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Department Chemie Lehrstuhl für Biotechnologie

(Prof. Buchner)

Bacterial HOCl resistance

Diploma Thesis

Athina Keramidiotou

Munich

Supervisor: Dr. Jeannette Winter

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Approved by the three members of the Examination Committee:

First examiner (Supervisor)
Dr. Winter Jeannette. Chair of Biotechnology, Research group “Oxidative Stress”. Department of Chemistry. Technische Universitaet Munich.

Second examiner
Prof. Kouretas Demetrios. Professor of Animal Physiology. Department of Biochemistry and Biotechnology. University of Thessaly.

Third examiner
Prof. Dr. Johannes Buchner. Chair of Biotechnology. Department of Chemistry. Technische Universitaet Munich.

Official Declaration

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Date **Signature**

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Περίληψη

Το γενικό θέμα της παρούσας διπλωματικής εργασίας είναι η ανθεκτικότητα των βακτηρίων στο υποχλωριώδες οξύ (HOCl). Αυτή η εργασία έχει τρεις στόχους: (1) την ανάλυση του ρόλου των ενζύμων αναγωγής σουλφοξειδίων μεθειονίνης, Msra και MsrB, (2) την κρυστάλλωση του ιδιοσύστατα ενεργού μεταλλάγματος του μεταγραφικού παράγοντα HypT (Hypochlorite specific Transcription factor), και (3) τον προσδιορισμό των γονιδίων που απαιτούνται για την αντοχή εξελιγμένων κυττάρων *E. coli* σε HOCl. Για να μελετηθεί, αν τα ένζυμα MsrA και MsrB παίζουν ρόλο στην ανθεκτικότητα των κύτταρων *E. coli* σε HOCl, διεξήχθησαν δοκιμές ανάπτυξης και βιωσιμότητας, συγκρίνοντας τρία στελέχη: ένα άγριου τύπου, ένα με απάλειψη του *hypT* και ένα με απάλειψη των γονιδίων *hypT* , *msrA* και *msrB*. Για τα συγκεκριμένα στελέχη *E. coli* σχεδιάστηκαν καμπύλες ανάπτυξης (growth curves) για να μελετηθεί πώς αναπτύσσονται αυτά τα στελέχη και πόσο γρήγορα θα ανακάμψουν από την κατεργασία με HOCl. Επίσης διεξήχθησαν, δοκιμές βιωσιμότητας προκειμένου να αναλυθεί ο αριθμός των βιώσιμων κυττάρων κατά τη διάρκεια της κατεργασίας με HOCl. Τα αποτελέσματα και από τα δύο πειράματα έδειξαν διαφορές μεταξύ του αγρίου τύπου και των μεταλλαγμάτων, κάνοντας σαφή την εμπλοκή του HypT και των Msr ενζύμων στην ανθεκτικότητα στο HOCl . Ο δεύτερος στόχος, ήταν η κρυστάλλωση του ιδιοσύστατα ενεργού μεταγραφικού παράγοντα HypT, όπου τρία από τα αμινοξέα μεθειονίνης του HypT (Met^{123}, Met^{206} και Met^{230}) έχουν υποκατασταθεί από γλουταμίνη. Προηγούμενες δοκιμές κρυστάλλωσης, για τον HypT αγρίου τύπου απέδωσαν καλά περιθλώμενους κρυστάλλους. Ωστόσο , για τον μεταλλαγμένο HypT, οι κρυσταλλώσεις απέδωσαν μόνο αδρανή υλικά και κρυσταλλικό ίζημα . Έτσι, προέκυψε η ανάγκη για τη σταθεροποίηση των συνθηκών προκειμένου να βελτιώσουμε την κρυστάλλωση. Αναλύσεις της θερμικής σταθερότητας της πρωτεΐνης πραγματοποιήθηκαν με σκοπό να εντοπιστούν οι συνθήκες που μπορούν να βελτιώσουν την θερμική σταθερότητα της μεταλλαγμένης πρωτεΐνης. Αυτές οι συνθήκες στη συνέχεια εφαρμόστηκαν στον επόμενο κύκλο απομόνωσης και καθαρισμού του μεταλλάγματος του HypT. Παρόλα αυτά, ακόμη και κάτω από αυτές τις συνθήκες η πρωτεΐνη μετατράπηκε πλήρως σε συσσωμάτωμα και δεν πραγματοποιήθηκαν νέες κρυσταλλώσεις. Έτσι, μέχρι στιγμής δεν έχουν ληφθεί κρύσταλλοι. Το τρίτο μέρος αυτής της εργασίας, αφορά τα γονίδια που εμπλέκονται στην ανθεκτικότητα σε HOCl. Η Jasmin Gundlach, πρώην μέλος του εργαστήριου, εξέλιξε κύτταρα *E. coli* με ισχυρή ανθεκτικότητα σε HOCl. Τα συγκεκριμένα βακτηριακά κύτταρα δείχνουν σημαντικές διαφορές στο πρωτέωμα τους, οι οποίες δεν οφείλονται σε χρωμοσωμικές μεταλλάξεις. Έχει παρατηρηθεί ότι, αυτά τα κύτταρα έχουν ένα διαφορετικό μοντέλο μεθυλίωσης της κυτοσίνης του DNA , η οποία είναι πιο έντονη, στα γονίδια dinF , *dppF* , *xdhD* , *mdtA* , *guaB* , *xylE* και *ompF*. Στόχος είναι να αναλυθεί ο ρόλος αυτών των γονιδίων στην ανθεκτικότητα σε HOCl διότι δεν είναι γνωστό, αν οι διαφορές στην μεθυλίωση της κυτοσίνης του DNA προκαλεί διαφορές στην έκφραση των γονιδίων. Ως εκ τούτου, δημιουργήθηκαν στελέχη απαλείφοντας κάθε φορά ένα από τα συγκεκριμένα γονίδια, προκειμένου να κατανοηθεί ο ρόλος τους στην ανθεκτικότητα σε HOCl. Πρώτα, εφαρμόστηκε η μέθοδος Datsenko και Wanner (2000) σε MC4100 και MC4100 (Δ*rpoH*) στελέχη, αλλά κλώνοι έχοντας τις απαλειφές ελήφθησαν μόνο για το στέλεχος MC4100 . Από κάθε έναν από τους κλώνους MC4100 που είχε την απαλειφή , παρήχθη κυτταρόλυμα φάγου το οποίο χρησιμοποιήθηκε για την διαδικασία μεταγωγής φάγου του στελέχους MC4100 (Δ*rpoH*), με την οποία ελήφθησαν κλώνοι του στελέχους MC4100 (Δ*rpoH*) με απαλειφές για τα γονίδια *dppF*, *ompF*, *xdhD* και *xylE*. Οι κλώνοι αυτοί υποβλήθηκαν σε διάφορους τύπους στρες (οξείδωση, άλατα, θερμότητα) και παρατηρήθηκαν πιθανές διαφορές στο φαινότυπο τους. Αυτές οι αναλύσεις δείχνουν ότι τα γονίδια *dppF*, *ompF*, και *xdhD* παίζουν ρόλο στην ανθεκτικότητα, καθώς η διαγραφή καθενός από αυτά τα γονίδια καθιστά τα κύτταρα πιο ανθεκτικά στο HOCl.

1. Summary

The general topic of the diploma thesis is bacterial HOCl resistance. This thesis has three aims: (1) analysis of the role of methionine sulfoxide reductases, MsrA and MsrB, (2) crystallization of the constitutively active HypT mutant, and (3) the identification of factors required for HOCl-resistance in evolved in *E. coli* cells. To understand if MsrA and MsrB play a role in HOCl resistance in *E. coli* cells, growth and viability assays were performed and compared for three strains: a wild type, a deletion strain for *hypT* and a deletion strain for *hypT*, *msrA* and *msrB*. Growth curves for these *E. coli* strains were performed to obtain an idea how these strains grow and how fast they recover from HOCl stress. Viability assays were performed in order to analyze the number of viable cells during HOCl stress. Results from both experiments showed differences between the WT and the mutants, unraveling a clear involvement of HypT and Msr enzymes in HOCl resistance. The second aim was the crystallization of the constitutively active transcription factor HypT, in which three methionine residues of HypT (Met123, Met206, and Met230) were substituted by glutamine. Initial crystallization screens were performed under conditions that yielded well-diffracting crystals for wild-type HypT. However, for the HypT mutant, crystallization screens yielded only aggregates and crystalline precipitate. Thus, the need for stabilizing conditions arose in order to change and improve the crystallization conditions. Thermostability assays performed in order to find conditions that may improve the thermostability of the mutant protein. These conditions were then applied for new purifications. However, the mutant protein aggregated completely under these conditions and new crystallization screens could not be conducted. So far, no crystals were obtained. The third part of this work concerns genes that are involved in HOCl resistance. Jasmin Gundlach, a former Ph.D. lab member, evolved strongly HOCl resistant E. coli cells. These show strong proteome differences but not due to chromosomal mutations. She observed that these cells have a different DNA cytosine methylation pattern, most strongly pronounced in the genes *dinF*, *dppF*, *xdhD*, *mdtA*, *guaB*, *xylE*, *ompF*. The aim is to analyze the role of these genes in HOCl resistance because we do not know, whether differences in DNA cytosine methylation cause differences in gene expression. Therefore, we created deletion strains in order to obtain an idea about their role in HOCl resistance. First, the Datsenko and Wanner (2000) method was applied to MC4100 and MC4100 (Δ*rpoH*) strains but clones were obtained only for MC4100. From each of the deletion clones obtained for MC4100, phage lysate was generated and used for phage transduction of MC4100 (Δ*rpoH*), by which deletion clones for the genes *dppF*, *ompF*, *xdhD* and *xylE* were obtained. These clones were subjected to different types of stress (oxidative, salt, heat) and potential differences in phenotype observed. These analyses show that *dppF*, *ompF,* and *xdhD* play a role in HOCl resistance as the deletion of each of these genes rendered cells more resistant to HOCl.

2. Introduction

2.1. Reactive oxygen species and Oxidative stress

The major sources of reactive oxygen species (ROS) are the electron leakage from mitochondrial and microsome electron transfer chains, phagocytosis, and the endogenous enzyme systems, like glucose oxidase, xanthine oxidase, thiol groups and flavins [1]. The reactive radicals produced from normal cell's metabolism or in response to an external signal (X-ray, UV, smoking) are known as pro-oxidants and play a dual role, sometimes beneficial to cells and organisms and sometimes harmful. The beneficial effects of the production of free oxygen radicals are observed at low/moderate concentrations and relate to normal roles in cellular response to stress, in signal transduction, in cell differentiation, gene transcription, the cell proliferation, inflammation and apoptosis [2, 4]. The harmful effects of reactive oxygen species exerted on biomolecules such as proteins, nucleic acids, and lipids can cause damage to cells and tissues, from which cells are protected by a series of antioxidants. Antioxidants are produced endogenously or are from external sources and include enzymes such as catalase, superoxide dismutase, glutathione reductase, glutathione peroxidase, vitamins A, C and E, and glutathione. Oxidative stress is defined as the imbalance between pro-oxidants and antioxidants in the cell and is due to either increased production of free radicals or a deficiency of cellular antioxidant mechanisms [2]. Oxidative stress is implicated in the pathophysiology of numerous diseases and in the aging process [3, 4].This is why it is particularly interesting to attempt to modify pharmacologically the response of organisms to oxidative stress.

The free radical is defined as an atom or molecule, which contains one or more unpaired electrons. The presence of unpaired electron gives to radicals a particular activity and can either give an electron or take an electron from other molecules, thus behaved as oxidizing or reducing substances. The free radicals produced by oxygen constitute the most important group of free radicals in living organisms. But there are some oxygen compounds, such as hydrogen peroxide and molecular oxygen, which although they are not free radicals behave as such because it is chemically very active and can lead to the formation free radicals.

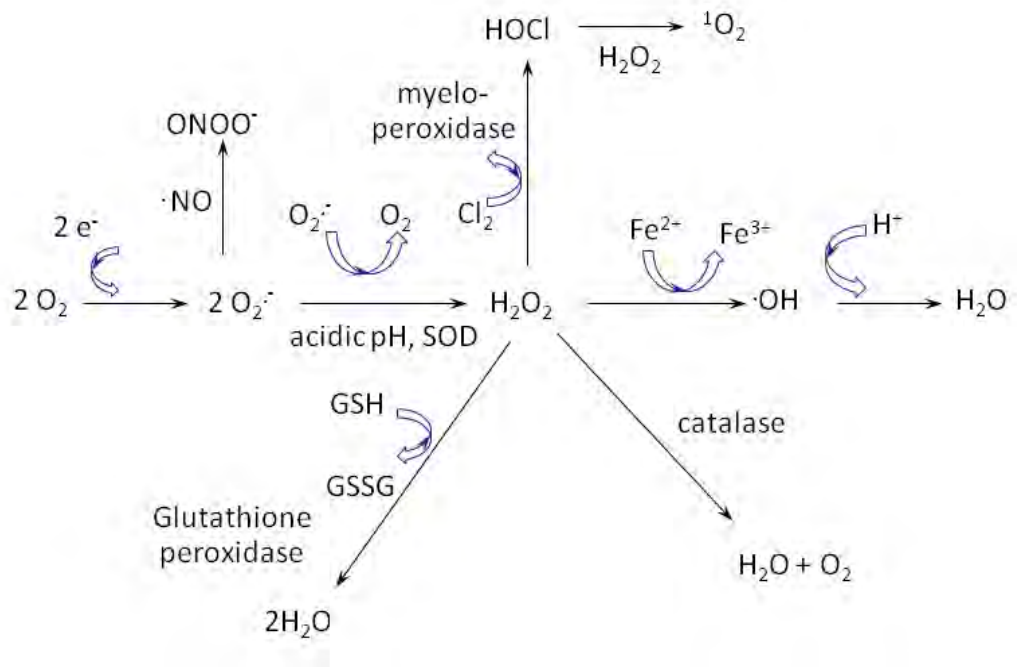


Figure 1. Formation and elimination of reactive oxygen species

ROS are created through a cascade. An electron is transferred to oxygen and superoxide is formed. Superoxide dismutase dismutates superoxide to hydrogen peroxide and molecular oxygen, under acidic pH. Through Haber-Weiss reaction, hydroxyl radicals are generated. If superoxide reacts with nitric oxide then peroxynitrite is formed which is highly reactive. Myeloperoxidase, which is released from macrophages, turns hydrogen peroxide to hypochlorous acid and singlet oxygen. Glutathione peroxidase catalyses the reduction of hydrogen peroxide through the oxidation of glutathione. Catalase catalyzes the formation of water and oxygen from hydrogen peroxide. This figure was taken from reference [3].

Table 1. Reactions that generate reactive oxygen species

Haber Weiss reaction	$2O_2\bullet^- + 2\ H+ \rightarrow H_2O_2 + O_2$ $O_2\bullet^- + Fe(III) \rightarrow O_2 + Fe(II)$ $H_2O_2 + Fe(III) \rightarrow HO\bullet + HO^- + Fe(II)$ $O_2\bullet^- + H_2O_2 \rightarrow HO\bullet + HO^- + O_2$
Fenton reaction	$H_2O_2 + Fe(II) \rightarrow HO\bullet + HO^- + Fe(III)$

2.2. Hypochlorous acid

Hypochlorous acid (HOCl) is the main ingredient of bleach, which is the most powerful disinfectant were used for cleaning purposes. HOCl is also produced by cells of the immune system during bacterial infection [6]. Phagocyte cells generate hydrogen peroxide (H_2O_2) and superoxide (O_2^-), and release the heme enzyme myeloperoxidase [5]. Myeloperoxidase produces HOCl from hydrogen peroxide (H_2O_2) and a chloride anion (Cl^-) (or the equivalent from a non-chlorine halide) during the phagocytes respiratory burst [5]. The importance of production of HOCl as a defense mechanism of the immune system was recognised by Ha et al. [7]. This study showed that in *Drosophila* the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme, dual oxidase (dDuox), is vital for the antimicrobial activity of the gut. When, dDuox was silenced in adult flies their gut is overgrown by bacteria leading to an increased mortality rate even if the infection was minor. After, specific reintroduction of dDuox, this was restored, showing that this oxidase through oxidative burst limits microbial proliferation in the gut. These results indicate that the oxidant mediated antimicrobial responses are not only characteristic of phagocytes but are common and among other cell types, like mucosal barrier epithelia.

The bactericidal effect of HOCl is due to its high reaction speed with many biomolecules, especially proteins [5], because they are the most abundant molecules in a cell. HOCl reacts with amino acids, preferentially with sulfur containing ones.

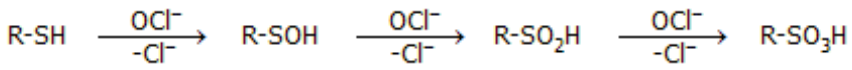


Figure 2. HOCl's reactions with thiol groups.

Typically the thiol group (-SH) is oxidized to a sulfenic (-SOH), which can further become oxidized to a disulfide bond (-S-S-), to sulfinic acid ($-SO_2H$), or to sulfonic acid ($-SO_3H$). The other thiol groups of a polypeptide can react with each of these intermediates, disturbing the protein conformation, which finally leads to the loss of protein function. This figure was taken from reference [8].

Moreover, chlorate substitutes hydrogen of amino groups. The formed unstable chloramine products interrupt the protein chain which is supported by the observation that 0.01M solution of HOCl is capable of breaking the proteins down accordingly to McKenna et.al [9].

Another mechanism is based on hydrolysis of monochloramine derivatives to aldehyde (Fig. 3).

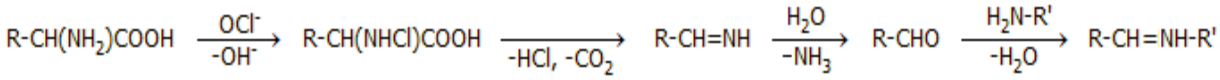


Figure 3. Hydrolysis of monochloramines derivatives to aldehyde.

Initially, HOCl reacts with the amino and carboxylic acid sites of a-carbon of the amino acids for the production of aldehyde. The hydrogen atom is substituted by chlorate. This leads to the formation of a monochloramine which is hydrolized to aldehyde. The aldehyde group can react with another amino group of the protein chain through the formation of a Schiff base, which changes the protein conformation. This figure was taken from reference [8].

Another major reason for the bactericidal activity of HOCl is that it leads to depletion of cellular energy in bacterial cells. HOCl oxidizes membrane components which could lead to rapid ATP hydrolysis. This is achieved by direct oxidation of a membrane-bound ATP-dependent enzyme or transport protein or oxidation of an ATP-independent membrane bound protein, which ends up in ATP hydrolysis by an ATP-dependent system that attempts to counterbalance the inefficiency of metabolism caused by the damage [10]. Barrette et al. have shown that in *E. coli* lethal doses of HOCl are accompanied by dramatically decreased ATP levels and proportionally increased AMP levels. This affected viability, as cells could not form colonies. In addition, the lower pH arising from HOCl addition changed the proton motive force and subsequently the respiration is inhibited leading to loss of energy, too [10].

Methionine is another major target of HOCl and from their reaction methionine sulfoxide (MetSO) is generated [11]. MetSO is a stable product and reduced back to methionine by methionine sulfoxide reductases in a slow rate enzymatic reaction [12]. Accordingly to Rosen et al., in uropathogenic *E. coli* high concentration of HOCl increased MetSO formation and reduced viability. The contribution of methionine oxidation by HOCl in bacterial killing was estimated by alternating the expression of methionine sulfoxide readuction enzymes, *msrA* and *msrB*. In double mutants increased bactericidal activity was observed, compared to the wild type. Additional support came from tests in *msrA* over-expressing strains where the bacterial survival was even higher than the wild type, indicating the direct role of methionine oxidation by HOCl in bacterial killing [13]. Proteins, as mentioned before, are the main target of HOCl, because of their cellular abundance. HOCl is proved to cause rapid oxidative protein unfolding in vitro and protein aggregation, which is irreversible, in vivo [17]. Bacteria under oxidative and heat stress use the chaperone Hsp33 as one of the defense mechanisms. This chaperone is redox regulated and in order to be activated requires unfolding conditions (e.g., high temperatures or HOCl) and oxidative stress at the same time. Only under HOCl is activated without the need for high temperature. The activation is achieved by oxidative unfolding

of Hsp33's C-terminal redox switch domain. As a result, activation of Hsp33 into a chaperone holdase protects cell's proteins from HOCl caused aggregation. Moreover, in the same study they tested the response of heat shock protein GrpE, disulfide oxireductase DsbA and citrate synthase (CS) under HOCl treatment. Depending on the HOCl:protein ratio, proteins lost their secondary structure. Results from mass spectrometry showed oxidative side chains modifications, and methionine oxidation too, which are not restored even after the addition of thiol reductants. This indicates that maybe the modifications are irreversible or they may require an advanced reducing system like methionine sulfoxide reductases [14]. Thus, HOCl was identified as a highly protein unfolding reagent, with major contribution to bacterial killing.

2.2.1. Protective mechanisms against HOCl

While HOCl is important in the accurate immune response and for public health maintenance, only little is known about how microorganisms react to this type of stress.

HOCl is toxic for bacteria, even in low concentrations [9]. Nevertheless, protective mechanisms exist in order to cope with this stress. Major bacterial responses include scavenging the generated reactive species and protecting against protein unfolding.

2.2.1.1. Methionine sulfoxide reductases

Methionine sulfoxide reductases (Msr) are major enzymes for the maintenance of protein function. These enzymes are conserved among archaea, bacteria and eukaryotes and their role is to reduce MetSO back to methionine with MsrA being specific for the S-form and MsrB reducing the R-form of MetSO [16]. The steps of this catalytic mechanism is first the production of a sulfenic intermediate and the release of repaired Met residue and then the recycling reduction of MsrA/MsrB in order to restore their activity [17]. The amino acid sequence and the structure differ between these enzymes and the only common feature is their catalytic mechanism. Also, there are differences related to their location on DNA and the copy number, which differs among prokaryotic organisms and among eukaryotes [17]. Studies have shown that MsrA/MsrB may act synergistically with chaperone and/or degradation pathway in order to relieve cell from damaged proteins [17]. One such example is in *Helicobacter pylori* where the repair, by Msr enzymes, of oxidized Met residues of catalase after treatment with HOCl could not restore the activity of catalase and only after the addition of GroEL the catalase was activated again [18]. In contrast to proteins that are *in*activated by Met oxidation, some recent examples exist where proteins are *activated* by methionine oxidation. The three so far known examples are calcium/calmodulin-dependent protein kinase II (CaMKII), which is activated after myocardial infarction in mice [19], HypT which become activated under HOCl oxidative stress[28], and actin, which disassemblies when is oxidized (by Mical1 and Mical2 proteins) and

assemblies when is reduced (by MsrB1) [20]. All of them are reversible oxidations and the reduction is accomplished by Msr enzymes.

2.2.1.2. Glutathione

Glutathione of proteobacteria and eukaryotes and other low molecular weight thiols of bacteria contribute actively in maintaining the redox balance of cells. These molecules either react directly with the oxidants preventing them from reacting with other cellular components or modify proteins to prevent further oxidation. One molecule of glutathione for instance reacts with four molecules of reactive chlorine species, which is supported by the fact that *E. coli* mutants lacking glutathione are more sensitive to HOCl exposure. Moreover, in *Bacillus spp*. methionine synthetase is regulated by bacillithiolation under HOCl stress, which causes halting of translation and protein synthesis probably allowing cells to recover during and after stress [15].

2.2.1.3. Hsp33

Hsp33 is a chaperone and, as mentioned before, activated under HOCl stress by unfolding of its C-terminal domain. Hsp33 has four cysteines that form two intramolecular disulfide bonds and release zinc upon oxidation and, as a result the C-terminal domain unfolds. It does not require nucleotide binding or hydrolysis in order to be activated. Thus, there is no need for ATP, which has low availability under HOCl stress, rendering Hsp33 a really important protective mechanism [15].

2.2.1.4. DNA repair enzymes

As HOCl damages DNA, DNA repair enzymes constitute an important defence mechanism. There are indications that chloramine (NH_2Cl) and other ROS generated from HOCl react with DNA causing its damage. However bacteria have evolved efficient methods such as systems of homologous recombination, repair of damaged nucleotides and DNA repair polymerases to prevent DNA damage. In mutants for homologous recombination genes, *recA* and *recB*, the damage by HOCl treatment was more severe and cells were more sensitive [21]. The DNA binding protein, Dps, offers also significant protection in wild type cells. Dps acts by oxidizing Dps bound iron, limiting the generation of hydroxyl radicals, and it forms DNA co-crystals [22]. According to Dukan et al. (1996) the protection of DNA by its coverage with Dps proteins may prevent DNA from access of reactive species [21].

2.2.1.5. Cell envelope protection

HOCl attacks at first the cell envelope decreasing the protection of the bacterial cell. That is why bacteria react by changing the synthesis and the structure of their outer membrane. Under HOCl the *ycfR* gene is induced causing a decrease in cell permeability and increase in hydrophobicity of the outer membrane [15]. *ycfR* mutants are more sensitive when treated with HOCl than the wild type [23]. Also outer membrane porin genes (*omp*) are downregulated under HOCl in *S.enterica* and

P.aeruginosa. Particularly in *S.enterica omp* mutants are more resistant in HOCl because the permeability of membrane was reduced [15].

2.2.1.6. NemR, HypR

NemR of *E. coli* is a conserved TetR family repressor, which senses the HOCl and N-chlorotaurine [25]. The oxidation of its cysteine residues by HOCl or N-chlorotaurine leads to a decrease of its DNA binding affinity. As a result, the downstream target gene expression is de-repressed. Michael J. et al. (2013) demonstrated that under HOCl stress the NemR regulated genes encoding for glyoxylase 1 and N-ethylmaleimide reductase are upregulated conferring resistance by detoxifying methylglyoxal and reactive electrophiles [25].

HypR is a MarR-type regulator of the OhrR and DUF24 families which sense organic peroxides, diamide, quinines or aldehydes through its cysteine-thiol group oxidation and the formation of a disulfide bond. HypR under stress conditions induces the transcription of flavin oxidoreductase HypO that confers protection against HOCl stress in *Bacillus subtilis* [26].

2.3. The transcription factor HypT

The first identified transcription factor specific to HOCl is HypT [28], formerly known as YjiE [24]. HypT is a LTTR that is conserved among proteobacteria and eukaryotes and protects *E.coli* against HOCl stress [24]. LTTRs are prokaryotic transcription factors regulating many different genes, featuring a DNA-binding domain and a co-inducer response domain, in which the co-inducer (product of metabolic pathway) binds and typically activates or represses the LTTRs [27].

HypT forms dodecameric ring-like structures, which probably serve as a storage form. Dodecamers dissociate into dimers and tetramers upon binding to DNA and constitute the DNA binding active species. HypT regulates genes that are involved in cysteine and methionine biosynthesis, sulfur metabolism and iron acquisition. The down-regulation of iron acquisition genes aims at decreasing free iron levels and consequently the impairment of the Haber Weiss and Fenton reactions. As a result, the oxidized metabolites are restored and the HOCl mediated production of ROS is decreased. HypT is activated by methionine oxidation to methionine sulfoxide (Met-SO) by HOCl [28]. Analysis of point mutants for Met showed that the most important Met residues are Met123, Met206, and Met230. Their substitution by isoleucine renders the protein inactive and unable to confer HOCl resistance [28]. The substitution of these Met residues by glutamine resulted in HOCl resistance and in regulation of the target genes *metN*, *cysH*, *metB*, and *fecD* independently of HOCl stress. This triple substitution of Met to Gln rendered HypT constitutively active.

Table2. Bacterial protection factors against HOCl

Factor	Description	Function
Methionine sulfoxide reductases (Msr)	Reductases of methionine sulfoxide to methionine	Protein repair
Glutathione	High reactivity with reactive chlorine species (RCS)	Acts as scavenger
Hsp33	Chaperone	Protects cellular proteins from HOCl-induced aggregation
RecA	Recombinase	Homologous recombination for DNA repair
Dps (DNA protecting under stationary phase)	Iron-sequestering DNA-binding protein	Protection of DNA
ycfR	Small outer membrane protein	Reduces the permeability and increases the hydrophobicity of the outer membrane
HypT / YjiE	Transcription factor	Upregulation of cysteine and methionine synthesis, downregulation of iron aquisition decreases intracellular iron levels / protects cells from damage
NemR (N-ethyl**m**aleimide reductase **r**epressor)	Transcription factor	Derepression of N-ethylmaleimide reductase and glyoxalase 1→ electrophiles detoxification
HypR	Transcription factor	Positively controls flavin oxidoreductase HypO → Confers protection against HOCl stress

Data were taken from reference [15].

2.4. Identification of factors required for HOCl resistance in evolved E.coli cells

A former member of the lab, Jasmin Gundlach, evolved strongly HOCl resistant *E. coli* strains. These strains show strong proteome differences, which probably contribute to HOCl resistance. Importantly, however, such alterations are not due to genomic mutations, which happen usually in response to growth limiting conditions [29]. Instead, she found out that these strains have a different DNA cytosine methylation pattern. DNA methylation is one component of epigentics and an important factor in gene regulation [30]. The genes with different methylation pattern are *dinF* (DNA damage inducible SOS response protein), *dppF* (dipeptide transporter), *xdhD* (catalyze oxidation of xanthine), *mdtA* (multidrug efflux system, subunit A), *guaB* (inosine-5'-monophosphate dehydrogenase involved in de novo guanine nucleotide biosynthesis), *xylE* (D-xylose/proton

symporter) and *ompF* (outer membrane porin). The strong HOCl resistance remained stable even when evolved cells were incubated in the absence of HOCl stress, indicating that they are beneficial at no stress conditions too. The RNA levels of these genes were constitutively altered, even though at not statistically significant level, and this may play role in HOCl resistance, either directly or by affecting other factors.

2.5. Objectives

The general topic of this diploma thesis is bacterial HOCl resistance, which includes three separate projects. The aim of the first project is to figure out the role of methionine sulfoxide reductases MsrA and MsrB, in HOCl stress. For this, three *E. coli* strains (wild type, *hypT*$^-$ and *hypT*$^-$ *msrA*$^-$ *msrB*$^-$*)* should be stressed with HOCl. The rate of their growth and the percentage of viability upon HOCl stress should be observed and then the differences between the strains analyzed. The second project concerns the HOCl specific transcription factor HypT. In this work the goal was the crystallization of the constitutively active mutant of HypT and the solution of its structure. In order to achieve this, the protein was purified by affinity and ionexchange chromatography in standard buffer and by size exclusion chromatography in MES buffer and salt. Finally, crystallization screens should be performed. The last project is the identification of factors required for HOCl-resistance in evolved in *E. coli* cells. This project is based on the work of Jasmin Gundlach (a former PhD member of the lab). She, subjected *E. coli* cells to successively increased HOCl and obtained strong HOCl-resistant cells for MC4100 (Δ*rpoH*) strain. Evolved lineages showed no mutations. Nevertheless, differences in DNA cytosine methylation were observed, compared to the ancestor, in stress related genes. Single deletion of each of these genes should be generated and the deletion strains subjected to various types of stresses in order to observe phenotypic differences and to obtain an idea about their role in HOCl stress.

3. Material and Methods

3.1 Materials used

3.1.1. Chemicals

Agar-Agar	Serva (Heidelberg, Germany)
Agarose	Serva (Heidelberg, Germany)
Ampicillin sodium salt	Roth (Karlsruhe,Germany)
Arabinose	Sigma-Aldrich (St. Louis, USA)
Chloramphenicol	Merck (Darmstadt, Germany)
Bromphenolblue S	Serva (Heidelberg,Germany)
Dithiothreitol (DTT)	Roth (Karlsruhe, Germany)
Imidazol	Sigma-Aldrich (St. Louis, USA)
Isopropyl-β-D-thiogalactopyranoside (IPTG)	Roth (Karlsruhe, Germany)
Kanamycin sulfate	Roth (Karlsruhe, Germany)
LB medium (Lennox), powder	Serva (Heidelberg,Germany)
Sodium hypochlorite solution, available chlorine 10-15 %	Sigma-Aldrich (St. Louis, USA)
Protease inhibitor Mix HP	Serva (Heidelberg,Germany)
Serva Blue G-250	Serva (Heidelberg,Germany)
Serva Blue R-250	Serva (Heidelberg,Germany)
Tris(hydroxymethyl)aminomethane	Roth (Karlsruhe, Germany)
Hydrogen peroxide, 30 wt. % in H_2O	Sigma-Aldrich (St. Louis, USA)

3.1.2. Proteins

Enzymes

Go-Taq polymerase	Promega (Madison, USA)
Phusion high-fidelity DNA-polymerase	New England Biolabs (Ipswich, USA)

3.1.3. Other Materials

Standards

peqGOLD 1 kb ladder Orange G	PEQLAB (Erlangen, Germany)

Kits

Wizard® *Plus* SV Minipreps DNA purification systems	Promega (Madison, USA)
Wizard® SV gel and PCR clean-up system	Promega (Madison, USA)

Chromatography Columns

HisTrap FF (5ml)	GE Healthcare (Amersham, UK)
HiTrap Q FF (5ml)	GE Healthcare (Amersham, UK)
Superdex-75 (330ml)	Amersham Bioscience (USA)

Filtration, Concentration and Dialysis or Desalting units

Amicon Ultra-30 (10000 MWCO) filter units	Millipore (Billerica, USA)
Amicon Ultra-10 (10000 MWCO) filter units	Millipore (Billerica, USA)
Syringe filter (0,22 µm)	Millipore (Billerica, USA)
Cellulose acetate filter (0,45µm)	Sartorius Stedim Biotech (Göttingen, Germany)

Cuvettes

Plastic half-micro cuvvettes	Sarstedt (Nümbrecht, Germany)
Gene Pulser® cuvvettes (0,1 cm)	Bio-Rad (München, Germany)

Tubes, microtiter plates and Petri dishes

PE tubes (50ml, 15ml)	Greiner&Söhne (Nürtingen, Germany)
PCR reaction tubes (0,2ml)	Ratiolab (Dreieich, Germany)
Sterile microtiter plates (96 well)	Sarstedt (Nümbrecht, Germany)
Petri dishes 120x120x16 mm	nerbe plus (Winsen/Luhe, Germany)

3.2. Materials of crystallization

96-well Crystallization plates (3-hole)	Art Robbins Instruments
	Dunn Labortechnik (Asbach, Germany)
Linbro SuperClear pregreased 24 Platten	Jena Bioscience (Jena, Germany)
Crystallization Screens	Hampton Research VWR (Ismaning, Germany)
Cryo Loops	Hampton Research VWR (Ismaning, Germany)

3.3. Equipment

UV-VIS-Spectrophotometer

Nanodrop ND-1000 spectrophotometer	PEQLAB (Erlangen, Germany)
Ultraspec 1100 pro	GE Healthcare (Amersham, UK)

Gel electrophoresis

45-2020 gel electrophoresis unit (Agarose gel)	PEQLAB (Erlangen, Germany)

Voltage sources

EPS 601	GE Healthcare (Amersham, UK)

Scales

Analytical balance BP 121 S	Sartorius (Göttingen, Germany)
Laboratory balance 1601 004	Sartorius (Göttingen, Germany)
Semi-micro balances BL 310 und BL 1500S	Sartorius (Göttingen, Germany)

Centrifuges

Avanti J26XP (JA-10/25 rotor)	Beckman Coulter (Krefeld, Germany)
Fresco17	Heraeus (Thermo Scientific,Waltham,USA)
Universal 16R	Hettich (Tuttlingen, Germany)

Chromatography

ÄKTA-FPLC System	GE Healthcare (Amersham, UK)
with a fraction collector Frac900	

Incubators

Thermomixer comfort	Eppendorf (Hamburg, Germany)
Shaking with Unimax1010 Inkubator1000	Heidolph (Schwabach, Germany)

Gel Documentation Systems

BioDoc-It® imaging system	PEQLAB (Erlangen, Germany)

Other devices

PCR Thermocycler Primus 25	PEQLAB (Erlangen, Germany)
Stratagene Mx3005 P QPCR System	Agilent Technologies (Santa Clara, USA)

Gene pulser Xcell elektroporation device	Bio-Rad (München, Germany)
Constant cell disruption system basic Z	Constant Systems (Northants, UK)
pH-meter multical pH 538	WTW (Weilheim, Germany)
SANOclav autoclave	Adolf Wolf (Bad Überkingen – Hausen, Germany)
Ice machine	Ziegra (Isernhagen, Germany)
Magnetic stirrer RCT basic	IKA Werke (Staufen, Germany)
MS2 minishaker (Vortexer)	IKA Werke (Staufen, Germany)
-80°C-cabinet U725 Innova	New Brunswick Scientific (Nürtingen, Germany)

3.4. Programms

Adobe Photoshop 9.0	Adobe Inc (San Jose, USA)
Adobe Reader 7.0	Adobe Inc (San Jose, USA)
Microsoft Office 2007	Microsoft (Redmon, USA)
Origin 8.0	Originlab (Northhampton, USA)

3.5. Buffer and Media

Solutions and buffers for microbiological work were autoclaved or sterile filtered immediately after their production (121°C, 30 min). Unless otherwise stated, the solutions were stored at room temperature.

3.5.1 Media

All media components were weighed, dissolved and autoclaved. Antibiotics were not added until shortly before use of the medium.

LB-Medium 20 g/l LB-Medium Pulver

(*lysogeny broth*)

To prepare the LB agar medium 15 g / l agar-agar was added. After autoclaving, the medium was cooled to a temperature below 50°C and then antibiotic added, if was necessary. Subsequently, the plates were poured and stored after solidification at 4°C. For the LB top agar medium 7.5 g / l agar was added and after autoclaving and cooling below 50°C, 10 mM MgSO 4 and 5 mM CaCl2 were added.

3.5.2 Antibiotic -Stock solutions

Ampicillin-stock solutions: 200 µg/ml in H_2O

Chloramphenicol-stock solutions: 35 mg/ml in ethanol

Antibiotics were stored at -20°C, unless otherwise noted, and added to the medium in the ratio 1:1000.

3.5.3 Buffers for molecular biology methods

50x TAE-Puffer:	2 M Tris/Acetat
	50 mM EDTA
	adjusted with acetic acid to pH 8.0
DNA-loading buffer:	50 % (v/v) glycerin
	0, 025 % (w/v) Xylencyanol
	0, 025 % (w/v) bromphenolblue
10x TBE-Puffer:	0, 89 M Tris
	0, 02 M EDTA
	0, 89 M boric acid

3.5.4. Buffers for preparative methods

Cell lysis buffer:	50 mM NaH_2PO_4 pH 7,5
	300 mM NaCl
	10 mM Imidazol
HisTrap buffer A:	50 mM Tris pH 7,5
	300 mM NaCl
	10 mM Imidazol
HisTrap buffer B:	50 mM Tris pH 7,5
	300 mM NaCl
	750 mM Imidazol
Dilution buffer:	10 mM NaH_2PO_4 pH 7,5
	5 mM EDTA
Q-Sepharose buffer A:	10 mM NaH_2PO_4 pH 7,5
Q-Sepharose buffer B:	10 mM NaH_2PO_4 pH 7,5
	600 mM NaCl
Gel filtration buffer:	20 mM MES pH 6, 200 mM NaCl

3.6. Oligonucleotides

Table 3: List of oligonucleotides used in this work

Name	Sequence 5´→ 3´
D xhdD_Fw	GGGGAGGAAGCCGTatgATCATCCACTTTATGTGTAGGCTGGAGCTGCTTC
D xhdD_Rv	CGGCCATTTATTATttaTATTTTTTCCAGCGCCATATGAATATCCTCCTTAG
D mdtA_Fw	GAAACTCTTAACGatgAAAGGTAGTTATATGTGTAGGCTGGAGCTGCTTC
D mdtA_Rv	TAACACCTGCAtcaGGAGCGTGCTCCATATGAATATCCTCCTTAG
D dinF_Fw	GAGACCGCGatgCCGCCTGGCGTCGTGTGTAGGCTGGAGCTGCTTC
D dinF_Rv	CAGAATTTTTAACCGtcaCGTTGCGGCAAACCACATATGAATATCCTCCTTAG
D ompF_Fw	GAGGGTAATAAATAatgATGAAGCGCAATATTTGTGTAGGCTGGAGCTGCTTC
D ompF_Rv	AAAGAGGTGTGCTAttaGAACTGGTAAACGCATATGAATATCCTCCTTAG
D guaB_Fw	CGA GATATTGCCCatgCTACGTA TCGCTATGTGTAGGCTGGAGCTGCTTC
D guaB_Rv	GGGCGAAGAGAAtcaGGAGCCCAGACGGCATATGAATATCCTCCTTAG
D dppF_Fw	GGA GGCCGACACTatgAGTACGC AAGAGGTGTGTAGGCTGGAGCTGCTTC
D dppF_Rv	CGGTTTTGTGAGttaACGCTGCGGATTTTCCATATGAATATCCTCCTTAG
D xylE_Fw	CTAAGGCAGGTCTGAatgAATACCC AGTATATGTGTAGGCTGGAGCTGCTTC
D xylE_Rv	TGGACAGGAAGAttaCAGCGTAGCAGTTTGCATATGAATATCCTCCTTAG
xdhD_ctr_Fw	GATGGCGTCG CCAGTAAAGCACTGC
xdhD_ctr_Rv	GGTCAGAACCTGCATGGTTTATATC
mdtA_ctr_Fw	CCTTTCGCC ATGCAAGCAGTCTATC
mdtA_ctr_Rv	GACATCCGGGCTGGCACCTGGGTAG
dinF_ctr_Fw	GGTCGTTGT CGCACGTATTGATGAC
dinF_ctr_Rv	CATCATCGGTCAGTTTGCCCCATTG
ompF_ctr_F	GTTACATATT TTTTCTTTTTGAAAC
ompF_ctr_Rv	GTAATGTTCTCAAACATGACGAGG
guaB_ctr_Fw	CTAACGGTA AAAATTGCAGGGGATTG
guaB_ctr_Rv	GTGCTTCTGTCACATCCCACGCCCAC

dppF_ctr_Fw	GCTCAGGACA AAGAACGTCTGGCG
dppF_ctr_Rv	GTGTTCTTTCAGTTGGGAAGCCCGT
xylE_ctr_Fw	CCCACTTACG ATAATTCTCTTTCGT
xylE_ctr_Rv	GCTACGTTACTTTGTCTATATTGGG

3.7. Strains

Table 4: List of strains used in this work

#	Parental strain	Genetic marker / resistance / derivative	Plasmid / insert	Resistance	Comments / source
AK1	MC4100	*xdhD*$^{-}$		Cm^{25}	This work
AK3	MC4100	*xylE-*		Cm^{25}	This work
AK4	MC4100	*dinF-*		Cm^{25}	This work
AK7	MC4100	*dppF-*		Cm^{25}	This work
AK8	MC4100	*xylE-*		Cm^{25}	This work
AK9	MC4100	*dppF-*		Cm^{25}	This work
AK11	MC4100	*ompF-*		Cm^{25}	This work
KMG232	C600	*yjiE-*		-	Drazic et al. 2013
HM15	C600	*yjiE-,msrA-,msrB-*		Cm^{10}, Kan^{50}	Drazic et al. 2013
JW181	C600	*yjiE*		-	Lab stock (Drazic et al. 2013)
JW356	BW25113		pKD3	Cm25	lab stock
JW342	BB7222		pKD46	Amp200	04/2006 Grow at 30C!, for Datsenko and Wanner, 2000 mutagenesis
JW012	BB7222	MC4100 ara+			Lab stock
JW013	BB7224	BB7222 *rpoH*::Km		Km20	
KMG91	MG1655	Δ*rpoH*	pKD46	Amp^{200}	Lab stock
AD 27	BL21(DE3)	*yjiE*(M123Q, M206Q, M230Q)	pET11a	Amp^{200}	Drazic 2013

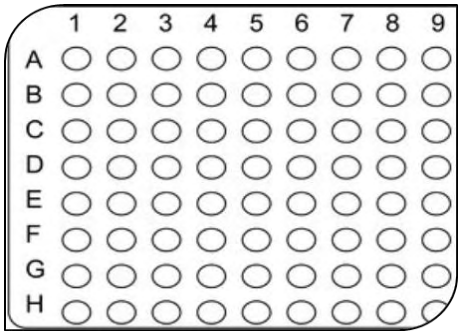
3.8. General methods

3.8.1. Growth Curves

In order to examine the role of MsrA and MsrB proteins in growth of bacteria, three different strain cultures were prepared in 10 ml LB media, incubated over night at 37°C, 135 rpm. The strains used were, *E. coli* C600 (WT), KMG232 (C600 *yjiE*-) and HM15 (C600 *yjiE*-, *msrA*-, *msrB*-). 150 µl from each culture were added to 30 ml LB media and incubated at 37°C, 135 rpm. OD measurements were taken every 30 min until OD_{600} ~0.8. Then, each culture was stressed with different concentrations of HOCl (2.5 mM, 3 mM, 3.5 mM) and incubated at 37°C, 135 rpm for 15 minutes. After stress, 5 ml of 1xLB were added in order to quench the HOCl and the OD was measured immediately using the spectrophotometer. Further measurements were taken every 30 min until OD_{600} ~0.8. LB was used to blank the spectrophotometer and the samples were diluted with water.

3.8.2. Viability Assay

For the viability assay the same three strains were used (WT, *yjiE*-, *msrA*- *msrB*-) in 10 ml LB media, incubated over night at 37°C, 135 rpm. 50 µl cells from each culture were inoculated into 10 ml LB and incubated at 37°C until OD was ~0.8. For the next step, nine flasks were prepared with 5 ml LB and 250 µl cells were added. Then the cells were stressed with HOCl at 2.75 mM, 3 mM, and 3.25 mM final concentration, respectively. Samples were taken every 30 minutes for 120 minutes in total, serially diluted and spotted onto LB plates, marked with time points 0', 30', 60', 90', 120' and the sample number (two plates for every time point: samples 1 to 5 in the one and samples 6 to 9 on the other plate). The plates were incubated at 37°C overnight.



Dilution of cells: LB
1:1
1:30
1:900
1:27000
1:810000

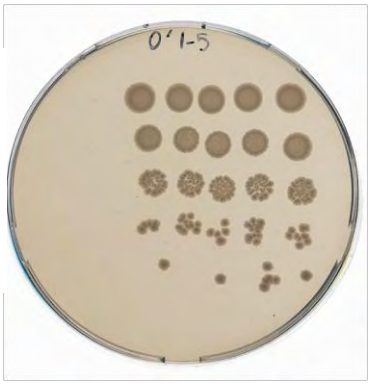


Figure 4. Example of dilution and spotting.

3.8.3 Protein purification

3.8.3.1. Expression and preparation of cell Lysate

The *E. coli* strain AD27 (BL21 (DE3) pET11a-*yjiE*$^{M123Q,\ M206Q,\ M230Q}$) was used for production and purification of the constitutively active HypT protein. For the culture, cells (glycerin stocks from -80^{o}C) were inoculated in 4×1.5 l LB media plus antibiotic (200 μg/ml ampicillin) and incubated over night at 37^{o}C without shaking. Then, cultures were incubated at 25^{o}C, 135 rpm for 1 hour. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.1 mM final concentration) was added to induce expression. After 4 hours at 25^{o}C shaking (135 rpm), cells were harvested by centrifugation (JA-10 rotor, 6,000 rpm, 4^{o}C, 10 minutes). The cell pellet was re-suspended in 15 ml of ice cold disruption buffer and protease inhibitor HP (1:400) was added. The cell pellet was stored in 50 ml Greiner tube at -80^{o}C. For the preparation of cells' lysate, cells were thawed, disrupted using a French press (two passages at 1.8 kbar), and centrifuged (20,000 rpm, 8^{o}C, 45 minutes). The supernatant was filtrated using a 0.2 μm sterile filter and used for purification.

3.8.3.2. Affinity chromatography

The His Trap FF column (5 ml column volume) was equilibrated with 5 column volumes of His Trap buffer A. The cell lysate was loaded onto the column via a 150 ml super loop. Then, the column was washed with His Trap buffer A to reduce unspecific bounding. Bound protein was eluted with a gradient to 100 % His Trap buffer C, collecting 3 ml fractions. Alarm pressure was set at 0.5 MPa and the flow rate at 5 ml/min. Fractions corresponding to the protein were pooled.

3.8.3.3. Ion exchange chromatography

A 5 ml Hi-Trap Q FF column was equilibrated with 5 column volumes of Q-Seph buffer A. Pooled fractions from the His Trap FF column were diluted to obtain 200 mM final salt concentration using dilution buffer. Then, the protein solution was loaded onto the column via a 150 ml super loop. The column was washed from unspecific bounding with Q-Seph buffer A until absorbance reached the baseline. Bound protein was eluted with a gradient to 100 % Q-Seph B buffer, collecting 2 ml fractions. Alarm pressure was set at 0.5 MPa and the flow rate at 5 ml/min. Fractions corresponding to the protein were pooled and stored at 4^{o}C until the next step.

3.8.3.4. Thermal shift assay

Thermal shift assay experiments were carried out in order to test the thermo stability of the protein under various conditions using a quantitative real-time PCR machine. Protein (1 μM, final) was assayed in 22 μl volume under 20 mM MES (pH 6) buffer plus additives dNTPs (0 -4 mM), NaCl (200 - 600 mM) and ATP (0 -20 mM) in a 96-wells plate. What it should be taken in consideration is that the protein used for the assay already contained 400 mM NaCl. So, in order to get a 600 mM NaCl final concentration only 200 mM NaCl final concentration was added into the sample and not 600 mM. SYPRO Orange (dilution 1:1.00) was added as a fluorescence probe. Samples briefly centrifuged (~1000 × g-force, 1 min) for the assay plate to mix compounds into the protein solution. Excitation and emission filters for the SYPRO-Orange dye were set at 465 and 590 nm, respectively. The temperature was raised at 1°C/min from 25°C to 80°C and fluorescence readings were taken at each interval. The results analyzed by Origin 8.0.

3.8.3.5. Gel filtration or size exclusion chromatography

A 330 ml Superdex-75 column was equilibrated with 330 ml of 20 mM MES buffer, pH 6 containing 200 mM NaCl. Protein solution was concentrated at 20 ml in order to load it in the column. Then, the protein solution was loaded onto the column via a 150 ml super loop. Bounded protein was eluted with a gradient of 100 % 20 mM MES buffer, collecting 95 fractions of 3.5 ml. Alarm pressure was set at 0.3 MPa and the flow rate at 2.5 ml/min. Fractions corresponding to the protein were pooled and stored at 4^{o}C until the next step.

3.8.3.6 Concentration of the protein solution

The protein concentrating procedure consists of several centrifuging steps. For this purpose an Amicon Ultra 30K device – 30,000 NMWL was used. The protein solution after the Gel filtration column was centrifuged at 3,500 rpm, 4^{o}C for 20 min, several times until a volume of 500 µl was reached. Afterwards, these 500 µl were transferred to a 10 K microcon, centrifuged (12,000 g, 4°C, 5 min) and then the concentration was measured with Nanodrop (e:32,43, MW:35,5). When the desired concentration was reached, the protein was transferred to a 1.5 ml Eppendorf tube. The protein was centrifuged (13,300 rpm, 4°C, 10 min) to remove aggregates and the supernatant transferred to a fresh 1.5 ml tube and the concentration measured again. Samples were stored on ice and used for crystallization screens.

3.8.4Construction of deletion strains

3.8.4.1. Deletion PCR

In order to study the role of genes *dinF, dppF, guaB, mdtA, ompF, xdhD and xylE*, deletion strains attempted to be generated for each of these genes. The chloramphenicol resistance cassette was amplified by PCR using pKD3 plasmid as template and the deletion primers from Table.3. The composition of the 50 µl PCR reaction is listed in Table.5. The pKD3 was isolated from *E.coli* strain BW25113 (JW356) and primers diluted in 1:10 with ddH_2O before usage. The primers used carry a ca. 30 bp sequence homologous to the gene to be deleted (ca. 15 bp up- and downstream of the ATG start) and a region homologous to the pKD3 plasmid. As a result, the PCR product contained the FRT-flanked chloramphenicol resistance gene flanked by gene specific sequences. After the PCR reaction, the products were mixed with 10 µl 6x DNA loading buffer and analysed on a 1.5% agarose gel stained with DNA Stain G. After verifying the correct size of the PCR products on the gel with UV light emission, the bands were cut out and purified with WizardSV Gel and PCR Clean up-system.

Table 5. Composition of PCR for the generation of deletion constructs

Component	Volume
Plasmid DNA	0.7 µl
10mM dNTPs	1 µl
Deletion Fw primer (1:10)	0.4 µl
Deletion Rv primer (1:10)	0.4 µl
DMSO	1.5 µl
5x GoTaq Buffer colorless	10 µl
ddH_2O	35.3 µl
GoTaq Polymerase	0.7 µl

Table 6. Cycling program of deletion PCR

Time	Cycles	Temperature
2min	1	98°C
10sec	5x	98°C
20sec		50°C
50sec		72°C
10sec	25x	98°C
20sec		55°C
50sec		72°C
5min	1	72°C
∞		4°C

After purification, the DNA was precipitated by ethanol. For precipitation, 1/10 volume of 3 M sodium acetate pH 5.5 was added to the PCR reaction and then 2.5 volumes of ethanol were added. After incubation at -20°C overnight, the sample was centrifuged (13,300 rpm, 4°C, 20 min) and the supernatant discarded. The pellet was washed with 70 % ethanol twice and the supernatant discarded. After the ethanol is evaporated and the DNA pellet was dried, it was re-suspended in 5 µl sterile water and kept on ice, in order to use it for the following bacterial cells transformation.

3.8.4.2. Preparation and transformation of competent cells

For the preparation of cells for the method of Datsenko and Wanner (2000), *E. coli* strains (glycerol stocks -80°C) MG1655, MC4100, and MC4100 (Δ*rpoH)* carrying the pKD46 plasmid were cultivated in 1xLB media, plus ampicillin 200 µg/ml at 30°C for 15 hours. Next, 0.8 ml from each overnight culture

was added to 19 ml 1xLB media supplemented with 10 % arabinose and incubated at 30°C, 135 rpm for 6 hours. The total volume of the cells was harvested. First, the cells were centrifuged (5,000 rpm, 8°C, 5 min). Then, cells were washed two times with 5 ml ice cold sterile water (Wanner method). For every washing step the cells where re-suspended and centrifuged again. After the last washing step, the cell pellet was re-suspended in 600 µl ice cold sterile water (Wanner method). For transformation, cells were mixed with the required DNA on ice. Electroporation was performed in electroporation cuvettes at 1.7 kV, 200 Ω, 25 µF. Afterwards, 500 µl LB medium was added to the transformation mixture, transferred to 1.5 ml tubes, incubated (30°C or 37°C, 500 rpm, 3 h), and plated on LB plates containing Cm 25 µg/ml.

3.8.4.3. Colony control PCR of deletion strains

The identification of the deletions was made by control PCR. The Cm resistance cassette was amplified using deletion control primers listed in Table.3 and using genomic DNA of resistant colonies as template. The composition of the 10 µl PCR reaction is listed in Table.6. The top of the resistant colonies was used as template and primers diluted in 1:10 with ddH_2O before usage. After the PCR reaction, the products were analysed on a 1.5% agarose gel stained with DNA Stain G. After verifying the correct size of the PCR products on the gel with UV light emission, the positive clones were picked in order to produce glycerol stocks.

Table 7. Composition of deletion control PCR

Component	Volume
Genomic DNA	Cells from colonies
10mM dNTPs	0.2 µl
Deletion Fw primer (1:10)	0.04 µl
Deletion Rv primer (1:10)	0.04 µl
DMSO	0.1 µl
5x GoTaq Buffer Green	2 µl
ddH_2O	7.57 µl
GoTaq Polymerase	0.05 µl

Table 8. Cycling program of deletion control PCR

Time	Cycles	Temperature
2min	1	95°C
30sec	30x	95°C
30sec		55°C
50sec		72°C
5min	1	72°C
∞		4°C

3.8.4.4. P1*vir* phage lysate production

By following the Datsenko and Wanner (2000) method was not possible to obtain MC4100 (Δ*rpoH*) deletion clones and for this reason the P1*vir* transduction method was performed. Phage transduction is used to move selectable genetic markers from one "donor" strain, which in this case are the deletion strains of MC4100, to another "recipient" strain, MC4100 (Δ*rpoH*). Nat Sternberg and other scientists used phage P1 to move genetic elements in *E. coli* and through the use of the Cre/Lox system from P1 to achieve controlled recombination. Phage P1 can package random sections of the host chromosome instead of its own genome generating "transducing particles". P1*vir* is a

mutant phage that enters the lytic cycle upon infection (ensuring replication and lysis). During the replication and lysis of the phage in a culture of bacteria, a small percentage of the phage particles will contain a genome segment that contains your gene of interest.

Firstly, for the production of phage lysate, *E. coli* strain (glycerol stock from -80^{o}C) MG1655 with pKD46 plasmid was incubated in 1xLB media, plus ampicillin 200 μg/ml (1:1000 dilution) at 30^{o}C, for 12 to 16 hours. After incubation, 5 ml of cells were centrifuged at 5,000 rpm for 10 min. The pellet was re-suspended in 500 μl 10 mM $MgSO_4$, 5 mM $CaCl_2$ buffer. Five samples were prepared. The first contained only cells, the second only lysate, the third cells and 1:100 lysate, the forth cells and 1:10 lysate and the last cells with lysate in 1:1 dilution. The samples were incubated at 37^{o}C for 30 min without shaking. After incubation, 2 or 3 ml of melted top agar, which contained 10 mM $MgSO_4$, 5 mM $CaCl_2$ final concentration, were added to each sample and immediately poured onto warm LB plates. After 5 min on the bench, plates were incubated at 37^{o}C for 3 to 5 hours, until plaques were observed. The top agar of the plaques-containing plate(s) was scoped with a spatula and transferred into a 15 ml Greiner tube which already contained 200 μl chloroform, in order to stop any bacteria from growing. Next, the tube was vortexed for 1 min and then centrifuged (5,000 rpm, 20 min). The supernatant was transferred to a Cryo tube and 100 μl of chloroform was added. The phage lysate was stored at 4^{o}C.

3.8.4.5. Determination of phage lysate title

An overnight culture of 10 ml LB was used for this experiment. Cells were centrifuged (5,000 rpm, 10 min) and then re-suspended in 500 μl 10 mM $MgSO_4$, 5 mM $CaCl_2$ buffer. 10 μl of the culture was added to 5 ml top agar and immediately poured onto an LB plate. After leaving the plate for 5 min on the bench the phage dilutions were placed on the plate. For the dilution, 10 μl of phage lysate was diluted until 10^{-8} using 10 mM $MgSO_4$, 5 mM $CaCl_2$ buffer. The incubation lasted over night at 37^{o}C. The plaques formed per unit were calculated with the formula,

$$\frac{Pfu}{ml} = \frac{n}{d}$$

Where, ***n*** is the number of plaques and ***d*** is the dilution of the phage lysate.

In this case, the phage lysate titer was 2.7 x 10^{11} pfu/ml.

This phage lysate was used for the generation of phage lysate from the deletion strains of MC4100 (*xdhD::Cm, ompF::Cm, dppF::Cm, dinF::Cm, xylE::Cm*), obtained formerly with Datsenko and Wanner (2000) method. The phage lysate generated from each of the deletion strains was used to obtain deletion clones for the MC4100 (Δ*rpoH*) strain, through P1*vir* transduction.

3.8.4.6. Phage Transduction

The *E. coli* strains MC4100and MC4100 (Δ*rpoH*) were cultivated in 1xLB media overnight at 37^{o}C and 30^{o}C respectively, 135 rpm. Then cells were centrifuged (5,000 rpm, 10 min) and re-suspended in 2 ml LB supplemented with 10 mM $MgSO_4$, 5 mM $CaCl_2$ final concentration. Five samples were prepared for every gene transduction of each *E. coli* strain and five different phage lysates were used (lysates generated from the five deletion strains of MC4100). The first sample contained 100 μl

phage lysate and 100 µl LB, the second 100 µl cells and 100 µl LB, the third 100 µl cells and 100 µl 1:3 phage lysate, the forth 100 µl cells and 100 µl 1:30 phage lysate and the last 100 µl cells and 100 µl 1:300 phage lysate. The samples were incubated at 30°C for MC4100 (Δ*rpoH*) strain and at 37°C for MC4100 strain, without shaking (for the attachment of the phage) for 30 min exactly. For stopping phage from further attachment on the cells, 1 ml of LB media supplemented and 200 µl 1 M Na-citrate, were added. Then cells were incubated at 30°C for MC4100 (Δ*rpoH*) and at 37°C for MC4100, at 200 rpm for 1 hour, allowing expression of the antibiotic resistance marker. After incubation, cells were centrifuged (6,000 rpm for 2 to 3 min) and re-suspended in 100 µl LB plus 100 mM Na-citrate. Cells were plated in Cm^{25}/100 mM Na-Citrate plates and incubated overnight at 30°C. Some of the surviving colonies were streaked out onto new Cm^{25}/10 mM Na-Citrate plates in order to ensure the phage re-movement and then confirmed by control PCR, if the targeted genes were replaced by Cm resistance gene or not. The MC4100 strain was used as positive control.

3.8.5. Deletion strains streak out for phenotypic analysis

Streak out of deletion strains onto various types of plates performed in order to observe differences between the deletion strains. Deletion strains of MC4100 (Δ*rpoH*) strain and the wild type, MC4100, were streaked out on an LB plate and incubated at 30°C over night. The plates tested contained HOCl (2, 3, 3.5, 4, 5 and 6 mM), H_2O_2 (0.2, 0.3, 0.4, 0.5 and 0.6 mM), NaCl (0.6, 0.7, 0.8 and 0.9 mM). Also, bile salts tested using Mc Conkey plates. LB plates functioned as control. In order to prepare HOCl plates, 25 ml warm LB/agar poured in 50 ml Greiner tube and the corresponding amount of HOCl added in order to achieve the right final concentration. The tube was mixed softly and poured in the Petri dish. The plate left 10 minutes on the bench in order to be solidified and then dried in an open position at 37°C for 20 minutes. The same procedure conducted for all of the plates except from Mc Conkey, which were prepared earlier and were kept out of light at 4°C. Only before using them, they were warmed at 49°C for 30minutes. Single colonies of each of the strains were picked from the overnight LB plate and streaked out on the plates. One colony was streaked onto one type of plates. Streak out started from the plate with the higher concentration of the stress factor, to ensure the presence of cells and was taken into consideration in the interpretation of the results. All plates incubated for 48 hours at 30°C. LB, NaCl and Mc Conkey plates incubated also at 37°C and 39°C to cause heat stress. The growth of deletion strains was compared to control in order to observe differences.

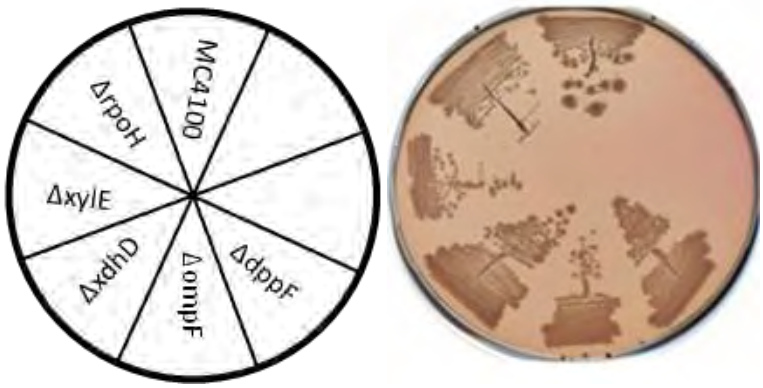


Figure 5. Example of a streak out onto Mc Conkey plate.

3.8.6. Deletion strains' viability assays

3.8.6.1. Viability assay under H_2O_2

For the viability assay under H_2O_2 the MC4100 (Δ*rpoH*) deletion strains (Δ*xylE,* Δ*xdhD,* Δ*ompF,* Δ*dppF*) and MC4100 (Δ*rpoH*) as control (glycerol stocks -80 ^{o}C) were used. Cells inoculated in 10 ml LB media and incubated at 37^{o}C, 135 rpm overnight. Then, 50 µl cells from each culture were inoculated into 10 ml LB and incubated at 37^{o}C until OD reached ~0.8. For the next step, five flasks were prepared with 5 ml LB and 250 µl cells were added. Then the cells were stressed with H_2O_2 3.5 mM final concentration. Samples were taken every 30 min for 120 min in total, serially diluted and spotted onto LB plates marked with time points 0', 30', 60', 90', 120' and the sample number. The plates were incubated at 30^{o}C overnight. The experiment performed in duplicate.

3.8.6.2. Viability assay under heat

For the viability assay under heat the MC4100 (Δ*rpoH*) deletion strains (Δ*xylE,* Δ*xdhD,* Δ*ompF,* Δ*dppF*) and MC4100 (Δ*rpoH*) as control (glycerol stocks -80 ^{o}C) were used. Cells inoculated in 10 ml LB media and incubated at 30^{o}C, 135 rpm overnight. Then, 50 µl cells from each culture were inoculated into 10 ml LB and incubated at 37^{o}C until OD reached ~0.8. For the next step, five flasks were prepared with 5 ml LB and pre-warmed at 47^{o}C for 30 minutes. Then 250 µl of cells were added in the flasks and the first sample was taken. Then, the cells were put back again at 47^{o}C. Samples were taken every 30 min for 120 min in total. Samples were serially diluted and spotted onto LB plates marked with time points 0', 30', 60', 90', 120' and the sample number. The plates were incubated at 30^{o}C overnight. The experiment performed in duplicate.

4. Results

4.1 Analysis of the role of *msrA* and *msrB* in HOCl resistance

4.1.1 Growth Curves and Viability Assays under HOCl stress

Growth curves for these *E. coli* strains were performed to obtain an idea how these strains grow and how fast they recover from HOCl stress, while viability assays were performed in order to analyze the number of viable cells. The *E. coli* strains C600 (WT), KMG232 (C600 Δ*hypT*), and HM15 (C600 Δ*hypT*, Δ*msrA*, Δ*msrB*) were used for these assays.

The first thing tested was the effect of HOCl on WT, regarding growth and viability. As can be seen in Fig. 6, HOCl inhibits growth and reduces viability. It should be noted that cells at OD_{600} ~0.8 were used for growth curves while cells for viability were diluted in LB (1:20) before addition of HOCl. Because of this fact, although a similar range of concentrations of HOCl was used, cells during viability assay did not manage to recover even after quenching the HOCl, and finally died.

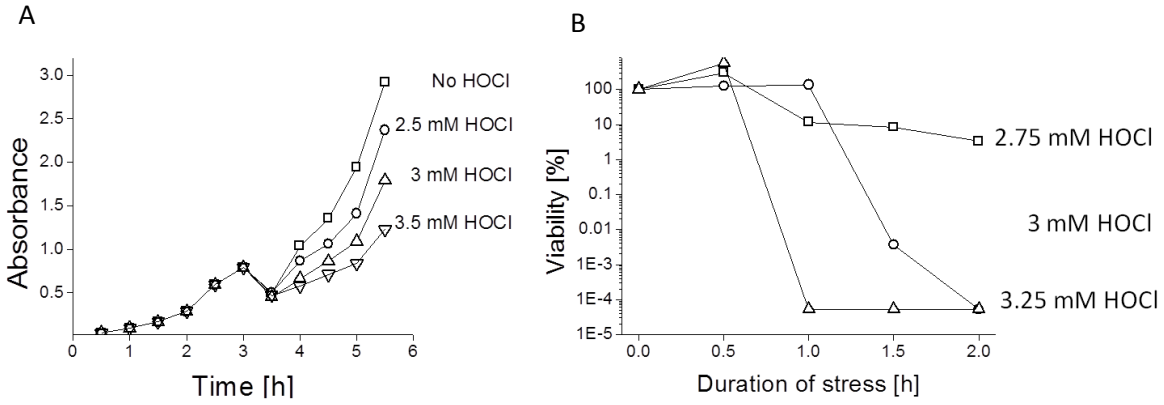


Figure 6. A) Analysis of growth and B) viability of the WT upon addition of various concentrations of HOCl. Represented data are from one experiment. Number of living cells on the plate (viability) was determined by considering 100% viability the number of colonies at time point 0' and dividing the number of colonies from the next time points with the number of cells at 0'.

In order to measure the growth of strains under HOCl, each of the three strains subjected to 0 mM, 2.5 mM, 3 mM, 3.5 mM of HOCl for 15 min and then the HOCl quenched with the addition of 1xLB. Every 30 minutes samples of the cells were taken and the OD_{600} measured with the spectrophotometer. Absorbance data in relation to time yielded the following growth curves (Fig. 7). The experimental procedure performed in triplicate, yielding reproducible results. After the growth curves, viability assays performed in order to analyze the number of viable cells. Again the three strains subjected to HOCl (2.75 mM, 3 mM, 3.25 Mm). Samples removed every 30 minutes, diluted in 96 well-plates contained 1xLB, spotted on LB plates and incubated at 37°C. The number of viable cells were counted and expressed upon percentage (%) (Fig.8). The WT strain recovered faster as indicated by growth curves and was more resistant to HOCl according to viability assay, than the mutated strains. Also, differences observed among the mutants, as the mutant for the single deletion grown better than the mutant for the double deletion. These results indicate that besides *hypT*, which is clear that is involved in HOCl resistance [26, 36]. *msrA* and *msrB* are also involved in HOCl resistance and their deletion renders cells sensitive to HOCl stress.

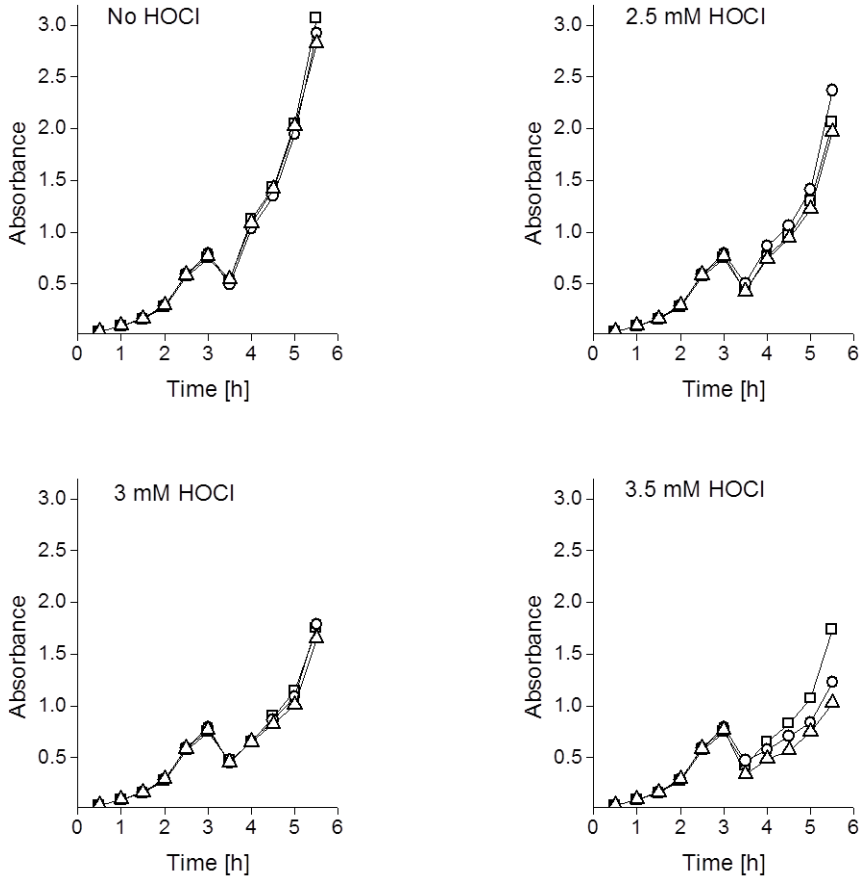


Figure 7. Growth curves evaluation. WT (squares), *yjiE*⁻ (circles), *yjiE*⁻, *msrA*⁻, *msrB*⁻ (triangles). Represented data are from one experiment. Number of living cells on the plate (viability) was determined by considering 100% viability the number of colonies at time point 0' and dividing the number of colonies from the next time points with the number of cells at 0'.

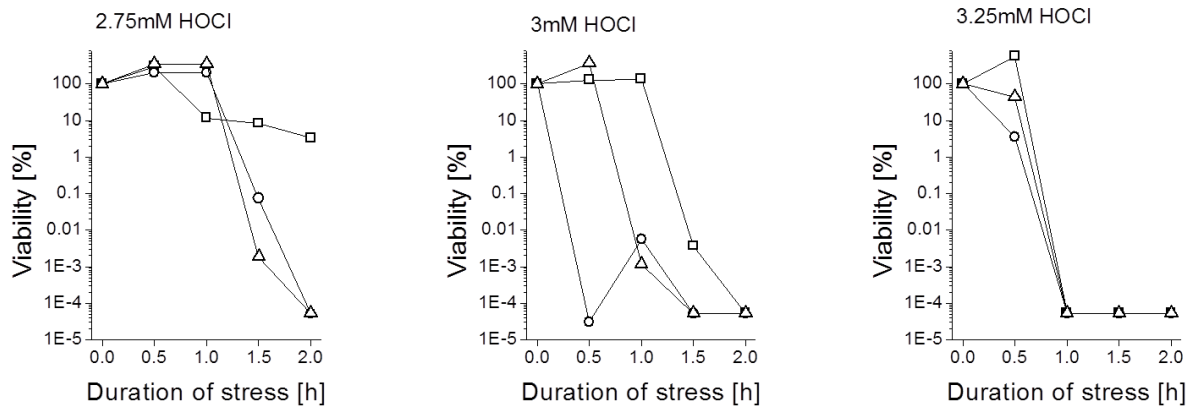


Figure 8. Viability assay evaluation. WT (squares), *yjiE*⁻ (circles), *yjiE*⁻, *msrA*⁻, *msrB*⁻ (triangles). Represented data are from one experiment. Number of living cells on the plate (viability) was determined by considering 100% viability the number of colonies at time point 0' and dividing the number of colonies from the next time points with the number of cells at 0'.

4.2. Crystallization of the constitutively active HypT mutant

The aim of this topic was the crystallization of the constitutively active HypT in order to solve its structure. Although many purifications and crystallization screens performed no crystals obtained so far. Thus, thermo stability assays (TSA) were performed. The aim was to determine the conditions that would improve the thermo stability of the protein and consequently improve the crystallization conditions.

During the first purification affinity and ionexchange chromatography performed, the protein was concentrated at 3.96 mg/ml and crystallization screens were performed. After two to three days, crystalline precipitate and aggregates were observed. A second purification was performed similarly, but the concentration of the protein was 6.89 mg/ml. At this concentration only aggregation was observed. Another purification and crystallization screen yielded the same results.

In order to improve the stability of the protein, a TSA was performed by Adrian Drazic. He found that 20 mM MES buffer, pH 6 and 600 mM salt were the conditions that HypT was most stable at, with Tm 70°C. In order to avoid high salt concentrations that interfere with crystallization, further TSA were performed with various additives such as ATP and dNTPs, which may also improve the thermo stability of the protein. Results from thermostability assay are represented in the following tables (Table 9, 10, 11). The addition of dNTPs, especially at 3 mM increased the melting temperature of the protein compared to the addition of salt (Table 9, 11). Also the addition of ATP seem to increase the melting temperature of the protein but the not all of the results could be evaluated and the obtained values do not give a clear conclusion about which concentration is better for the stability of the protein. Results about salt addition are not in accordance with previous assays that Adrian Drazic performed as at 600 mM salt the melting temperature was expected around 70°C, while at this assay the melting temperature of the protein was around 45°C. This difference is due to the fact that in the first case the pH of the MES buffer used for the assay was 6, while in the second case the pH was 8, which is an important factor that affects the behavior of the protein. These facts lead to the conclusion that the protein is more stable at pH 6.

The next purification procedures were performed in standard buffer and the size exclusion chromatography was performed using 20 mM MES, pH 6 and 200 mM NaCl, with no addition of any additive. Already during the purification protein started to aggregate and was completely aggregated during concentration. Complete protein aggregation happened in both of the purifications that were tried. In the third purification using a PD-10 desalting column (Sephadex™ G-25 Medium) instead of the gel filtration column, the protein was not completely aggregated and reached a concentration of 1.5 mg/ml. Because of the low amount of protein crystallization screens did not yield crystals. Concluding, the MES buffer used to improve the thermo stability of the HypT mutant had no positive effect on the protein purification and crystallization outcome, which means that more screens should be performed in order to improve the purification and crystallization conditions.

Table 9. Thermostability assay results upon dNTPs addition

dNTP concentration	Tm (measurement 1)	Tm (measurement 2)
1 mM	50°C	52°C
2 mM	55°C	52°C
3 mM	55°C	56°C
4 mM	48°C	48°C

Table 10. Thermostability assay results upon ATP addition

ATP concentration	Tm (measurement 1)	Tm (measurement 2)
4 mM	44°C	46°C
6 mM	44°C	46°C
8 mM	54°C	55°C
20 mM	53°C	54°C

Table 11. Thermostability assay results upon NaCl addition

NaCl concentration	Tm (measurement 1)	Tm (measurement 2)
400	47°C	47°C
420	48°C	45°C
440	49°C	46°C
460	46°C	43°C
480	44°C	46°C
500	45°C	48°C
520	45°C	55°C
540	45°C	44°C
560	46°C	45°C
580	53°C	44°C
600	45°C	45°C

4.3. Identification of factors required in HOCl resistance in evolved *E. coli*

4.3.1. Construction of deletion strains

Jasmin Gundlach evolved HOCl resistant MC4100 (Δ*rpoH*) strains of which analyzed the underlying mechanism. The results showed no mutations in the chromosomal DNA but revealed differences in cytosine's methylation pattern. The genes *xdhD*, *xylE*, *mdtA*, *dinF*, *guaB*, *dppF* and *ompF* showed the strongest differences in cytosine methylation compared to the control (MC4100 (Δ*rpoH*)). To analyze the role of these genes in HOCl resistance, deletion strains should be generated using the method of Datsenko and Wanner (2000). First, the Cm resistance cassette was amplified with primers, thus generating an 1100bp product, where Cm is flanked by ~30bp surrounding the ATG start site of the respective gene to be deleted (Fig. 10).

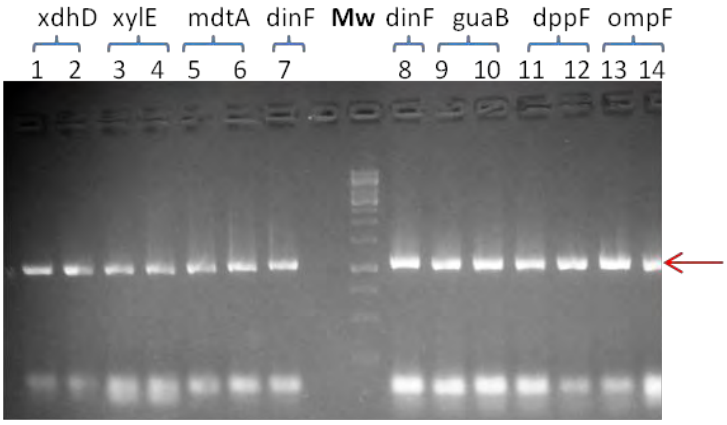


Figure 9. Agarose gel stained with DNA Stain G. Cm resistance cassette amplified with each of the deletion primers. 1-2: D_xdhD_primers, 3-4: D_xylE_primers, 5-6: D_mdtA_primers, 7-8: D_dinF_primers, 9-10: D_guaB_primers, 11-12: D_dppF_primers, 13-14: D_ompF_primers. Red arrows indicate the bands with the correct length.

After the homologous recombination between the amplified Cm resistance cassette and the genomic DNA, the target gene is replaced by the Cm resistance cassette. The resulting control PCR yields a 1500bp product (Fig 11).

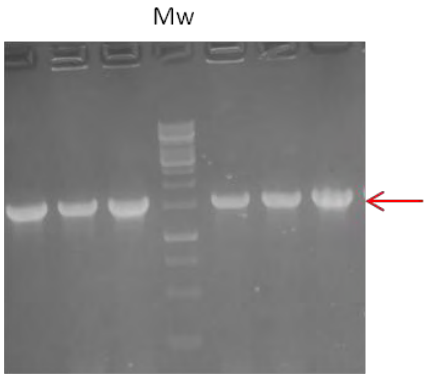


Figure 11. Agarose gel stained with DNA Stain G. This gel is an example of deletion colony control PCR of MC4100 strain, to test for the deletion of *xylE*. Deletion control primers (D_ctr_primers) used for this PCR are listed in Table 3. A clear band at 1500bp indicates successful deletion, which can additionally confirmed by a second control PCR using a primer specific for the Cm resistance cassette.

As a further control, a PCR was performed that should yield a 500 bp product if the clones are positive for the deletion and no product at all if the clones are negative for the deletion (Fig. 12). Deletion control forward primers used are listed in Table 3 and as reverse primer C1 was used, which anneals inside the sequence of Cm resistance cassette. Red arrows indicate the bands with the correct size of PCR product. Glycerol stocks of the positive clones (MC4100 and MC4100 (Δ*rpoH*)) were generated and stored at -80°C.

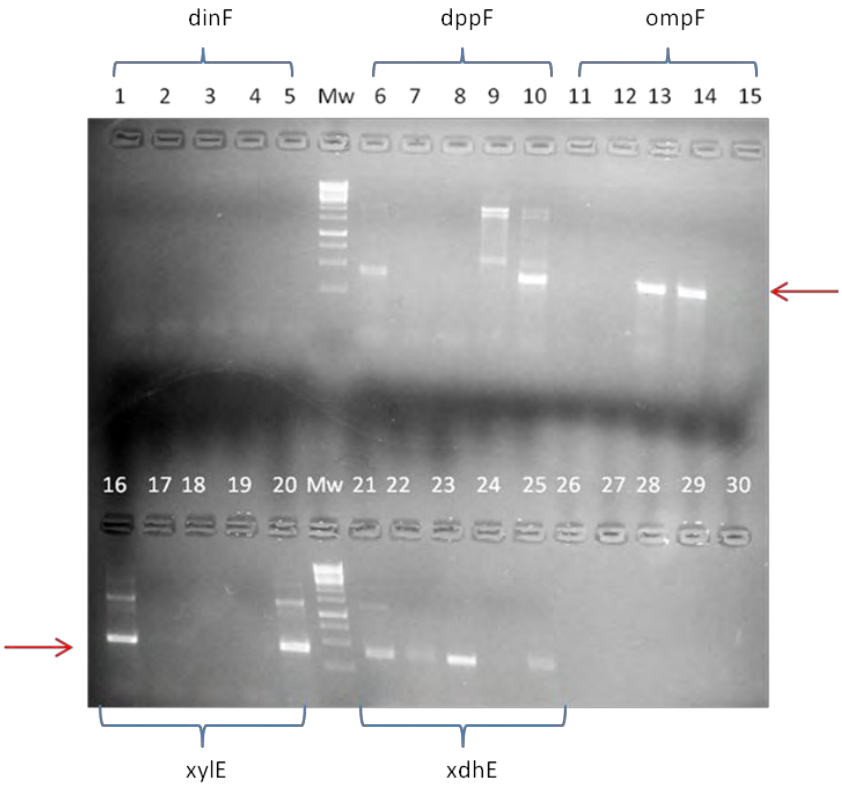


Figure 12. Agarose gels stained with DNA Stain G. Second deletion colony control PCR of MC4100 strain using deletion control primers (D_ctr_primers) and the primer specific for Cm resistance cassette. 1-5: D_ctr_dinF, 6-10: D_ctr_dppF, 11-15: D_ctr_ompF, 16-20: D_ctr_xylE, 21-25: D_ctr_xdhD.

Using the Datsenko and Wanner method, deletion clones were obtained for the MC4100 strain. However, no clones were obtained for *mdtA* and *guaB*. For the MC4100 (Δ*rpoH*) strain also no clones were obtained. In order to obtain deletion clones for MC4100 (Δ*rpoH*), the P1*vir* phage transduction method was followed. For phage transduction preparation, phage lysates were produced from each of the MC4100 deletion strains. The phage lysate contains "transducing particles", which are viruses carrying random sections of the MC4100 deletion strains' chromosomal DNA instead of its own genome. Some of these viral particles could contain the genome section that contains the gene of interest, which is the Cm resistance cassette. These phage lysates, produced from deletion strains, were used in order to infect MC4100 (Δ*rpoH*) strain. The chromosomal DNA of the virus could be transferred to MC4100 (Δ*rpoH*) through homologous recombination with the use of Cre/Lox system of the P1*vir*. Thus, it may be transferred in this way the gene in which we are interested in and the original gene in this position would be deleted. The deletion clones of MC4100 (Δ*rpoH*) confirmed by following the same procedure for the confirmation of MC4100 deletion clones.

4.3.2. Analysis of the growth of MC4100 Δ*rpoH* deletion strains on HOCl plates

Evolved *E. coli* cells are HOCl resistant [36]. These cells have proteome differences and different DNA cytosine methylation pattern for some genes, compared to the ancestor [36]. To analyze the role of these genes (*xdhD*, *xylE*, *mdtA*, *dinF*, *guaB*, *dppF* and *ompF*) in HOCl resistance, single deletion strains and the respective controls (MC4100 and MC410 (Δ*rpoH*)) were streaked on HOCl containing plates and their ability to form colonies was analyzed (Fig. 13, Table 9). HOCl inhibits growth of all strains. When comparing the control and deletion strains, it becomes obvious that Δ*xdhD*, Δ*ompF*, and Δ*dppF* grow better than Δ*rpoH* (Fig. 13, Table 12). This indicates that deletion of these genes confers HOCl resistance.

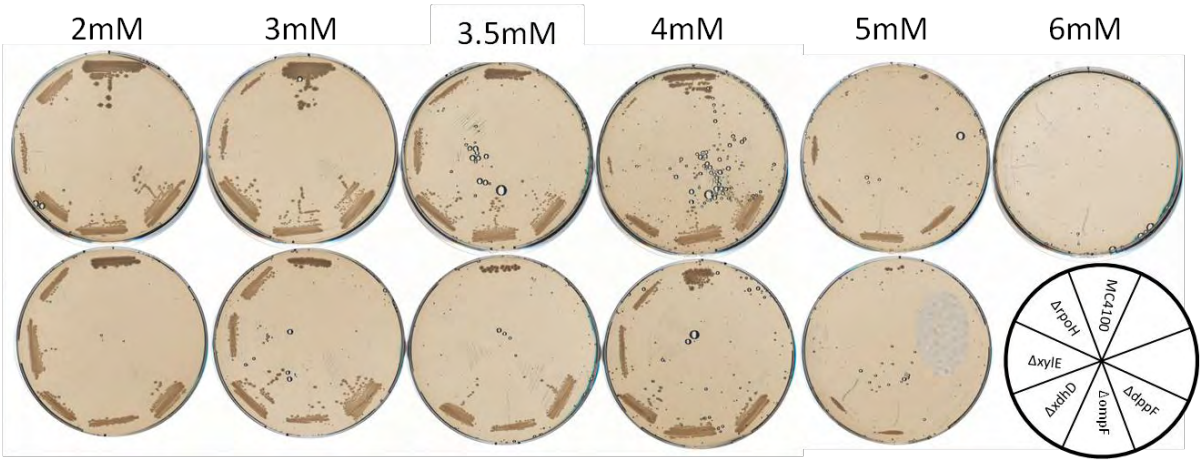


Figure 13. Streak out of the indicated control and deletion strains on HOCl plates. The agar contained the indicated concentration of HOCl. After the streak out, plates were incubated for 48 hours at 30°C. As shown here, the experiment was performed in duplicate. The differences in growth between the plates of the first and the second line are due to the fact that the plates of the first line prepared earlier than the second and they stayed longer on the bench. As a result more HOCl evaporated as chloramines and the concentration of HOCl reduced. This is the reason why colonies of the first line grow better than in the second.

Table 12. Evaluation of HOCl streak out.

Strain	2mM HOCl	3mM HOCl	3,5mM HOCl	4mM HOCl	5mM HOCl	6mM HOCl
(WT)	+	+	+	+	-	-
Δ*rpoH*	+	+	-	+	-	-
Δ*xylE*	+	+	-	+	-	-
Δ*xdhD*	++	++	+++	++	+	-
Δ*ompF*	+	+	+	++	+	-
Δ*dppF*	+++	+++	+++	++	-	-

** Four pluses indicate normal growth, formation of single colonies after the dilution of cells and a homogenous culture. Three pluses indicate the sameas four pluses but with smaller colonies. Two pluses are for cultures with reduced growth, forming much less single colonies after the dilution of the*

cells and being sometimes inhomogeneous. One plus indicates sick cells with little growth and being inhomogeneous. Minus indicates no growth at all.

4.3.3. Analysis of the growth of MC4100 Δ*rpoH* deletion strains on H_2O_2 plates

Further, the role of the genes in H_2O_2 resistance was analyzed. Similar to the HOCl plates shown above, agar plates containing H_2O_2 were generated and cells streaked onto these plates. Because it was found that the evolved *E. coli* cells were strongly resistant to different types of stress, including stress upon H_2O_2, plates contained H_2O_2 were tested. Increasing concentrations of H_2O_2 cause slower growth to all the strains. The comparison of the strains shows that Δ*xylE* and Δ*ompF* are more resistant from MC4100 (Δ*rpoH*) and even more resistant than MC4100. On the other hand Δ*xdhD* and Δ*dppF* seem to be more sensitive than the control. However, it can not be made a clear conclusion as the observed sensitivity and the differences between the different strains maybe are due to the little amount of cells may transferred to the plate for the streak out (Fig. 14, Table 13).

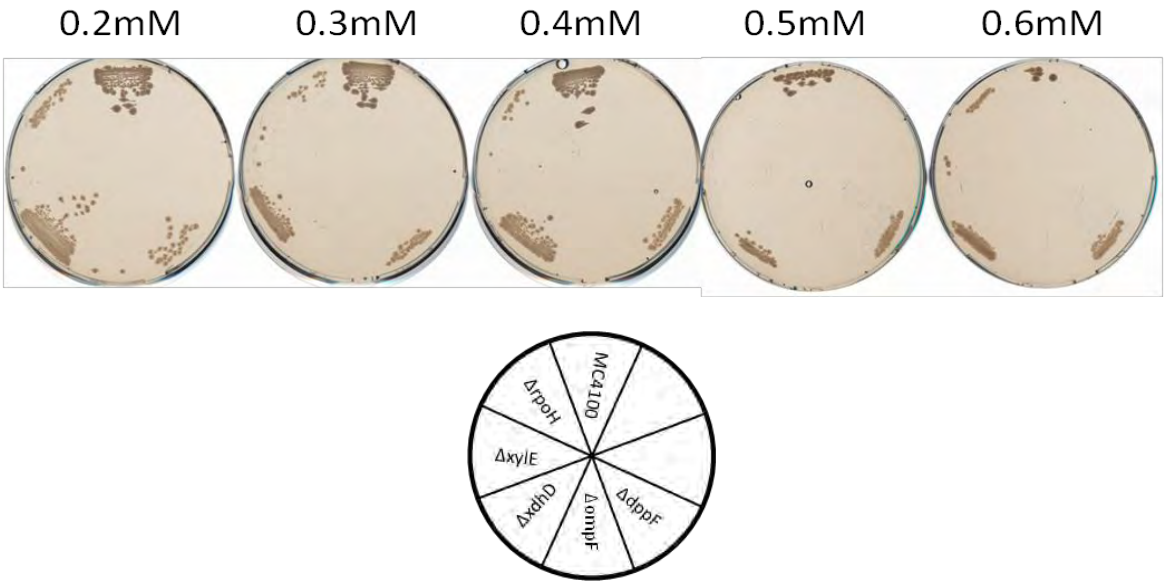


Figure 14. Streak out of H_2O_2 plates after 48 hours incubation at 30°C. The agar contained the indicated concentration of H_2O_2. After the streak out, plates were incubated for 48 hours at 30°C.

Table 13. Evaluation of H_2O_2 streak outs.

Strain	0.2mM H_2O_2	0.3mM H_2O_2	0.4mM H_2O_2	0.5mM H_2O_2	0.6mM H_2O_2
WT	+++	+++	+++	++	+
Δ*rpoH*	+	+	+	-	+
Δ*xylE*	-	+	-	-	+
Δ*xdhD*	+++	++	++	+	+
Δ*ompF*	-	-	-	-	-
Δ*dppF*	+	+	+	+	+

4.3.4. Analysis of the growth of MC4100 Δ*rpoH* deletion strains on agar plates containing high salt concentrations and at different temperatures

As mentioned, evolved *E. coli* cells are strongly resistant to various stress conditions. That is why salt and heat were also tested. The deletion of the genes that are analyzed do not seem to have an effect upon salt stress, as the increasing concentration of NaCl did not cause differences in growth of the deletion strains compared to the control. Also, under heat stress there are not strong differences between the deletion strains and the Δ*rpoH*. Differences there only between the deletion strains and the MC4100, as deletion strains are generated from MC4100 (Δ*rpoH*) which is thermosensitive and they do not grow, under these temperatures, so fast as the wild type (MC4100). The various NaCl stress condition together with heat stress did not have strong effect on the deletion strains as all of them grown in all types of conditions.

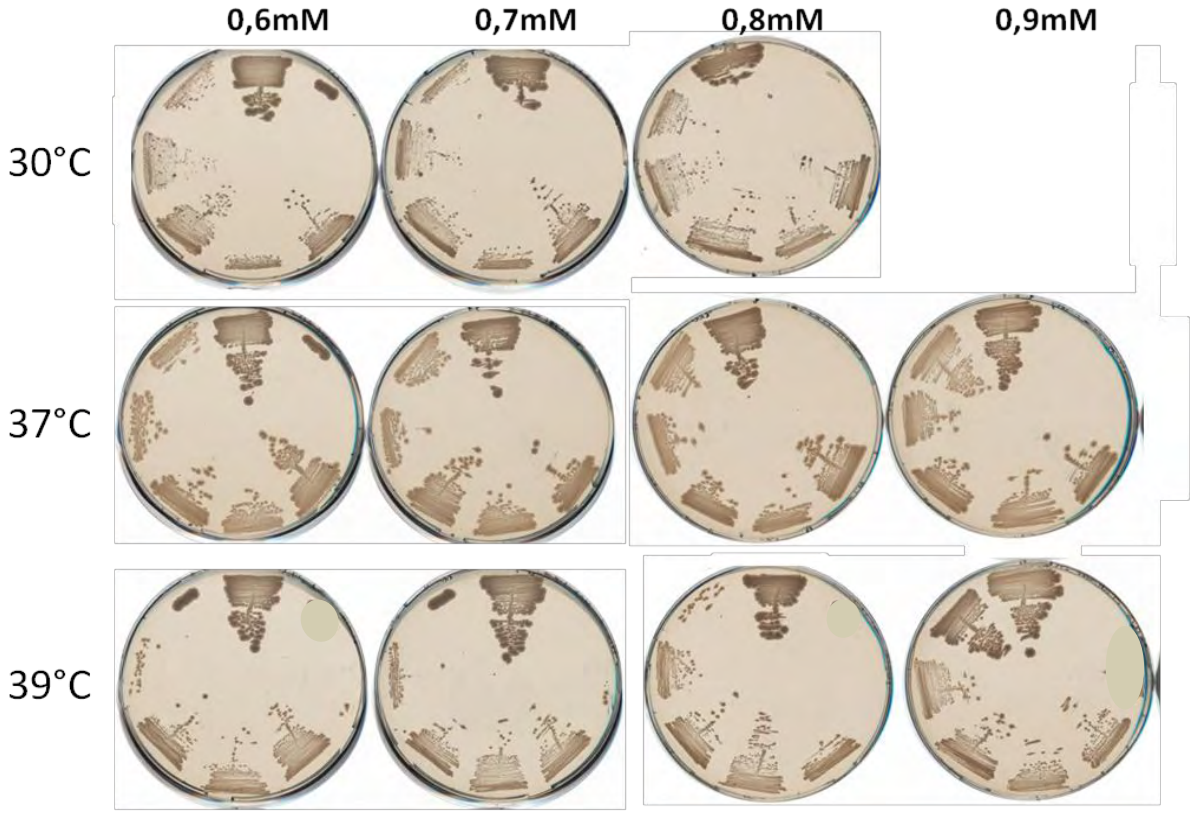


Figure 15. Streak out of NaCl plates after 48 hours incubation. The agar contained the indicated concentration of NaCl. After the streak out, plates were incubated for 48 hours at 30°C, 37°C, 39°C.

Table 14. Evaluation of NaCl streak outs.

Strain	0.6mM NaCl			0.7mM NaCl			0.8mM NaCl			0.9mM NaCl		
	30°C	37°C	39°C	30°C	37°C	39°C	30°C	37°C	39°C	30°C	37°C	39°C
WT	++++	++++	++++	++++	++++	++++	++++	++++	++	++++	++++	
Δ*rpoH*	++	++	+	++	++	+	+++	+	+++	+++	++++	
Δ*xylE*	++	+	++	++	+	++	+++	++	+++	+++	+++	
Δ*xdhD*	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Δ*ompF*	++	+++	+	++	+++	+	++	+++	+++	+++	+++	
Δ*dppF*	++++	+++	+++	++	+++	+++	+++	++	+++	+++	++	

4.3.5. Analysis of the growth of MC4100 Δ*rpoH* deletion strains on Mc Conkey plates

Mc Conkey plates used in order to test if there is resistance against bile salts. LB plates functioned as control. In Mc Conkey and LB plates nice and homogenous growth observed at 30°C and 37°C. At 39°C, growth was slower for Δ*xylE*, Δ*ompF* and Δ*dppF*, in Mc Conkey plates which indicates that these deletions may render cells more sensitive to bile and heat stress. Like heat stress of NaCl plates, the growth differences observed in LB plates, under increased temperatures, between the deletion strains and the MC4100 are due to the thermosensitivity of the deletion strains as they were generated from MC4100 (Δ*rpoH*) which is thermosensitive and they grow slower than the wild type.

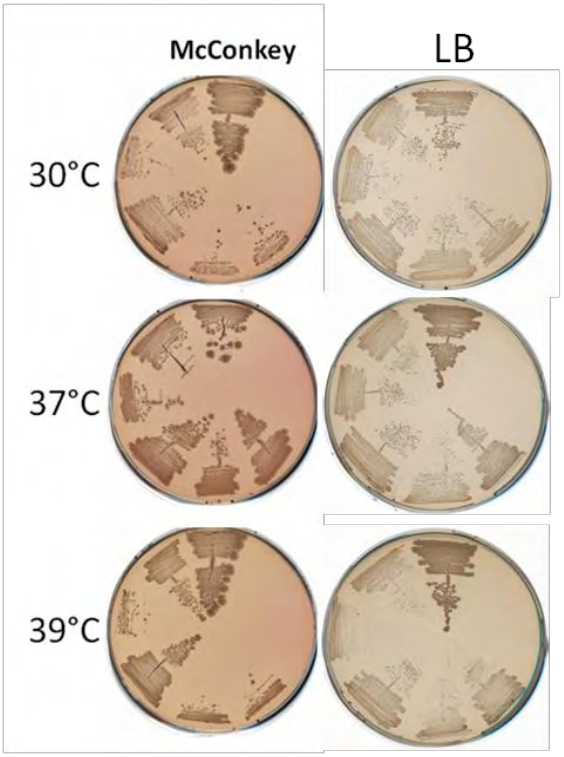


Figure 16. Streak out of McConkey and LB plates after 48 hours incubation. After the streak out, plates were incubated for 48 hours at 30°C, 37°C, 39°C.

Table 15. Evaluation of Mc Conkey and LB streak outs.

Strain	Mc Conkey			LB		
	30°C	37°C	39°C	30°C	37°C	39°C
WT	++++	+++	++++	++++	++++	+++
Δ*rpoH*	+++	+++	+++	+++	+++	+++
Δ*xylE*	+++	+++	++	+++	++	++
Δ*xdhD*	+++	+++	+++	+++	+++	+++
Δ*ompF*	+++	+++	++	+++	++	++
Δ*dppF*	+++	+++	++	+++	+++	+++

4.3.3. Viability assays of MC4100 (ΔrpoH) deletion strains

Additionally to the streak out and the phenotypic analysis, viability assays for the deletion strains under heat and H_2O_2 were performed. For these experiments MC4100 (Δ*rpoH*) deletion strains were used (listed in Table 3.). MC4100 (Δ*rpoH*) strain with no extra deletions was used as control.

For the viability assay upon heat stress, the deletion strains were exposed to heat (47°C), aliquots were removed at indicated time points, diluted in a 96 well-plate, spotted on LB plates and incubated at 30°C for 24 hours. The number of viable colonies was counted and analyzed. The effect of deletion at heat stress is not clear as the viability of the deletion clones is similar to the control. Because at this temperature no killing, neither differences among the strains were observed it is suggested that maybe higher temperatures should be tested in order to make better observations and make clearer conclusion (Fig.17).

For the viability assay upon H_2O_2, the deletion strains were exposed to 3.5 mM H_2O_2 and samples removed at indicated time points and treated like is mentioned in heat viability assay. Analysis of the viability led to the conclusion that viability reduced upon extended exposure to H_2O_2. Specifically Δ*xdhD* and *ΔdppF* strains were slightly benefited by short term exposure. Nevertheless, the deletion of these genes finally led to negative effect on viability of the cells upon H_2O_2. Δ*ompF* also had reduced viability of cells compared to control (Δ*rpoH*) under H_2O_2 [38]. The deletion of *xylE* had similar effect on viability with control indicating that is not involved in H_2O_2 stress response (Fig.18).

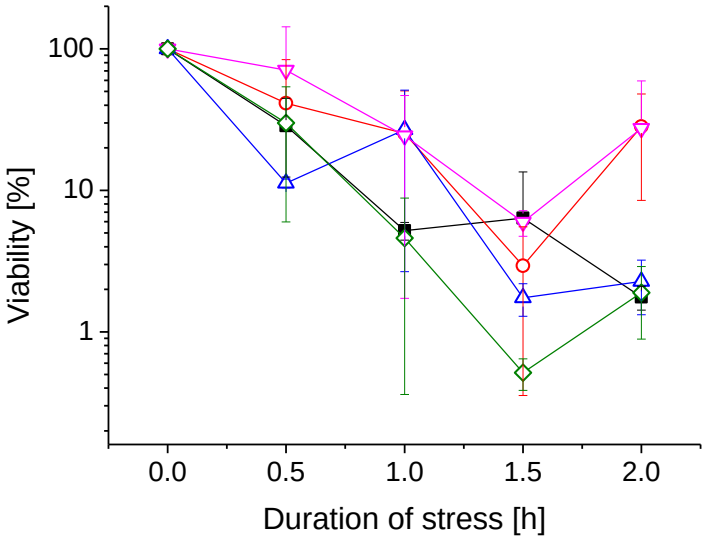


Figure 17. Viability assay under heat stress (47°C). Δ*rpoH* (squares), Δ*xylE* (circles), Δ*xdhD* (triangles up), Δ*ompF* (triangles down), Δ*dppF* (diamonds). MC4100 (Δ*rpoH)* with no extra deletion was used as control. Number of living cells on the plate (viability) was determined by considering 100% viability the number of colonies at time point 0' and dividing the number of colonies from the next time points with the number of cells at 0'.

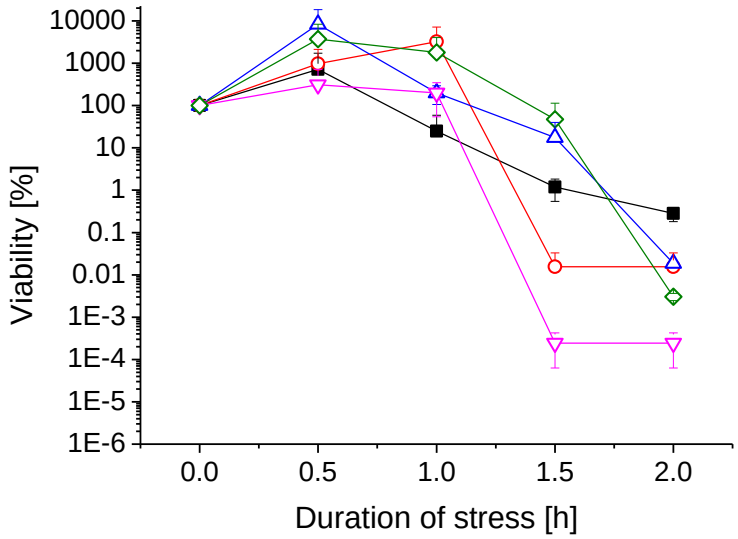


Figure 18. Viability assay under H_2O_2 stress (3,5Mm). Δ*rpoH* (squares), ΔxylE (circles), ΔxdhD (triangles up), ΔompF (triangles down), ΔdppF (diamonds). MC4100 (Δ*rpoH)* was used as control. Number of living cells on the plate (viability) was determined by considering 100% viability the number of colonies at time point 0' and dividing the number of colonies from the next time points with the number of cells at 0'.

5. Discussion

5.1. Role of MsrA and MsrB in HOCl resistance

The first part of this work concerning the role of MsrA and MsrB in HOCl resistance suggests that these enzymes play a role in HOCl resistance in *E. coli* cells. Under HOCl stress, triple deletion for *hypT msrA msrB* rendered cells more sensitive to HOCl than the wild type. Small differences were also observed between the triple mutant compared to the strain with the single deletion of *hypT*. Although, the differences were small they indicate the involvement of these enzymes in HOCl resistance. Their involvement in HOCl resistance is also supported by the work of Rosen et al. (2009) in an uropathogenic *E. coli* strain. The authors showed that over expression of *msrA* increased the viability of cells under HOCl stress, as cells survived even better than the wild type [13]. Also, Moskovitz et al. performed experiments with an *msrA* deletion strain. This mutant was more sensitive than the wild type under oxidative stress, suggesting that methionine oxidation of proteins play an important role upon oxidative stress [35]. The fact that Msr are involved in HOCl resistance was expected, as Msr enzymes are thought to be the primary system for the reduction of oxidized methionines. However, larger differences were expected between the strains, as *E. coli* has one copy of *msrA* and *msrB* [17]. It is speculated that maybe there are no big differences between the mutant with the deletion of *hypT* and the mutant with the deletions of *hypT*, *msrA* and *msraB*, because maybe these genes are involved in the same pathway, so the deletion of one of these genes has almost the same effect in the growth and the resistance of cells. One limiting factor is that we did not have a strain with deletion only for *msrA* and *msrB*. Experiments with this type of strain are going to give a better view about the role of Msr in HOCl resistance.

5.2. Crystallization of the constitutively active HypT mutant

The second part of the thesis, which is about the crystallization of the constitutively active mutant HypT, did not yield crystals at all. However, there are crystals for the wild type HypT, obtained during a previous work (Gebendorfer Katharina, Ph.D. Thesis). This shows that crystallization conditions may differ for different mutants of one protein and need to be adjusted even for point mutants. The thermo stability assays performed, proposed that 20 mM MES buffer and 200 mM NaCl at pH 6 are the conditions where the protein's melting temperature is raised and the protein is more stable. These conditions were applied to the size exclusion chromatography, but the protein completely aggregated during this procedure. This shows that even though MES buffer may increase the thermal stability of the HypT mutant, it is not useful for the purification procedure. One suggestion to overcome this problem could be to add 20 mM MES in the standard buffers used for the affinity and ionexchange chromatographies and then to try again to perform the size exclusion chromatography or PD-10 column. Thus, if better stabilizing conditions are achieved this probably would improve the crystallization outcome, too.

5.3 Identification of factors required for HOCl resistance in evolved E. coli cells

In the third part of the thesis, the genes *xdhD*, *dppF* and *ompF*, were identified to play a role in HOCl resistance in *E. coli.* These genes were found to have increased extent of DNA cytosine methylation (Jasmin Gundlach, 2012, data not published yet) and the hypothesis was that if altered cytosine methylation leads to altered gene expression then these genes are going to be up-regulated or down-regulated. Here, we hypothesize that one of these conditions leads to HOCl resistance. Deletion of each of these genes in MC4100 (Δ*rpoH*) *E. coli* strain led to HOCl resistance, which supports the hypothesis that maybe methylation of these genes leads to their down-regulation and

HOCl resistance. DppF is an ATP-binding component of the dipeptide ABC transporter and OmpF is an integral membrane protein located in the outer membrane of *E. coli.* ABC transporter isresponsible for the export and the import of small ions to macromolecules and requires DppF for import function [32]. OmpF allows the passive diffusion of small, polar molecules (600-700 Da in size) through the cell's outer membrane. Such molecules include water, ions, glucose, and other nutrients as well as waste products [33]. Thus, the deletion of one of these genes may result in reduced HOCl permeability through cell membrane, protecting in this way the cells. XdhD is a xanthine oxidase and belongs to the molybdoenzyme family [34]. It catalizes purine degradation, hypoxanthine metabolism and xanthine conversion to uric acid generating at the same time superoxides [34]. By the deletion of *xdhD,* cells became more resistant to HOCl. This may have happened due to the lower amount of superoxides generated, less damage happened by endogenously produced reactive species and in this way cells have less oxidative stress to account for. This may be an explanation why deletion of *xdhD* renders cells more resistant to HOCl. Results from phenotypic analysis of deletion strains upon H_2O_2 were not clear. Δ*ompF* and Δ*xylE* strains did not grow at all and seem to be more sensitive than the control (Δ*rpoH*). However, this sensitivity maybe is due to the little amount of cells transferred to the plate for the streak out. Δ*xdhD* and Δ*dppF* strains seem resistant compared to the control (Δ*rpoH*), but again there is a possibility that little amount of cells transferred on the plate for Δ*rpoH.* That is why Δ*xdhD* and Δ*dppF* seem to grow better. Additionally, the viability assay performed under 3.5 mM H_2O_2 showed that all the deletion strains are more sensitive than the control under long term incubation. For salt stress all the deletion strains grown similar compared to the control. For heat stress a Δ*ompF* and Δ*xylE* seem to be sensitive compared to the control, while the other deletion strains grow similar to the control. The viability assay performed under 47^{o}C showed no differences between the deletion strains and the control, too. These results, about general stress responses, are different from the results obtained for evolved *E. coli* cells. Evolved cells were strongly resistant to different stress conditions (HOCl, H_2O_2, heat and salt) [31], while here resistance to HOCl for some genes, is what was observed. Thus, maybe these genes play no role in general resistance as observed in HOCl evolved cells. Finally, the results may suggest that, at least not as single gene and solely, these genes are not responsible for all the characteristics of HOCl evolved cells.

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