Inactivation of *Escherichia coli* Using Electrochemical Disinfection with Potassium Periodate (KIO_4)

by

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Abstract

Electrochemical disinfection of water has drawn attention in recent years as an alternative for conventional chlorine-based water treatment, due to the generation of toxic disinfection by-products (DBP) during chlorination, and increased antibiotics resistant, chlorine resistant and virulent *E. coli* strains found in chlorine treated wastewater. This study aims to investigate the stress factors involved in electrochemical disinfection using KIO_4 as an experimental oxidizing reagent for bacteria inactivation. H_2O_2 and NaClO were selected as reference reagents. *E. coli* mutants that lost specific genetic functions against environmental stress, or those with the addition of locus of heat resistance (LHR) were selected as tools for evaluation of this treatment method. *E. coli* strains were treated with oxidative stress and pH stress, separately or combined, to observe their patterns of behaviour. The results were then compared to electrochemical treatment. Reactive oxygen species (ROS) generated in each experimental group were measured using corresponding fluorescent probes. It was found that levels of oxidative compounds generated by KIO_4 depended on the pH. In addition, a shift in pH enhanced the effect of KIO_4 when disinfecting *E. coli*, especially in alkaline pH. During electrochemical disinfection, electro-generated H_2O_2 by KIO_4 was higher on the cathode chamber compared to that of anode, which resulted in higher inactivation of *E. coli* on the cathode. Chromosomally integrated LHR-positive mutant was found to have a protective effect against alkaline stress. Current research provides supportive evidence that KIO_4 catalyzes the generation of reactive oxidative components such as •OH, $^1O_2$ and H_2O_2 that can inactivate *E. coli* and facilitate the process of electrochemical disinfection.

Preface

This thesis is an original work by TingTing Liu. No part of this thesis has been previously published.

Acknowledgement

I would like to thank my parents for helping me become who I am today. Before the age of 12, I was the most spoiled kid in the world. My dad would take my mom and me to a park or a road trip every single weekend. When I was a toddler, he would dance and flip in front of a dining table just to get me laugh so that my mom can feed me when I was reluctant to eat. He was the one taught me how to treat people respectfully, treat things objectively, but he was also the one told my mom that we were eating healthy while she was away from home, and took me for some KFC chicken. After he passed away, my mom became the strongest woman I have ever known. She endured my rebellious stage, supported me through all the lows in my life and encouraged me to get higher education when I have the chance. She is always there listening to my complaints and confusions in life and giving me all the support that I needed. Thank you, mom and dad, for teaching me what love is and how to love. I love you both forever.

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Table of contents

1 Introduction 1

1.1 Foodborne disease caused by *Escherichia coli* 1

1.2 Concerns of *E. coli* in water 2

1.3 Disinfection of *E. coli* in water treatment 3

1.4 Electrochemical disinfection compared to chlorination. 5

1.5 *E. coli* defense system against environmental stress 6

1.5.1 Oxidative stress response 7

1.5.2 Acid stress and alkaline stress 9

1.5.3 Osmotic stress response 10

1.5.4 Heat resistance 11

1.6 Purpose of the study, hypothesis, objectives 13

2 Materials and Methods 15

2.1 Bacterial strains and growth conditions. 15

2.1.1 Bacterial strains and plasmids. 15

2.1.2 Growth conditions, media and antibiotics. 16

2.2 Procedures for determining efficiency of applying oxidative stress alone. 17

2.3 Procedures for determining efficiency of applying acid/base stress alone. 18

2.4 Procedures for exposing strains to a combination of acid/alkaline and oxidative stress. 19

2.5 Procedures for determining efficiency of disinfecting strains using electrochemical approach. 20

2.6 Measurement of reactive oxygen species (ROS) using fluorescence probes. 22

2.6.1 Detecting hypochlorite ion (-OCl) using APF/HPF. 22

2.6.2 Detecting hydrogen peroxide (H_2O_2) using Amplex® Red and horseradish peroxidase. 22

2.6.3 Detecting singlet oxygen ($^1O_2$) using SOSG. 23

2.7 Statistical Analysis. 23

3 Results 25

3.1 Determining efficiency of applying acid or alkaline stress alone. 25
3.2 Determining efficiency of applying oxidative stress alone. 27
3.3 Determining the efficiency of combining acid stress and oxidative stress. 31
3.4 Determining the efficiency of combining alkaline stress and oxidative stress. 34
3.5 Determining the efficiency of disinfecting strains using electrochemical approach. ... 37
4 Discussion 40
References 52

List of Tables

Table 1 Bacterial strains used in this study. 16

Table 2 Antibiotics used in this study. 17

Table 3 Fluorescence assay of 490mM H_2O_2, 8mM NaClO and 9mM KIO_4 in neutral PBS. 29

Table 4 Fluorescence assays of 490mM H_2O_2 and 9mM KIO_4 in acidic PBS 32

Table 5 Fluorescence assays of 8mM NaClO and 9mM KIO_4 in alkaline PBS 35

Table 6 Fluorescence assay of electrolytes in both electrodes after 5min treatment 38

List of Figures

Figure 1 Activation of transcription regulators and SOS response in *E. coli* against environmental stress.....13

Figure 2 Electrolytic cell to disinfect *E. coli* MG1655 and its mutants.....21

Figure 3 Effect of acid/alkaline stress on *E. coli* MG1655 and its mutants.....26

Figure 4 Effect of oxidative stress (490mM H_2O_2, 8mM NaClO or 9mM KIO_4) on *E. coli* MG1655 and its mutants.....31

Figure 5 Effect of applying acid stress and oxidative stress (490mM H_2O_2 or 9mM KIO_4) combined on *E. coli* MG1655 and its mutants.....33

Figure 6 Effect of applying alkaline stress and oxidative stress (8mM NaClO or 9mM KIO_4) combined on *E. coli* MG1655 and its mutants.....36

Figure 7 Effect of electrochemical disinfection method on *E. coli* MG1655 and its mutants.....38

List of Abbreviations

ANOVA	Analysis of variance
APF	Aminophenyl fluorescein
CFA	Cyclopropane fatty acid
CFU	Colony forming unit
DBP	Disinfection by-product
DNA	Deoxyribonucleic acid
EHEC	Enterohaemorrhagic *E. coli*
HGT	Horizontal gene transfer
HPF	Hydroxyphenyl fluorescein
HUS	Hemolytic uremic syndrome
LB	Luria-Bertani
LHR	Locus of heat resistance
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
PBS	Phosphate buffer saline
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SOSG	Singlet oxygen sensor green
UPEC	Uropathogenic *E. coli*
UTI	Urinary tract infection

1 Introduction

1.1 Foodborne disease caused by *Escherichia coli*

Escherichia coli are facultative anaerobic, gram-negative, rod-shaped bacteria that belong to the family of *Enterobacteriaceae*. It is commonly found in gastrointestinal tract of humans and warm-blooded animals (George & Garrity, eds, 2005). Most *E. coli* strains are harmless commensal, some even have beneficial effects on host health, by producing vitamin K_2, stimulating immune response against *Salmonella* infection and preventing other pathogenic bacteria to colonize the host intestines (Bentley & Meganathan, 1982; Schieber et al, 2015; Reid, Howard & Gan, 2001). However, some strains are pathogenic and cause serious foodborne illnesses, such as vomiting, diarrhea, and more severely, hemolytic uremic syndrome (HUS) and urinary tract infections (UTI). Diarrhoeagenic *E. coli* are categorized into 8 pathotypes: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroaggregative hemorrhagic *E. coli* (EAHEC), enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli* (DAEC), and *Shigella* spp. (Kaper, Nataro, & Mobley, 2004; Jafari, Aslani & Bouzari, 2012). *E. coli* strains causing infections outside of the gastrointestinal tract are termed extraintestinal pathogenic *E. coli* (ExPEC), where uropathogenic *E. coli* (UPEC) is a prominent member that causes UTIs (Johnson & Russo, 2005; Zhang & Foxman, 2003). Due to the extremely low infectious dose of fewer than 100 cells, foodborne outbreaks associated with EHEC have been reported globally, for example, O157:H7 serotype in ground beef in North America. The most recent outbreaks of EHEC in the United States, are identified to be caused by contamination of *E. coli* O26 in flour, O103 in ground beef and O157 in romaine lettuce (CDC, 2019). These EHEC strains share a common virulence factor, Shiga toxin, which cleaves ribosomal RNA, inhibits protein synthesis and induces apoptosis in intestinal epithelial cells, and is

encoded on lambdoid prophages (Melton-Celsa, Darnell & O'Brien, 1996; Jones et al., 2000). Genomic islands, plasmids and bacteriophages such as lambdoid prophages are referred to the mobile genetic elements of *E. coli* (Asadulghani et al., 2009). Mobile genetic elements facilitate evolution of *E. coli* through horizontal gene transfer (HGT), resulting in acquisition of new accessory genes and virulence factors, thus allow bacteria to develop new traits for niche adaptation and pathogenicity (Croxen et al., 2013).

1.2 Concerns of *E. coli* in water

Transmission of EHEC strains is through fecal-oral route, where the pathogenic *E. coli* frequently found in animal guts can contaminate drinking water through feces. Exposure to contaminated water during vegetable growing and food processing poses an additional threat to public health. It was recently reported that 6 EHEC strains were isolated in two water sources in Switzerland, including O157, O26, O104 and O103 (Baumgartner, Niederhauser, Diston, & Moor, 2016). *E. coli* O157:H7 can survive in various water sources, such as rivers and lakes for over 2 months (Avery et al., 2008). EHEC was also detected frequently in wastewater effluents in Turkey, suggesting strains adaptation or resistance towards wastewater treatment process (Ayaz, Gencay & Erol, 2014). Whole genome comparison of *E. coli* strains isolated from multiple wastewater treatment plants in Switzerland and in North America revealed that the genomes of remotely related *E. coli* strains are highly similar, indicating the global distribution of these *E. coli* strains (Zhi et al. 2019). It is also evidenced that *E. coli* strains isolated from wastewater possess virulence genes found in both UPEC and EHEC which are not shared between the two pathotypes, suggesting horizontal gene transfer among strains (Zhi et al., 2019). *E. coli* has been found to be resistant to chlorine treatment through the formation of biofilms and increased in porin protein OmpC (Mah & O'Toole, 2001). Moreover, wastewater treated with chlorine dioxide leads to selection for antibiotic resistant genes in *E. coli* strains, demonstrating the ability of strains to selectively

survive in stressful conditions (Liu et al., 2018). Chlorine treatment commonly used in water disinfection has also been linked to increased tetracycline resistance (Huang et al., 2013). Hence, conventional chlorine-based treatments for *E. coli* inactivation in water may not be sufficient when strains have achieved resistance through mutation, adaptation to new genomic characteristics via HGT and biofilm formation (Chapman, 2003). Moreover, the increased existence of antibiotic- and chlorine-resistant, virulent strains of *E. coli* in drinking water and wastewater poses new health risks to human, animal and the environment.

1.3 Disinfection of *E. coli* in water treatment

Current mainstream water disinfection methods besides physical removal of inorganic compounds is using chlorine as a primary or secondary disinfectant for inactivation of microbials. Alternatives include chloramine, chlorine dioxide, ozone and UV radiation. Since the first proposal of using chlorinated solutions for washing hands in hospitals in 1847 by Ignaz Semmelweis, chlorination of water has been implemented predominantly in a large scale around the globe for over 150 years because of its low cost, ease of use and effectiveness (Wyklicky & Skopec, 1983). However, while effectively disinfecting water-borne pathogens, generation of carcinogenic disinfection by-products (DBPs) during the treatment process raised other health concerns, such as miscarriages, bladder cancer and respiratory diseases (Sedlak & Gunten, 2011). Chloroform was the first reported compound found in drinking water caused by chlorination in 1974 (Rook, 1974). As a substitution of chlorine, replacement with chloramine has been found to decrease the risk of bladder cancer (McGeehin et al., 1993). It is a less reactive chlorine derivative formed by adding excessive ammonia in the presence of free chlorine. However, by applying chloramines to treat water, it creates other unexpected DBPs, most importantly, *N*-nitrosodimethylamine (NDMA), which is a mutagenic compound that can be metabolized through human liver and triggers tumor growth (Sedlak & Mitch, 2002; Tricker & Preussmann, 1991). Other hazardous DBPs generated

during reactions between free chlorine or chloramine and organic compounds, such as halogenated chemicals: haloquinones, halo-cyclopentene, and halonitriles, can be transported through blood and cause damages to other tissues (Bull et al., 2011; Lin et al., 1999). In addition, the concentration of lead has been found at elevated levels when switching from free chlorine to chloramine, due to the corrosion of paint on plumbs (Edwards & Dudi, 2004). Halogenated by-products and other toxic compounds such as chlorite and chlorate, are also identified in the treatment of chlorine dioxide (Richardson, 1994). Due to the generation of numerous harmful by-products from chlorination, as well as increased stress resistant bacteria found in drinking water and wastewater effluents, some water treatment plants have opted for ozonation and UV radiation as alternative disinfection methods. However, bromate, which is formed as a major DBP during water treatment with ozone, has been listed as a potential carcinogen by the International Agency for Research on Cancer (WHO, 1990). Although ozonation is considered an effective practice that inactivates a broad spectrum of organisms, certain bacteria require higher doses of ozone for ideal inactivation, where the dose needed for inactivating *Clostridium perfringens* is over 40 mg/L, exceeding the economically reasonable level of 20mg/L (Gehr et al., 2003). Additionally, disadvantages of using ozone also include requirements on advanced technology, more complex equipment, and high operational costs which may limit many water treatment plants for choosing it. UV disinfection has been proven effective against a wide range of microbials and used extensively for pharmaceutical and medical purposes for many years. However, there are drawbacks when using UV radiation for decontaminating water. Based on the characteristic of UV method, no residuals would be found in water post treatment, unlike chlorine-based treatment which maintains its presence in water and thus has a long-term disinfecting effect on microorganisms. It was found that *E. coli* treated with UV successfully maintained their functions coded on plasmids, and revived during photoreactivation (Guo et al., 2012). Furthermore, the turbidity of water may interfere with UV penetration, where UV light gets

absorbed during purification process and results in ineffective bacteria inactivation (Liu & Zhang, 2006).

1.4 Electrochemical disinfection compared to chlorination.

Due to the rise of health concerns by chlorination, more facilities have been seeking alternatives. Several substituting methods have gained increased acceptance, such as ozonation and UV irradiation. However, some methods are not suitable for complete replacement of chlorination, for example, high intensity pulsed electric fields, titanium dioxide photocatalysis, and UV light, because of their lack of ability to generate residuals to continuously disinfect water after primary disinfection (Martínez-Huitle & Brillas, 2008). Residual disinfection plays an important role against bacterial regrowth after the initial disinfection that the remaining active chemicals in the water assure the water quality during transportation from the treatment facility to consumers. On the other hand, electrochemical disinfection as a recently emerging treatment method, has been developed aiming to replace chlorination in primary and residual water disinfection. The most common mechanism is through electro-generation of reactive oxygen species (ROS). During the treatment, movement of electrons between electrodes allows formation of oxidative compounds, where the amount and type of oxidants generated depend on the selection of electrode material, electrolytes and voltage applied. Saline water has been found to generate reactive chlorine compounds, such as Cl_2, HOCl, and OCl^- (Diao et al., 2003; Jeong, Kim, & Yoon, 2009). This method is known as electrochlorination, which solves the problems of transporting and storing chlorine gas, instead, it generates active chemicals on-site (Rajeshwar & Ibanez, 1997). Furthermore, electrochlorination has higher efficacy compared to free chlorine when inactivating *E. coli* and *Bacillus subtilis*, due to the formation of other ROSs such as •OH, H_2O_2 and ozone (Son et al., 2004). Despite its effectiveness, similar chlorinated by-products are generated during electrochlorination (Moreno-Andrésab &Peperzakb, 2019). Thus, several chlorine-free electrochemical systems were

proposed recently. Candidates including sulfate, phosphate, carbonate and acetic acids are used for the formation of oxidative radicals, with specific electrode materials (Sirés et al., 2014). Electro-generated potassium periodate (KIO_4) from iodate (IO_3^-) has also been found to effectively inactivate *E. coli* (Okochi et al., 2005). However, the mechanism of using this chemical for disinfection is unclear. Selection of electrode materials is also a contributing factor to generate reactive compounds, where the materials need to have an oxygen overpotential to prevent the consumption of electricity to produce oxygen as a side product (Jeong, Kim, & Yoon, 2009). Currently, most opted electrode materials include PbO_2, TiO_2, boron-doped diamond (BDD) and activated carbon fiber (Martínez-Huitle & Brillas, 2008).

1.5 *E. coli* defense system against environmental stress

In the natural environment, *E. coli* must maintain its cellular function to survive in stressful conditions. Environmental stresses may include limited nutrients, pH change, extreme temperature, osmotic stress, and oxidative stress. Through evolution, *E. coli* has developed a complex defensive system against these stressors by sensing external stimuli and regulating its metabolic functions via transcriptional factors (Janga et al., 2007; Gama-Castro et al., 2008). The stress responses can be achieved by induction of corresponding proteins initiated through global regulators such as sigma factors or other transcription factors (Timmermans & Melderen, 2010). Among all global regulators, sigma factors (σ) allow recruitment of RNA polymerase in the form of holoenzyme, at promotor regions of specific DNA binding sites, depending on types of stressors. To date, seven sigma factors have been identified in *E. coli*, being σ_A for house-keeping; σ_{FecI} for ion transportation; σ_E in response to extreme temperature; σ_F for flagellum biosynthesis; σ_H for heat shock response; σ_N for nitrogen regulation; σ_S for general stress response (Shimizu, 2014). Global regulators control gene expression at multiple levels. For example, sigma factors, H-NS (histone-like nucleotide structuring protein) and cAMP (cyclic-AMP) regulate at transcriptional level; CsrA (carbon storage regulator A) and small noncoding RNAs (sRNAs) regulate at

post-transcriptional level by acting on mRNAs during translation; proteases and chaperones regulate functional proteins at post-translational level (Timmermans & Melderen, 2010). Another type of transcription factor is the two-component signal transduction system, which consists of a transmembrane histidine protein kinase sensor responsible for sensing extracellular signals, and a target transcription factor response regulator that results in physiological and morphological changes (Seshasayee et al., 2006).

1.5.1 Oxidative stress response

Reactive oxygen species (ROS) such as superoxide radicals ($O_2^{\bullet-}$), hydroxyl radicals (•OH), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) are generated routinely through aerobic cell respiration. Excessive levels of these compounds could cause severe cell damage. For example, H_2O_2 binds with intracellular Fe^{2+} and generates •OH through Fenton reaction. •OH is one of the most reactive oxidizing reagents that can lead to destruction of proteins, cell membranes and DNA. However, low levels of these ROSs have been found to actively engaged in cell metabolism. Therefore, defense system against oxidative stress in *E. coli* is to maintain the ROSs at a harmless level (Shimizu, 2014; Flint, Tuminello & Emptage, 1993).

SoxR/S is a two-component regulatory system responsible for oxidative stress, where SoxR is a cytoplasmic sensor protein that has subunits containing [2Fe-2S] clusters. Under non-stress conditions, inactivated state of SoxR is maintained by reduced form of these clusters. However, upon oxidation of ROSs such as superoxide, nitride oxide and hydrogen peroxide, or redox-cycling agents, the clusters are oxidized, leading to conformational change in SoxR, which can in turn act as a transcription factor and enhance gene expression of *soxS* by up to 100-fold (Pomposiello, Bennik & Demple, 2001; Ding, Hidalgo & Demple, 1996). Thus, SoxS is able to regulate over 100 genes and regulons, including *sodA* (Manganese superoxide dismutase) (Fawcett & Wolf, 1995). SoxR is then reduced by NADPH-

dependent SoxR reductase and returns to its redox state, which represses *soxS* expression (Watanabe et al., 2008). SoxR may be activated not only by superoxide radicals, instead, its oxidation depends on multiple oxidizing factors (Chiang & Schellhorn, 2012). As a downstream gene of SoxR/S regulon, *sodA* codes for MnSOD, which is not synthesized under anaerobic conditions, but only induced upon exposure to oxygen and in turn catalyzes dismutation of superoxide radicals (Hassan & Fridovich, 1977; Carlioz & Touati, 1986).

OxyR is another global regulator that has protective effects against hydrogen peroxide toxicity, heat stress, near-UV, singlet oxygen lipid peroxidation-mediated cell damage, and neutrophil-mediated killing (Chiang & Schellhorn, 2012). OxyR regulates approximately 40 genes and negatively regulates the expression of its encoding gene, *oxyR* (Schell, 1993). In the presence of H_2O_2, OxyR is transformed into oxidized state by forming reversible disulfide bond between the two cysteines on each of the four subunits (Zheng, Aslund & Storz, 1998). Transcriptional induction of OxyR-dependent promotors is achieved by positive or negative regulation through the oxidized and reduced forms of OxyR (Tao, Fujita & Ishihama, 1993). Promotor region of the gene encoding DNA binding protein (*dps*) is also activated by OxyR during exponential growth phase (Altuvia et al., 1994). OxyR plays an important role in the glutaredoxin and thioredoxin pathways, through up-regulation of *gorA* (glutathione reductase), *grxA* (glutaredoxin 1), *trxB* (thioredoxin reductase) and *trxC* (thioredoxin 2), and thus facilitates the reduction of oxidized proteins (Chiang & Schellhorn, 2012; Zheng, Aslund & Storz, 1998; Prieto-Álamo et al., 2000). While the oxidative stress is absent, OxyR-dependent genes *gorA* and *grxA* are up-regulated and in turn transform OxyR back to its reduced state through glutaredoxin pathway (Zheng, Aslund & Storz, 1998).

SOS response is a post-stress DNA repair system of *E. coli* (Radman, 1975). During non-stress conditions, SOS genes are repressed by a repressor protein, LexA. Upon stress, accumulation of single-

stranded DNA due to DNA damage activates the DNA recombination/repair protein RecA. Induction of RecA in turn negatively regulates the SOS response repressor, LexA and promotes the DNA repair process (Nelson & Cox, 2005). Polyphosphate (polyP) is an essential inorganic compound involved in regulating expression of genes coding DNA repair enzymes, such as *recA*. Synthesis of polyP is converted from ATP by polyP kinase, PPK (Walker, 1984). In a *ppk* null mutant, when PPK is insufficient, the cell shows increased sensitivity to H_2O_2, heat shock and osmotic stress (Crooke et al., 1994).

1.5.2 Acid stress and alkaline stress

RpoS, RNA polymerase for stationary phase, is a general stress regulator, which belongs to the sigma factor family as σ_S/σ_{38} (Lange & Hengge-Aronis, 1991). It is a global regulator activated not only during oxidative stress, but also under pH challenge. About 200 genes are regulated by RpoS when the cell is under oxidative stress, including genes encoding for hydrogenperoxidase II (*katE*), DNA-binding protein (*dps*), and superoxide dismutase (*sodC*). (Chiang & Schellhorn, 2012; Gort, Ferber & Imlay, 1999). When the cell is undergoing acid attack, a different set of RpoS-dependent genes are positively regulated. These genes include: *gadC*, being part of the glutamate-dependent low pH resistance systems; *hdeAB*, encoding pH-regulated periplasmic chaperones that are responsible for suppressing aggregated proteins under acid stress; and *cfa*, coding for cyclopropane fatty acid synthesis. High CFA content in membrane lipids stabilizes membrane structure through conversion of unsaturated fatty acids by CFA synthase (Yoshida, Ueguchi & Mizuno, 1993; Battesti, Majdalani & Gottesman, 2011; Chen & Gänzle, 2016). Glutamate-dependent response system is another pathway of *E. coli* against acid stress. In acidic environment, *gadA* and *gadB* encoding for glutamate decarboxylase isozymes and *gadC* encoding for glutamate/ γ-amino butyric acid (GABA) are upregulated. Glutamate is then reduced by glutamate decarboxylase with consumption of excessive intracellular proton to decrease the internal pH and

maintain pH homeostasis (Shimizu, 2014; Foster, 2004). Urease activity has been found at elevated levels in acid-tolerant organisms, such as *Helicobacter pylori*, *Streptococcus salivarius*, and *Yersinia enterocolitica* against acidic pH (Chen et al., 1998; De Koning-Ward & Robins-Browne, 1995; Mobley, Island & Hausinger, 1995). Upon acid attack, urea is hydrolyzed by urease to carbonic acid and ammonia, which then spontaneously binds with intracellular H^+ and becomes ammonium to stabilize the internal pH (Smith, 2003). Genes encoding for urease has also been found in *E. coli* O157: H7, but not in non-pathogenic *E. coli* K-12 strains (Heimer, et al., 2001). On the other hand, *E. coli* have also developed strategies against alkaline stress. A variety of proteins and enzymes is increased at alkaline pH. For example, MalE encoding for maltose/maltodextrin-periplasmic protein regulates an acid-generating carbohydrate. This mechanism is similar to the regulation of glutamate decarboxylase which alkalinizes the internal pH under acidic conditions (Stancik, et al., 2002; Richard & Foster, 2004). At genetic level, *nhaA* encoding Na^+/H^+ antiporter, has been found to contribute to survival of *E. coli* in alkaline conditions, and is regulated by RpoS in stationary phase (West & Mitchell, 1974; Dover & Padan, 2001). Upon alkaline challenge, it is crucial that Na^+/H^+ antiporter is electrogenic, instead of electroneutral, in which exchange of cytoplasmic Na^+ and extracellular H^+ is 1:1. Electrogenicity of the NhaA antiporter helps the cell transport H^+ inward that is two times higher compared to the level of Na^+ pumped outward, to neutralize internal alkaline pH (Padan, et al., 2005).

1.5.3 Osmotic stress response

One of the major porin gene regulators is EnvZ/OmpR two-component system, where EnvZ is a membrane sensor kinase, OmpR is the corresponding cytoplasmic regulator (Shimizu, 2014). Upon environmental signals, OmpR is phosphorylated by EnvZ to form OmpR-P, resulting in increased binding affinity towards the promotor regions of porin genes *ompF* and *ompC*. OmpF and OmpC are the most abundant porin proteins responsible for glucose uptake. At low osmolarity, kinase activity of

EnvZ is decreased and in turn decreases the amount of OmpR-P. OmpR-P then binds to specific sites of OmpF and induces latter to higher level compared to OmpC, which is due to the higher binding affinity towards OmpF. Because of the porin size of OmpF being slightly larger than OmpC, enhanced OmpF induction accelerates the nutrient intake. In contrast, at high osmolarity, abundant OmpR-P binds to both porin genes, where the binding of itself to low affinity *ompC* negatively regulates the gene expression of *ompF*. Therefore, the small porin protein OmpC becomes the predominant outer membrane porin to limit ion exchange (Feng et al., 2003; Egger, Park & Inouye, 1997). It was also reported that deletion in *ompR* leads to higher sensitivity to acid exposure (Stincone et al., 2011).

1.5.4 Heat resistance

The heat shock response in *E. coli* has been found mediated by σ_H/σ_{32} transcription factor encoded by *rpoH* gene (Gross et al., 1990). It was found that induction of heat shock proteins reaches its peak level at 5-10min after heat treatment, where the RNA polymerase core enzyme binds to σ_H, and in turn transcribes heat shock genes (Tilly et al., 1986; Skelly et al., 1987; Grossman, Erickson & Gross, 1984). Three major chaperones Hsp60, Hsp70 and Hsp 90 coded by *groEL*, *dnaK*, *htpG* are under regulation of σ_H, to prevent protein aggregation and facilitate protein refolding (Shimizu, 2014). In the DnaK chaperone system, conformation of DnaK is determined by its binding with ATP and ADP (Mayer et al., 2000). When heat stress is introduced to the cell, DnaJ detects hydrophobic segments of a protein, and promotes ATP hydrolysis to ADP, which binds to DnaK to form a closed site for trapping and unfolding polypeptide substrate. GrpE then releases ADP from the DnaK binding site, which allows ATP to bind with DnaK to form an open site to release the unfolded substrate for spontaneous refolding (Peng et al., 2011). Another “damage control” mechanism involves proteases, such as Clp, FtsH and Lon. These proteases are ATP-fueled and contribute to translocation and degradation of misfolded proteins caused by heat shock (Yura, Nagai & Mori, 1993). Small heat shock proteins such as IbpA and IbpB also

protect denatured proteins from irreversible aggregation (Kitagawa, 2002). Recently, a genomic island with 16 open reading frames encoding heat shock proteins and proteases, was discovered in highly heat resistant beef isolate, *E. coli* AW1.7 (Mercer et al., 2015). This genomic island is named locus of heat resistance (LHR). *E. coli* strains carrying this sequence confer high heat tolerance and have D_{60} value of more than 6min. The frequency of LHR in published genomes of *E. coli* is about 2%, indicating the ecological trade-off for having LHR, might result in the decrease of overall cell fitness (Mercer et al., 2015). However, 59% of *E. coli* samples collected in wastewater treated by chlorination were detected to be LHR positive strains. In addition, 36% of *E. coli* isolates from raw milk cheese were also found to be LHR positive which could be due to the thermization process of heating at 60 ºC for 30min (Zhi et al., 2016). These observations support the explanation that selection for LHR strains is not limited to heat treatment, but also other stressor, such as chlorine or oxidative stress. In such environmental conditions where LHR strains are selected, it provides the opportunity of acquisition of LHR sequence by pathogenic or antibiotic resistant strains through horizontal gene transfer (Mercer et al., 2017).

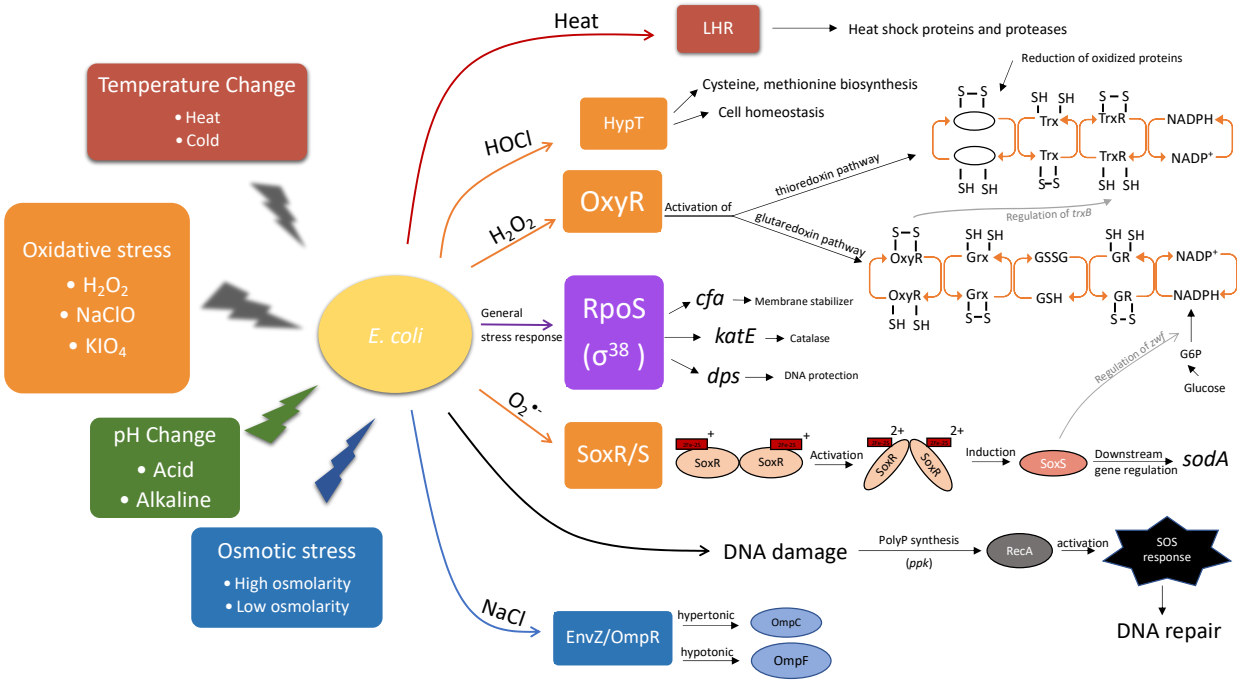


Figure 1 Activation of transcription regulators and SOS response in *E. coli* against environmental stress. Trx: thioredoxin, encoded by *trxA* and *trxC*; TrxR: thioredoxin reductase, encoded by *trxB*. Grx: glutaredoxin, encoded by *grxA*; GR: glutathione reductase, encoded by *gorA*. All five genes involved in thioredoxin pathway and glutaredoxin pathway are upregulated upon oxidation of OxyR. Under oxidative stress, SoxR/S facilitates formation of NADPH through induction of *zwf*.

1.6 Purpose of the study, hypothesis, objectives

In summary, due to the selection of stress resistant strains by chlorination of water, as well as the generation of harmful disinfection by-products which raises health concerns, it is necessary to study further on the mechanism of electrochemical disinfection. Inactivation of bacteria using electrochemical approach could be a potential substitution for conventional water treatments, serving as primary and residual disinfection method. Electrochemical disinfection using iodine-based chemicals was poorly studied. Therefore, this research aimed to investigate the efficacy of electrochemical disinfection using KIO_4, and to discover the potential stress factors involved in this mechanism. As a candidate for water treatment, KIO_4 is able to remain in the water to generate ROSs continuously, before it decomposes to

KIO_3. H_2O_2 and NaClO were used as reference treatment. *E. coli* MG1655, which was selected as a control strain for this study, is a non-pathogenic laboratory strain derived from *E. coli* strain K-12. Baba et al. (2006) constructed the Keio Collection mutants from *E. coli* MG1655, where each mutant has a single-gene deletion of non-essential genes. The complete loss of specific gene functions in Keio Collection mutants allows for using these mutants as a tool for analysis of bactericidal mechanism. To understand whether the electrochemical disinfection involves oxidative stress or pH stress, or both, mutants having defects in the stress defensive system were chosen to observe specific stress response towards each stressor. *E. coli* MG1655 mutants used in this study were strains with single-gene deletions in global regulators, *rpoS*, *oxyR*, *soxR*; downstream genes regulated by the global regulators, *sodA*, *katE*, *cfa*, *dps*, *trxB*; porin regulator, *ompR*; HOCl-specific transcription factor, *hypT*, which potentially links to chlorine resistance; gene codes for polyphosphate kinase contributing to SOS reponse, *ppk*; plasmid-coded LHR-positive strain, pLHR and its empty plasmid control, pRK767; chromosomally integrated LHR-positive strain, *lacZ:LHR*.

Therefore, it is hypothesized that the electrochemical disinfection with KIO_4 involves generation of reactive oxygen species and pH shifts which can cause bactericidal effects on *E. coli*.

The objectives of this study were to:

1) Determine the effect of acid/alkaline treatment.
2) Determine the effect of reagent KIO_4 using H_2O_2 and NaClO as reference reagents.
3) Determine the combine effect of extreme pH and oxidative stress.
4) Investigate the efficacy of electrochemical disinfection using KIO_4.

2 Materials and Methods

2.1 Bacterial strains and growth conditions.

2.1.1 Bacterial strains and plasmids.

Strains and plasmids used in this study are listed in Table 1.

E. coli MG1655 is a non-pathogenic, lab generated derivative of *E. coli* K-12 strain (Blattner et al, 1997). Keio Collection consists of 3985 mutants with single-gene knockout of nonessential genes in *E. coli* K-12. Mutants were created by replacing open-reading frame regions with a kanamycin resistant cassette flanked by FLP recognition target sites (Baba et al., 2006). *E. coli Δcfa* was constructed by in-frame deletion of gene *cfa* without antibiotics resistance (Chen & Gänzle, 2016). *E. coli lacZ:LHR* was constructed using no-SCAR (Scarless Cas9 Assisted Recombineering) method to select for short insertion in one-step (Wang, Simpson & Gänzle, unpublished; Reisch & Prather, 2015). Mutants chosen for this study had single deletions in genes that code for proteins that are known to contribute to resistance against environmental stress including osmotic stress, heat stress, oxidative stress and pH change.

Table 1 Bacterial strains used in this study.

Strains	Description	Antibiotics resistance	Reference
MG1655	K-12 lab strain	-	Guyer et al., 1981
ΔrpoS	RpoS deficient derivative of MG1655 (Keio collection)	Kanamycin	Baba et al., 2006
ΔsodA	SodA deficient derivative of MG1655 (Keio collection)	Kanamycin	Baba et al., 2006
ΔkatE	KatE deficient derivative of MG1655 (Keio collection)	Kanamycin	Baba et al., 2006
Δcfa	CFA deficient derivative of MG1655	-	Chen & Gänzle, 2016
ΔoxyR	OxyR deficient derivative of MG1655 (Keio collection)	Kanamycin	Baba et al., 2006
ΔompR	OmpR deficient derivative of MG1655 (Keio collection)	Kanamycin	Baba et al., 2006
Δdps	Dps deficient derivative of MG1655 (Keio collection)	Kanamycin	Baba et al., 2006
ΔtrxB	TrxB deficient derivative of MG1655 (Keio collection)	Kanamycin	Baba et al., 2006
ΔhypT	HypT deficient derivative of MG1655 (Keio collection)	Kanamycin	Baba et al., 2006
Δppk	Ppk deficient derivative of MG1655 (Keio collection)	Kanamycin	Baba et al., 2006
ΔsoxR	SoxR deficient derivative of MG1655 (Keio collection)	Kanamycin	Baba et al., 2006
pRK767	Low copy number plasmid (empty)	Tetracycline	Gill & Warren, 1988
pLHR	Full length LHR in pRK767	Tetracycline	Mercer et al., 2015
lacZ:LHR	Insertion of full length LHR on MG1655 chromosome	-	Wang, Simpson & Gänzle, unpublished

2.1.2 Growth conditions, media and antibiotics.

Strains were incubated in Luria-Bertani (LB) medium (BD, Fisher Scientific, Ottawa, CA) at 37°C for 16h with agitation at 200 rpm when in liquid medium. LB agar plates were prepared with LB broth and 1.5% granulated agar (BD Difco, Fisher Scientific, Ottawa, CA). LB broth was prepared with sterile tap water. Antibiotics were added to plates to select for antibiotics resistant strains. Keio collection strains

were grown in LB agar plates with kanamycin-sulfate, to prevent contamination. Strains with cloned plasmids were selected by adding tetracycline-HCl to maintain the plasmids. Antibiotics and concentrations are listed in Table 2.

Stock cultures were stored at -80 °C in LB medium with 20% glycerol (*v/v*). Inoculation was performed by aseptic streaking from stock onto LB agar plates supplemented with antibiotics accordingly, for overnight incubation, followed by sub-culturing of single colonies into 10mL LB broth. During the experiment, incubation was extended to 48h when no growth was observed on plates after the first 24h, to confirm the complete elimination of the strains by observing no further growth.

Table 2 Antibiotics used in this study.

Antibiotic	Solvent	Stock concentration (g/L)	Working concentration (mg/L)
Kanamycin-sulfate	water	25	25
Tetracycline-HCl	ethanol (70%)	10	10

2.2 Procedures for determining efficiency of applying oxidative stress alone.

Each strain was exposed to three oxidizing reagents: sodium hypochlorite (NaClO), hydrogen peroxide (H_2O_2) and potassium periodate (KIO_4) individually. Cells were harvested after overnight incubation at their stationary phase by centrifugation, followed by cell wash three times with 10mM potassium phosphate buffer saline (PBS) (pH = 7.4). Cell suspensions were remained at room temperature before treatment.

To prepare potassium periodate in liquid form for further treatment, 0.042 g of KIO_4 (99.8%, Sigma-Aldrich, Germany) was dissolved in 10mL 10mM PBS which was prepared with sterile tap water. The amount of KIO_4 added was limited to its solubility at 20°C, 0.42g/100mL. Solution was prepared within half an hour each time prior to the treatment.

During the treatment, 100μL of KIO_4 solution was pipetted and mixed with 100μL of washed cell culture, with a final reactive concentration 9mM. Exposure time was controlled at 5 min by adding 0.2% sodium thiosulfate (*w/v*) (Sigma-Aldrich, Germany) at the end of the reaction. Plating was done after serial dilution of the mixture. Plates were then placed in a 37°C incubator overnight and cell counts were collected the next day.

To treat the strains with sodium hypochlorite, 1.5μL of 4% NaClO (Sigma-Aldrich, Germany) was pipetted and mixed with 100μL of washed cell culture at a final concentration of 8mM. After 5 min reaction, 0.2% $Na_2S_2O_3$ was added before plating.

With H_2O_2, 5μL of 30% H_2O_2 (Sigma-Aldrich, Germany) was added to 100μL of washed cell culture with final concentration of 490mM, allowed for 5 min of reaction before plating.

Concentration of each reagent used above was determined by preliminary survival test based on the cell count of control group (*E. coli* MG1655 wild type). Each set of experiment was done with three biological replicates.

2.3 Procedures for determining efficiency of applying acid/base stress alone.

In this process, stationary phase cell cultures were harvested by centrifugation, and washed three times with 10mM neutral PBS (pH = 7.4). After washing, cell pellets were kept at room temperature before acid/alkaline treatment.

To prepare the acidic solution, a 10mM acidic PBS was made by adding 4g NaCl, 0.1g KCl, 0.64g KH_2PO_4 and 340μL of 85% H_3PO_4 (Sigma-Aldrich, Germany) to 500mL distilled water with a final pH of 2.1. To prepare the alkaline solution, the 10mM alkaline PBS was prepared by adding 4g NaCl, 0.1g KCl, 0.79g K_2HPO_4 and 0.98g K_3PO_4 (Sigma-Aldrich, Germany) per 500mL distilled water with a final pH of 12.7. During treatment, cell pellets were resuspended in acidic/alkaline PBS, respectively, where

reaction was allowed for the next 5min before serial dilution. Plating was done immediately after the treatments and cell counts were collected the next day after overnight incubation.

2.4 Procedures for exposing strains to a combination of acid/alkaline and oxidative stress.

In this experiment, strains were treated with the following oxidative compounds in acid or alkaline pH environments, respectively:

1. 8mM NaClO in alkaline PBS for 1min;
2. 490mM H_2O_2 in acid PBS for 5min;
3. 9mM KIO_4 in acidic PBS for 5min;
4. 9mM KIO_4 in alkaline PBS for 1min.

NaClO with alkaline. During this step, cell cultures were harvested and washed three times using 10mM neutral PBS. Cell pellets were remained at room temperature before treatment. Due to the hypersensitivity of *E. coli* cells towards alkaline stress, reaction time was set to 1min for better observation of countable survival rate. Cells were resuspended in alkaline PBS, followed by adding 1.5μL 4% NaClO to 100μL cell cultures. When the time reached 1min mark, serial dilution and plating were done instantly to prevent from further reaction.

H_2O_2 in acid. During this process, cell cultures were harvested by centrifugation and washed three times using 10mM neutral PBS, followed by resuspension in acidic PBS. 5μL of 30% H_2O_2 were added to 100μL cell suspensions and reacted for 5 min. Serial dilution and plating were done after 5min mark was reached.

KIO_4 with acid. During this step, 0.042g KIO_4 was weighted and dissolved in 10mL acidic PBS. Cells were collected by centrifugation and washed in 10mM neutral PBS buffer three times. Pellets were resuspended in acidic PBS before treatment. During the reaction, 100μL of KIO_4 acidic solution was

added to 100μL cell cultures to allow them for contact for 5 min. This step was followed by serial dilution and plating.

KIO_4 with alkaline. During this process, the procedure was consistent to the treatment of KIO_4 with acid, except acidic PBS was replaced with alkaline PBS when dissolving KIO_4 and resuspending cell pellets. And reaction time was set to 1min which was consistent to treatment of NaClO in alkaline solution.

2.5 Procedures for determining efficiency of disinfecting strains using electrochemical approach.

In this process, an electric field was generated using a power supply which gave the electric circuit 12V of voltage. Within the circuit, anode and cathode were separated by linking the electrodes from the power supply to two Corning™ Falcon 50mL Conical Centrifuge Tubes (Fisher Scientific, Ottawa, CA) using copper wires and expanded graphite. Cell cultures of a same strain sub-cultured from a common single colony were contained in the tubes of both electrodes, to observe differences of pH change, level of generation of ROS, and cells survival. To close the circuit, electrodes were connected by a multimeter that was set to amperage measurement. Due to the lack of suitable materials, the multimeter was used for electric readings as well as a salt bridge between two electrodes. Because of the resistance generated in voltmeter was large enough to block the electricity from going through the circuit, multimeter was initially set to voltage reading, once the reading confirmed the voltage was set to 12V, it was turned to amperage reading to work as a salt bridge (Fig. 1).

Cell cultures were incubated in 15mL LB broth overnight, then harvested by centrifugation with three times cell wash using same amount of 10mM neutral PBS (pH = 7.4). To minimize the variable factors, the ratio between solutions of cell cultures and KIO_4 was maintained at 1:1, which was consistent with the condition in treating cells with KIO_4 solely. After washing, cell pellets were resuspended in 15mL neutral 10mM PBS. 15mL of KIO_4 solution were added to both tubes instantly prior to the

electrochemical treatment, to avoid the killing effect caused by KIO_4 itself. Original cell counts were collected before the treatment. During the treatment, cells were treated at 12V for 5 min. Serial dilution and plating were done when the treatment reached 5 min mark. pH values of solutions on both electrodes were measured after the treatment. Preliminary experiments using KI and KIO_3 as electrolytes were conducted, no reduction of cell counts was observed.

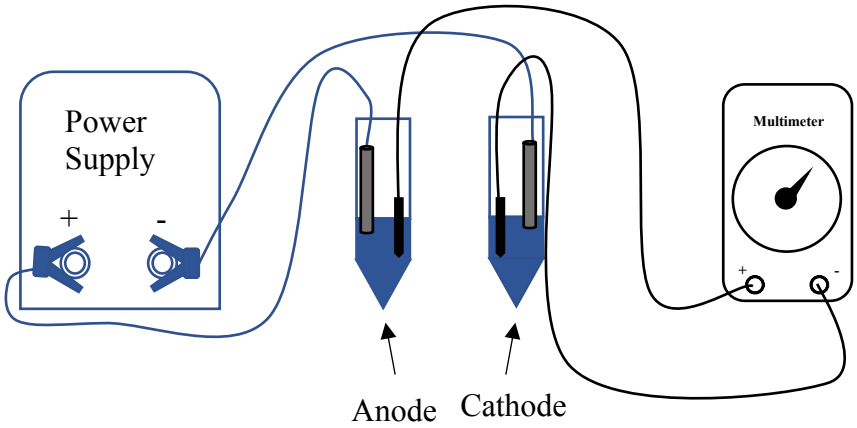


Figure 2 Electrolytic cell to disinfect *E. coli* MG1655 and its mutants.

2.6 Measurement of reactive oxygen species (ROS) using fluorescence probes.

To validate if ROS was generated by different oxidizing reagents during the treatment of cells, fluorescence intensities of chemical solutions used in the experiments were measured using four specific probes through a fluorescence spectrophotometer (Varioskan Flash, Thermo Scientific). Each reagent (H_2O_2, NaClO and KIO_4) was tested by the four probes respectively, to analyze the cause of oxidative stress to the strains.

2.6.1 Detecting hypochlorite ion (-OCl) using APF/HPF.

Two probes used in this assay, Aminophenyl fluorescein (APF) and hydroxyphenyl fluorescein (HPF) (Molecular Probes Inc., USA), emit green fluorescence at 515nm, with excitation at 490nm. HPF and APF are initially nonfluorescent unless react with specific ROS including hydroxyl radical (•OH) or peroxynitrite anion (ONOO-) to become fluorescent. In addition, APF is also able to react with -OCl, which indicates that combining these two probes can selectively identify -OCl (Setsukinai et al., 2003). Original stock concentration for both dyes was 5mM in dimethylformamide (DMF). Working concentration of 10μM APF and HPF was made with 0.1M PBS (pH = 7.4), respectively. According to the product manual given by Molecular Probes™, recommended final concentration of 3μM of -OCl was added to the working solution which served as positive control in a 96-well microplate (Fisher Scientific, Ottawa, CA) during the assay. Plus, 0.1M PBS solution with no added dye or oxidizing reagent (NaClO) served as blank, where working solution (contained APF or HPF in 0.1M PBS) alone served as negative control.

2.6.2 Detecting hydrogen peroxide (H_2O_2) using Amplex® Red and horseradish peroxidase.

H_2O_2 was measured using Amplex® Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) and horseradish peroxidase (HRP) (Molecular Probes Inc., USA). With HRP, Amplex® Red reagent is oxidized by H_2O_2 and emits red fluorescence at 590 ± 17.5 nm with excitation wavelength of 530 ± 12.5 nm.

A final concentration of 50μM of Amplex® Red reagent and 0.1U/mL HRP were added to the reaction plate with H_2O_2 at 0.49M (same concentration as previous treatment to the strains) in 10mM PBS as sample. Same concentration of Amplex® Red reagent and HRP were added in 10mM PBS without H_2O_2 as negative control and 10mM PBS alone being blank.

2.6.3 Detecting singlet oxygen ($^1O_2$) using SOSG.

Singlet Oxygen Sensor Green reagent (SOSG) (Molecular Probes Inc., USA) has much higher response to $^1O_2$ compared to other ROSs (hydroxyl radicals or superoxide) (Rota, Chignell & Mason, 1999). With the presence of $^1O_2$, SOSG is transformed to SOSG endoperoxide (SOSG-EP), exhibiting bright green fluorescence at 525nm (excitation at 504nm) compared to its original weak blue fluorescence at 395 or 416nm.

According to the reaction of various concentration of SOSG with $^1O_2$ carried out by Lin et al. (2013), reaction rate reached plateau when concentration of SOSG was at 6μM. SOSG in a 100μg vial was dissolved in 330μL methanol to make a stock solution of 500μM. During the assay, 1.2μL of the stock solution was pipetted into 100μL PBS to be diluted to a working concentration of 6μM. With reagents added to the mixture, amount of $^1O_2$ generated was measured, where the mixture alone served as a negative control, and PBS alone as blank.

2.7 Statistical Analysis.

Strains carrying plasmids have a slower growth rate and are less competitive to strains without plasmids (Anderson, 1974; Melling, Ellwood, & Robinson, 1977). In addition, the DNA sequence responsible for tetracycline resistance is easier to lose during growth compared to other antibiotics resistance genes (Godwin & Slater, 1979). Therefore, to rule out the impact of having plasmids or carrying different types of drug resistance when observing the survival of cells, in this study, strains were divided into three groups for statistical analysis based on their properties of antibiotic resistance. *E. coli* MG1655,

Δcfa and *lacZ:LHR* were incubated on LB plates without antibiotics, hence one-way ANOVA was used for their comparisons. *E. coli* pRK767 and pLHR were plated on tetracycline-LB plates, therefore analyzed by independent t-test. The rest of the mutants were from Keio Collection which had kanamycin resistance and were grouped for another one-way ANOVA analysis. Experiments were repeated at least three times with independent replicates. Cell counts data were converted to $\log_{10}$ and analyzed using one-way analysis of variance (ANOVA) or independent t-test for dual-strain comparison through SPSS® Statistics. Significant differences among strains were calculated using Tukey's HSD (honestly significant difference) test with $P\ value < 0.05$.

3 Results

To investigate the potential stress factors involved in an electrochemical disinfection system, the bactericidal effect of acidity, alkalinity, oxidation (H_2O_2, NaClO, KIO_4) was evaluated using *E. coli* MG1655 and its mutants. The experiments were divided into 5 groups to perform a break-down measurement of each stressor: 1) Treating the cells with acid or alkaline stress alone; 2) treating the cells with oxidative stress alone, using KIO_4, and H_2O_2, NaClO as comparison ; 3) Treating the cells with a combination of acid and oxidative stress ; 4) Treating the cells with a combination of alkaline and oxidative stress; 5) Treating the cells with the electrochemical disinfection system.

Reduction of cell counts were measured after 1min of applying alkaline and oxidative stress combined, or 5min for other groups based on the stress factors that were chosen for each experimental group. Experimental conditions were determined by preliminary tests to reduce the cell counts of the wildtype, *E. coli* MG1655 to have 1-6 $\log_{10}$ (CFU/mL) reduction.

In the following graphs, dotted lines were added accordingly at reduction of 7 $\log_{10}$(CFU/mL) benchmark, indicating the detection limit. Bars sitting below that line with error bars touching the dotted line, indicating the cell counts were below detection limit on one or two replicates.

3.1 Determining efficiency of applying acid or alkaline stress alone.

E. coli can survive at extremely low pH (= 2.5) for at least 2h (Gorden & Small, 1993), and up to 4h at pH=9.8, but the survival decreased dramatically when pH>10.2 (Small, Blankenhorn, Welty, Zinser, & Slonczewski, 1994). To setup a baseline for better comparison towards the groups of combining acid/alkaline stress and oxidizing reagents, the strains were treated solely with acidic or alkaline PBS, at pH = 2 and 12, respectively, for 5min. As shown in Fig. 3, all strains showed acid resistance in 5min treatment and their cell counts were reduced by less than 2 $\log_{10}$. However, *E. coli* MG1655 pLHR showed increased resistance when compared to its empty plasmid control strain, *E. coli* MG1655

pRK767. In contrast of low pH treatment groups, cell counts for most of the mutants were reduced to below detection limit at high pH except for the wildtype *E. coli* MG1655 and mutants *E. coli ΔtrxB*, *E. coli lacZ:LHR*. Cell counts on *E. coli lacZ:LHR* was lower than that of the wildtype by 3 $\log_{10}$. Data indicates that addition of LHR not only protects the cell from heat stress, but also provides resistance in alkaline environment.

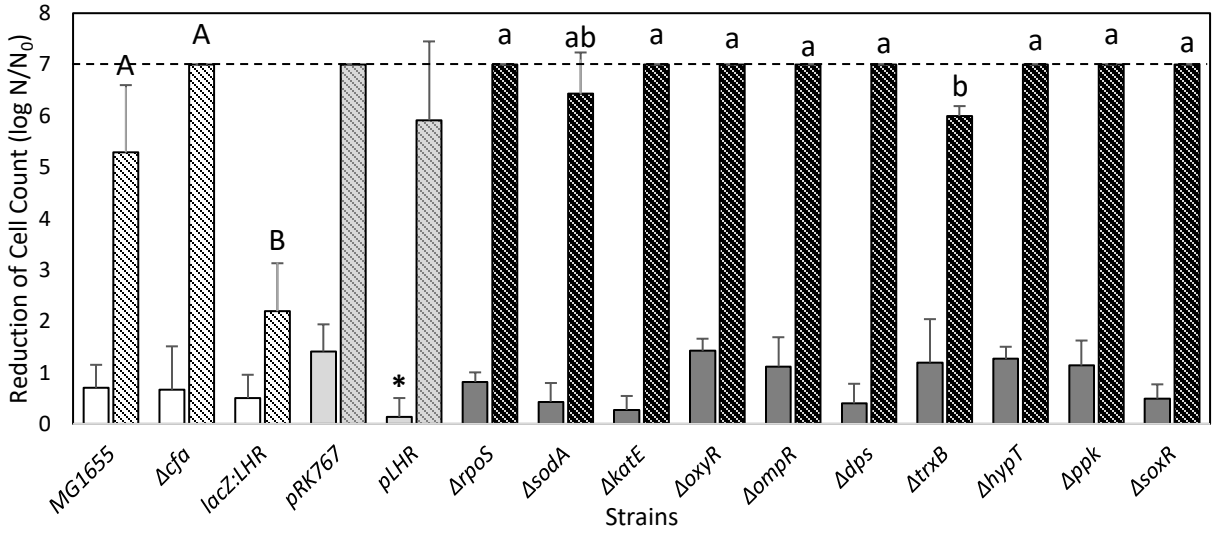


Figure 3 Effect of acid/alkaline stress on *E. coli* MG1655 and its mutants. Cells were treated with acidic (pH=2) or alkaline (pH=12) PBS for 5min, respectively. Dotted line on 7 $\log_{10}$ represents detection limit. White bars represent strains incubated on LB plates; light bars represent strains incubated on tetracycline-LB plates; dark bars represent strains incubated on kanamycin-LB plates. plain bars represent treatments at pH 2; hatched bars represent treatments at pH 12. Statistical analysis for these categories was done separately based on the supplementation of antibiotics. White bars and dark bars were analyzed by one-way ANOVA, respectively; light bars were analyzed by independent t-test. Each analysis was done within the same pH groups. Data represent means ± standard deviation of three independent replicates. Significant differences ($P < 0.05$) among strains are shown as different letters or asterisks. Bars without annotations indicate no significant differences.

3.2 Determining efficiency of applying oxidative stress alone.

H_2O_2 and NaClO have been studied extensively for disinfection of *E. coli* (Rahman, Jin, & Oh, 2011). However, the efficacy of using KIO_4 as a disinfectant is not well documented. To evaluate the efficiency of KIO_4, H_2O_2 and NaClO were used as reference treatments.

It was suggested that cell counts of *E. coli* treated with 650mM H_2O_2 at 50 ºC for 90s had reduction by 4 log_{10} (Lin, Moon, Doyle, & McWatters, 2002), also cell counts was observed to have reduction by 3 log_{10} when treated with 50mM H_2O_2 for 20min (Chen & Gänzle, 2016). Therefore, concentration of 490mM was used to treat the cells for 5min to observe reduction of cell counts for the wildtype by 2-4 log_{10} (Fig. 4A). In this treatment, *E. coli lacZ:LHR* showed increased resistance compared to MG1655. *E. coli* pLHR also showed increased resistance compared to its control, pRK767. Also, data showed that the addition of LHR, either on the plasmid or the chromosome, led to similar or higher resistance compared to the wildtype. On the other hand, single-gene deletions of *rpoS, sodA, dps* and *soxR* resulted in higher sensitivity. It is supported that *Δdps* is more sensitive to oxidative stress especially when concentration of H_2O_2 is over 200mM (Nair & Finkel, 2004). Through fluorescence assay (Table 3), it could be concluded that H_2O_2 was the only reactive compound during treatment, since Amplex® Red does not respond to the generation of $O_2^{\bullet-}$ (Gomes, Fernandes, & Lima, 2005).

It was suggested that NaClO reacts with amino acids and proteins more rapidly than H_2O_2, and causes lipid peroxidation (Noguchi, Nakada, Itoh, Watanabe, & Niki, 2002). Therefore, for same period of time, the effective dose used in this study for NaClO (8mM) was much lower than that of H_2O_2 (490mM). To observe comparable result at neutral and alkaline pH, same amount of NaClO was used for both groups. In Fig. 3B, *E. coli* MG1655, *Δcfa* and *lacZ:LHR* responded to the treatment similarly, being resistant. Strain pLHR had higher resistance when compared to pRK767. Null mutant of *dps* and *soxR* showed higher sensitivity compared to the rest of Keio mutants.

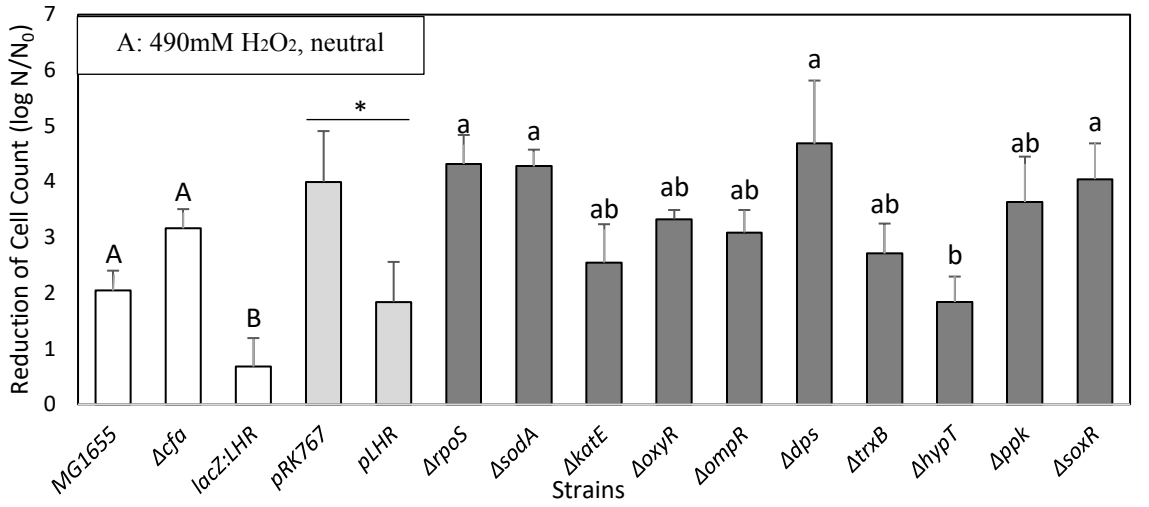
Fluorescence assay (Table 3) showed that H_2O_2 was generated by adding NaClO, however, no reactive OCl^- was detected.

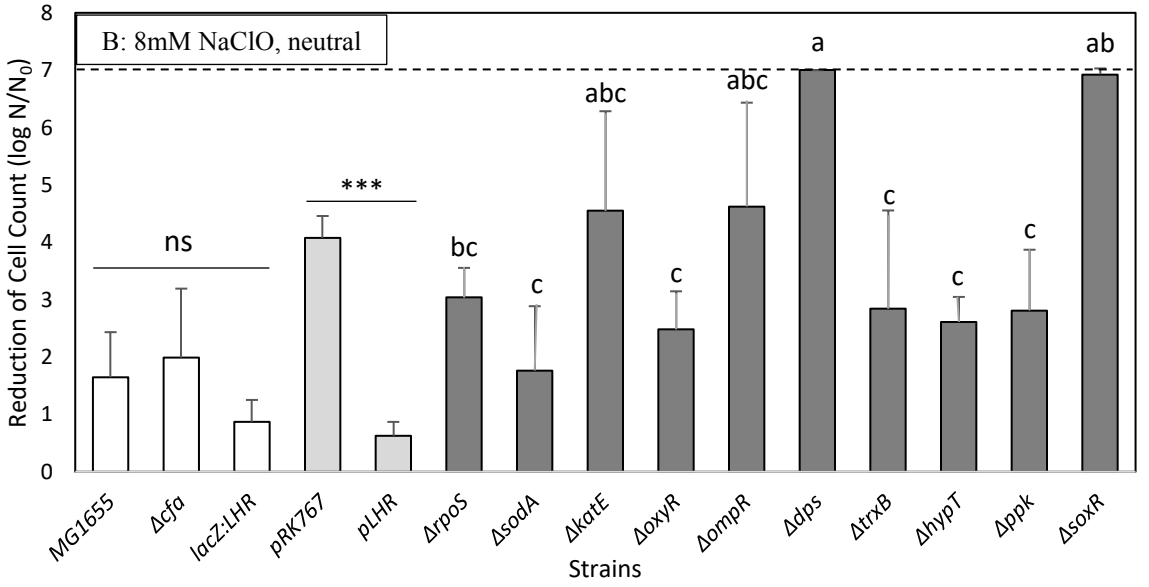
For elucidation of the mechanism of the electrochemical approach to disinfect *E. coli*, where several stress factors could be involved, potential stress factors need to be broken down to single-factor, or two-factor groups to establish standards for further experiments. In this group, cells were treated with KIO_4 solely in neutral PBS for 5min. Due to the limitation of KIO_4 solubility at 25 ºC (0.42g/100mL), maximal dissolvable amount of KIO_4 was added to the solution to observe its bactericidal effect. Fluorescent data in Table 3 showed that by adding KIO_4, various ROSs were generated during the treatment, not only limited to $^1O_2$, but also •OH, ^-OCl and H_2O_2, indicating that the strains were confronting multiple levels of oxidative attack. The source of ^-OCl could be the tap water used to prepare the 10mM PBS. Fluorescent reading of ^-OCl was determined by APF, after subtracting •OH reading from HPF. Interestingly, in Fig. 4C, consistent with data shown in Fig. 4A, *E. coli lacZ:LHR* showed higher resistance than the wildtype. In addition, *E. coli* pLHR was also more resistant compared to pRK767, indicating the protective effect of LHR against oxidative stress. Within comparisons of Keio mutants, *E. coli ΔsodA, ΔkatE* and *ΔtrxB* showed higher sensitivity than others.

When the effects of three oxidizing reagents were compared, strains in the treatment group with 8mM NaClO showed more differential responses than that in the H_2O_2 and KIO_4 treatments. Some mutants were either higher in sensitivity (*E. coli Δdps, ΔsoxR*) or higher in resistance (*E. coli* pLHR) compared to when using the other two reagents. Plus, based on the dosage, NaClO was more effective than H_2O_2 and KIO_4 in disinfecting *E. coli* when oxidizing reagent was used alone. However, generation of ROSs at different levels by the three reagents (Table 3) indicated that the interaction between the cells and the reagents might be through different defensive mechanisms that were regulated by multiple transcription regulators. This was based on the inconsistency of how the mutants behaved towards each reagent.

Table 3 Fluorescence assay of 490mM H_2O_2, 8mM NaClO and 9mM KIO_4 in neutral PBS (pH=7.4). Solutions containing corresponding reagents were measured with fluorescence probes HPF (for detection of •OH), APF (in addition to HPF, it detects -OCl), SOSG (for detection of $^1O_2$) and Amplex Red (for detection of H_2O_2). Controls contained corresponding fluorescence probes but without the reagents. Control of Amplex Red was with Amplex Red and horseradish peroxidase.

Fluorescence	**Control**	**490 mM H_2O_2**	**8mM NaClO**	**9mM KIO_4**
HPF	2.6±0.3	21.4±15	0.1±0	315.5±16
APF	1.4±0.2	36.4±4	0.2±0	747.8±30
SOSG	15.0±1	20.5±3	1.5±0.7	124.1±10
Amplex Red	414.3±56	1530.76±86	929.0±89	1041.8±153


A: 490mM H2O2, neutral
Reduction of Cell Count (log N/N0)
Strains
MG1655
Δcfa
lacZ:LHR
pRK767
pLHR
ΔrpoS
ΔsodA
ΔkatE
ΔoxyR
ΔompR
Δdps
ΔtrxB
ΔhypT
Δppk
ΔsoxR
A
A
B
*
a
a
ab
ab
ab
a
ab
b
ab
a


B: 8mM NaClO, neutral
Reduction of Cell Count (log N/N0)
Strains
MG1655
Δcfa
lacZ:LHR
pRK767
pLHR
ΔrpoS
ΔsodA
ΔkatE
ΔoxyR
ΔompR
Δdps
ΔtrxB
ΔhypT
Δppk
ΔsoxR
ns
***
bc
c
abc
c
abc
a
c
c
c
ab

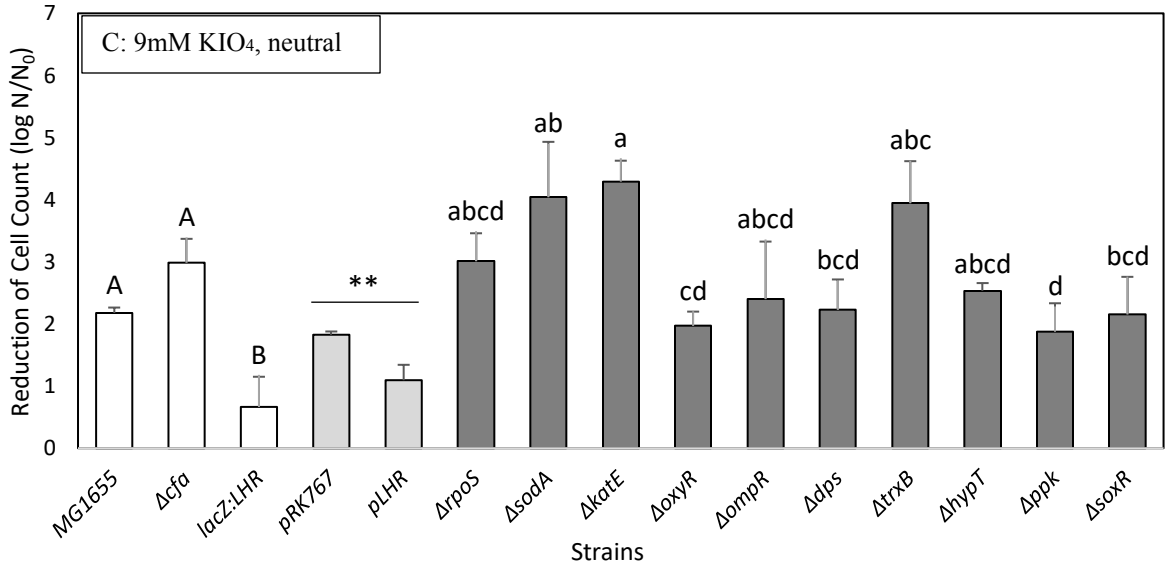

C: 9mM KIO4, neutral
Reduction of Cell Count (log N/N0)
Strains
MG1655
Δcfa
lacZ:LHR
pRK767
pLHR
ΔrpoS
ΔsodA
ΔkatE
ΔoxyR
ΔompR
Δdps
ΔtrxB
ΔhypT
Δppk
ΔsoxR
A
A
B
**
abcd
ab
a
cd
abcd
bcd
abc
abcd
d
bcd

Figure 4 Effect of oxidative stress (490mM H_2O_2, 8mM NaClO or 9mM KIO_4) on *E. coli* MG1655 and its mutants. Cells were treated with three reagents in neutral PBS for 5min, respectively. Fig. 4A: H_2O_2; Fig. 4B: NaClO; Fig. 4C: KIO_4. Dotted line on 7 $\log_{10}$ represents detection limit. White bars represent strains incubated on LB plates; light bars represent strains incubated on tetracycline-LB plates; dark bars represent strains incubated on kanamycin-LB plates. Statistical analysis for these categories was done separately. White bars and dark bars were analyzed by one-way ANOVA, respectively; light bars were analyzed by independent t-test. Data represent means ± standard deviation of three independent replicates. Significant differences ($P < 0.05$) among strains are shown as different letters or asterisks. Bars annotated with ns stand for no significant differences.

3.3 Determining the efficiency of combining acid stress and oxidative stress.

In an electrochemical reactor, due to the accumulation of H^+ on anode and OH^- on cathode, pH change was overserved on the electrodes. To investigate whether the pH plays a role in disinfecting *E. coli* cells, a combination of acid and oxidizing chemicals was applied to the cells, without electricity. The combination of acid and H_2O_2 was used as a reference for the treatment with KIO_4 in acidic solution. The fluorescence assay (Table 4) did not show significant response to any of the ROSs. However, the result might not accurately reflect the actual level of H_2O_2, since the optimal working pH for Amplex® Red is 7.5 – 8.5. Furthermore, superoxide radicals were not tested by fluorescent probes in this study. In Fig. 5A, sensitivity of *E. coli* pRK767 was higher when compared to that of pLHR. In addition, the cell counts of pLHR and *lacZ:LHR* were differed by 3 $\log_{10}$, indicating that presence of antibiotics could also become an external stressor that increases strain sensitivity. *E. coli ΔrpoS* showed hypersensitivity that its cell counts were reduced by 6 $\log_{10}$, indicating that the lack of RpoS as a general stress regulator, may result in the lethality of acid and oxidative stress.

In order to better understand the mechanism of electrochemical disinfection, prior to integrating all stress factors into one reaction chamber, sensitivity of the strains was being evaluated by treating them with KIO_4 in acidic and alkaline PBS, respectively. In this experiment, cells treated with KIO_4 in acidic

PBS were compared to the combination of acid and H_2O_2 to determine its effectiveness. In Fig. 5B, strains with single-gene deletions in *rpoS*, *sodA*, *ompR*, showed increased sensitivity compared to other Keio mutants. The sensitivity of *ΔsodA*, which encodes for superoxide dismutase, could be an indicator of the generation of superoxide radicals. Mutant *ΔompR* also showed higher sensitivity in this treatment compared to its performance in neutral treatment groups. Furthermore, *E. coli Δcfa* was more sensitive than the wildtype MG1655, but *E. coli lacZ:LHR* did not show increased resistance, unlike treatment groups previously discussed. However, *E. coli lacZ:LHR* was still more resistant than *E. coli* pLHR. In Table 4, none of the fluorescent probes had recognizable response to their corresponding ROSs in both treatment groups.

Table 4 Fluorescence assays of 490mM H_2O_2 and 9mM KIO_4 in acidic PBS (pH=2). Acidic solutions containing corresponding reagents was measured with fluorescence probes HPF (for detection of •OH), APF (in addition to HPF, it detects -OCl), SOSG (for detection of $^1O_2$) and Amplex Red (for detection of H_2O_2). Controls contained corresponding fluorescence probes but without the reagents. Control of Amplex Red was with Amplex Red and horseradish peroxidase.

Fluorescence	**Control**	**490mM H_2O_2 (acidic)**	**9mM KIO_4 (acidic)**
HPF	2.1±0.4	0.2±0	4.4±0.3
APF	1.8±0.3	0.9±0.1	3.5±0.1
SOSG	0.5±0.2	0.5±0.2	0.4 ± 0
Amplex Red	0.6±0.2	2.6±0.7	6.1±0.8

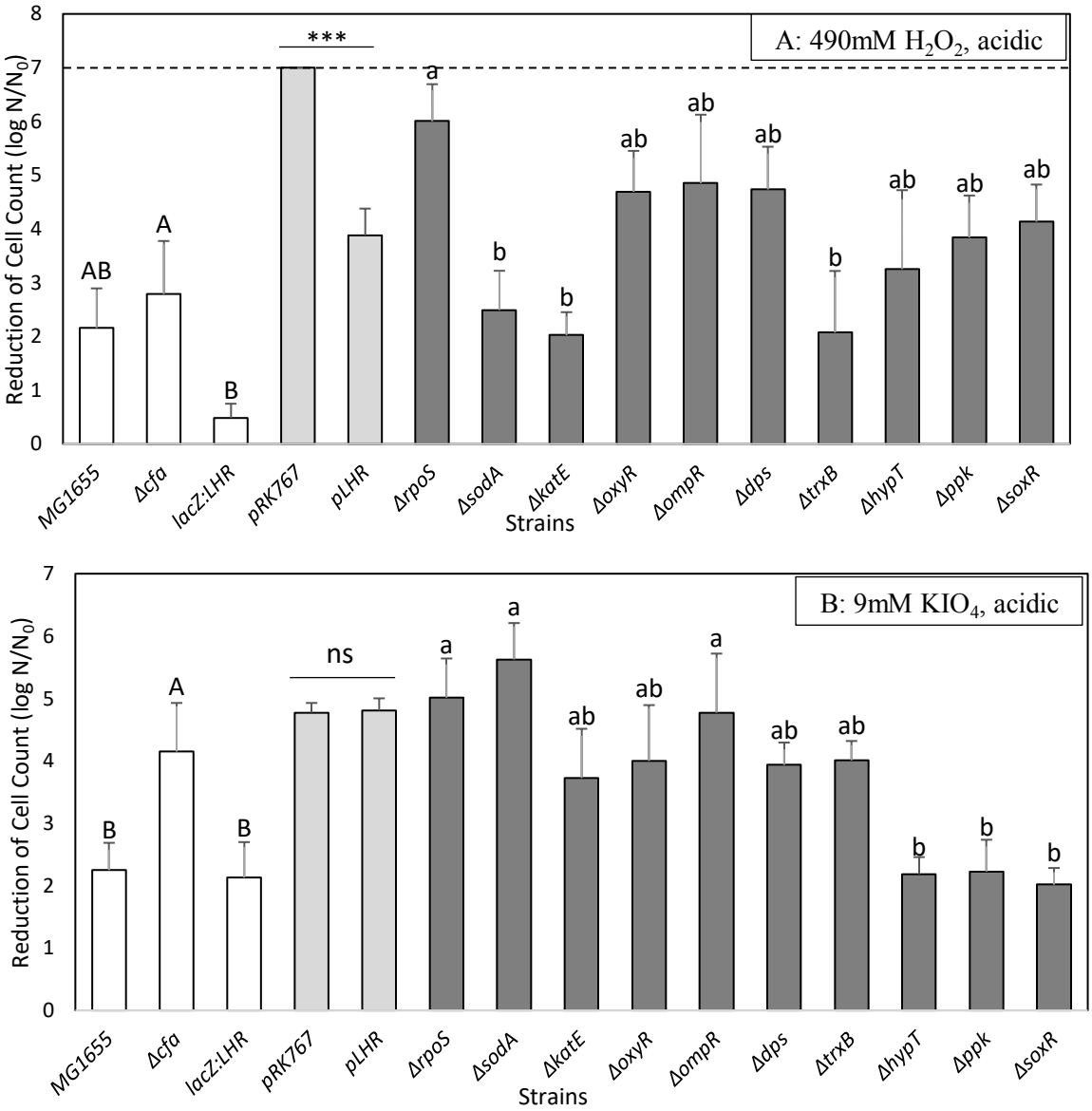


Figure 5 Effect of applying combined stress of acid and oxidative compounds of A: 490mM H_2O_2 or B: 9mM KIO_4 on *E. coli* MG1655 and its mutants. Cells were treated with two reagents in acidic PBS for 5min, respectively. Dotted line on 7 $\log_{10}$ represents detection limit. White bars represent strains incubated on LB plates; light bars represent strains incubated on tetracycline-LB plates; dark bars represent strains incubated on kanamycin-LB plates. Statistical analysis for these categories was done separately. White bars and dark bars were analyzed by one-way ANOVA, respectively; light bars were analyzed by independent t-test. Data represent means ± standard deviation of three independent replicates. Significant differences ($P < 0.05$) among strains are shown as different letters or asterisks. Bars annotated with ns stand for no significant differences.

3.4 Determining the efficiency of combining alkaline stress and oxidative stress.

In Fig. 6A, *E. coli lacZ:LHR* showed higher resistance compared to MG1655 where the difference on the cell counts between the two strains was 2 $\log_{10}$. However, the other LHR-positive strain pLHR, showed increased sensitivity under the combination of alkaline and oxidative stresses, compared to treatment with oxidative stress alone. In addition, *E. coli* pRK767 and *Δcfa* also showed higher sensitivity compared to the wildtype. Fluorescence assay in Table 5 showed that a small amount of •OH was generated during the treatment. Also, much higher level of $^{-}$OCl was detected through combining APF and HPF readings. Hypochlorite ion as the conjugate base of hypochlorous acid, was stabilized in this highly alkaline environment. The level of SOSG indicated that during the treatment, part of $^{-}$OCl was converted to $^{1}O_2$.

In Fig. 6B, more strains exhibited higher sensitivity, including *ΔkatE, ΔoxyR, ΔompR, Δcfa,* pRK767, and pLHR, compared to MG1655. In opposite, *lacZ:LHR* was the only mutant that showed similar resistance compared to the wildtype. *E. coli* pLHR was more sensitive compared to *lacZ:LHR*, but performed similarly to its control strain pRK767. In Table 5, fluorescence data showed that high level of hypochlorite ion was generated in this treatment group, along with much lower but still noticeable amount of hydroxyl radical and singlet oxygen. Amplex® Red was not used due to the limit of its working pH range. When the two treatment groups were compared, though NaClO showed higher effectiveness in neutral treatment group, KIO_4 in alkaline PBS had higher level of fluorescent response indicating that ROSs generated in this group was higher than that of NaClO group.

Table 5 Fluorescence assays of 8mM NaClO and 9mM KIO_4 in alkaline PBS (pH=12). Solutions containing corresponding reagents were measured with fluorescence probes HPF (for detection of •OH), APF (in addition to HPF, it detects ^{-}OCl), SOSG (for detection of $^1O_2$) and Amplex Red (for detection of H_2O_2). Controls contained corresponding fluorescence probes but without the reagents. *SOSG only slightly increased when increase pH from 7 to 10 (Gollmer et al., 2011).

Fluorescence	**Control**	**8mM NaClO (alkaline)**	**9mM KIO_4 (alkaline)**
HPF	1.4±0.5	51.9±3	154.2±7
APF	0.7±0.3	1271.3±51	2761.1±65
SOSG	14.1±1*	50.5±3	91.2 ± 2

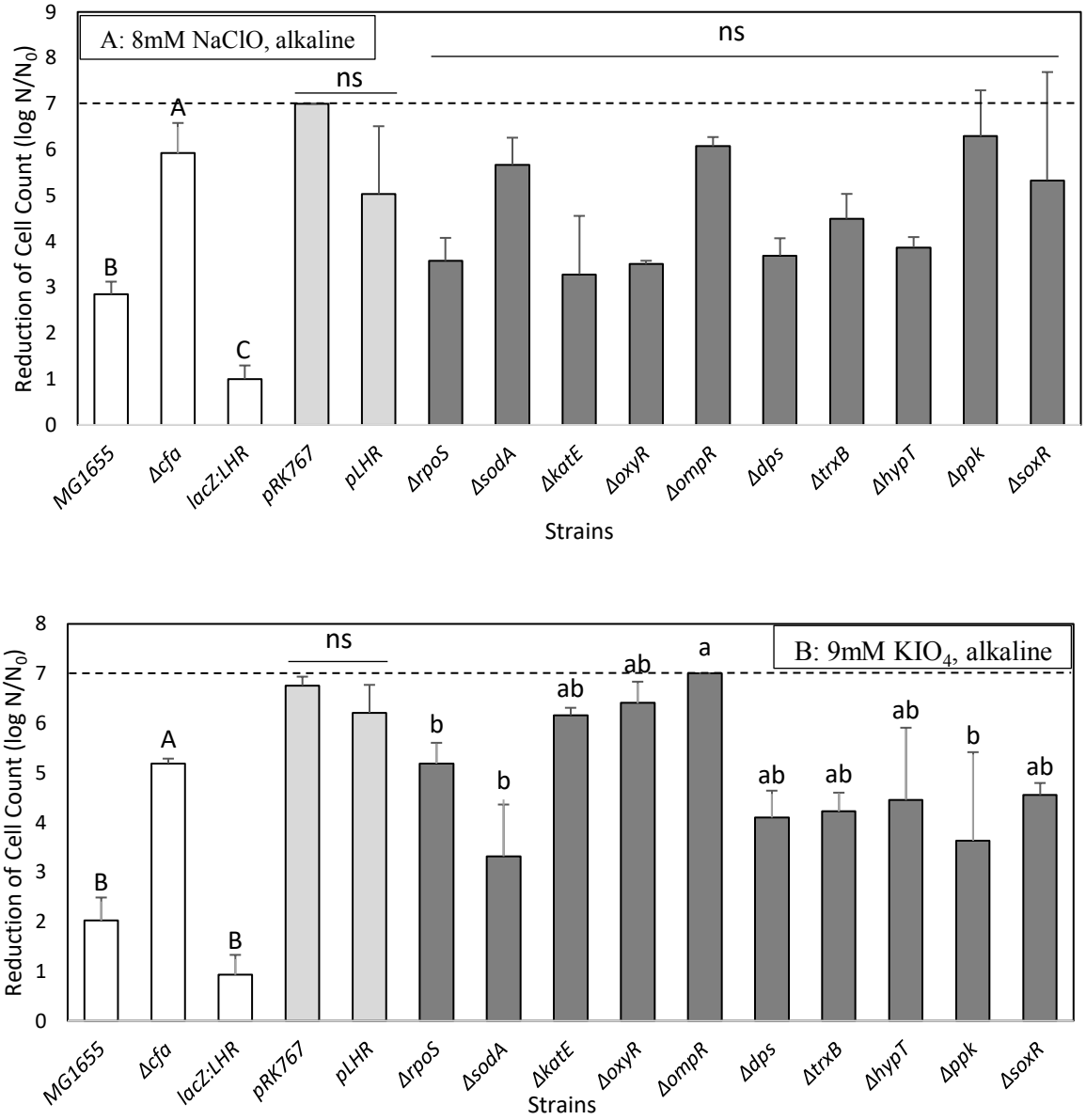


Figure 6 Effect of applying combined stress of alkaline and oxidative compounds of A: 8mM NaClO or B: 9mM KIO_4 on *E. coli* MG1655 and its mutants. Cells were treated with two reagents in alkaline PBS (pH =12) for 1min, respectively. Dotted line on 7 $\log_{10}$ represents detection limit. White bars represent strains incubated on LB plates; light bars represent strains incubated on tetracycline-LB plates; dark bars represent strains incubated on kanamycin-LB plates. Statistical analysis for these categories was done separately. White bars and dark bars were analyzed by one-way ANOVA, respectively; light bars were analyzed by independent t-test. Data represent means ± standard deviation of three independent

replicates. Significant differences ($P < 0.05$) among strains are shown as different letters or asterisks. Bars annotated with ns stand for no significant differences.

3.5 Determining the efficiency of disinfecting strains using electrochemical approach.

Electrochemical disinfection has obtained increased recognition by water treatment industries over the years because of its low cost compared to UV radiation or ozonation (Diao et al., 2004). In addition, it has less harmful by-products compared to conventional methods such as chlorination (Jeong et al., 2007). Therefore, to have an overall assessment of inactivating *E. coli* using electrochemical disinfection method where multiple stress factors were combined, this experiment was designed that 12V of electricity was applied to cell cultures with KIO_4 neutral PBS as electrolyte. pH value on anode was at ~6.5 and that of cathode being ~8. In Fig. 7, *E. coli lacZ:LHR* showed increased resistance compared MG1655 on anode, but not on cathode. In addition, *E. coli lacZ:LHR* on cathode was more sensitive than that of anode. *E. coli* pRK767 was more sensitive compared to *E. coli* pLHR on both electrodes. Within the Keio mutants, *E. coli ΔrpoS* was the only strain that showed substantially higher sensitivity that its cell counts were reduced by 6 $\log_{10}$ on both electrodes, where other mutants were observed to have higher sensitivity on cathode. Mutants *E. coli ΔrpoS, ΔoxyR, ΔtrxB* and *ΔhypT* showed sensitivity on the cathode. Differential lethal effects to the same strain caused by anode and cathode was observed in some mutants: *lacZ:LHR, ΔkatE, ΔoxyR, Δdps, ΔtrxB, ΔhypT* and *ΔsoxR*, where *ΔrpoS* being sensitive on both electrodes.

In Table 6, data showed that through applying voltage to the system, H_2O_2 was generated on both electrodes, where the amount generated on cathode being slightly higher compared to that of anode. No other ROSs was detected.

Table 6 Fluorescence assay of electrolytes in both electrodes after 5min treatment (pH=7.4). Solutions containing electrolytes was measured with fluorescence probes HPF (for detection of •OH), APF (in addition to HPF, it detects -OCl), SOSG (for detection of $^1O_2$) and Amplex Red (for detection of H_2O_2). Controls contained corresponding fluorescence probes but without the reagents. Control of Amplex Red was with Amplex Red and horseradish peroxidase.

Fluorescence	**Control**	**Anode**	**Cathode**
HPF	2.6±0.3	2.4±1.3	4.7±0.9
APF	1.4±0.2	0.9±1	1.6±1
SOSG	15.0±1	1.22±0.1	2.82±0.7
Amplex Red	414.3±56	810.5±9	978.5±98

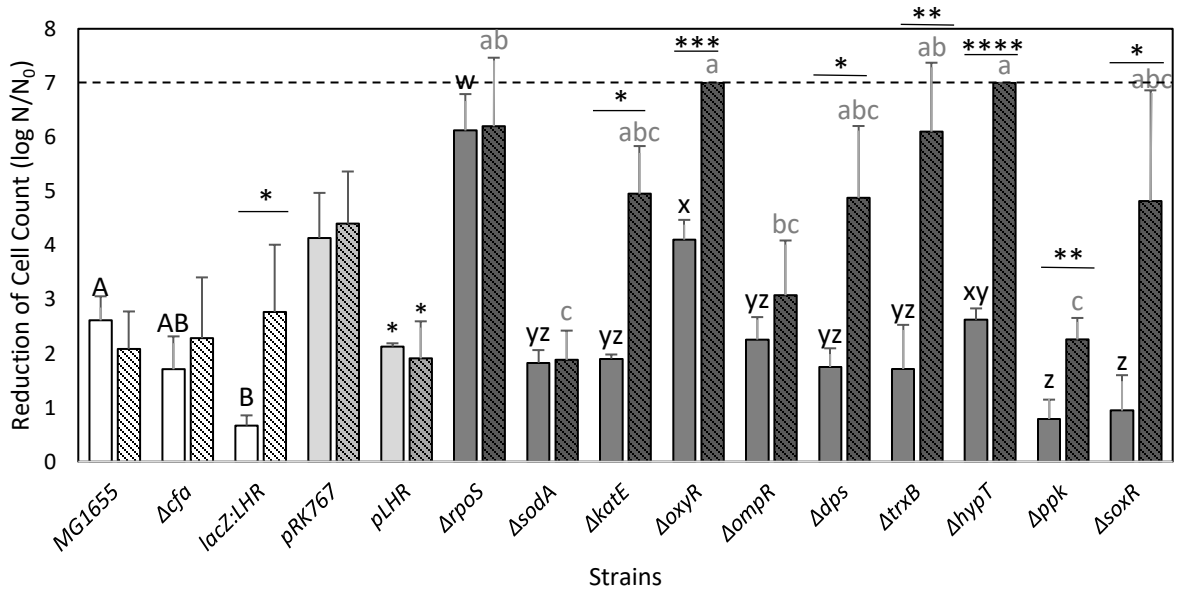


Figure 7 Effect of electrochemical disinfection method on *E. coli* MG1655 and its mutants. Cells were treated with 12V electricity and 9mM KIO_4 in for 5min. Light bars are cells treated in anode and dark bars are cells treated in cathode. pH value at anode was ~6.5, and that of cathode was ~8. Dotted line on 7 log_{10} represents detection limit. White bars represent strains incubated on LB plates; light bars represent strains incubated on tetracycline-LB plates; dark bars represent strains incubated on kanamycin-LB plates. Plain bars represent treatments at anode; hatched bars represent treatments at cathode. Statistical analysis for these categories was done separately based on the supplementation of antibiotics. White bars and dark bars were analyzed by one-way ANOVA, respectively; light bars were analyzed by independent t-test. The difference between anode and cathode for each strain was also evaluated through independent t-test. Data represent means ± standard deviation of three independent

replicates. Significant differences ($P < 0.05$) among strains are shown as different letters or asterisks. Underlined asterisks represent the differences between anode and cathode; asterisks on annotation of pLHR represent the differences between pRK767 and pLHR on same electrodes.

4 Discussion

Through the comparison of using oxidative stress, acid stress, alkaline stress and electrochemical disinfection on *E. coli* MG1655 and its mutants, the effect of each stressor and combined effect of multiple stressors were evaluated. It was found that mutants with single-gene deletions exhibited sensitivity when oxidative stress and pH stress were combined. Chromosome-coded LHR-positive strain *E. coli lacZ:LHR* exhibited resistance against alkaline pH despite the presence of oxidizing reagents. Bacterial resistance against H_2O_2 is dependent on pH, where the less favourable of pH for growth of cells, the greater the bactericidal effect of H_2O_2 (Jackett, Aber & Lowrie, 1978). It was found in this study that bacterial inactivation by KIO_4 was enhanced in alkaline condition but not in acidic condition. It was suggested that when treated with aqueous chlorine solutions, inactivation of *E. coli* was maximal between pH 2.7-7, but not in alkaline pH (Nakagawara, et al., 1998). However, this study observed that mutants were more sensitive to NaClO at alkaline pH compared to neutral pH. In addition, the strains showing increased sensitivity to NaClO at neutral pH did not show the same level of sensitivity in alkaline pH, instead, strains resistant in neutral became sensitive in alkaline pH. Reactive oxidative compounds generated in the neutral treatment with KIO_4,, were not found or found in lower levels in treatments with H_2O_2 and NaClO, such as •OH, -OCl and $^1O_2$. The bactericidal effect of KIO_4 was also enhanced by applying additional pH stresses. When using electrochemical disinfection through electrogenerated hydroxyl radicals, bacterial inactivation due to the pH shift within 5-9 is negligible (Cong, Wu & Li, 2008). *In-situ* generated H_2O_2 in an electrochemical reactor is dependent on pH, and that slightly acidic condition is more effective compared to slightly alkaline condition, when 0.1M PBS is used as electrolyte (Cho, et al., 2010). However, pH-dependence of the efficacy of electrochemical disinfection using KIO_4 has not yet been discovered. In this study, it was found that inactivation of most of the target strains was more effective on the weakly alkaline cathode (pH 8) but not on weakly acidic

anode (pH 6.5). This observation was supported by the fluorescence data in Table 6, that electro-generated H_2O_2 was higher on cathode. Furthermore, the addition of antibiotics in the media for selected mutants could act as another source of stress for the organisms. Therefore, the strains grown on the media with different antibiotics were not compared.

Reactions of NaClO and H_2O_2 in acid, neutral and alkaline environment. In neutral environment (pH =7.4), when NaClO dissociates to Na^+ and ^-OCl, where ^-OCl as the conjugate base of weak acid HOCl, the following reaction would occur: $^-OCl + H_2O \rightleftharpoons HOCl + OH^-$. The fluorescence data in Table 3 shows that no ^-OCl was detected, it could be thus concluded that majority of the ^-OCl was converted to HOCl. HOCl partially reacts with water to form H_2O_2 (Lister, 1952), which was confirmed by H_2O_2 measurement using Amplex Red. However, none of the fluorescent probes used in this study was able to detect HOCl. In alkaline environment, ionic compounds exist in the solution mainly as ^-OCl, which was confirmed through comparing Table 3 & 5. In acidic environment, due to the reaction between the two compounds: $NaClO + HCl \rightarrow Cl_2 + H_2O + NaCl$, reactive chlorine compounds were transformed to chlorine gas, which escaped from the solution prior to treatment and resulted in ineffective disinfection. Based on the preliminary tests, the solution containing NaClO in acidic PBS (pH=2) did not damage the cells. Therefore, experiment with this combination was not selected. Using H_2O_2 in acidic environment, following reactions may occur: $H_2O_2 + H^+ + Cl^- \rightarrow HOCl + H_2O$; $H_2O_2 + Cl^- \rightarrow {}^-OCl + H_2O$, indicating generation of multiple reactive compounds (Murray, Koch & van Eldik, 2014). However, due to the limitation of fluorescent probes, ROSs were not detectable in acidic pH. H_2O_2 decomposes in alkaline environment via reaction with NaOH, therefore, this combination was not selected (Guo, et al., 2012).

Acid and alkaline stress response of *cfa* mutant. Gene *cfa* encodes for cyclopropane fatty acids (CFA), which is found at a higher level in acid resistant strains of *E. coli* and stabilizes cytoplasmic membrane lipids (Zhang & Rock, 2008; Chen & Gänzle, 2016). In neutral treatments, despite the

chemicals used, *E. coli Δcfa* did not show increased sensitivity compared to MG1655. However, when treated with acid or alkaline in the presence of oxidative reagents, *E. coli Δcfa* was more sensitive. The mutant was even more sensitive in alkaline treatment compared to that of acid treatment based on the reaction time being 1min and 5min while achieving similar inactivation. It was observed in Chen's study (2016) that *cfa* mutant showed sensitivity after 3h exposure to pH 2.5. In this study, cross-stress challenge increased the sensitivity in three out of four of the extreme pH groups with oxidative compounds, except for when treated with H_2O_2 in acid. When comparing the treatments of oxidative stress in neutral and in extreme pH, the difference between the cell counts of *E. coli* MG1655 and *Δcfa* was increased from less than 1 $\log_{10}$ to 2-3 $\log_{10}$. This observation indicates that regulation of CFA also involves in protection against acid and alkaline stress.

Contribution of LHR to resistance against oxidative stress and alkaline stress. The presence of LHR has been directly linked to increased heat resistance in *E. coli*, where the difference of cell counts on LHR-positive strains compared to LHR-negative strains was 4 $\log_{10}$ upon cooking to 71°C in ground beef (Mercer et al., 2017). LHR-mediated heat resistance was enhanced by higher salt concentrations (Pleitner et al., 2012). However, LHR-positive strains against other environmental stresses have not yet been documented. In this study, I investigated the resistance of LHR strains against oxidative, acid and alkaline stress. In the treatment of solely applying oxidative stress to the strains, *E. coli* pLHR exhibited resistance throughout the three treatment groups with H_2O_2, NaClO and KIO_4 compared to pRK767. However, pLHR-mediated resistance appeared to contribute only to resistance against oxidative stress. Interestingly, a protective effect of plasmid-coded LHR was not observed in either acid or alkaline treatment. *E. coli* pLHR in both acid treatments with H_2O_2 and KIO_4 and in both alkaline treatments with NaClO and KIO_4 showed higher sensitivity than the neutral treatments. Sensitivity of *E. coli* pLHR in alkaline treatments with NaClO and KIO_4 was even higher since its reduction of cell counts was at the

same level as acid treatments with H_2O_2 and KIO_4, within much shorter time period. On the other hand, the chromosomally-integrated strain, *E. coli lacZ:LHR* was resistant in all treatment groups. In some treatments, it was more resistant compared to its LHR-negative wildtype strain, *E. coli* MG1655, such as in neutral with H_2O_2 and KIO_4 and in alkaline with NaClO. The differential behaviour of strains integrated with LHR on plasmid and on chromosome, may be attributed to the maintenance of additional engineered plasmid which might change the metabolic flux of the cell and cause increased energy expenditure, therefore affect the overall fitness (Baltrus, 2013; Diaz Ricci & Hernández, 2000). As seen in comparison of *E. coli* MG1655 and pRK767, the latter was more sensitive than the former in almost all cases. It was also observed that by introducing pLHR to an LHR-negative *Enterobacter cloacae* strain, its heat resistance was lower than that of LHR-positive wildtype *E. cloacae* (Mercer et al., 2017). Hence, keeping the plasmid by growing *E. coli* pRK767 and pLHR on antibiotics supplemented plates could be another stressor for the cells which makes them more vulnerable in extreme conditions.

Similarities and differences among Keio mutants in neutral H_2O_2 and NaClO. It was suggested that HOCl increases cell membrane permeability, inhibits glucose uptake, increase ATP expenditure and reacts with cells more rapidly compared to H_2O_2 (Ochoa, Waypa, Mahoney, Rodriguez, & Minnear, 1997). Therefore, when comparing the reagents H_2O_2 and NaClO, it was found that NaClO (8mM) can cause similar lethal effect to cell cultures as H_2O_2 when the dose of latter was used at a much higher level (490mM). Performance of *E. coli Δdps* and *ΔsoxR* were comparable in neutral groups with H_2O_2 and NaClO. Sensitivity for both strains was higher than other Keio mutants. Dps has been reported to have peak expression at stationary-phase that can act as a primary target for ROS (Nair & Finkel, 2004). Additionally, in the presence of H_2O_2 at high concentration, it can protect the cells from single-strand DNA breakage through directly binding to their DNA (Martinez & Kolter, 1997). Moreover, because the generation of H_2O_2 after dissolving NaClO in neutral solution, it could also explain the sensitivity of *dps*

depleted strain during this treatment. On the other hand, *soxRS* regulon that activates transcription of enzymes in the presence of ROS, has previously been shown to only respond to accumulation of superoxide radicals by regulating superoxide dismutase (*sodA*) (Storz & Imlay, 1999; Chiang & Schellhorn, 2012; Manchado, Michan, & Pueyo, 2000). However, a study suggested that H_2O_2 can also induce *soxRS* enzymes through oxidizing SoxR to trigger the signaling response that activates SoxS (Zheng et al., 2001). Moreover, HOCl or free chlorine could also interfere with the formation of reduced SoxR by limiting its energy source, such as NADPH (Dukan, Dadon, Smulski, & Belkin, 1996). In addition, SoxR is the sensor protein that activates the transcription of *sox* gene which encodes for protein SoxS. Thus, under stress, the two-component system SoxRS can control the expression of over 17 genes (Amabile-Cuevas & Demple, 1991; Liochev, Hausladen & Fridovich, 1999). In comparison, inactivation of *E. coli ΔrpoS* and *ΔsodA* was more effective when treated with H_2O_2 compared to that of NaClO. The protecting effect of *rpoS* was extensively studied that cells exposed to low concentration of H_2O_2 during exponential phase exhibits higher resistance compared to exposure to H_2O_2 in stationary phase without pretreatment (Demple & Halbrook, 1983). Thus, it explains the sensitivity of *E. coli ΔrpoS* in high concentration of H_2O_2 in stationary phase. However, similar effect of inactivation of *E. coli* in NaClO treatment was not observed. It was suggested that when treated with NaClO, deletion in *rpoS* of *Salmonella* Enteritidis did not result in increased sensitivity compared to the wildtype, but *dps* mutant was more sensitive than these two strains (Ritter et al., 2012). Therefore, the involvement of *dps* and σ_S induction might be through different mechanisms. The other strain showing higher sensitivity in H_2O_2 than in NaClO, *ΔsodA*, a superoxide radical scavenger, was found to be activated by SoxRS regulon (Greenberg, Monach, Chou, Josephy, & Demple, 1990). It could be an indication that the level of superoxide radicals generated during H_2O_2 treatment, was higher compared to NaClO group, which compensated the limit of detecting superoxide radicals through fluorescent probes. Another Keio

mutant, *ΔkatE* that encodes for hydroperoxidase II, was not found sensitive in the H_2O_2 treatment. Though *katE* was considered as the major catalase producer in stationary phase, it was found that single mutation on *katE* has little contribution to the challenge of H_2O_2. Instead, double mutant *katE katG* shows exceptionally higher sensitivity towards oxidative stress where *katG* encodes for hydroperoxidase I (Imlay & Linn, 1987; Dukan, Dadon, Smulski, & Belkin, 1996; Chiang & Schellhorn, 2012). The unexpected resistance observed in *ΔoxyR*, was supported that the activity of OxyR is suppressed by RpoS regulator to avoid extra energy cost (Altuvia, Weinstein-Fischer, Zhang, Postow, & Storz, 1997). Thus, to maximize the efficiency of cell metabolism against toxic compounds, elimination of *oxyR* could be an advantage for survival under certain stressors in the presence of *rpoS*.

Distributions of reduction on Keio mutants with H_2O_2 treatment in acidic pH. In this group of treatments, *E. coli* strains has undergone extreme conditions of free radicals and acid attack. Therefore, the differential behaviour of strains compared to in neutral, could be due to the additional acid stress. It was observed on *ΔrpoS* that the lethal effect of combining oxidative and acid stress caused its cell counts to reduce by over 5 $\log_{10}$ in both acidic treatment groups which showing higher sensitivity than both reagents in neutral pH. This indicated that this gene encoding the sigma S is necessary to support the growth of *E. coli* in extreme low pH. RpoS has been studied extensively and recognized as the general stress regulon (Lange & Hengge-Aronis, 1991), which not only helps with the pH homeostasis, but also regulates other genes that are found to be important against oxidative stress, such as *dps* and *katE* (Chiang & Schellhorn, 2012). Moreover, *rpoS* induces the expression of three sRNAs, DsrA, RprA and ArcZ. Deletion in any of the three sRNAs leads to higher acid sensitivity (Bak, Han, Kim, & Lee, 2014). Hence, the deletion of the activator *rpoS* can cause major deficiency against acid stress. *E. coli ΔompR* also showed similar tendency that the sensitivity of *ΔompR* with H_2O_2 in acid pH was higher compared to their corresponding neutral treatments (Fig.4). In EnvZ-OmpR two component system, upon pH

stress, EnvZ leads to conformational change of OmpR which contributes to porin regulation by binding to the promotor region of porin genes *ompF* and *ompC* (De la Cruz et al., 2007; Stincone et al., 2011). Therefore, deletion in *ompR* disrupts porin genes expression which could result in higher sensitivity in acidic environment. Another strain showing different behaviours, *ΔsodA*, could be a tool for detecting generation of superoxide radicals in acidic KIO_4 but not in H_2O_2.

Comparison of Keio mutants with NaClO in alkaline and neutral pH. Acid resistance response system was studied extensively, while information on the mechanisms of resistance of *E. coli* to alkaline environment is not nearly as detailed. However, alkaline resistance is as important for the bacteria to achieve cytoplasmic pH homeostasis. In this experimental group, genes that encode for proteins against oxidative stress and contribute to acid resistance were investigated aiming to study the mechanism of survival in alkaline environment. Due to the double stress attack of oxidizing reagents and alkaline, to determine whether the change in behaviour of strains were caused by oxidative stress, alkalinity, or combination of both, this group of treatments were compared to that in neutral. In alkaline NaClO treatment, according to Table 5, ^{-}OCl was at a much higher level in alkaline pH, accompanied by generation of small amount of $^1O_2$ and •OH. *E. coli ΔsodA* showed higher sensitivity when treated with NaClO in alkaline compared to that in neutral, where the treatment time at alkaline pH was 80% shorter than that in neutral. Furthermore, superoxide dismutase (SOD) binds with metal ions, such as Cu, Fe and Mn, and utilizes ^{-}OH to reduce superoxide radicals (Miller, 2012). Thus, the deletion of *sodA* could result in disadvantage in scavenging $O_2^{\cdot-}$ and consuming ^{-}OH. Another mutant that showed higher sensitivity with NaClO in alkaline was *Δppk* that encodes for polyphosphate kinase. Polyphosphate hydrolysis has been found to positively relate to restoring cytoplasmic pH balance in alkaline pH. In addition, polyphosphate kinase helps accumulate polyphosphate (Pick et al., 1990). Hence, deletion in *ppk* can result in reduction on hydrolysis, in turn, disrupts the process of recovering internal pH

homeostasis. In fluorescence assay (Table 5), the types of ROSs generated by NaClO and KIO_4 were identical but at different levels. Both compounds generated a high level of $^{\cdot}OCl$, where the amount detected in KIO_4 was two times higher compared to that in NaClO. However, the order of ROSs being released is unknown. Additionally, because of the treatment time was set to as short as 1min to achieve viable cell counts, the contrasting distribution of strains between the two treatments could only be explained that the mutants responded to any of the ROSs whichever was generated prior to others.

Effect of KIO_4 in neutral, acidic and alkaline pH. Though KIO_4 is considered a strong oxidizing reagent, it has not been used in large-scale for disinfection of bacteria until recently. This could be due to its low solubility in water compared to other oxidizers. KIO_4 is the highest oxidation state of iodine, considering as the salt of periodic acids, H_5IO_6 and HIO_4 (Jackson, 1944). It exists in aqueous solution in various forms depending on the pH. It has two forms of anions: IO_4^- and IO_6^{5-}, which can react with water through hydrolysis and forms periodic acid in a series of dissociation equilibriums (Wiberg, Wiberg & Holleman, 2001).

$$H_4IO_6^- \rightleftharpoons IO_4^- + 2H_2O$$

$$H_5IO_6 \rightleftharpoons H_4IO_6^- + H^+ \quad pK_a = 3.29$$

$$H_4IO_6^- \rightleftharpoons H_3IO_6^{2-} + H^+ \quad pK_a = 8.31$$

$$H_3IO_6^{2-} \rightleftharpoons H_2IO_6^{3-} + H^+ \quad pK_a = 11.60$$

Also, it can be easily decomposed to KIO_3 and O_2 upon heating (Takriti & Duplâtre, 1988). In this study, the effectiveness of KIO_4 on inactivation of *E. coli* was investigated at different pH values. Fluorescent assays for these groups showed that unlike the acid treatment, ROSs generated in neutral and alkaline groups were more similar, where the amount of $^{\cdot}OCl$ in alkaline group being much higher. Generation of $^{\cdot}OCl$ could be due to the reaction between KIO_4 and available Cl^- in PBS, where other free radicals serving as intermediate. Also, both $^{\cdot}IO_4$ and $^{\cdot}OCl$ are the conjugate bases of two weak

acids, HIO_4 and HOCl where the pK_a value of HOCl being slightly lower, indicating that ^{-}OCl is more dissociated in water compared to IO_4^-. Alkaline pH helps the equilibrium to shift, that, even more ^{-}OCl is able to stabilize in water. On the other hand, in acid treatment group, no active ROSs were detected, which could be caused by limited pH range of fluorescent dyes. In neutral, fluorescence data showed that several ROSs were generated by adding KIO_4. However, more strains showed higher resistance in KIO_4 than in NaClO. Differential behaviour of Keio mutants were observed among acid, neutral and alkaline pHs. *E. coli ΔrpoS* exhibited sensitivity in both alkaline and acid treatments in the presence of oxidative stress compared to that in neutral, where the reaction time of 1min in alkaline pH, being much shorter than that in acid and neutral (5min). This observation was supported that *rpoS* contributes to both acid and alkaline resistance where in general, tolerance of alkalinity for *E. coli* is lower than its acid resistance (Small et al., 1994). Sensitivity of *E. coli ΔompR* was observed in extreme pH groups, indicating that porin regulation involves in both acid and alkaline resistance. Through cross-comparison between KIO_4 and the reference reagents, H_2O_2 and NaClO at neutral, acidic and alkaline pH, it was observed that in neutral, fewer strains exhibited sensitivity in KIO4. Additionally, the strains showing sensitivity in KIO_4 treatment were not the same strains showing sensitivity in H_2O_2 or NaClO treatment. In acidic pH, more mutants exhibited higher sensitivity in KIO_4 treatment, compared to that in H_2O_2. In alkaline pH, strains with higher sensitivity in KIO_4 treatment did not match the strains with higher sensitivity to NaClO treatment. Therefore, it was suggested that the attack mechanism of KIO_4 towards the cells might be different from H_2O_2 or NaClO. KIO_4 does not seem as powerful as H_2O_2 and NaClO in neutral, but KIO_4 may serve as a catalyst that depends strongly on pH change and releases •OH, ^{-}OCl, $^1O_2$ and H_2O_2 to cause stress response in the cells. However, the generation rate of these oxidative compounds was not measured.

Differential killing effects on anode and cathode of electrochemical disinfection. *E. coli* can be inactivated by electric fields of 220V/cm within 6min (Machado et al., 2010). In this study, *E. coli* was treated at a much lower voltage, 12V, along with oxidizer KIO_4 for 5min, aiming to understand further on protective mechanism of the cells. The total energy input over the 5min treatment was ~3.6kJ, being less than one-quarter of 1kcal when 12V of power was applied. During the inactivation of *E. coli* using electrochemical method, reduction of cell counts was observed to be higher on cathode than on anode for most mutants. It was first thought that the sensitivity of these strains under such condition could be due to a combined effect of pH change and oxidative stress. However, while conducting the experiment, it was shown that pH affected by the electric field was within 1U on each electrode. When comparing treatments with KIO_4 in neutral and in electric field, mutants showed higher sensitivity on cathode. Hence, it must be considered that electricity itself somehow involves in destroying the cells. In an electric field of 50V/cm, cell membranes have been shown to have structural damage that electropores increase in sizes (Neumann et al., 1999; Machado et al., 2010). Peptidoglycans, which is the layer in between outer and inner membrane, are majorly comprised of N-acetylglucosamine (NAG). It was found that the concentration of NAG increases outside the cell after 5min exposure of *E. coli* to an electric field, indicating destruction of outer membrane of the cell (Boudjema, Kherat, Drouiche, & Mameri, 2019). Electric field can further disrupt the structure of peptidoglycans and cause pore formation that increases cell permeability (Boudjema et al., 2014). When compared the fluorescence assays in Table 6, it was observed that H_2O_2 was produced in both groups of treating the cells with KIO_4 alone and electrochemical disinfection with KIO_4. On anode, electron (e^-) and H^+ are released to the solution. In consequence, the pH value of anode is slightly acidic. Electron then travels to the cathode chamber, reacts with H_2O and forms OH^- which gives the cathode alkaline pH reading. In addition,

according to the data shown in Table 3, hydroxyl radicals (•OH) were formed in the presence of KIO_4. Therefore, the reaction among compounds was considered as:

$\bullet OH + H_2O + H^+ + e^- \rightarrow H_2O_2 + 2H^+ + 2e^-$, (Viswanathan, Hansen, & Nørskov, 2015).

The reaction explains the detection of H_2O_2 in electrochemical group. Also, due to the consumption of •OH in this reaction, the fluorescent probe HPF did not show any positive indications on the existence of •OH (Table 6). Existence of H_2O_2 in electric field also explains the observation on cathode, that deletions in either of the two major stress regulons in *E. coli*, *ΔrpoS* and *ΔoxyR* resulted in hypersensitivity (Zheng, Aslund & Storz, 1998; Loewen & Hengge-Aronis, 1994). Another mutant, *E. coli ΔtrxB*, where *trxB* encodes for thioredoxin reductase, also showed higher sensitivity on cathode. Thioredoxin has been located at the outer and inner membrane of *E. coli* and found to involve in reductions of various proteins (Bayer, Bayer, Lunn, & Pigiet, 1987). Though *trxB* is not the only gene responsible for expression of thioredoxin reductase, its absence leads to increase in thioredoxin oxidation (Bessette, Aslund, Beckwith, & Georgiou, 1999). Therefore, upon charging, absence of *trxB* and increase in cell permeability possibly lead to further cell destruction. Hypersensitivity of *E. coli ΔhypT* is unclear. However, due to its selective response to HOCl (Drazic et al., 2013), it could potentially indicate the generation of chlorine compound.

Conclusion

This study investigated the stress factors involved in electrochemical disinfecting system by applying multiple stresses to *E. coli* MG1655 and selected mutants, separately or combined, to observe differential behaviour of the strains. Potential stressors include oxidative stress, acid stress, alkaline stress and electrochemical stress. Two well studied disinfectants, H_2O_2 and NaClO were used as controls to evaluate the effectiveness of a new oxidizing reagent, KIO_4. It was found that levels of oxidative compounds generated by KIO_4 depended strongly on the pH. In addition, shift in pH enhanced the effect

of KIO_4 when disinfecting *E. coli*, especially in alkaline pH, indicating that KIO_4 might serve as a source for generation of ROS, instead of acting on cells directly. It was also found that electrical generated KIO_4 has bactericidal effect on *E. coli* cells (Okochi, Lim, Nakamura, & Matsunaga, 1997). However, within the time frame determined by preliminary experiments, killing effect observed when KI or KIO_3 were added to electric field (data not shown) was negligible. During electrochemical disinfection, electro-generated H_2O_2 by KIO_4 was higher on the cathode chamber compared to that of anode, which resulted in higher inactivation of *E. coli* on the cathode. Current research provides supportive evidence that KIO_4 catalyzes the generation of reactive oxidative components such as •OH, $_1O_2$ and H_2O_2 that can inactivate *E. coli* and facilitate the process of electrochemical disinfection. Moreover, it was found that chromosomally integrated LHR exhibited resistance throughout all treatment groups. Addition to the heat resistance discovered by Mercer et al. (2015), protective effect of LHR against alkaline was identified in this study. For future research, adjustments could be made on selection of electrode materials, such as PbO_2 and boron-doped diamond (BDD) to allow for higher efficiency by increasing the oxygen overpotential, thus maximizing the generation of reactive oxygen species; electrolyte composition, where ionized water replaces tap water to eliminate the involvement of chlorine. Pathogenic bacteria such as *E. coli* O157: H7 and other antibiotic resistant strains should be tested to determine whether the electrochemical disinfection is still effective and can be applied to industrial use.

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