohsC, ldrD and rdlD* mutants and wild type grown in LBK medium at pH 8

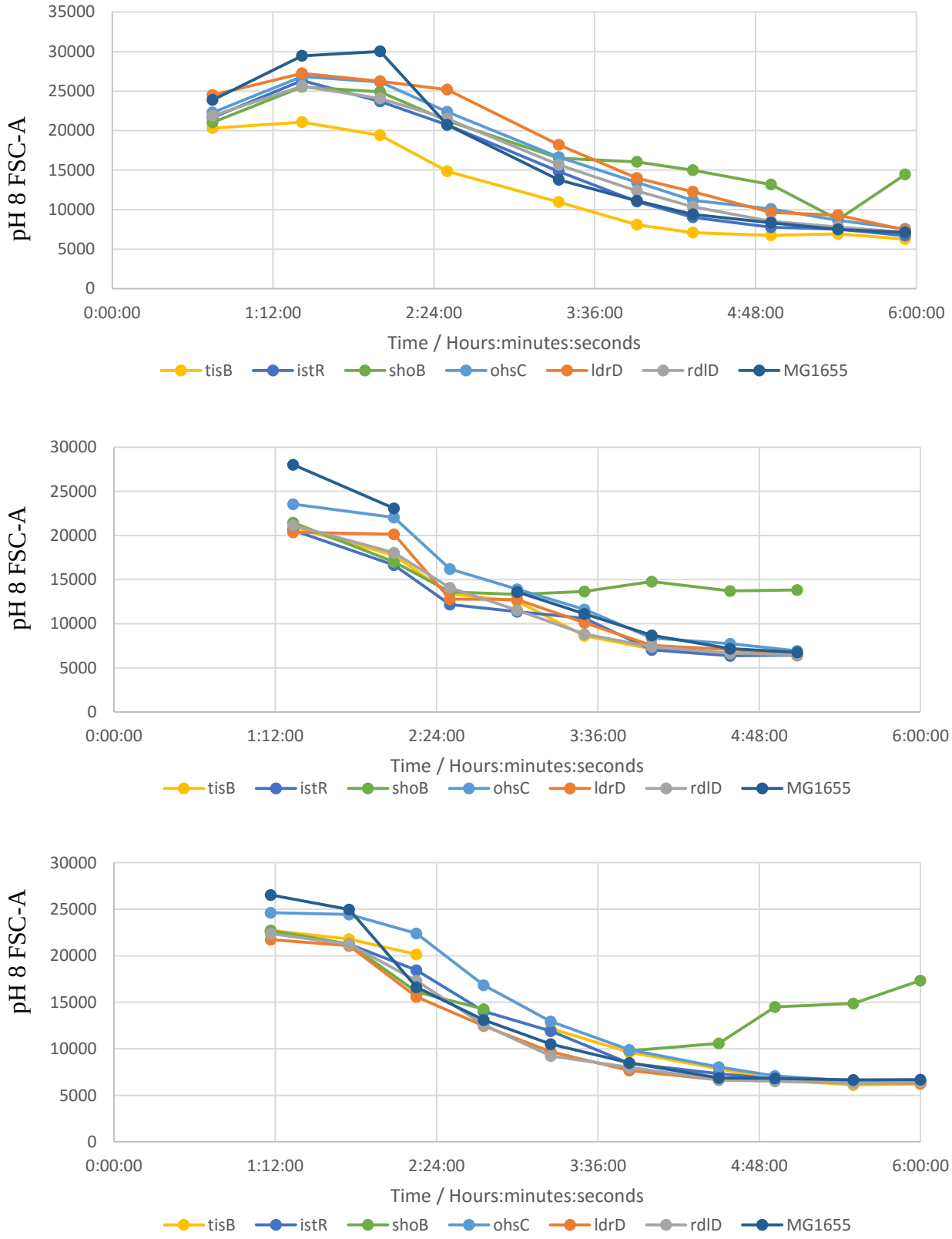


Figure 2: Three independent forward scatter (FSC) measurements of *tisB, istR, shoB, ohsC, ldrD and rdlD* mutants and wild type grown in LBK medium at pH 8.

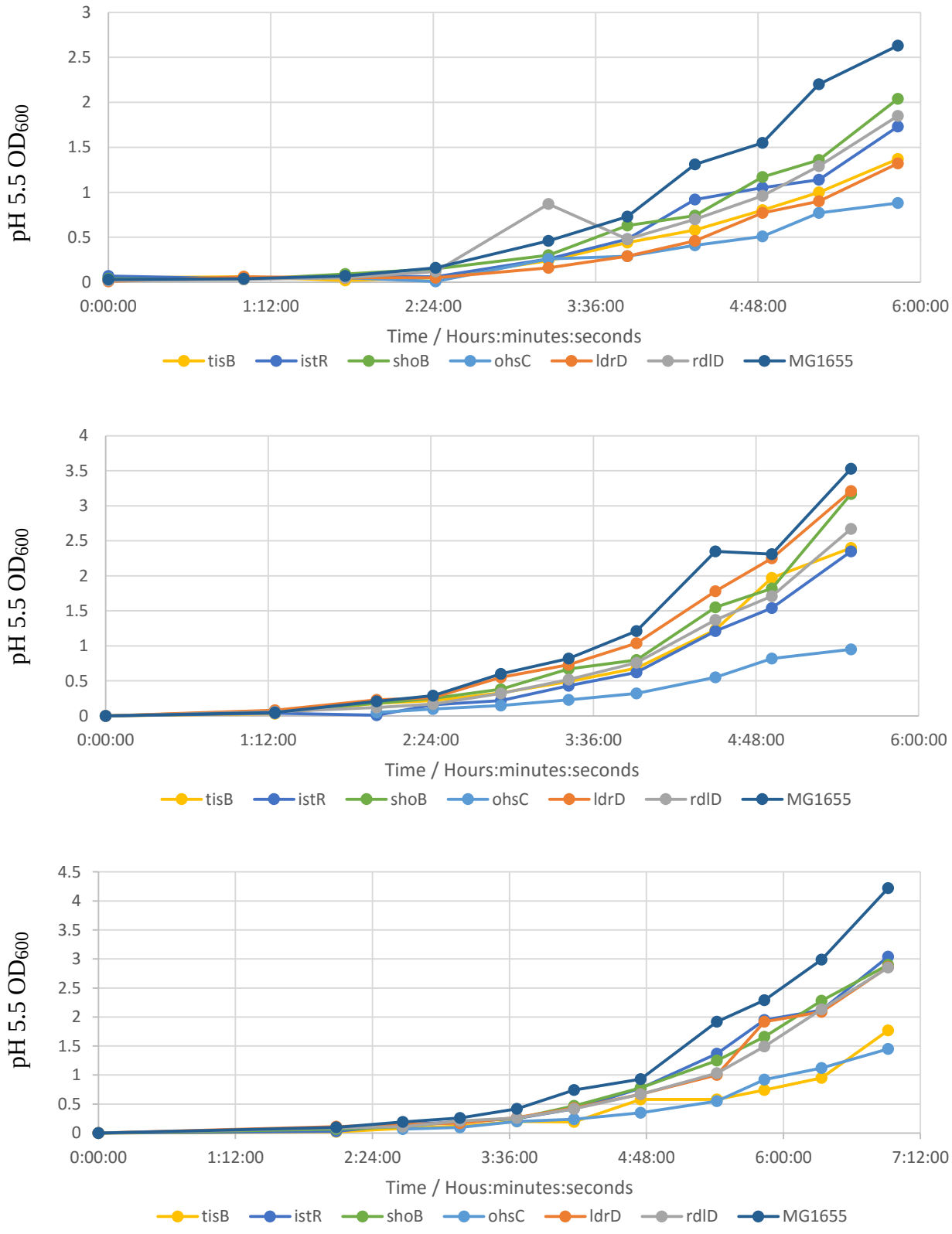


Figure 3: Three independent OD_{600} measurements of *tisB, istR, shoB, ohsC, ldrD and rdlD* mutants and wild type grown in LBK medium at pH 5.5.

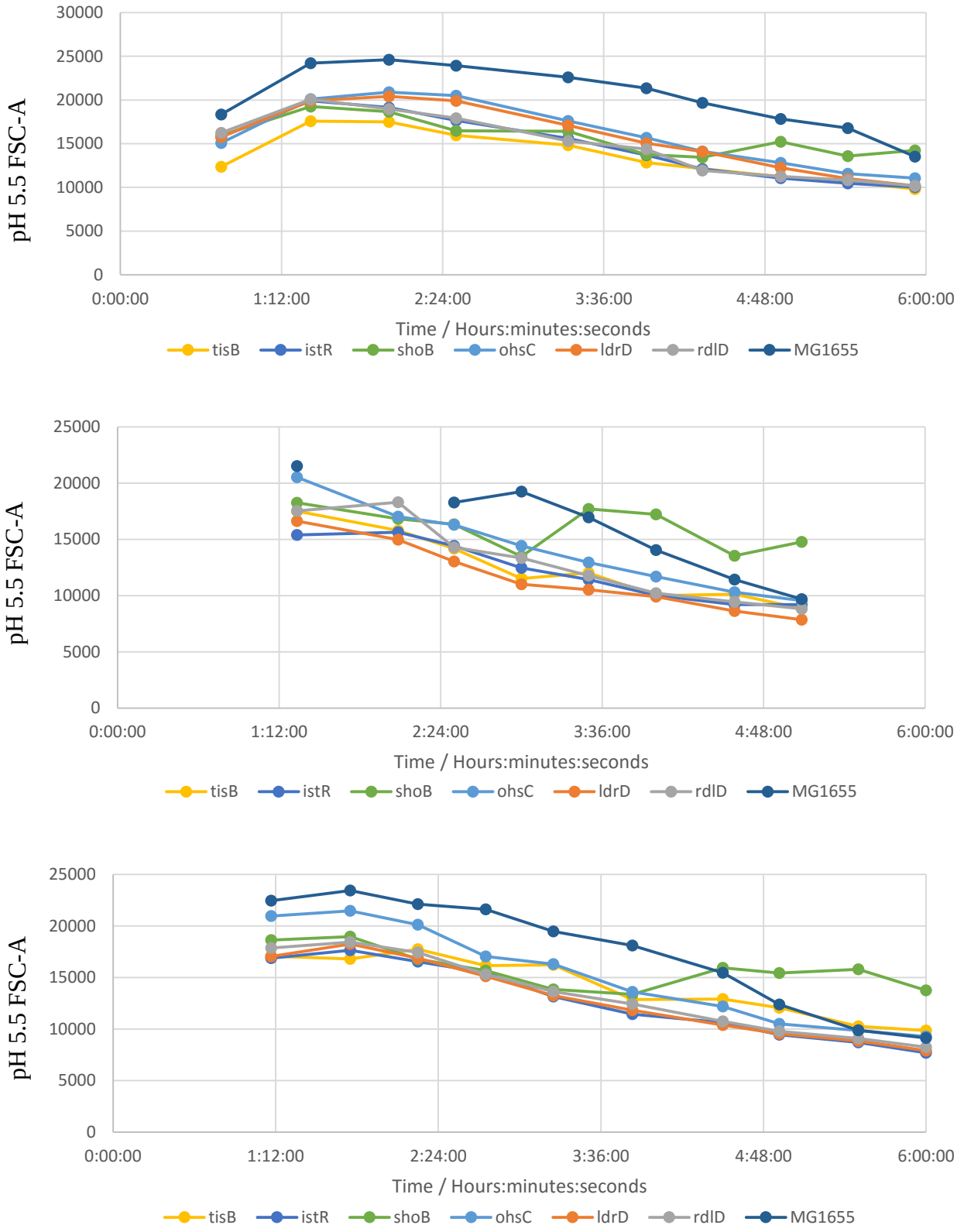


Figure 4: Three independent forward scatter (FSC) measurements of *tisB, istR, shoB, ohsC, ldrD and rdlD* mutants and wild type grown in LBK medium at pH 8.0.

Appendix I: Raw data from flow cytometry and OD_{600} measurements for growth curve

In three independent experiments, the mutants *tisB, istR, shoB, ohsC, ldrD, rdlD* and wild type were grown in LBK medium adjusted to pH 5.5 and 8. OD_{600} and forward scatter light measurements were made with 30-minute intervals in between. The data below is from the first experiment.

pH 5.5 FSC-A

	09:50:00	10:30:00	11:05:00	11:35:00	12:25:00	13:00:00	13:25:00	14:00:00	14:30:00	15:00:00
	00:45:00	01:25:00	02:00:00	02:30:00	03:20:00	03:55:00	04:20:00	04:55:00	05:25:00	05:55:00
tisB	12366.97	17582.31	17492.74	15950.72	14842.73	12852.93	12137.4	11225.69	10639.68	9799.39
istR	15798.03	19893.36	19149.91	17656.01	15601.91	13704.64	12107.66	11063.06	10456.61	10041.5
shoB	15936.25	19243.03	18653.28	16473.28	16406.46	13758.74	13436.77	15238.11	13586.79	14231.78
ohsC	15089.82	20094.46	20886.12	20502.99	17612.95	15679.84	14125.77	12817.39	11580.96	11054.1
ldrD	15779.24	19893.22	20426.9	19901.88	17111	15046.51	14094.37	12254.05	10994.81	10186.03
rdlD	16240.49	20072.38	18980.3	17918.69	15309.35	14382.11	11938.35	11251.6	10817.84	10169.53
MG1655	18340.94	24226.3	24627.08	23947.38	22591.81	21339.41	19673.2	17836.92	16790.54	13525.06

pH 8 FSC-A

	09:50:00	10:30:00	11:05:00	11:35:00	12:25:00	13:00:00	13:25:00	14:00:00	14:30:00	15:00:00
	00:45:00	01:25:00	02:00:00	02:30:00	03:20:00	03:55:00	04:20:00	04:55:00	05:25:00	05:55:00
tisB	20323.81	21073.93	19402.44	14862	10960.59	8068.13	7076.63	6734.01	6933.7	6273.77
istR	21661.03	26344.09	23693.27	20718.4	14812.56	11014.63	9004.35	7758.3	7520.51	6718.34
shoB	21026.41	25529.84	24903.02	21246.37	16522.37	16033.59	14971.76	13158.53	8738.34	14446.11

ohsC	22277.58	26842.12	26141.08	22369.88	16633.04	13436.26	11163.67	10081.21	8635.68	7553.43
ldrD	24491.79	27228.4	26236.55	25165.79	18196.62	13992.72	12272.7	9643.48	9292.23	7413.47
rdlD	21881.4	25632.29	24065.29	21502.76	15667.22	12366.26	10355.9	8533.3	7799.7	7109.4
MG1655	23866.28	29454.86	30013.74	20727.79	13772.75	11122.38	9387.75	8331.29	7481.21	7129.49

pH 5.5 OD600

	09:05:00	10:05:00	10:50:00	11:30:00	12:20:00	12:55:00	13:25:00	13:55:00	14:20:00	14:55:00
	00:00:00	01:00:00	01:45:00	02:25:00	03:15:00	03:50:00	04:20:00	04:50:00	05:15:00	05:50:00
tisB	0.05	0.06	0.02	0.05	0.24	0.44	0.58	0.8	1	1.37
istR	0.07	0.04	0.07	0.06	0.26	0.48	0.92	1.05	1.14	1.73
shoB	0.04	0.03	0.09	0.15	0.3	0.63	0.74	1.17	1.36	2.04
ohsC	0.02	0.05	0.05	0.01	0.26	0.29	0.41	0.51	0.77	0.88
ldrD	0.01	0.06	0.05	0.05	0.16	0.29	0.46	0.77	0.9	1.32
rdlD	0.02	0.03	0.05	0.12	0.87	0.48	0.7	0.96	1.29	1.85
MG1655	0.03	0.04	0.07	0.16	0.46	0.73	1.31	1.55	2.2	2.63

pH 8 OD600

	09:05:00	10:05:00	10:50:00	11:30:00	12:20:00	12:55:00	13:25:00	13:55:00	14:20:00	14:55:00
tisB	0.03	0.1	0.32	0.59	1.48	1.7	2.05	2.37	2.52	3
istR	0.02	0.02	0.16	0.32	0.72	1.36	1.72	1.87	2.17	2.59
shoB	0.05	0.04	0.1	0.2	0.5	1.0	1.52	1.72	1.92	2.29
ohsC	0.01	0.04	0.14	0.23	0.55	0.98	1.3	1.68	1.72	1.84
ldrD	0.01	0.02	0.07	0.1	0.42	1.35	1.22	1.68	1.71	2.09
rdlD	0.04	0.03	0.1	0.18	0.55	1.12	1.5	1.61	2.01	2.37
MG1655	0.01	0.04	0.2	0.34	0.9	1.44	1.89	2.0	2.39	2.83

The data below is from the second experiment.

pH 5.5
FSC-A

	11:20:00	12:05:00	12:30:00	13:00:00	13:30:00	14:00:00	14:35:00	15:05:00
tisB	17504.62	15783.94	14175.46	11506.76	12009.5	9995	10125.46	8850.41
istR	15394.08	15629.64	14421.3	12449.74	11426.53	10020.03	9199.68	9201.82
shoB	18248.59	16818.97	16345.31	13468.67	17700.82	17220.59	13539.95	14779.09
ohsC	20531.68	17009.47	16291.74	14413.89	12936.59	11694.29	10297.55	9542.05
ldrD	16633.29	14975.47	13040.43	10997.61	10523.77	9906.26	8635.32	7845.9
rdlD	17522.72	18293.81	14286.55	13343.07	11781.22	10213.82	9451.78	8819.88
MG1655	21504.19		18270.71	19240.29	16953.78	14052.18	11428.27	9689.15

pH 8
FSC-A

tisB	20954.67	17732.98	13363.23	12541.53	8625.81	7148.66	6613.66	6384.04
istR	20583.75	16649.43	12178.31	11376.08	10610.11	7025.98	6347.41	6450.14
shoB	21467.14	17000.33	13591.34	13332.52	13646.57	14784.48	13718.26	13834.84
ohsC	23546.89	22031.1	16198.44	13902.94	11612.7	8386.17	7741.51	6932.91
ldrD	20371.72	20144.33	12789.08	12733.81	10102.93	7541.5	7060.21	6503.75
rdlD	21151.53	18041.23	14063.22	11543.58	8821.67	7359.75	6628.03	6475.43
MG1655	28021.12	23075.28		13601.66	11115.8	8669.63	7158.5	6746.5

pH 5.5
OD600

	11:15:00	12:00:00	12:25:00	12:55:00	13:25:00	13:55:00	14:30:00	14:55:00
	01:15:00	02:00:00	02:25:00	02:55:00	03:25:00	03:55:00	04:30:00	04:55:00
tisB	0.03	0.18	0.22	0.33	0.49	0.68	1.23	1.97
istR	0.04	0.01	0.16	0.22	0.43	0.62	1.21	1.54
shoB	0.07	0.18	0.25	0.38	0.67	0.8	1.55	1.82
ohsC	0.05	0.10	0.15	0.32	0.55	0.82	0.95	1.5
ldrD	0.08	0.23	0.26	0.55	0.73	1.04	1.78	2.25
rdlD	0.06	0.12	0.17	0.32	0.52	0.76	1.37	1.71
MG1655	0.05	0.21	0.29	0.6	0.82	1.21	2.35	2.31

pH 8
OD600

	11:45:00	12:30:00	12:55:00	13:25:00	13:55:00	14:25:00	15:00:00	15:25:00
tisB	0.06	0.26	0.45	0.73	1.03	1.82	1.99	2.2
istR	0.05	0.26	0.43	0.85	1.28	1.84	1.97	2.34
shoB	0.11	0.31	0.45	0.9	1.51	1.73	2.07	2.49
ohsC	0.07	0.12	0.24	0.45	0.67	1	1.59	1.67
ldrD	0.05	0.12	0.24	0.42	1.14	1.36	1.69	2.08
rdlD	0.08	0.24	0.4	0.65	0.94	1.51	1.87	2.27
MG1655	0.05	0.19	0.31	0.52	0.87	1.21	1.72	2.13

The Data below is from the third experiment.

pH 5.5
FSC-A

	11:10:00	11:45:00	12:15:00	12:45:00	13:15:00	13:50:00	14:30:00	14:55:00	15:30:00	16:00:00
	01:10:00	01:45:00	02:15:00	02:45:00	03:15:00	03:50:00	04:30:00	04:55:00	05:30:00	
tisB	17,051.67	16,801.68	17,737.09	16,140.64	16,223.64	12,861.75	12,894.69	12,057.36	10,253.05	9,839.48
istR	16,867.54	17,633.12	16,540.10	15,451.67	13,132.44	11,428.56	10,617.61	9,444.59	8,690.20	7,679.83
shoB	18,622.24	18,957.88	16,768.37	15,664.73	13,843.95	13,348.41	15,920.72	15,414.70	15,787.83	13,750.79
ohsC	20,941.98	21,465.95	20,104.76	17,036.21	16,277.24	13,585.40	12,185.22	10,494.18	9,839.82	9,270.79
ldrD	17,037.23	18,232.03	16,855.14	15,108.55	13,235.06	11,818.93	10,369.36	9,541.71	8,827.21	7,895.00
rdlD	17,850.11	18,437.86	17,415.01	15,299.09	13,613.22	12,415.73	10,742.07	9,763.51	9,077.45	8,226.31
MG1655	22,447.15	23,439.84	22,094.15	21,611.59	19,461.98	18,092.52	15,455.89	12,359.47	9,881.46	9,115.96

pH 8
FSC-A

tisB	22,719.72	21,790.63	20,162.48		12,164.63	9,639.00	7,843.49	6,676.94	6,112.35	6,214.30
istR	22,596.67	21,245.36	18,460.30	14,035.47	11,919.76	8,427.04	7,336.87	6,759.64	6,337.77	6,342.96
shoB	22,719.77	21,254.69	16,126.20	14,259.02		9,780.82	10,590.72	14,516.96	14,882.00	17,346.98
ohsC	24,640.93	24,454.89	22,400.26	16,829.79	12,943.25	9,896.71	8,035.51	7,097.24	6,532.97	6,406.45
ldrD	21,736.81	21,076.04	15,592.79	12,469.05	9,678.41	7,665.23	6,670.40	6,595.24	6,392.66	6,307.02

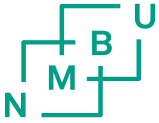
rdlD	22,373.77	21,248.80	17,323.21	12,563.34	9,241.13	8,024.95	6,652.50	6,516.95	6,288.51	6,425.64
MG1655	26,540.89	24,971.89	16,615.42	13,109.37	10,497.23	8,502.13	6,867.31	6,813.79	6,657.17	6,698.13

pH 5.5 OD600

	11:05:00	11:40:00	12:10:00	12:40:00	13:10:00	13:45:00	14:25:00	14:50:00	15:20:00	15:55:00
tisB	0.02	0.08	0.12	0.2	0.19	0.58	0.58	0.74	0.95	1.77
istR	0.03	0.15	0.19	0.25	0.42	0.77	1.37	1.95	2.12	3.04
shoB	0.07	0.11	0.2	0.25	0.47	0.78	1.25	1.66	2.28	2.9
ohsC	0.07	0.1	0.2	0.24	0.35	0.55	0.92	1.12	1.45	2.06
ldrD	0.11	0.15	0.17	0.27	0.43	0.67	1	1.92	2.09	2.86
rdlD	0.09	0.11	0.21	0.26	0.42	0.67	1.03	1.49	2.13	2.85
MG1655	0.1	0.19	0.26	0.42	0.74	0.93	1.92	2.29	2.99	4.22

pH 8 OD600

	11:05:00	11:40:00	12:10:00	12:40:00	13:10:00	13:45:00	14:25:00	14:50:00	15:20:00	15:55:00
tisB	0.07	0.13	0.27	0.52	0.8	1.41	1.88	2.03	2.48	3.06
istR	0.06	0.17	0.37	0.58	1.08	1.52	2.01	2.3	2.82	3.41
shoB	0.11	0.25	0.45	0.82	1.43	2.63	2.48	2.64	3.73	3.66
ohsC	0.12	0.16	0.24	0.42	0.81	1.42	1.52	2.64	3.73	3.68
ldrD	0.12	0.19	0.49	0.79	1.48	1.65	2.13	2.67	3.1	3.48
rdlD	0.18	0.22	0.4	0.73	1.36	1.72	2.2	2.61	3.02	3.49
MG1655	0.14	0.29	0.53	0.79	1.29	1.79	2.2	2.52	3.07	3.71



Norwegian University
of Life Sciences

Postboks 5003
NO-1432 Ås, Norway
+47 67 23 00 00
www.nmbu.no