Eva Boritsch

Virulence gene regulation in *Vibrio cholerae*: Modulation of ToxR activity by ToxS

Masterarbeit

Zur Erlangung des akademischen Grades einer Master of Science
an der Fakultät für Technische Chemie, Verfahrenstechnik und Biotechnologie
der Technischen Universität Graz

Durchgeführt am Institut für Molekulare Biowissenschaften
unter der Betreuung von
Univ.-Prof. Dr.rer.nat. Joachim Reidl

Graz, 2012

Danksagung

Ich möchte mich bei allen bedanken, die mich in diesem Lebensabschnitt begleitet und unterstützt haben. Dieser Dank gebührt vor allem meiner Familie und meinen Freunden, die in freudigen, wie auch sorgenvollen Zeiten an meiner Seite standen, mit mir gelacht und geweint haben.

Besonders bedanke ich mich bei Joachim Reidl und Stefan Schild, sowie dem Rest der Arbeitsgruppe für ein Jahr voller Erfahrungen, Erkenntnisse und Bereicherungen, vor allem aber bei Vera für die zahlreichen gemeinsam verbrachten Stunden im Labor und überhaupt, für ihre bemerkenswerte Geduld, für geteilte Freuden über funktionierende Experimente und das geteilte Leid, wenn nach unzähligen Versuchen und Abänderung aller erdenklichen Parameter noch immer keine Banden am Blot sichtbar waren; Sarah ohne deren tatkräftige Unterstützung ich vermutlich noch heute klonieren würde; sowie Mothe, Philipp und Sabine für ihren unerschütterlichen Einsatz an der FM4-Front - und für so vieles mehr.

Vor allem aber danke ich meinen Eltern: meiner Mama und besten Freundin, die immer für mich da war und mich nie daran zweifeln ließ, dass sie stolz auf mich war. Ohne dich wäre ich so schnell nicht wieder aufgestanden. Wolfi, der immer an mich geglaubt und mich unterstützt hat; und meinem Papa, der, auch wenn geographisch weit entfernt, trotzdem irgendwie hier war.

Ein besonderer Dank gebührt Georg, der es dank seines willkommenen Desinteresses an der Molekularbiologie immer geschafft hat mich auf andere Gedanken zu bringen, der stets mit Rat und Tat Kreativität in mein Leben bringt und ohne den Vieles wohl nicht einmal nur halb so schön wäre.

Danke!

Table of Contents

I Abstract V

II Introduction 1

1 Cholera 1

2 *Vibrio cholerae* - Biology and life cycle 2

3 Virulence gene regulation in *V. cholerae* 3

3.1 The main players 3

3.2 The regulatory network behind 4

4 The transcription factor ToxR 7

5 The transmembrane protein ToxS 11

6 Aim of this study 13

III Material and Methods 14

1 Material 14

1.1 Instruments and devices 14

1.2 Bacterial strains, plasmids and oligonucleotides 17

1.3 Media, supplements, buffers and solutions 20

1.3.1 Media 20

1.3.2 Media supplements 21

1.3.3 Buffers and solutions 22

2 Methods 25

2.1 Molecular biological and genetic methods 25

2.1.1 Chromosomal DNA preparation 25

2.1.2 Plasmid DNA preparation 25

2.1.3 Polymerase chain reaction (PCR) 26

2.1.4 Agarose gel electrophoresis 27

2.1.5 Digestion of DNA with restriction enzymes 27

2.1.6 Purification of DNA fragments and ligation 27

2.1.7 Construction of expression plasmids 27

2.2 Microbiological methods 28

2.2.1 Culture conditions 28

2.2.2 Preparation of electrocompetent cells and transformation 28

2.2.3 Induction of pBAD18 expression plasmids 29

2.2.4 Induction of pFLAG-MAC expression plasmids..... 29
2.3 Biochemical methods..... 29
2.3.1 Preparation of whole cell extracts..... 29
2.3.2 SDS-PAGE..... 30
2.3.3 Immunoblot analysis..... 30
2.3.4 ToxR stability assay..... 31
2.3.5 Outer membrane protein preparation..... 31
2.4 Phenotypical assays..... 32
2.4.1 Detection of virulence gene expression..... 32
2.4.1.1 CTX-KmΦ transduction assay..... 32
2.4.1.2 G_{M1}-ELISA (CT ELISA)..... 32
2.4.1.3 *In vitro* and *in vivo* competition assays..... 33
IV Results..... 35
1 ToxS influence on ToxR activity..... 35
1.1 Construction of ptoxS..... 35
1.2 $ToxR^{CC}$ and Δ*toxS* double mutants show additive defect on ToxR activity..... 36
2 ToxS influence on ToxR conformations..... 40
2.1 Construction of pFLAGtoxRS, pFLAGtoxRS(Δ264) and pFLAG$toxR^{CC}$S..... 40
2.2 ToxS alters ToxR conformations..... 42
2.3 No heterodimer visible in a *V. cholerae* background..... 45
2.4 ToxRS heterodimerization and ToxR operator binding act competitively..... 47
3 ToxS influence on ToxR stability..... 49
V Discussion..... 53
VI References..... 61
VII Appendix..... 71
1 Abbreviations..... 71

Zusammenfassung

Das Gram-negative Bakterium *Vibrio cholerae* ist ein fakultatives Humanpathogen und der Erreger der Durchfallserkrankung Cholera, welche durch Infektion des Dünndarms und massiven Wasserverlust gekennzeichnet ist. In den letzten Jahren wurde ein Anstieg der Cholera-, wie auch der Todesfälle, die auf Cholera zurückzuführen sind, vermerkt. Somit stellt Cholera auch heute noch ein ernstzunehmendes globales Gesundheitsproblem dar. Die Hauptvirulenzfaktoren Choleratoxin und Toxin-coregulierter Pilus werden durch ein komplexes regulatorisches Netzwerk, dem sogenannten ToxR-Regulon, kontrolliert, in dem die Hauptakteure AphAB, TcpPH, ToxRS und ToxT einer streng koordinierten Kaskade unterliegen.

Der OmpR-artige Transkriptionsfaktor ToxR ist maßgeblich an der Virulenzgenregulation von *V. cholerae* beteiligt. ToxR ist ein Transmembranprotein, das sich aus einer cytoplasmatischen DNA Bindedomäne und einer periplasmatischen Signalsensordomäne zusammensetzt. Natives ToxR kann verschiedene Konformationen einnehmen, die auf dem Redox-Zustand der beiden periplasmatischen Cysteinreste an den Positionen 236 und 293 basieren. Somit kann ToxR in einer i) oxidierten monomeren Konformation, ausgehend von einer intramolekularen Disulfidbindung, ii) einer reduzierten, iii) einer homodimeren Form aufgrund der Ausbildung von intermolekularen Disulfidbrücken, sowie in einer iv) oligomeren Konformation, in der mehrere ToxR Moleküle hochmolekulare Komplexe ausbilden, vorliegen.
Für die Aktivierung von ToxR regulierten Genen, wie zum Beispiel dem Hauptvirulenz-transkriptionsaktivator *toxT*, sowie den beiden Porinen *ompU* und *ompT*, sind zwei oder mehr ToxR Proteine nötig. Zusätzlich wird ein weiteres Transmembranprotein, ToxS, für ToxR Wildtyp-Aktivität benötigt. *toxS* Deletionsmutanten weisen verringerte Virulenzgenexpression auf, die genaue Funktion von ToxS für ToxR ist jedoch nicht bekannt. Diese Arbeit befasst sich mit den molekularen Mechanismen, die zur Aktivierung von ToxR führen. Dies beinhaltet die Charakterisierung der Heterodimerisierung von ToxR und ToxS, bezugnehmend auf die Cystein-abhängigen ToxR Konformationen und ToxR Aktivität.

Die Ergebnisse verschiedener Expressions- und Interaktionsstudien mittels Immuno Blot ergaben, dass Coexpression von ToxS die Bildung von Cystein-abhängigen hochmolekularen ToxR Formen zugunsten von ToxRS Heterodimerisierung in *Escherichia coli* Stammhintergründen unterdrückt. Überraschenderweise war in *V. cholerae* die Ausbildung von Heterodimeren nicht sichtbar, wenngleich die hochmolekularen ToxR Formen ebenso reduziert

waren wie in *E. coli*. Durch Einbringen von ToxR Operatorsequenzen auf den *toxRS*-Expressionssystemen kam es jedoch zur Abnahme von Heterodimeren auch in *E. coli*, was darauf hindeutet, dass ToxRS Heterodimerisierung und ToxR Operatorbindung miteinander kompetitieren.

Im Weiteren konnte gezeigt werden, dass eine ToxR Cys/Ser Doppelmutante ($ToxR^{CC}$), die verminderte transkriptionelle Aktivität aufweist, auch weniger effizient mit ToxS interagiert. Die Abwesenheit von ToxS hatte jedoch einen noch stärkeren negativen Effekt auf die Transkriptionsaktivität von $ToxR^{CC}$, was durch ein verändertes Expressionsprofil der Porine, sowie durch stark verminderte *in vitro* Expression der Virulenzgene ersichtlich wurde. Degradierungs-Assays ergaben, dass ToxS ToxR in der stationären Phase vor Proteolyse schützt, jedoch gleichzeitig die Degradierung von falsch-gefaltetem ToxR einleitet, da in der Anwesenheit von ToxS in der stationären Phase nur mehr prozessiertes $ToxR^{CC}$ detektiert werden konnte.

Die im Zuge dieser Arbeit erhaltenen Ergebnisse, deuten daher daraufhin, dass ToxS zwei unterschiedliche Funktionen erfüllen kann: Die eine ist eine Chaperon-artige, wobei ToxS aktive ToxR Konformationen bis zur Bindung an die Operatoren stabilisiert, die andere involviert Einleitung des Abbaus von falsch-gefaltetem ToxR.

I Abstract

Vibrio cholerae is a Gram-negative, facultative human pathogen, which causes the diarrheal disease cholera, resulting in infection of the small bowel and massive loss of water. In recent years, an increase of cholera incidents and deaths resulting from this disease was observed, underlining that cholera still is an important global public health problem. The main virulence factors cholera toxin and toxin-coregulated pilus are under the control of a complex regulatory network, named the ToxR regulon, with the main players AphAB, TcpPH, ToxRS and ToxT.

The OmpR-like transcription factor ToxR is among the major actors in virulence gene regulation of this bacterium and controls expression of about 150 genes. ToxR is a 296 aa transmembrane protein and consists of a cytoplasmic winged helix-turn-helix DNA binding domain, a short transmembrane domain and a periplasmic signal sensor domain. Native ToxR can have different conformations based on the redox-state of the two periplasmic cysteine residues at positions 236 and 293. So, ToxR can exist either in an (i) oxidized monomeric form, where the two cysteine residues form an intra-chain disulfide bond, in a (ii) reduced monomeric form, in a (iii) homodimeric conformation, where two ToxR molecules build up inter-chain disulfide bonds, and in a recently detected (iv) oligomeric conformation, where several ToxR molecules form high molecular complexes.
For activation of ToxR regulated genes, like e.g. the main virulence transcription factor *toxT* or the porins *ompU* and *ompT,* two or more ToxR molecules are required. Yet, it remains unknown how ToxR as a transcription factor is activated itself. For wildtype ToxR activity another inner membrane protein, ToxS is needed. *toxS* deletion mutants show decreased ToxR activity for activation of virulence gene expression, but the exact role of ToxS in the virulence gene regulatory cascade is largely unknown. This study addresses the molecular mechanisms leading to ToxR activation, including characterization of heterodimerization of ToxR and ToxS with regard to cysteine dependent ToxR conformations and ToxR activity.

Results of different expression and interplay studies via immunoblot analysis of ToxR showed that coexpression of ToxS suppresses formation of the cysteine dependent high molecular ToxR species in favor of ToxRS heterodimerization in *Escherichia coli* backgrounds. Yet, the same experimental setup in *V. cholerae* backgrounds did not yield ToxRS heterodimerization, even though ToxR high order complexes were similarly reduced as in *E. coli*. However, by providing ToxR operator sites in *E. coli*, heterodimers were absent, indicating that ToxRS

heterodimerization and ToxR operator binding act competitively. This observation provides an important new aspect of when ToxRS interaction occurs.

Moreover, a ToxR Cys/Ser double mutant ($ToxR^{CC}$), which had been shown before to have decreased transcriptional activity, was found to be not as efficient in forming heterodimers with ToxS as native ToxR. Yet, absence of ToxS even enhanced the transcriptional defect, which is displayed by an altered porin expression profile and more importantly by deficient virulence gene expression *in vitro*. Additionally, in degradation assays it could be observed that ToxS protects ToxR from proteolysis in stationary phase cultures, however at the same time was found to be able of initiating degradation of potentially misfolded ToxR species, as no full-length $ToxR^{CC}$ could be detected anymore in stationary phase cultures when ToxS was present.

This study therefore proposes a model in which ToxS possesses two distinct roles: one is to act as a chaperone in order to stabilize properly folded ToxR and keep it active until operator binding, the other is to act as a kind of proteolytic targeting factor where it promotes proteolysis of mutant ToxR in order to remove misfolded, transcriptionally inactive ToxR species.

II Introduction

1 Cholera

Cholera is a severe diarrheal disease that is characterized by massive loss of water, which leads to dehydration, acidosis, hypovolemic shock and subsequently, if untreated, to death [Childers and Klose, 2007; Klose, 2001]. In recent years, an increase of cholera incidents, as well as an increase of about 50% in deaths resulting from cholera could be observed. This increased death rate resulted from an outbreak in Haiti in 2010, which shifted the proportion of cholera cases reported worldwide from Africa and Asia to the Americas. An overall number of 317.534 cases and 7.543 deaths worldwide was reported to the WHO in 2010, pointing up that cholera still is an important global public health problem [WHO, 2011].

The causative agent, *Vibrio cholerae,* is acquired through ingestion of contaminated water or food, such as raw shellfish and fish, as well as raw vegetables or fruits, with a relatively high infection dose of about 10^6 to 10^{11} colony forming units for healthy persons and higher risks for immunocompromised or malnourished individuals [Childers and Klose, 2007; WHO, 2008; López-Gigosos et al., 2011]. The incubation period of two hours to five days is short and contributes to its rapid spread. Upon contact with the pathogen, only about 20% of infected individuals display severe dehydration symptoms, the remaining part develop no or only mild watery diarrhea [WHO, 2008]. Treatment includes rehydration therapy by application of oral rehydration salts (ORS) for mild cases or administration of intravenous fluids for severe cases. An additional application of antibiotics, such as azithromycin, ciprofloxacin or norfloxacin, shortens the duration of the disease and the spread of the pathogen [WHO, 2008; López-Gigosos et al., 2011]. The current vaccination strategy consists of two killed oral vaccines, of which only one, Dukoral®, is pre-qualified by WHO and thus internationally available. Even though Dukoral® has been shown to be safe and efficacious, it displays only moderate protection against one *V. cholerae* serogroup (O1) and is not recommended for children under two years [WHO, 2010; López-Gigosos et al., 2011; Clemens, 2011]. Thus, development and improvements of new vaccination strategies are still of major interest and importance.

2 *Vibrio cholerae* - Biology and life cycle

Vibrio cholerae belongs to the family of *Vibrionaceae* and is a Gram-negative, comma shaped, facultative human pathogen, which shows an environmental lifestyle in aquatic habitats as well as an infectious lifestyle in the human host [Wachsmuth et al., 1994]. It was first described by Filippo Pacini in 1854, however, it was Robert Koch in 1884, who gained recognition for the identification of *V. cholerae* as being the causative agent of cholera [Bentivoglio and Pacini, 1995; Howard-Jones, 1984; Koch, 1884]. So far, more than 200 serogroups of *V. cholerae* have been described, a distinction which is mainly based on the immunogenic O antigen of the outer membrane component lipopolysaccharide (LPS). Yet, one single serogroup, O1, seems to be responsible for the seven cholera pandemics that have occurred since 1817. In 1992, a new serogroup, O139, emerged in India and Bangladesh through gene conversion, which is said to mark the beginning of a potential eighth pandemic and thus abolished the notion that only *V. cholerae* O1 strains are toxigenic [Childers and Klose, 2007; Faruque et al., 1998; Reidl and Klose, 2002; Dziejman et al., 2002; Bik et al., 1995]. Serogroup O1 can be distinguished into two major, Ogawa and Inawa, and into one rather rare serotype, Hikojima [Faruque et al., 1998; Kaper et al., 1995]. Based on phenotypical and biochemical differences, such as susceptibility to polymyxin B or ability to agglutinate chicken erythrocytes, these serotypes can be sub-divided into the two biotypes classical and El Tor. El Tor strains seem to be better adapted to survival in the environment and the human host, hence causing milder forms of infection compared to the classical biotype [Safa et al., 2010; Murley et al., 1999; Kaper et al., 1995].

V. cholerae constitutes a natural free-living bacterium in marine and estuarine environments, with non-O1 and non-O139 strains being more frequently isolated from aquatic areas than O1 or O139 strains [Kaper et al., 1995]. In its aquatic lifecycle *V. cholerae* is able to adhere to chitin of marine organisms, such as zooplankton, phytoplankton, crustacean or insects, where it was shown to form biofilms in order to better persist in harsh environmental conditions [Reidl and Klose, 2002; Peterson, 2002]. O1 strains were found to exist as so-called ‘conditionally viable environmental cells’ (CVEC), which form dormant cell aggregates that become viable again under proper culture conditions, e.g. in the intestines of adult rabbits. Infected human individuals shed multicellular biofilm-like progenitor forms of CVEC, which were shown to be hyperinfectious [Faruque et al., 2006]. In areas with poor sanitation and water pollution, infection of the human host with pathogenic *V. cholerae* strains is facilitated. Upon consumption of

contaminated water or food, the bacteria first have to survive the acidic environment of the stomach before they can pass the mucus lining that covers the small intestine and attach to receptors on the surface of the intestinal epithelia [Childers and Klose, 2007; Freter et al., 1976]. In the course of colonization the bacteria encounter numerous stress factors, such as changes in osmolarity and temperature, growth inhibitory substances like bile salts, as well as components of the immune system of the host, which they have to sustain [Reidl and Klose, 2002]. After attachment *V. cholerae* produce cholera toxin (CT), which leads to (i) efflux of Cl^- and HCO_3^- out of the cells and (ii) inhibited absorption of chloride and sodium ions. This results in formation of an osmotic gradient that is responsible for the immense secretion of water from the cells into the intestinal lumen, which is the cause of the watery diarrhea [Field et al., 1972]. Adults can lose up to 20 l of body fluid per day, which can lead to death within 12 h if untreated [Banwell et al., 1970]. Bacteria are subsequently shed into the aquatic environment in a hyper-infectious state (see above), which contributes to a facilitated transmission to new hosts [Merrell et al., 2002; Faruque et al., 2006]. Moreover, it was found that already in the late phase of infection, genes are switched on which augment the survival upon re-entering into the aquatic habitats and thus create optimal conditions for an enhanced fitness in the changing environment [Schild et al., 2007].

3 Virulence gene regulation in *V. cholerae*

3.1 The main players

The main virulence factors of *V. cholerae* are cholera toxin (CT), which is, as described already, responsible for the acute water loss, and toxin-coregulated pilus (TCP), which is crucial for intestinal colonization. The ADP-ribosylating CT is a member of the AB-toxins, with an A and a B subunit [Finkelstein and LoSpalluto, 1969; Lönnroth and Holmgren, 1973]. While the pentameric B subunits are involved in attachment to the cells by interacting with the GM_1 ganglioside [van Heyningen, 1974] and subsequent transport of the A subunit into the host cells, the A subunit displays the toxigenic properties by ADP-ribosylating an arginine residue of the $G_{s\alpha}$ protein, which is responsible for regulation of adenylate cyclase. This leads to activation of the G protein and thus, constant activation of adenylate cyclase, which catalyzes the transformation of ATP to cyclic AMP (cAMP). The increased level of cAMP in the cells enhances activation of cAMP-

dependent protein kinase and alters ion transport, which results in secretory diarrhea (see chapter 2) [Moss and Vaughan, 1977; Kaper et al., 1995]. CT is encoded by the genes *ctxA* and *ctxB*, which are located on the genome of the lysogenic filamentous bacteriophage CTXϕ and thus seem to have been acquired through horizontal gene transfer, most likely within the intestines of the host. CTXϕ needs TCP in order to attach to *V. cholerae* and transfer its genome into the *Vibrio* cells, which subsequently become toxigenic [Waldor and Mekalanos, 1996; Davis and Waldor, 2003]. TCP belongs to the type IV bundle-forming pili and is further essential for colonization of the intestinal epithelia by *V. cholerae* [Taylor et al., 1987; Herrington et al., 1988], even though a bacterial-host interaction has not been demonstrated so far [Childers and Klose, 2007]. Its essential role in pathogenicity may be due to bacterial interactions that result in aggregation of *V. cholerae* and micro-colony formation and thus facilitate colonization [Kirn et al., 2000]. The main component of TCP is the pilin subunit TcpA, which is encoded on the *Vibrio* pathogenicity island (VPI) together with other virulence associated genes, such as the accessory colonization factor genes (*acfA, acfB, acfC, acfD*), the main virulence transcription factor *toxT*, as well as the regulatory genes *tcpPH* [Kovach et al., 1996; Karaolis et al., 1998]. Thus, acquisition of the two mobile genetic elements, CTXϕ genome and VPI, was crucial for the evolution of *V. cholerae* pathogenicity.

3.2 The regulatory network behind virulence gene expression

Both, CT and TCP, are under the control of a complex network of regulators, named the ‘ToxR regulon’ after the first identified positive regulator [Miller and Mekalanos, 1984]. The main players in this regulation cascade include ToxRS, TcpPH, ToxT and AphAB (see Fig. 1). Under the anaerobic conditions that are encountered in the intestine, the regulator protein AphB gets activated through modification, i.e. reduction of one of its cysteine residues and subsequent dimerization [Liu et al., 2011]. This results in an increased expression of the transcription factor TcpP, which is under the control of AphB and a second regulator, AphA [Kovacikova et al., 1999; 2004; Skorupski and Taylor, 1999]. AphA links virulence gene regulation and quorum-sensing, since it is repressed by the main regulator of quorum-sensing, HapR [Kovacikova and Skorupski, 2002]. At low cell density *hapR* mRNA is destabilized, hence *aphA* transcription and consequently virulence gene expression is active [Matson et al., 2007]. Recently, it was shown that AphB could also bind to and activate the *toxR* promoter in stationary growth phase, thus

adding an additional layer of control to the complex regulation of virulence genes [Xu et al., 2010].

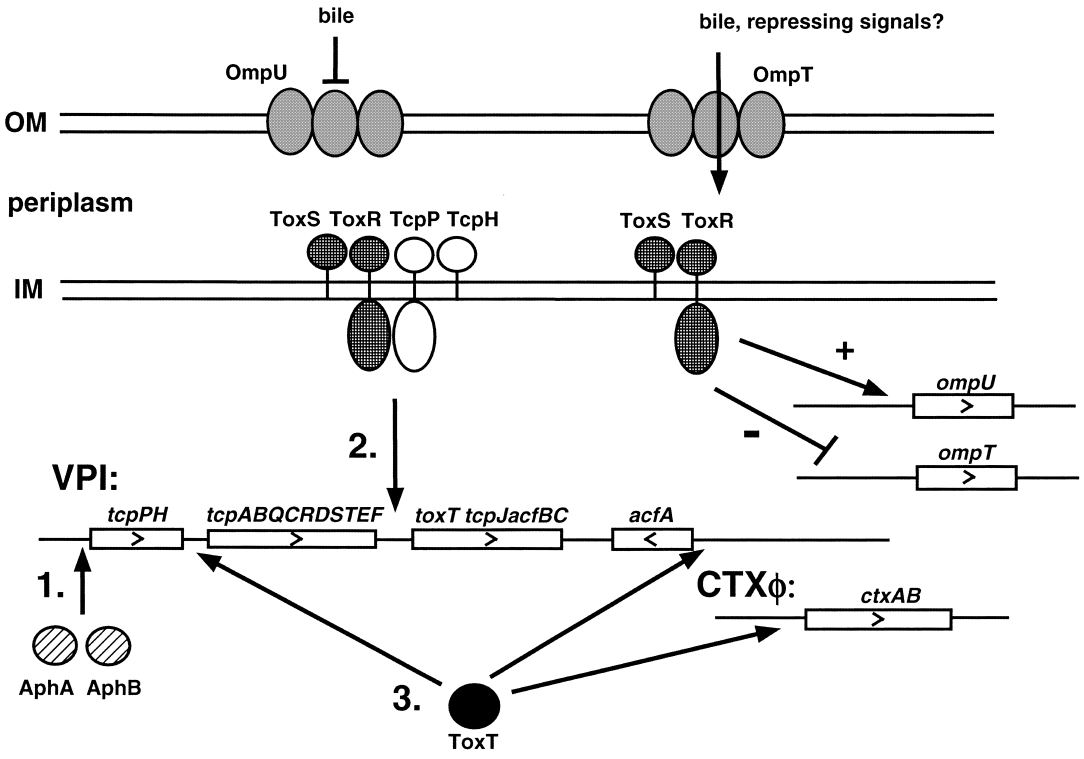

Fig. 1: Schematic illustration of virulence gene regulation in *V. cholerae* [Klose, 2001]. AphA and AphB activate expression of TcpPH (1). The membrane bound transcription factors TcpPH and ToxRS play together in order to activate gene expression of the main virulence transcription activator ToxT (2), which subsequently initiates transcription of the virulence factors, such as cholera toxin (*ctxAB*) or toxin-coregulated pilus (*tcpA*) (3). Independently from TcpP, ToxRS further regulate the porins *ompU* and *ompT*, which are among other functions involved in response to stress induced by bile salts. For detailed description see text.

The OmpR-like transcription factors TcpP and ToxR, of which the latter is encoded on the ancient *Vibrio* chromosome, play together in order to activate expression of the main virulence transcription factor ToxT. Both, TcpP and ToxR, are membrane localized and both need the presence of another specific membrane bound factor, TcpH or ToxS, respectively, for proper activity [Matson et al., 2007]. While the role of ToxS for ToxR is not yet understood (see chapter 5), TcpH was found to stabilize TcpP, since in its absence TcpP gets degraded rapidly by the protease YaeL [Beck et al., 2004; Matson and DiRita, 2005]. In contrast to ToxR, which seems to

be constitutively expressed, transcription of TcpP is activated only under conditions when expression of virulence genes is favored, e.g. under low oxygen levels sensed by AphB (see above). Upon overexpression of TcpP, ToxR is not required anymore for transcription of *toxT*, whereas the opposite is not true [Krukonis et al., 2000; Goss et al., 2010]. Hence, a model was suggested where ToxR recruits TcpP to the *toxT* promoter and facilitates interaction of TcpP with the RNA polymerase [Krukonis et al., 2000; Krukonis and DiRita, 2003; Goss et al., 2010; Morgan et al., 2011]. Upon activation, ToxT, an AraC like transcription factor [Higgins et al., 1992], is expressed and regulates transcription of the major virulence genes, which are located on the CTXϕ genome - *ctxAB* - and the VPI - *tcpPH, tcpA*, *acf* genes, as well as *toxT* itself [Brown and Taylor, 1995; Yu and DiRita, 1999] by binding to specific binding sites, the so-called toxboxes, in the promoter regions of these genes [Withey and DiRita, 2006]. Fatty acids were found to modulate ToxT activity by regulating ToxT dimerization, which is indispensable for activation of virulence gene expression [Childers et al, 2011]. Moreover, ToxT activity is enhanced in the presence of bicarbonate, which can be found in high doses in the small intestine and which constitutes an additional environmental signal that controls activation of virulence genes [Abuaita and Withey, 2009; Hogan et al., 1994]. Apart from the ToxT-dependent transcriptional regulation of virulence genes, ToxR was also shown to be able to activate CT expression in *E. coli* independently from ToxT [Miller et al., 1987], as well as regulate the porins *ompU* and *ompT* in *V. cholerae* [Miller et al., 1988]. OmpU and OmpT have been found to be involved in bile resistance and intestinal colonization. While decreased expression of OmpU is associated with an increase in bile sensitivity, OmpT was found to facilitate transport of anionic detergents through the outer membrane, which renders the cytoplasmic membrane more susceptible to membrane disrupting substances such as bile [Provenzano and Klose, 2000; Provenzano et al., 2000]. Furthermore, presence of bile was found to have a negative effect on expression of virulence genes, which demonstrates the significance of porin regulation for pathogenicity of *V. cholerae* [Gupta and Chowdhury, 1997]. Since *ompU* and *ompT* are like *toxR* encoded on the ancient *Vibrio* chromosome, outside the VPI region, regulation of the porins might represent an ancestral role of ToxR before acquisition of the mobile genetic elements VPI and the CTXϕ genome [Provenzano et al., 2001]. Moreover, it was demonstrated recently that termination of virulence gene expression is initiated by proteolysis of ToxT and TcpP in late stages of infection prior to leaving the host [Abuaita and Withey, 2011]. Yet, how and if ToxR is also involved in switching virulence genes on and off has not been described so far.

Influence of environmental signals on virulence gene transcription vary among the different strains, given that the *in vitro* virulence inducing conditions of classical strains compared to El Tor strains differ immensely. While virulence genes in classical strains are induced in LB medium at pH 6.5 and 30˚C, El Tor strains need a special rich medium that contains bicarbonate and are first grown under anaerobic conditions for 4 h, followed by 4 h of aerobic cultivation at 37˚C [Kaper et al., 1995; Iwanaga and Yamamoto, 1985]. Some of these differences arise from the variance in control of ToxT expression and activity [DiRita et al., 1996], like e.g. low oxygen activates AphB or bicarbonate enhances ToxT activity in El Tor strains, some arise from the differences in transcriptional activation of *tcpPH* by AphB based on specific base-pairs at the positions -65 and -66 in the promoter regions of classical and El Tor strains, respectively. A single base-pair exchange of these residues results in a switch of activation in such a way that El Tor strains behave like classical strains and *vice versa* [Kovacikova and Skorupski, 2000]. This explains most of the variances in transcriptional activation. However some differences, like for example the requirement for the host-unrelated temperature of 30˚C for induction of virulence genes in classical strains, still remain to be determined.

4 The transcription factor ToxR

An involvement of ToxR in virulence gene regulation has first been reported in 1984 when Miller and Mekalanos screened for genes that could activate gene transcription of a *lacZ* genetic fusion that was under the control of the *ctx* promoter. There they showed that a gene called *toxR* was able to activate CT expression more than 100 fold in *E. coli* as well as in *V. cholerae* [Miller and Mekalanos, 1984]. It was later found that ToxR is an about 32 kDa membrane bound transcription factor with a cytoplasmic winged helix-turn-helix (wHTH) DNA binding domain at its N-terminal end, comprising 182 aa, a short transmembrane domain of about 16 aa and a periplasmic domain of about 96 aa [Miller and Mekalanos, 1987]. ToxR has been described as a one-component signal transduction system [Kolmar et al., 1995], showing sequence homologies in its DNA binding motif to members of the OmpR family, of which most members act as effector proteins of two-component systems, such as OmpR itself, PhoP or VirG [Miller and Mekalanos, 1987; Martinez-Hackert and Stock, 1997]. Substitutions in amino acids of ToxR that are conserved among the listed regulatory proteins, either result in failure to bind to ToxR promoter elements and subsequently to activate gene transcription or in the deficiency to activate transcription, yet displaying unaffected promoter binding [Ottemann et al., 1992]. However, no

evidence was obtained that ToxR has a phosphoacceptor site or kinase activity itself [Pfau and Taylor, 1998]. Furthermore, most members of the OmpR family are soluble proteins with their DNA binding domain on the carboxy terminal end [Martinez-Hackert and Stock, 1997]. Exceptions apart from ToxR include TcpP of *V. cholerae* [Hase and Mekalanos, 1998], PsaE, a transcription factor involved in fimbria regulation and haemagglutination in *Yersinia pestis* [Yang and Isberg, 1997] and CadC, a transcriptional activator of the lysine decarboxylase system in *E. coli* [Watson et al., 1992].

Whole genome microarray studies suggested that ToxR is involved in regulation of about 150 genes, including the virulence genes such as *toxT*, cholera toxin or toxin-coregulated pilus, the porins *ompU* and *ompT*, as well as genes involved in energy metabolism, chemotaxis or motility [Bina et al., 2003]. However, so far direct interaction of ToxR with promoter regions could only be demonstrated for the genes *toxT, ompU*, *ompT* as well as *ctxAB* in *E. coli*, whereby it was seen that ToxR regulates transcription differently at each of these promoters [Crawford et al., 2003; Morgan et al., 2011]. ToxR binds to three different sites within the *ompU* promoter, ranging from region -238 to -139, as well as -116 to -58 and -53 to -24, where it was found to interact directly with RNA polymerase. While ToxR does not need other co-activators apart from ToxS for activation of *ompU* [Crawford et al., 1998], TcpP is needed for activation of ToxT expression. Both transcription factors, ToxR and TcpP, bind to distinct regions within the *toxT* promoter, with ToxR interacting further upstream within three inverted repeats in the region from -172 to -49 and with better efficiency than TcpP [Higgins and DiRita, 1994; Krukonis et al., 2000; Krukonis and DiRita, 2003]. In contrast, the porin OmpT is negatively regulated by ToxR (see Fig. 1). ToxR binding to the promoter herein interferes with transcription activation of *ompT* by cyclic AMP receptor protein (CRP), since the ToxR binding sites overlap with the one of CRP [Li et al., 2000; 2002]. Even though no consensus ToxR binding sequence could be determined for ToxR regulated genes, ToxR binding sites are characterized by their A/T richness and inverted repeats and the promoters display large sizes that are protected by ToxR in footprinting studies, suggesting that several ToxR proteins bind the promoters in order to activate gene transcription [Miller and Mekalanos, 1987; Li et al., 2000]. Additionally, Dziejman et al. suggested that ToxR binds DNA in a co-operative manner, since λ repressor - ToxR fusion proteins were able to dimerize and regulate a λ repressor controlled reporter system [Dziejman et al., 1999].

The membrane localization of ToxR raised the questions whether the periplasmic domain is involved in signal sensing and dimerization and whether gene transcription is subsequently activated by a cleaved soluble form, by membrane bound ToxR or by ToxR homo- or

heterodimers. Thus, numerous works focused on ToxR activity studies with various ToxR truncations as well as studies on potential interactions of the periplasmic region with different proteins including ToxR itself. Results of the different groups were somewhat contradictory though. Crawford et al., for example, stated that while membrane localization was necessary for activation of *toxT*, it was not required for regulation of the porins *ompU* and *ompT,* since a cytoplasmic version of ToxR containing only the conserved wHTH motif expressed from a plasmid was able to activate *ompU* and repress *ompT.* Thus, they concluded that membrane localization was only required for ToxR in order to interact with TcpP, even though the type of periplasmic domain was irrelevant for this interaction [Crawford et al., 2003]. Ottemann and Mekalanos in turn observed that a chromosomally encoded cytoplasmic ToxR chimera, consisting of the first 170 aa of ToxR fused to the leucine zipper sequence of GCN4, was not able to regulate porin expression [Ottemann and Mekalanos, 1995]. Similar results were obtained by Pfau and Taylor, who found that proteolytically stable cytoplasmic versions of ToxR failed to interact with the *ctx* promoter [Pfau and Taylor, 1998]. Furthermore, it was shown that ToxR fusion proteins, where the periplasmic domain of ToxR was replaced by that of the alkaline phosphatase PhoA, which is known to function as a dimer [Akiyama et al., 1988], could activate gene transcription to levels comparable to wildtype ToxR [Miller et al., 1987; Kolmar et al., 1995; Dziejman et al., 1999]. Additionally, replacement of the periplasmic domain of ToxR with PhoA resulted in a deficiency to respond to certain environmental signals, such as osmolarity [Miller et al., 1987]. In contrast, fusion proteins where the periplasmic domain was replaced by that of a monomeric protein, such as MalE or Bla, resulted in a decreased ToxR transcriptional activity when heterologously expressed in *E. coli*, however not necessarily in *V. cholerae* [Kolmar et al., 1995; Dziejman et al., 1999].

Biochemical cross-linking experiments showed that ToxR was able to form homodimers through inter-chain disulfide bonding of its two cysteine residues, which are harbored in the periplasmic domain at positions 236 and 293. Moreover, when expressed at normal levels ToxR was found to mainly form heterodimers with its co-transcribed partner ToxS by simultaneously suppressing ToxR homodimers (see chapter 5). Replacement of one of the cysteine residue at position 236 by serine still lead to formation of ToxR homodimers, yet, unlike wildtype ToxR, which was also found to form intra-chain disulfide bonds, $ToxR^{C236S}$ only existed in a reduced conformation, which was less active for regulation of CT expression. Thus, ToxR seems to be able to form different conformations based on the redox-state of the cysteine residues: (i) an oxidized monomeric form, where the two cysteine residues form an intra-chain disulfide bond, (ii) a reduced monomeric form, (iii) a homodimeric conformation, where two ToxR molecules build up

inter-chain disulfide bonds [Ottemann et al., 1996], and a recently detected (iv) oligomeric conformation, where several ToxR molecules form high molecular complexes [Fengler et al., unpublished data] (see Fig. 2).

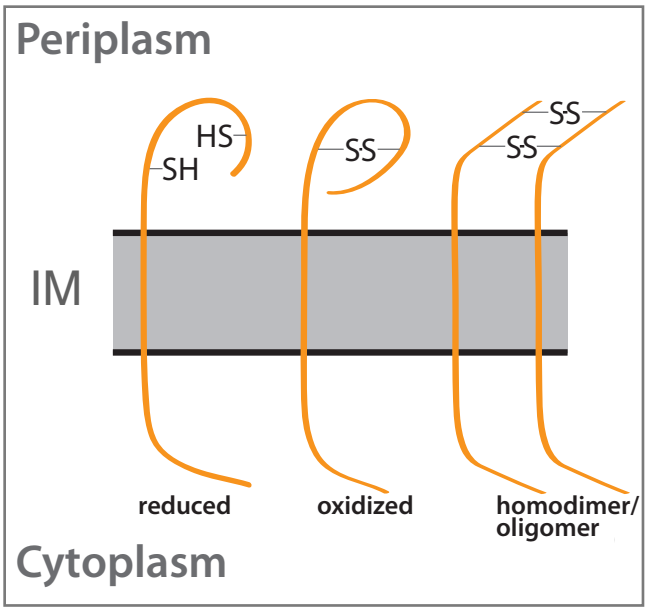

Fig. 2: Schematic illustration of possible ToxR conformations based on the redox-state of the cysteine residues at positions 236 and 293 in the periplasmic domain. Shown are a reduced and an intra-chain disulfide bonded (oxidized) monomeric form, as well as an inter-chain disulfide bonded ToxR homodimer (from left to right). ToxR oligomers are not specifically illustrated.

In conclusion, most obtained data from different research groups argue for ToxR being active in a homo- or heterodimeric conformation, however, the question of whether the periplasmic domain of ToxR acts as a signal sensor and whether dimerization occurs upon certain environmental signals, still needs to be answered. Even though ToxR activity on virulence gene regulation initially seemed to be affected by certain environmental and growth factors, such as osmolarity, pH and growth temperatures [Miller et al., 1988; Ottemann and Mekalanos, 1996], none of these conditions could be linked to a change in homo- or heterodimerization [Ottemann et al., 1996]. Yet, it was found later that most of these environmental signals acted on other factors involved in virulence gene regulation, like AphB, TcpP or ToxT [Liu et al., 2011; Abuaita and Withey, 2009; Häse and Mekalanos, 1998], leaving the question of a potential regulative signal for control of ToxR activity unanswered. A mix of certain amino acids (NRES; asparagine, arginine, glutamate and serine), heat shock response regulators, as well as the upstream

virulence transcription factor AphB were shown to influence ToxR expression levels and thus ToxR regulated genes [Mey et al., 2012; Parsot et al., 1990; Xu et al., 2010], however, the underlying mechanisms of ToxR regulation, as well as the function of the coexpressed protein ToxS on ToxR activity remain unresolved.

5 The transmembrane protein ToxS

In 1989 Miller et al. found that the classical strain 569B, which was less virulent than other *V. cholerae* strains, yet synthesized more cholera toxin *in vitro*, carried a 1.2 kbp genetic deletion downstream of *toxR*. They further found that this particular gene, which they called *toxS*, was in one operon with *toxR* and a *trans*-acting activator of the latter. However, ToxS did not activate the promoter of *toxR*, but rather had an influence on ToxR activity [Miller et al., 1989].

ToxS is a 19 kDa transmembrane protein, however, unlike ToxR, only has a short cytoplasmic domain of about 6 aa with the major part of its 173 aa residing in the periplasm [DiRita and Mekalanos, 1991]. No homologies to other proteins have been found so far, even though ToxS topology resembles the one of TcpH [Häse and Mekalanos, 1998]. Furthermore, the exact role of ToxS and its mechanism of action is not understood. ToxS does not have an influence on the levels of ToxR, neither does it affect binding of plasmid encoded ToxR to DNA as seen in a challenge phage assay, but in its absence ToxR fails to activate gene transcription [Pfau and Taylor, 1998]. However, presence of ToxS requires about 4 times less ToxR protein to shift *ctx* promoter DNA, indicating that ToxS does facilitate ToxR promoter binding at low ToxR concentrations [Ottemann et al., 1992].

ToxS interacts with the C-terminal periplasmic domain of ToxR, since ToxR truncations, where the periplasmic domain was either deleted or entirely replaced by alkaline phosphatase (PhoA), failed to show a ToxS dependent increase of transcriptional activity on the *ctx* promoter in *E. coli.* Since overexpression of ToxR obviates ToxS dependence, as does fusion of ToxR with PhoA, which is known to form dimers, it was suggested that ToxS stabilizes spontaneously forming ToxR homodimers and by doing so reduces the concentration of ToxR that is needed for transcriptional activity [DiRita and Mekalanos, 1991]. This model was supported by studies showing that truncated versions of ToxR which were fused to λ repressor were only able to dimerize and thus be active in the presence of ToxS [Dziejman and Mekalanos, 1994; 1999].

However, as already mentioned earlier and in conflict with these results, ToxS was found to form heterodimers with ToxR by simultaneously suppressing ToxR homodimerization under wiltype expression levels. This raised the question whether ToxRS heterodimers were also able to activate gene transcription and ToxR homodimerization was thus not necessary or whether the ToxR homodimers that were formed in the presence of ToxS could not be detected with the methods that were used in that study [Ottemann and Mekalanos, 1996].

Another question that was debated was whether ToxS is involved in stabilization of ToxR. In contrast to TcpH, which inhibits degradation of TcpP dramatically, ToxS did not show any effect on ToxR stability in classical strains in LB medium at 30˚C and pH 6.5 [Beck et al., 2004]. However, most strikingly, in stationary phase *toxS* deletion mutants no ToxR proteins could be detected, despite unchanged *toxR* transcription levels [Xu et al., 2010]. Also, a ToxR-PhoA fusion protein, where PhoA was fused to Pro286, which is 8 aa from the C-terminal end of ToxR, and a ToxR-λ-repressor fusion protein happened to be degraded in the absence of ToxS [DiRita and Mekalanos, 1991; Dziejman and Mekalanos, 1994]. Periplasmically truncated versions of ToxR were degraded in the presence of ToxS though, as was wild-type ToxR coexpressed with a ToxS L33S mutant, resulting in a proteolytic form of ToxR of about 22 kDa [Pfau and Taylor, 1998]. This again indicates that ToxR and ToxS interaction occurs through their periplasmic domains and is abolished even when short periplasmic parts are deleted. Still, ToxR periplasmic mutants and wild-type ToxR with the ToxS L33S mutant were able to bind to DNA, yet unable to activate transcription. Because of the enhanced degradation of ToxR seen in the presence of ToxS L33S mutant it was suggested that ToxS alters the periplasmic domain of ToxR, which leads to a higher susceptibility of ToxR to proteases, and that ToxS thus displays a chaperone-like activity [Pfau and Taylor, 1998]. Taken these data together, ToxR and ToxS seem to interact with each other through their periplasmic regions, however, the exact mechanism and function of this interaction for ToxR activity still needs to be unravelled.

6 Aim of this study

The membrane bound transcription factor ToxR is crucial for porin and virulence gene regulation in *V. cholerae* [Miller and Mekalanos, 1984]. ToxR harbors two cysteine residues in its periplasmic domain, which were found to be able to form intra- and inter-chain disulfide bonds. ToxR mutants that lacked one cysteine residue showed decreased transcriptional activity [Ottemann and Mekalanos, 1996]. Moreover, ToxR requires coexpression of another transmembrane protein, ToxS, for wildtype transcriptional activity. *toxS* deletion mutants showed decreased ToxR activity for activation of virulence gene expression [Miller et al., 1989], but the exact role of ToxS in the virulence gene cascade is largely unknown. While studies focused on activity of ToxR, ToxS was neglected. Still, understanding its influence on ToxR activity might help in unravelling the mechanisms that lead to activation of ToxR itself, which little is known of, and thus contribute to a better understanding of the molecular biology that underlies virulence gene regulation in *V. cholerae*.

In order to elucidate the influence of ToxS on cysteine dependent ToxR activity and its mechanism of action, ToxR transcriptional activity on porin regulation, as well as on virulence gene expression *in vitro* and *in vivo* should be tested for ToxR double cysteine mutants ($ToxR^{CC}$) in the absence of ToxS. For subsequent complementation of the mutants' phenotypes arabinose inducible plasmids carrying *toxS* should be constructed.

A second aim focused on ToxS influence on the different ToxR conformations that were based on the redox-state of the periplasmic cysteine residues. Ottemann and Mekalanos already showed in 1996 that ToxS did change disulfide bonded ToxR forms [Ottemann and Mekalanos, 1996], however, no further studies on the topic of ToxR conformations in dependence of ToxS, as well as ToxRS heterodimerization were attempted thereafter. Therefore, plasmids encoding FLAG-tagged *toxR* coexpressed with *toxS* should be constructed and the different conformations should be detected via immunoblot analysis. Furthermore, impact of ToxR DNA binding sites on ToxR and ToxS conformations should be analyzed by introducing *ompU* and *toxT* promoter elements into the expression systems carrying *toxRS*.

Finally, the question whether ToxS was involved in protection of ToxR from proteolysis should be brought up again. Consequently, degradation assays of ToxR and $ToxR^{CC}$ in the presence and absence of ToxS should be performed.

III Materials and Methods

1 Material

1.1 Instruments and devices

The instruments and devices used in this study are described in **table 1**. If not stated otherwise materials were purchased from the following companies: BD Biosciences, Carl Roth GmbH + Co, Eppendorf AG, Fisher Scientific GmbH, Greiner Bio-One GmbH, Merck KGaA, Neo Lab Migge Laborbedarf-Vertriebs GmbH, PEQLAB Biotechnologie GmbH, Sarstedt AG + Co, Scherf Praezision Europa GmbH, VWR International GmbH and Sterilin Limited.

Table 1: Instruments and devices used in this work

Instrument	Description	Manufacturer
Agarose gel documentation System	Bio-Vision CN-3000-WL	Vilber Lourmat
Analytical balance	BP121S	Sartorius AG
Anesthesia device	Combi-vet system TEC 3 ISOFLURANE	Rothacher Medical
Autoclave	Systec V-150	Systec GmbH
Balance	GP3202	Sartorius AG
Bunsen burner	Teclu-Safety Burner	Juchheim Laborgeräte
Centrifuge	Avanti® J-26 XP Series	Beckman Coulter
Certoclave	Multicontrol 1020	CertoClav Sterilizer GmbH
Clean bench	Type EF/SB	Clean Air Techniek
Cuvette for electroporation	Gap width: 1 mm Filling quantity:100 µl	Eppendorf AG
Electrophoresis unit for agarose gels	Wide Mini-Sub Cell GT	Bio-Rad Laboratories, Inc.
Electroporator	Elektroporator 2510	Eppendorf AG
Eppendorf centrifuge	Centrifuge 5810R	Eppendorf AG
Fiber-optic ultra-micro measuring cell	TrayCell	Hellma

Instrument	Description	Manufacturer
Freezer (-20°C)	Freezer	Allectric
Freezer (-70°C)	958	Thermo Scientific Forma
Heating block	Digital Heatblock	VWR International
Homogenizer	Tissue-Tearor	BioSpec Products
Ice machine	MF 30	Scotsman Ice
Imager	Molecular Imager ChemiDoc XRS System	Bio-Rad Laboratories, Inc.
Incubator	BD 115	BINDER GmbH
Magnetic stirrer	MR Hei-Mix S	Heildolph Instruments
Microcentrifuge	5415R	Eppendorf AG
Micro plate reader	MicroPlateReader 550 FLUOstar Omega	BMG-Labtech
Multichannel pipette	Research	Eppendorf AG
pH-Meter	Metrohm 632	Metrohm AG
Pipette (Gilson)	PIPETEMAN Neo P1000N, P200N, P20 N	Gilson
Pipette (Eppendorf)	Research (variable) 0.1 – 2.5 µl	Eppendorf AG
Pipettor	Accu-jet	BRAND
Plate reader	MicroPlateReader 550, FLUOstar Omega	BMG Labtech
Power supply	Power Supply PowerPac Basic	Bio-Rad Laboratories, Inc.
Refrigerated table centrifuge	5810R	Eppendorf AG
Refrigerated table microcentrifuge	5415R	Eppendorf AG
Refrigerator	UMKS 3600	Allectric
Rocker (RT)	Stuart Gyro-Rocker SSL3	Bibby Scientific
Rocker (4°C)	Promax 2020	Heidolph Instruments

Instrument	Description	Manufacturer
SDS-PAGE electrophoresis unit	Mini-PROTEAN Tetra cell	Bio-Rad Laboratories, Inc.
Shaker	VKS 75 A control	Edmund Bühler GmbH
Sonifier	Sonifier 250A	Branson Ultrasonics Corp.
Spectral photometer	Life Science UV/Vis - Spectrophotometer DU 730	Beckman Coulter
Still	Fontavapor 260	BÜCHI Labortechnik
Table microcentrifuge	Mikrocentrifuge 5415D	Eppendorf AG
Tank blot unit	TE22	Hoefer
Thermal cycler	C1000 Thermal Cycler ThermoStat plus	Bio-Rad Laboratories, Inc.
Thermomixer	Thermomixer compact	Eppendorf AG
Touchmixer	Vortex-Genie 1 Touch-Mixer	Scientific Industries
Touchmixer (cell disruptor)	Disruptor-Genie 1 Touch Mixer	Scientific Industries
UV chamber	GS Gene Linker	Bio-Rad Laboratories
UV transilluminator	TFX-20M	Vilber Lourmat
Vacuum pump	Vacuum gas pump	VWR International
Water bath	1003	GFL

1.2 Bacterial strains, plasmids and oligonucleotides

The strains and plasmids used in this study are listed in **tables 2** and **3**, respectively.

Table 2: Strains used in this study

	Strains	Description	Reference
E. coli	XL1-Blue	F`::Tn10 *proA*$^{+}$*B*$^{+}$ *lac*^{q} Δ(*lacZ*)M151 *recA1 end A1 gyrA46* (Nal^{r}) *thi hsdR17* ($r_K^-m_K^+$) *supE44 relA1 lac*	NEB
	DH5αλpir	F^{-} Φ80*dlacZ*Δ*M15*Δ(*argF lac*)*U169 deoR recA1 endA1 hsdR17* ($r_K^-m_K^+$) *supE44 thi-1 gyrA69 relA1* λrecA::RPA-2-Te::Mu λ*pir*R6K, km^{r}	Hanahan, 1983
	SM10λpir	*thi thr leu tonA lacY supE recA*::RPA-2-Te::Mu λpirR6K, km^{r}	Miller and Mekalanos, 1988
V. cholerae	SP27459	O1 Inaba, El Tor, clinical isolate, Bangladesh 1976, spontaneous sm^{r}	Pearson et al., 1993
	SP27459 Δ*toxR*	Deletion in *toxR*, sm^{r}	V. Fengler
	SP27459 Δ*toxS*	Deletion in *toxS*, sm^{r}	V. Fengler
	SP27459 Δ*toxRS*	Deletion in *toxRS*, sm^{r}	V. Fengler
	SP27459 Δ*toxR*::FLAG*toxR*	*toxR* replaced by FLAG*toxR*, sm^{r}	V. Fengler
	SP27459Δ*toxR*::FLAG *toxR* Δ*toxS*	*toxR* replaced by FLAG*toxR*, deletion in *toxS*, sm^{r}	V. Fengler
	SP27459 Δ*toxR*::FLAG*toxR*^{CC}	*toxR* replaced by FLAG*toxR*^{C236SC293S}, sm^{r}	V. Fengler
	SP27459 Δ*toxR*::FLAG*toxR*^{CC} Δ*toxS*	*toxR* replaced by FLAG*toxR*^{C236SC293S}, deletion in *toxS*, sm^{r}	V. Fengler

Table 3: Plasmids used in this work

Plasmids	Description	Reference
pFLAG-MAC™	Expression vector with N-terminal FLAG-Tag, IPTG inducible, ap^r	Sigma-Aldrich
pBAD18	Expression vector, arabinose inducible, km^r	Guzman et al., 1995
pMMB67EH	IncQ broad-host-range low copy-number-cloning-vector, IPTG inducible, ap^r	Morales et al., 1991
pFLAGtoxR	*toxR* of SP27459 in pFLAG-MAC™, ap^r	V. Fengler
pFLAGtoxRS	*toxR* and *toxS* of SP27459 in pFLAG-MAC™, ap^r	This study
pFLAGtoxRS_ompU	*toxR*, *toxS* and operator region of *ompU* of SP27459 in pFLAG-MAC™, ap^r	This study
pFLAGtoxRS_toxT	*toxR*, *toxS* and operator region of *toxT* of SP27459 in pFLAG-MAC™, ap^r	This study
pFLAGtoxRS(Δ264)	pFLAGtoxRS carrying a 264 bp deletion in *toxS* generated by two internal AccI sites, ap^r	This study
pFLAGtoxRS_ompU(Δ264)	pFLAGtoxRS_ompU carrying a 264 bp deletion in *toxS* generated by two internal AccI sites, ap^r	This study
pFLAGtoxRS_toxT(Δ264)	pFLAGtoxRS_toxT carrying a 264 bp deletion in *toxS* generated by two internal AccI sites, ap^r	This study

Plasmids	Description	Reference
pFLAGtoxR^{CC}	*toxR* ^{C236SC293S} point mutant of SP27459 in pFLAG-MAC™, ap^r	V. Fengler
pFLAGtoxR^{CC}toxS	*toxR* ^{C236SC293S} point mutant and *toxS* of SP27459 in pFLAG-MAC™, ap^r	This study
pFLAGtoxR^{CC}toxS_ompU	*toxR* ^{C236SC293S} point mutant, *toxS* and operator region of *ompU* of SP27459 in pFLAG-MAC™, ap^r	This study
pFLAGtoxR^{CC}toxS_toxT	*toxR* ^{C236SC293S} point mutant, *toxS* and operator region of *toxT* of SP27459 in pFLAG-MAC™, ap^r	This study
ptoxS	*toxS* of SP27459 in pBAD18, km^r	This study
pMMBtoxS	*toxS* of SP27459 in pMMB67EH, amp^r	V. Fengler

Oligonucleotides were purchased from Invitrogen Life Technologies and resuspended in ddH_2O (Fresenius Kabi GmbH). Oligonucleotides used in this work are listed in **table 4**.

Table 4: Oligonucleotides used in this study

Oligonucleotides	Sequence (5‘ - 3‘)[a]
KpnI_toxRS_5’_FLAG	AATGGTACCCATGTTCGGATTAGGACACAACTCA
BglII_toxRS_3’_FLAG	TTAAGATCTTTAAGAATTACTGAACAGTACGGT
BamHI_ompU_5’	ATTGGATCCTCCTAAATCGGGTCGGGTT
BamHI_ompU_3’	AATGGATCCGGCTCAGCCATTTTCGTGGC
BamHI_toxT_5’	TTAGGATCCGTATAGCAAAGCATATTCAGAGA
BamHI_toxT_3’	ATTGGATCCTAAATAAACGCAGAGAGCCATC
XbaI_toxS_5’	ATAGAGCTCGATGCCATCAAAGTGTGTGAG
SacI_toxS_3’	TTATCTAGATTAAGAATTACTGAACAGTACGGT

[a] restriction sites are underlined

1.3 Media, supplements, buffers and solutions

If not stated otherwise media components and chemicals were used from the following companies: Bio-Rad Laboratories Inc., Carl Roth GmbH + Co.KG, Fermentas, Fresenius Kabi GmbH, Invitrogen, Merk KGaA, neoLab Migge Laborbedarf-Vertriebs GmbH, PEQLAB Biotechnologie GmbH, Serva GmbH, Sigma-Aldrich Inc.

T4-Ligase, Taq-Polymerase, Phusion-Polymerase, Antarctic Phosphatase, SacI, XbaI, BamHI, AccI and KpnI were purchased from New England Biolabs, Inc., (Ipswich, USA), Lysozyme from Sigma-Aldrich, Inc. and Proteinase K from Carl Roth GmbH + Co. KG.
The following kits were used in this work: QIAprep Spin Miniprep Kit, QIAquick PCR Purification Kit und QIAquick Gel Extraction Kit from QIAGEN GmbH (Düsseldorf, Germany).

1.3.1 Media

Components of media and agar plates were purchased from BD Biosciences (Franklin Lakes, USA).

LB medium	10 g tryptone
	5 g yeast extract
	10 g NaCl
	ad 1 l ddH_2O, autoclave
LB agar	10 g tryptone
	5 g yeast extract
	10 g NaCl
	16 g agar
	ad 1 l ddH_2O, autoclave
M9 medium	100 ml 10 x M9-salts
	20 ml 20% glycerol
	10 ml 0.01 M $CaCl_2$
	ad 1 l with sterile ddH_2O
	2 ml 1 M $MgSO_4$

AKI-Medium	15 g peptone 4 g yeast extract 5 g NaCl ad 1 l ddH_2O, autoclave 0.3% $NaHCO_3$- solution was freshly prepared and added before use

1.3.2 Media supplements

Media supplements were added to media and LB agar after autoclaving. Antibiotics were purchased from Carl Roth GmbH + Co. KG and Sigma-Aldrich, Inc.

streptomycin	100 mg/ml in ddH_2O (stock solution) end concentration: 100 µg/ml
ampicillin	100 mg/ml in ddH_2O (stock solution) (storage -20°C) end concentration: 100 µg/ml or 50 µg/ml if used in combination with other antibiotics
kanamycin	50 mg/ml in ddH_2O (stock solution) end concentration: 50 µg/ml
chloramphenicol	30 mg/ml in 96% ethanol (stock solution)
glucose	20% in ddH_2O end concentration: 0.2%, sterile filtered
glycerol	20% in ddH_2O end concentration: 0.4%, sterile filtered
arabinose	20% in ddH_2O end concentration: 0.2%, sterile filtered
IPTG (Isopropyl-β-thiogalactopyranosid)	1 M in sterile ddH_2O
X-Gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside)	30 mg/ml in DMSO (stock solution) end concentration: 20 µg/ml

1.3.3 Buffers and solutions

Buffers and solutions used in this work are listed in **table 5.**

Table 5: Composition of buffers and solutions

Media components	
10 x M9 salts	60 g Na_2HPO_4 x H_2O
	30 g KH_2PO_4
	5 g NaCl
	10 g NH_4Cl
	ad 1 l ddH_2O, autoclave
Chromosomal DNA preparation	
TNE buffer	10 mM Tris/HCl pH 8
	10 mM NaCl
	10 mM EDTA
TNEX buffer	10 mM Tris/HCl pH 8
	10 mM NaCl
	10 mM EDTA
	1% TritonX 100
Plasmid DNA preparation	
S1 buffer	50 mM Tris/HCl pH 8
	10 mM EDTA pH 8
	100 µg/ml RNAse A
S2 buffer	200 mM NaOH
	1% SDS
S3 buffer	2.8 M KAc pH 5,1
DNA agarose gel electrophoresis	
50x TAE buffer	242 g Tris
	57.1 ml glacial acetic acid
	100 ml 0.5 mM EDTA pH 8
	ad 1 l ddH_2O

6x loading dye	10 mM Tris/HCl pH 7,6 60 mM ETDA pH 8 60% glycerol 0.03% xylene cyanol FF 0.03% bromophenol blue
SDS -PAGE	
10x SDS-PAGE running buffer	30.2 g Tris 188 g glycine ad 900 ml dH_2O, dissolve add 100 ml 10% SDS, pH 8.3 (verify only, do not set)
Stacking gel (stock solution)	100 ml 1M Tris/HCl pH 6.8 8 ml 10% SDS 92 ml ddH_2O
Separating gel (stock solution)	300 ml 2 M Tris/HCl pH 8.8 16 ml 10% SDS 84 ml ddH_2O
Stacking gel (4%)	3.75 ml stacking gel (stock solution) 1.95 ml 30% acrylamide-/0.8% bisacrylamide stock solution (Roth Gel30) 9.21 ml ddH_2O 120 µl 10% APS 45 µl 99% TEMED (Roth)
Separating gel (15%)	7.5 ml separating gel (stock solution) 15 ml 30% acrylamide-/0.8 % bisacrylamide stock solution (Roth Gel30) 7.35 ml ddH_2O 158 µl 10% APS 45 µl 99% TEMED (Roth)

Laemmli buffer	1.1 g SDS 0.41 g EDTA 0.17 g $Na_2H_2PO_4$ x 2 H_2O dissolve in ddH_2O, adjust pH to 7.2 and fill up with ddH_2O to 10 ml 10 ml 50% glycerol 0.2% bromophenol blue optional: 1.1 ml β-mercaptoethanol
Staining solution after Kang	5% aluminiumsulfat-hexadecahydrat 10% EtOH (96%) 0.02% Coomassie Blue G-250 2% ortho-phosphoric acid
Immunoblot analysis	
CAPS buffer	10 mM CAPS 10% MeOH pH 11
TBS	20 ml 1 M Tris/HCl pH 7.5 30 ml 5 M NaCl ad 1 l with ddH_2O
TBS-Tween/Triton	20 ml 1 M Tris/HCl pH 7.5 50 ml 5 M NaCl 2 ml Triton X100 500 µl Tween 20 ad 1 l with ddH_2O
High sensitivity ECL	Immun-Star™ WesternC™ Kit (Bio-Rad, Vienna)
ELISA	
PBS	137 mM NaCl 2.7 mM KCl 8.1 mM Na_2HPO_4 1.76 mM KH_2PO_4 pH 7.4, autoclave

PBS-T	PBS 0.05% Tween-20

2 Methods

2.1 Molecular biological and genetic methods

2.1.1 Chromosomal DNA preparation

Chromosomal DNA preparation was based on the protocol of Grimberg et al. 2 ml of ONC was harvested (5,200 g, 5 min at RT) and pellet was washed with 1 ml TNE and further resuspended in 270 µl TNEX. After 20 min incubation at 37˚C with 30 µl lysozyme (5 mg/ml in ddH_2O), 15 µl proteinase K (20 mg/ml in ddH_2O) was added, mixed by inverting and suspensions were incubated for at least 2 h at 65˚C. As a next step the samples were mixed with 400 µl saturated phenol (in TE pH 8, Roth), again mixed by inverting and centrifuged at 13,000 g for 10 min at RT. 200 µl of aquatic phase was collected and centrifuged again. Finally 150 µl of aquatic phase were mixed with 30 µl NaCl (5 M) and 750 µl ice cold ethanol (96%) for precipitation of chromosomal DNA. For a higher yield samples were incubated at -20˚C for 15 min and then centrifuged at 13,000 g for 30 min at 4˚C. Pellet was washed with 1 ml ice cold ethanol (70%) and dried before dissolving in 75 µl ddH_2O. Pellets were stored at -20˚C.

2.1.2 Plasmid DNA preparation

Pure plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (QIAgen) following the Protocol ‘Plasmid DNA Purification Using the QIAprep Spin Miniprep Kit and a Microcentrifuge’ (Quiagen, Hilden).

Additionally a slightly modified version of the plasmid isolation method by Birnboim and Doly was used. 4 ml ONC were harvested (5,000 g, 5 min, RT), resuspended in 300 µl S1 buffer and mixed with 300 µl S2 by inverting. After incubation for 5 min at RT 300 µl S3 buffer were added, mixed by inverting and incubated on ice for 30 min. The suspension was centrifuged at 17,900 g for 30 min at 4˚C and supernatant was transferred into new reaction tube. Plasmid DNA was precipitated with 0.8 volume of ice cold isopropyl alcohol (17,900 g, 30 min, RT) and pellet was washed with ice cold ethanol (70%) at 17,900 g for 15 min at RT. Pellet was dried at 37˚C and resuspended in 30 µl ddH_2O. DNA was stored at -20˚C.

2.1.3 Polymerase chain reaction (PCR)

For generating fragments used for cloning Phusion high-fidelity (Finnzyme, Espoo) and for verifying obtained constructs Taq DNA polymerase (New England Biolabs, Ipswich) was used. Oligonucleotides used in this work are listed in **table 4** and were purchased from Invitrogen Life Technologies (Invitrogen, Lofer). Annealing temperature for the PCR was determined by multiplying A and T bases of the oligonucleotides by two (2˚C) and G and C bases by four (4˚C) and subsequent addition of the obtained degrees. For the final hybridization temperature, 5˚C were subtracted from this sum. In case of different annealing temperatures of oligonucleotides used for one reaction, the lower temperature was used. The elongation temperature was 72˚C and the time was based on the length of the fragment to be amplified. As denaturation temperature 95˚C was used for Taq DNA polymerase and 98˚C for Phusion high-fidelity polymerase reactions. The number of cycles was 30.
Colony PCR was conducted in order to verify correct clones. Colonies were picked with pipette tips, resuspended in ddH_2O and boiled for 7 min.

The composition of the reaction mixes are listed in **table 6**.

Table 6: PCR reaction mix for either Taq DNA polymerase or Phusion high-fidelity polymerase reactions

	Polymerase used for PCR	
Reagents (for 25 µl)	Taq DNA polymerase	Phusion high-fidelity polymerase
polymerase	0.25 µl	0.25 µl
reaction buffer	2.5 µl (10x ThermoPol Reaction Buffer)	5 µl (5x HF Buffer)
dNTPs [10 mM]	0.5 µl	0.5 µl
5’ oligonucleotide [10 µg/ml]	0.5 µl	0.5 µl
3’ oligonucleotide [10 µg/ml]	0.5 µl	0.5 µl
template	4 µl (boiled colony)	1 µl (1:10 or 1:20 chromosomal DNA or plasmid DNA)
ddH_2O	16.75 µl	17.25 µl

Correct sizes of PCR products were checked on 0.8% agarose gels.

2.1.4 Agarose gel electrophoresis

0.8% agarose gels were used to separate DNA fragments according to their size. peqGOLD Universal-Agarose (PEQLAB Biotechnologie GmbH) was dissolved in 1x TAE buffer and 1 µl ethidium bromide per 100 ml agarose was added. DNA samples were mixed with 6x loading dye to a final concentration of 1x. 2-log DNA Ladder Standard (New England Biolabs, Ipswich) served as a reference to determine correct sizes of the fragments. 1x TAE was used as a running buffer.

2.1.5 Digestion of DNA with restriction enzymes

DNA fragments and plasmids were digested with restriction enzymes purchased from New England Biolabs. Optimal working conditions such as appropriate buffers, temperature and possible hindrance of methylation sites were determined using New England Biolabs handbook.

2.1.6 Purification of DNA fragments and ligation

In order to avoid re-ligation of empty plasmids, 1 µl Antarctic Phosphatase was added to the plasmid samples after restriction reactions and plasmids were incubated at 37˚C for 30 min. Before ligation DNA samples were purified by cutting fragments out of agarose gels and further purification with the QIAquick Gel Extraction Kit (QIAgen). PCR fragments were purified with QIAquick PCR Purification Kit (QIAgen) in order to remove ligation inhibiting salts.

For ligation of PCR products with plasmids 1 µl T4 DNA Ligase and 1 µl T4 DNA Ligase Reaction Buffer (10x) was added to an end volume of 10 µl reaction mix. An excess of insert (7.5 or 8 µl) lead to higher yields of ligated end products. Reactions were incubated at 16˚C over night and inactivated at 65˚C for 10 min.

2.1.7 Construction of expression plasmids

Inserts of interest for generating pFLAG-MAC™ and pBAD18 expression plasmids were amplified by PCR with the oligonucleotides listed in **table 4**. For construction of pFLAGtoxRS and pFLAGtoxR^{CC}S oligonucleotides KpnI_toxRS_5'_FLAG and BglII_toxRS_3'_FLAG were used with chromosomal DNA of SP27459 Δ*toxR*::FLAG*toxR* or SP27459 Δ*toxR*::FLAG*toxR*^{CC} as templates. PCR products and pFLAG-MAC™ plasmid were digested with KpnI and BglII and subsequently ligated. *ompU* and *toxT* operator fragments were integrated into the obtained

expression plasmids by digesting the plasmids with BamHI. Oligonucleotides BamHI_ompU_5' and BamHI_ompU_3', or BamHI_toxT_5' and BamHI_toxT_3', respectively, were used for amplifying *ompU* and *toxT* operator sites and PCR products were also digested with BamHI. Additionally, all expression plasmids were cut with AccI in order to generate a truncated version of *toxS*, where 264 bp were deleted out of its periplasmic encoding domain.

For construction of ptoxS the oligonucleotides XbaI_toxS_5' and SacI_toxS_3' were used with the vector pMMBtoxS as template. The PCR products as well as the pBAD18 plasmid were digested with XbaI and SacI and subsequently ligated.

Electrocompetent *E. coli* XL1-Blue cells were transformed with the expression plasmids and plated on LB agar plates containing ampicillin (pFLAG-MAC™ plasmids) or kanamycin (pBAD18 plasmids) and 0.2% glucose. Glucose represses expression of the proteins and was therefore added to all media for cultivation of cells containing these expression plasmids. Transformants were tested via Colony PCR and plasmids were isolated by using the QIAprep Spin Miniprep Kit (QIAgen). Finally, electrocompetent *V. cholerae* cells were transformed with the obtained expression plasmids. Constructs were confirmed by DNA sequencing after the method of Sanger [Sanger et al., 1977] with an automated DNA sequencer (LGC Genomics, Berlin).

2.2 Microbiological methods

2.2.1 Culture conditions

E. coli and *V. cholerae* cells were grown in LB media at 37˚C and 180 rpm over night. For growth on agar plates cells were either plated in different dilutions or streaked for single colonies and incubated at 37˚C over night. Glycerol stocks of strains were made by mixing LB overnight cultures in 1:3 dilutions with 70% glycerol and subsequent storage at -70˚C.

2.2.2 Preparation of electrocompetent cells and transformation

For preparation of electrocompetent cells 200 - 1,000 ml LB were inoculated with overnight cultures of *E. coli* or *V. cholerae* and cells were incubated at 37˚C and 180 rpm until they reached an OD_{600} of 0.8 - 1. Afterwards they were kept on ice for at least 30 min and subsequently washed twice with the same volume and once with 0.5 volume of ice cold ddH_20 for *E. coli* and 2 mM $CaCl_2$ for *V. cholerae* (5,000 g, 10 min at 4˚C). Cells were resuspended in 0.2 volume 15% glycerol (*E. coli*) or 15% glycerol in 2 mM $CaCl_2$ (*V. cholerae*) and centrifuged

again. Supernatant was discarded and cells were resuspended in remaining volume. Aliquots of 80 -120 µl were stored at -80˚C until used for electroporation.

For transformation electrocompetent cells were thawn on ice, mixed with 5 - 10 µl of DNA and transferred into a chilled cuvette for electroporation. *E. coli* cells were electroporated with an electric potential of 2.5 kV and *V. cholerae* with 1.8 kV. Immediately after electroporation 1 ml LB media was added to suspension and cells were incubated for 1 h at 37ºC. 10 µl, 100 µl and concentrated remaining cells were plated on LB plates with appropriate antibiotics and incubated overnight at 37ºC.

2.2.3 Induction of pBAD18 expression plasmids

For outer membrane preparation of cultures containing pBAD18 expression plasmids LB medium with sm/km was inoculated to an OD_{600} of 0.005. 0.2 - 1% arabinose was added directly and cultures were grown at 37˚C at 180 rpm for 18 h.

For induction of pBAD18 plasmids under virulence inducing conditions (see chapter 2.4.1) 0.1% arabinose was added to AKI medium and cultures were incubated for 8 h (4 h anaerobic and 4 h aerobic).

2.2.4 Induction of pFLAG-MAC expression plasmids

LB medium was inoculated with overnight cultures of *E. coli* or *V. cholerae* cells carrying pFLAG-MAC™ expression plasmids with amp and 0.2% glucose. Cultures were grown to an OD_{600} of 0.4 (37˚C, 180 rpm) and induced with either 0.005 or 0.05 mM IPTG for 60 to 90 min.

2.3 Biochemical methods

2.3.1 Preparation of whole cell extracts

For preparation of whole cell extracts equal amounts of cells (1 ml OD_{600} of 1.5) were harvested by centrifugation (5,000 g, 5 min) and cell pellets were washed with LB, resuspended in 50 µl sample buffer with or without β-mercaptoethanol and boiled for 10 min. Samples were either loaded directly on 15% SDS gels or stored at -20˚C.

2.3.2 SDS-PAGE

10 µl of whole cell extracts were loaded on 15% polyacrylamide gels (Mini-PROTEAN Tetra cell; Bio-Rad, Vienna) and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Until samples reached the separating gel 70 V were applied, for further separation a current of 15-25 mA per gel was used. 1x SDS running buffer served as running buffer and Prestained Protein Marker, Broad Range (7-175 kDa; New England Biolabs, Ipswich) as standard for all SDS-PAGEs.

For staining of proteins, gels were washed twice with dH_2O for 10 min after SDS-PAGE and subsequently incubated in Coomassie brilliant blue [Kang et al., 2002] over night. Destaining was done by washing the gels in dH_2O and pictures of gels were taken with the Biorad ChemiDoc™ detection system.

2.3.3 Immunoblot analysis

After separation of proteins via SDS-PAGE gels were washed twice with dH_2O for 10 min and afterwards incubated in CAPS transfer buffer for 20 min for subsequent transfer onto a Hybond™-C nitrocellulose membrane (Amersham-Bioscience, Freiburg) at 220 mA for 90 min. After the transfer membranes were dried at 37˚C in order to reduce background noise on the membranes and then washed twice in TBS for 5 min each. Non-specific binding sites were blocked at 4˚C over night with skim milk (10%) in TBS. In order to remove the blocking solution, membranes were again washed twice in TBS for 5 min each. Membranes were incubated with primary antibody (mouse monoclonal anti-FLAG M2 antibody (Sigma-Aldrich, Taufkirchen)) diluted 1: 2,000 in skim milk (10%) in TBS for 2 h at room temperature. Membranes were washed twice in TBS-Tween/Triton and once in TBS for 10 min each. Bound antibodies were detected with horseradish peroxidase-conjugated goat anti-mouse (Dianova GmbH, Hamburg) in a 1: 10,000 dilution in skim milk (10%) in TBS for 1 h at room temperature.

After washing the membranes three time in TBS-Tween/Triton and once in TBS, bound secondary antibodies were detected via chemiluminescence with the Immun-Star™ WesternC™ Kit (Bio-Rad, Vienna) and exposure in a Molecular Imager ChemiDoc™ XRS System (Bio-Rad, Vienna).

2.3.4 ToxR stability assay

Stability of ToxR was measured by adding chloramphenicol to the cultures and observing ToxR degradation over time. Chloramphenicol (cm) is a bacteriostatic microbicide that interferes with protein translation by binding to the 50S subunit of the ribosome [Schwarz et al., 2004].

50 ml LB medium containing sm/amp were inoculated to an OD_{600} of 0.1 with an ONC and incubated at 37˚C until an OD_{600} of 0.4 was reached. Cultures were induced with 0.05 mM IPTG for 30 min. Subsequently 30 ml were transferred into a new pre-warmed flask and protein translation was stopped by addition of cm (100 µg/ml). The remaining 20 ml culture served as a control. Whole cell extracts were prepared at the time points 0, 45, 90, 120, 150, 180 and 210 min after addition of cm and resuspended in a volume of Laemmli that was equal to 50 µl Laemmli for 1 ml of OD_{600} 1.5. After 210 min a sample was taken from the control without cm. Samples were boiled for 10 min and loaded onto 15% SDS gels for further immunoblot analysis.

2.3.5 Outer membrane protein preparation

Outer membrane protein (OMP) preparations were performed as described previously [Carlone et al., 1986]. *V. cholerae* strains were grown on LB medium and 10 ml cells with OD_{600} of 2 were harvested by centrifugation (4,000 g, 20 min, RT). After washing with 5 ml HEPES pH 7.5 (10 mM; 4,000 g, 15 min, RT), cells were lysed on ice by standard protocol sonification (Branson Sonifier 250A, Branson Ultrasonics Corp. Danbury) and samples were centrifuged (13,000 g, 8 min, RT) to settle lysed cells. The supernatants were centrifuged again (20,000 g, 30 min, RT) to pellet membrane fraction. Pellets were resuspended in HEPES pH 7.5 (10 mM) with sarcosyl (1%), incubated for 30 min at room temperature and again centrifuged (20,000 g, 30 min, RT). The outer membrane fraction was washed in HEPES pH 7.5 (10 mM) at 20,000 g for 30 min at RT and finally resuspended in 50 µl HEPES pH 7.5 (10 mM) supplemented with protease inhibitor (CompleteTM, Boehringer Mannheim). Protein amounts were determined via spectrophotometric measurements at 260 and 280 nm using TrayCell. According to the protocol of Warburg and Christian [Warburg and Christian, 1941] protein concentrations were calculated with the equation:

$$\text{protein in mg/ml} = [(1.55 * E_{280}) - (0.76 * E_{260})] * 10.$$

4 µg proteins per sample were mixed with Laemmli buffer, boiled for 10 min and finally loaded on 15% polyacrylamide gels for separation of proteins. Proteins were detected by staining the gels with Coomassie brilliant blue after Kang [Kang et al., 2002].

2.4 Phenotypical assays

2.4.1 Detection of virulence gene expression

Expression of virulence genes in the tested strains was either determined by the phage transduction frequency between phage CTX-kmΦ and toxin coregulated pili (TCP) expressing *V. cholerae* cells or expression of cholera toxin (CT) via CT-ELISA. For induction of virulence genes AKI medium was inoculated with an ONC of *V. cholerae* in a 1:100 dilution and incubated at 37˚C, first under anaerobic conditions for 4 h, following 4 h of aerobic growth (180 rpm).

2.4.1.1 CTX-KmΦ transduction assay

After induction of virulence genes 1 ml of *V. cholerae* cultures was incubated with 1 ml CTX-kmΦ lysate for 30 min and mixtures were subsequently plated in different dilutions (10^{-5}, 10^{-6}, 10^{-7}) on LB plates with sm as well as 100 µl and 1 ml (5,000 g, 5 min) of undiluted mixtures on sm/km plates for detection of transduction frequency. Only those *V. cholerae* cells that expressed TCP could interact with CTX-kmΦ and therefore grow on plates containing km. As negative controls 100 µl CTX-kmΦ lysate was plated on sm plates, as well as 1 ml *V. cholerae* culture mixed with 1 ml LB on LB plates containing sm/km.

Aliquots of lysogenic phage strain O395 CTX-kmΦ were gained using the protocol by Waldor and Mekalanos (1996) and stored at 4°C.

2.4.1.2 G_{M1}-ELISA (CT ELISA)

CT expression in culture supernatants was determined by the G_{M1}- ganglioside enzyme-linked immunosorbent assay (G_{M1}-ELISA) after Svennerholm and Holmgren (1978). Cells were grown under virulence inducing conditions and subsequently pelleted by centrifugation (5,000 g, 10 min, RT). Supernatants were transferred into new reaction tubes and again centrifuged at 13,000 g for 10 min at RT. Supernatants were stored at -20˚C. ELISA plates (BD Falcon, Heidelberg) were coated with 50 µl G_{M1} ganglioside (10 µg/ml) in Na_2CO_3 (60 mM, pH 10) (Sigma-Aldrich, Taufkirchen) for 4 h at 37°C and stored at 4˚C until use. After washing the plates four times with PBS-T, free binding sites were blocked with 100 µl BSA (4 mg/ml in PBS) for 1 h at RT. Plates were again washed four times with PBS-T and CT containing supernatants were loaded in duplicates in 1: 5 dilutions with PBS. A standard curve was generated for each plate

by loading known concentrations of purified CT in PBS (Sigma-Aldrich, Taufkirchen) in duplicates onto the plates (100, 80, 60, 40, 20, 10 and 5 ng/ml). PBS served as a negative control. Plates were incubated for 1 h at RT and washed again as described above. As a next step, plates were incubated with 50 µl primary antibody (anti-CT antibody produced in rabbit, Sigma Aldrich, Taufkirchen) in a 1: 2,000 dilution in PBS containing 4 mg/ml BSA for 1 h at RT. After washing four times with PBS-T, plates were incubated with 50 µl secondary antibody (goat anti-rabbit with conjugated horseradish peroxidase, Amersham-Biosiences, Freiburg) in a 1: 5,000 dilution in PBS containing 4 mg/ml BSA for 1 h at RT. Plates were washed again and bound secondary antibodies were detected by adding 100 µl TMB Substrate Reagent Set (BioLegend, Vienna). Reaction was stopped with 100 µl H_3PO_4 (1 M) after blue coloration of samples and plates were measured at 450 nm via microplate reader (FLUOstar Omega BMG LABtech, Vienna).

2.4.1.3 *In vitro* and *in vivo* competition assays

Keeping of mice was performed according to the regulations of the Institute of Molecular Biosciences and the recommendations in the Guide for the Care and Use of Laboratory Animals of the “Bundesgesetzblatt für die Republik Österreich” and the National Institutes of Health. The protocol was accepted by the Committee on the Ethics of Animal Experiments of the University of Graz and the Austrian Federal Ministry for Science and Research BM.W-F (Permit Number 39/158 ex 2000/10).

Mutant and wildtype (containing a *lacZ* deletion) were streaked on LB plates containing sm and grown at 37˚C for 16 h. Cells were resuspended in 1 ml LB medium and OD_{600} was set to 0.2. 5 µl of mutant was mixed with 5 µl of wildtype and 5 µl food coloring in 985 µl LB.

5, 50 and 200 µl of a 1: 1,000 dilution of this infection mix were plated on LB plates containing sm and X-Gal and incubated at 37˚C over night. Colonies were counted in order to define accurate quantities of input. Since wildtype cells carried a *lacZ* deletion, colonies showed a white color on X-Gal plates, whereas mutant cells were able to cleave the substrate, resulting in a blue colony color.

In parallel, 5 µl of the infection mix were used for inoculation of 5 ml LB and were incubated at 37˚C for 24 h. OD_{600} was again set to 0.2 and 5, 50 and 200 µl of a 1: 100,000 dilution were plated on LB plates containing sm and X-Gal and incubated at 37˚C over night. Colonies were counted and the *in vitro* competitive index was determined by calculating the ratio of mutant and

wildtype and subsequent normalization to the ratio of the input. For each input experiments were done in duplicates.

In vivo experiments were done according to the well established infant mouse model [Schild et al., 2007; Moisi et al., 2009]. 50 µl of infection mix was used for infection of six to seven days old C57BL/6 mice, which had been separated from their mother and kept at 37˚C 60 min prior infection. The mix was injected via a tube through the esophagus into the stomach. Mice were then kept in an incubator at 37˚C for 24 h. The remaining infection mix was mixed with half the volume 70% glycerol and stored at -80˚C.
24 h after infection, mice were sacrificed via cervical dislocation, small intestines were isolated and homogenized in LB containing half the volume 70% glycerol. 100 µl of 10^{-4}, 10^{-5}, 10^{-6} dilutions as well as undiluted suspension were plated on LB plates containing sm and X-Gal and incubated at 37˚C over night. For each small intestine suspension dilutions and subsequent plating were done in duplicates. Remaining suspension were frozen away at -80˚C. Next day, colonies were counted and the *in vivo* competitive index was determined by calculating the ratio of mutant and wildtype and subsequent normalization to the ratio of the input.

IV Results

1 ToxS influence on ToxR activity

1.1 Construction of ptoxS

To define the influence of ToxS on ToxR activity, *toxS* deletion mutants were tested for expression of ToxR regulated proteins. In order to complement Δ*toxS* phenotypes, an arabinose inducible plasmid that encoded ToxS had to be constructed. For this *toxS* was amplified by PCR with pMMBtoxS as template. The generated PCR product as well as the pBAD18 plasmid were digested with the restriction enzymes XbaI and SacI and subsequently ligated. Plasmids were amplified in *E. coli* XL-1 Blue and transformants selected on LB plates containing km and 0.2% glucose. Positive transformants were further verified by Colony PCR (**Fig. 3**). A colony of *V. cholerae* SP27459 wildtype served as a positive control, which generated a product of about 520 bp (lane 18). H_2O was used as a negative control (lane 19). ptoxS from clone 4 was isolated and used for transformation of *V. cholerae* SP27459 Δ*toxS.*

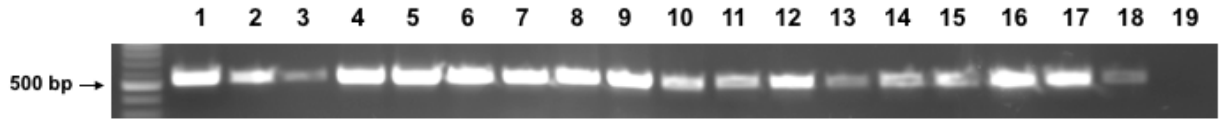

Figure 3: Colony PCR for verification of ptoxS in *E. coli* XL-1-Blue. Samples were separated on a 0.8% agarose gel, 2-log DNA Ladder served as standard. 1-17: Clones 1-17; 13: PC SP27459 wildtype; 14: NC H_2O.

ToxR activity in *toxS* deletion mutants was analyzed by preparation of the outer membrane fraction of the strains and further separation of the proteins on 15% SDS gels. Outer membrane profiles of *V. cholerae* are used for determining ToxR activity, since OmpU expression is activated and OmpT expression repressed by ToxR. This makes these two porins convenient reporters for ToxR transcriptional activity [Miller and Mekalanos, 1988]. As can be observed in **Fig. 4** SP27459 Δ*toxS* carrying only the empty pBAD18 plasmid (lane 2), as well as SP27459 Δ*toxS* carrying non induced ptoxS (0% arabinose, lane 3) showed decreased ToxR activity compared to wildtype (lane 1). This phenotype could be restored for OmpU expression, yet only

partially for OmpT when ToxS was reintroduced into the cells on the pBAD18 plasmid (0.2% arabinose, lane 4). This already indicates the importance of an exact ratio of ToxR and ToxS molecules for correct transcriptional activity, since transcriptional control of ToxR regulated genes is highly sensitive.

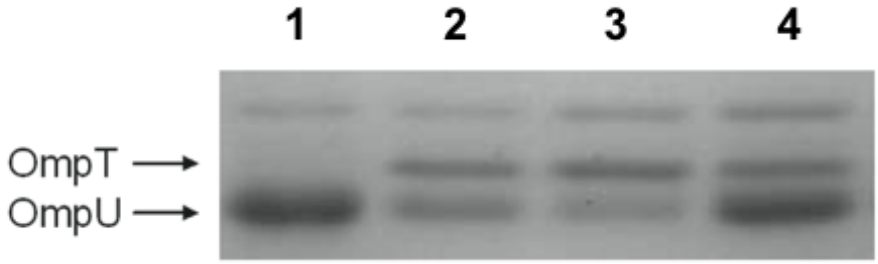

Figure 4: Outer membrane profile of *V. cholerae* wildtype, Δ*toxS* and complementation with ptoxS. Cells were grown in LB and induced with arabinose at a starting OD_{600} of 0.005 and incubated at 37˚C for 18 h. Outer membrane extracts of samples were prepared and loaded on a 15% SDS gel. 1: SP27459 pBAD18 1% arabinose; 2: SP27459 Δ*toxS* pBAD18 1% arabinose; 3: SP27459 Δ*toxS* ptoxS 0% arabinose; 4: SP27459 Δ*toxS* ptoxS 0.2% arabinose.

1.2 ToxR^{CC} and Δ*toxS* double mutants show additive defect on ToxR activity

In order to see if ToxS influence on ToxR activity is cysteine dependent, outer membrane profiles of SP27459 Δ*toxR*::FLAG*toxR*^{CC} Δ*toxS* were compared to those of SP27459 Δ*toxR*::FLAG*toxR*^{CC} and SP27459 Δ*toxR*::FLAG*toxR* Δ*toxS.* ToxR^{CC} showed a deficient activity on porin regulation compared to native ToxR (**Fig. 5** lanes 1 and 2), which could also be demonstrated on a transcriptional level by qRT-PCR by V. Fengler [Fengler et al., unpublished data]. In the absence of ToxS, activity of ToxR^{CC} decreased even more (lane 4). This phenotype could be restored to the OmpU and OmpT levels seen for SP27459 Δ*toxR*::FLAG*toxR*^{CC} with chromosomally encoded *toxS* (lane 2) when ToxS was reintroduced on the pBAD18 plasmid in strain Δ*toxR*::FLAG*toxR*^{CC} Δ*toxS* (lane 6). These results suggest that ToxS influence on ToxR activity is not cysteine dependent, since a ToxR^{CC} Δ*toxS* double mutant shows an additive defect of ToxR activity on OmpU expression compared to the single mutants.

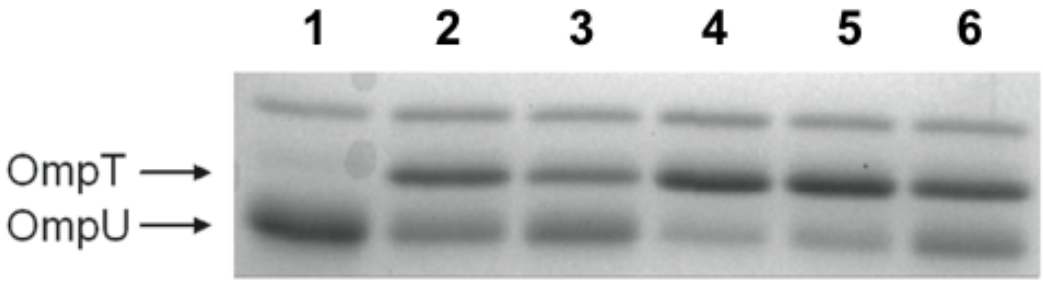

Figure 5: Outer membrane profile of *V. cholerae* Δ*toxR*::FLAG*toxR*, Δ*toxR*::FLAG*toxR*^{CC}, Δ*toxR*::FLAG*toxR* Δ*toxS*, Δ*toxR*::FLAG*toxR*^{CC} Δ*toxS* and complementation with ptoxS. Cells were grown in LB and induced with arabinose at a starting OD_{600} of 0.05 and incubated at 37˚C for 18 h. Outer membrane extracts of samples were prepared and loaded on a 15% SDS gel. 1: SP27459 Δ*toxR*::FLAG*toxR*; 2: SP27459 Δ*toxR*::FLAG*toxR*^{CC}; 3: SP27459 Δ*toxR*::FLAG*toxR* Δ*toxS*; 4: SP27459 Δ*toxR*::FLAG*toxR*^{CC} Δ*toxS*; 5: SP27459 Δ*toxR*::FLAG*toxR*^{CC} Δ*toxS* pBAD18 0.2% arabinose; 6: SP27459 Δ*toxR*::FLAG*toxR*^{CC} Δ*toxS* ptoxS 0.2% arabinose.

We further wanted to know whether a ToxR^{CC} Δ*toxS* double mutant was also more defective for virulence gene regulation, i.e. affected cholera toxin (CT) and toxin coregulated pili (TCP) production. Both, CT and TCP underlie the control of the ToxR regulon, thus deficient ToxR activity leads to a decrease in expression of these proteins.

Cholera toxin production can be measured by G_{M1}-ELISA [Svennerholm and Holmgren, 1977], whereby CT in collected culture supernatants binds to immobilized G_{M1} ganglioside receptors on ELISA plates. Bound CT can be detected with specific CT-antibodies and subsequent binding of peroxidase-conjugated secondary antibody to the CT-primary antibody complex. CT that was present in culture supernatants could be quantified by the intensity of blue coloration due to the color change of the TMB Substrate mediated by peroxidase (for detailed experimental description see Material and Methods chapter 2.4.1.2).

For quantification of TCP production, the CTX-kmΦ transduction assay was used. Since the phage CTX-kmΦ uses TCP as an attachment site for infection of *V. cholerae*, transduction rate of the km resistance gene and subsequent capability of *V. cholerae* cells to grow on LB plates containing km, can be employed to determine TCP production.

Table 7 shows that both, CT and TCP production in the double mutants are significantly decreased compared to wildtype. CT levels in Δ*toxR*::FLAG*toxR*^{CC} Δ*toxS* were 73 ng/ml compared to 7,338 for wildtype ToxR (Δ*toxR*::FLAG*toxR*), 5,012 for ToxR^{CC} and 1,529 for

Δ*toxS. toxR* deletion mutants produced 92 ng/ml CT and thus resembled Δ*toxR*::FLAG*toxR*^{CC} Δ*toxS.* Same effects can be observed for production of TCP, whereby transduction rates of Δ*toxR* and Δ*toxR*::FLAG*toxR*^{CC} Δ*toxS* are below the limit of detection of 5 x 10^{-8} and rates for wildtype and single mutants range from 3 x 10^{-5} to 10^{-6}. Hence, virulence gene regulation is severely impaired when controlled by ToxR^{CC} in the absence of ToxS, whereas no significant difference on CT and TCP levels could be detected between wildtype ToxR and ToxR^{CC} in the presence of ToxS, making ToxS to the decisive factor for virulence gene regulation in these ToxR mutants. CT and TCP production for Δ*toxR*::FLAG*toxR,* Δ*toxR,* Δ*toxR*::FLAG*toxR*^{CC} and Δ*toxR*::FLAG*toxR* Δ*toxS* was measured by V. Fengler.

Table 7: Virulence gene expression of SP27459 FLAG-tagged *toxR*, *toxR* deletion and cysteine mutants and *toxS* deletion mutants.

Strain	CT production [ng/ml][a]	ctxΦ transduction rate [cfu/ml][a]
Δ*toxR*::FLAG*toxR*[b]	7,338 (4,341 - 12,280)	4.76 x 10^{-5} (1.06 x 10^{-5} - 1.56 x 10^{-4})
Δ*toxR*[b]	92 * (77 - 127)	< LOD
Δ*toxR*::FLAG*toxR*^{CC} [b]	5,012 (2,554 - 10,530)	3.83 x 10^{-5} (2.48 x 10^{-5} - 6.84 x 10^{-5})
Δ*toxR*::FLAG*toxR* Δ*toxS*[b]	1,529 * (896 - 2,389)	1.06 x 10^{-6} (5.79 x 10^{-7} - 1.04 x 10^{-5})
Δ*toxR*::FLAG*toxR*^{CC} Δ*toxS*	73 * (69 - 85)	< LOD

[a] median and interquartile range of at least 7 independent experiments
[b] reference: V. Fengler
* significant by Kruskal-Wallis test followed by Dunn's test of selected pairs of columns with $P < 0.05$
< LOD below limit of detection of 5 x 10^{-8}

Reintroduction of ToxS on the pBAD18 plasmid into Δ*toxR*::FLAG*toxR*^{CC} Δ*toxS* and induction with 0.1% arabinose lead to a significant increase of cholera toxin production (554 ng/ml) compared to those strains carrying only the empty pBAD18 plasmid (27 ng/ml; **table 8**). Still, CT levels of ToxR^{CC} with chromosomally encoded *toxS* (5,012 ng/ml; **table 7**) could not be obtained. Of the several tested arabinose concentrations (data not shown) 0.1% delivered the

best results. This partial complementation points out again the importance of the precise interplay between exact levels of ToxR and ToxS for proper regulation of gene transcription. Complementation of Δ*toxR*::FLAG*toxR*^{CC} Δ*toxS* for TCP production could not be analyzed, since the pBAD18 plasmid contains a km resistance cassette, which interferes with the CTX-KmΦ transduction assay.

Table 8: Virulence gene expression of SP27459 *toxR* cysteine mutants and *toxS* deletion mutant and complementation with ptoxS.

Strain	CT production [ng/ml][a]
Δ*toxR*::FLAG*toxR*^{CC} Δ*toxS* pBAD18	27 (26 - 30)
Δ*toxR*::FLAG*toxR*^{CC} Δ*toxS* ptoxS	554 * (462 - 837)

[a] median and interquartile range of at least 7 independent experiments
* significant by Kruskal-Wallis test followed by Dunn's test of selected pairs of columns with $P < 0.05$

In order to see if virulence genes in Δ*toxR*::FLAG*toxR*^{CC} Δ*toxS* mutants were also downregulated *in vivo*, 6-7 days old C57BL/6 mice were infected with a mix of mutant and a wildtype that was lacking *lacZ* and tested for colonization. The infant mouse model is used, since in this model *V. cholerae* is effective in colonizing the small intestine, unlike in adult mice, where the innate gut microflora impedes attachment of *V. cholerae* [Klose, 2000]. The competitive index (CI) that was calculated for Δ*toxR*::FLAG*toxR*^{CC} Δ*toxS* comprises the ratio of the mutant colonization capability to that of the wildtype. Comparable capabilities equate to the value of 1. The *in vitro* CI was determined to include the possibility of a growth defect of the mutants *per se* into the calculation of the *in vivo* CI and averages the value of 0.65 (6 independent measurements). The *in vivo* CI constitutes an average value of 0.27 (6 mice). Thus, by considering the *in vitro* CI Δ*toxR*::FLAG*toxR*^{CC} Δ*toxS* is about 2.4 fold attenuated *in vivo* (calculated by the Mann Whitney test with $P < 0.05$). Yet, given the results of the *in vitro* virulence gene expression determined via CT-ELISA and CTX-KmΦ transduction assay, where Δ*toxR*::FLAG*toxR*^{CC} Δ*toxS* showed defects of about 100 fold, the mechanisms that underlie this defect *in vitro* may not be as crucial for *in vivo* virulence regulation. Furthermore, it was observed that *toxS* deletion mutants that carried chromosomally FLAG-tagged *toxR* showed a

difference of about 12 fold for *in vivo* colonization compared to *toxS* deletion mutants which expressed untagged ToxR (data not shown). Also, FLAG-tagged ToxR generally yields slightly higher expression levels of ToxR regulated genes than untagged ToxR does, which suggests that the FLAG-tag itself contributes to gene regulation to some extent, e.g. by causing a more stable binding of ToxR to the DNA. Thus, strains carrying FLAG-tagged ToxR seem not to be appropriate for *in vivo* colonization studies.

Table 9: Colonization competition of Δ*toxR*::FLAG*toxR* vs Δ*toxR*::FLAG*toxR*^{CC} Δ*toxS.* Results of 6 independent studies are shown.

CI[a] (Δ*toxR*::FLAG*toxR* vs. Δ*toxR*::FLAG*toxR*^{CC} Δ*toxS)*	
CI (*in vitro*)	0.65
CI (*in vivo*)	0.27

[a] competitive index

2 ToxS influence on ToxR conformations

2.1 Construction of pFLAGtoxRS, pFLAGtoxRS(Δ264) and pFLAGtoxR^{CC}S

In order to determine a potential difference of ToxR conformations in the presence of ToxS, an experimental setup had to be established which was close to the wildtype expression conditions in *V. cholerae*. Due to the low expression levels of chromosomally encoded *toxR* and the lack of an efficient ToxR antibody, plasmid encoded FLAG-tagged *toxR* coexpressed with *toxS* under one promoter had to be constructed. The pFLAG-MAC™ vector already carries the amino sequence of the FLAG-tag (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Asp-Lys) [Hopp et al., 1988], so that *toxRS* were fused in frame to the FLAG sequence. This was done by first amplifying *toxRS* from the *V. cholerae* SP27459 chromosome, following restriction of PCR fragments and pFLAG-MAC™ vector with KpnI and BglII. Vector and inserts were ligated and amplified in *E. coli* XL1-Blue cells. As it was observed in previous studies (unpublished data), continual expression of proteins from the pFLAG-MAC™ vector could only be retained when expression of proteins was repressed, e.g. by addition of glucose to media, for times in between experiments when protein expression was not desired (in ONCs, glycerol stocks...).

Positive transformants were selected on LB plates containing amp and 0.2% glucose and further verified by Colony PCR (**Fig. 6**). Chromosomal SP27459 Δ*toxR*::FLAG*toxR* DNA served as positive control and generated a product of about 1500 bp, which is the size of *toxR* and *toxS* together. Clone 9 was used to transform *V. cholerae* SP27459 Δ*toxRS* and sent for sequencing to LGC Genomics, Berlin.

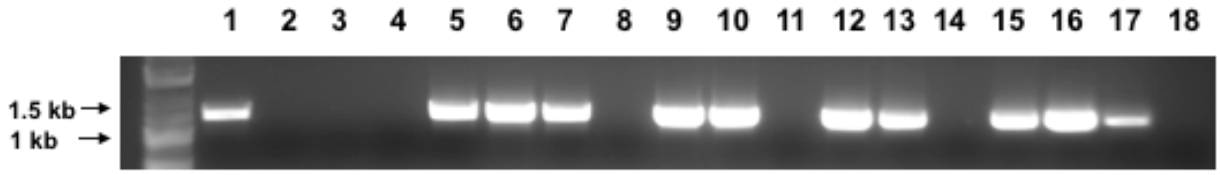

Figure 6: Colony PCR for verification of pFLAGtoxRS in *E. coli* XL-1 Blue. Samples were separated on a 0.8% agarose gel, 2-log DNA Ladder served as standard. 1-16: Clones 1-16; 17: PC SP27459 Δ*toxR*::FLAG*toxR* chromosomal DNA; 18: NC H_2O.

Results of the first studies (see chapter 2.2) made the generation of a *toxS* deletion in the plasmid pFLAGtoxRS necessary. Therefore, pFLAGtoxRS was digested with the restriction enzyme AccI, which lead to a 264 bp (88 aa) in frame deletion in the periplasmic domain of ToxS, removing amino acids 60 to 150. pFLAGtoxRS(Δ264) was again amplified in *E. coli* XL-1 Blue before transformation of *V. cholerae* SP27459 Δ*toxRS.* Transformants were verified by Colony PCR (**Fig. 7**). pFLAGtoxRS(Δ264) that had been isolated from *E. coli* served as a positive control (lane 6), chromosomal SP27459 Δ*toxR*::FLAG*toxR* DNA as a negative control (lane 7), since the primers were complementary to sequences around the start codon of ToxR and the stop codon of ToxS. Periplasmic truncated version of ToxS could thus be detected by a 264 bp shift on agarose gels in comparison to wildtype (lanes 1- 5).

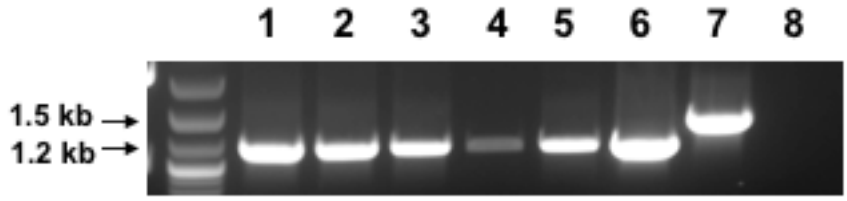

Figure 7: Colony PCR for verification of pFLAGtoxRS(Δ264) in *V. cholerae* SP27459 Δ*toxRS*. Samples were separated on a 0.8% agarose gel, 2-log DNA Ladder served as standard. 1-5: Clones 1-5; 6: PC pFLAGtoxRS(Δ264) isolated from *E. coli*; 7: NC SP27459 Δ*toxR*::FLAG*toxR* chromosomal DNA; 8: H_2O.

In addition to coexpressed FLAG-tagged ToxR with ToxS, a plasmid was constructed where the two cysteine residues of ToxR were replaced by serines. For this *toxR*^{CC}*toxS* was amplified by PCR with chromosomal SP27459 Δ*toxR*::FLAG*toxR*^{CC} DNA as template. All other steps were identical to the construction of pFLAGtoxRS. pFLAGtoxR^{CC}S from *E. coli* clone 6 was isolated and used for transformation of *V. cholerae* SP27459 Δ*toxRS* **(Fig. 8)**.

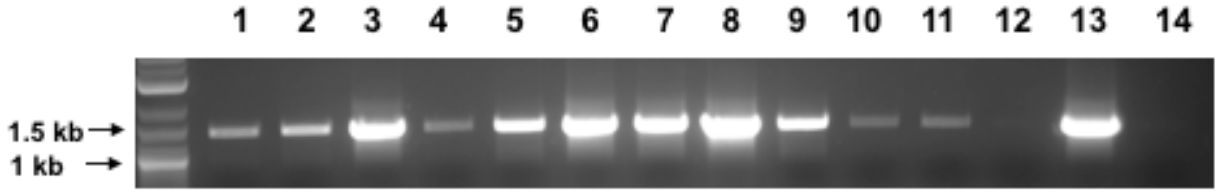

Figure 8: Colony PCR for verification of pFLAGtoxR^{CC}S in *E. coli* XL-1 Blue. Samples were separated on a 0.8% agarose gel, 2-log DNA Ladder served as standard. 1-12: Clones 1-12; 13: PC SP27459 Δ*toxR*::FLAG*toxR*^{CC} chromosomal DNA; 14: NC H_2O.

2.2 ToxS alters ToxR conformations

Native ToxR can have different conformations based on the redox-state of the two periplasmic cysteine residues at the positions 236 and 293 [Ottemann and Mekalanos, 1996]. So, ToxR can exist either in an (i) oxidized monomeric form, where the two cysteine residues form an intra-chain disulfide bond, in a (ii) reduced monomeric form, in a (iii) homodimeric conformation, where two ToxR molecules build up inter-chain disulfide bonds, and in an (iv) oligomeric

conformation, where several ToxR molecules form high molecular complexes [Fengler et al., unpublished data]. These different conformations can be detected on SDS gels under cysteine-non-reducing conditions (without β-mercaptoethanol) and subsequent immunoblot analysis.

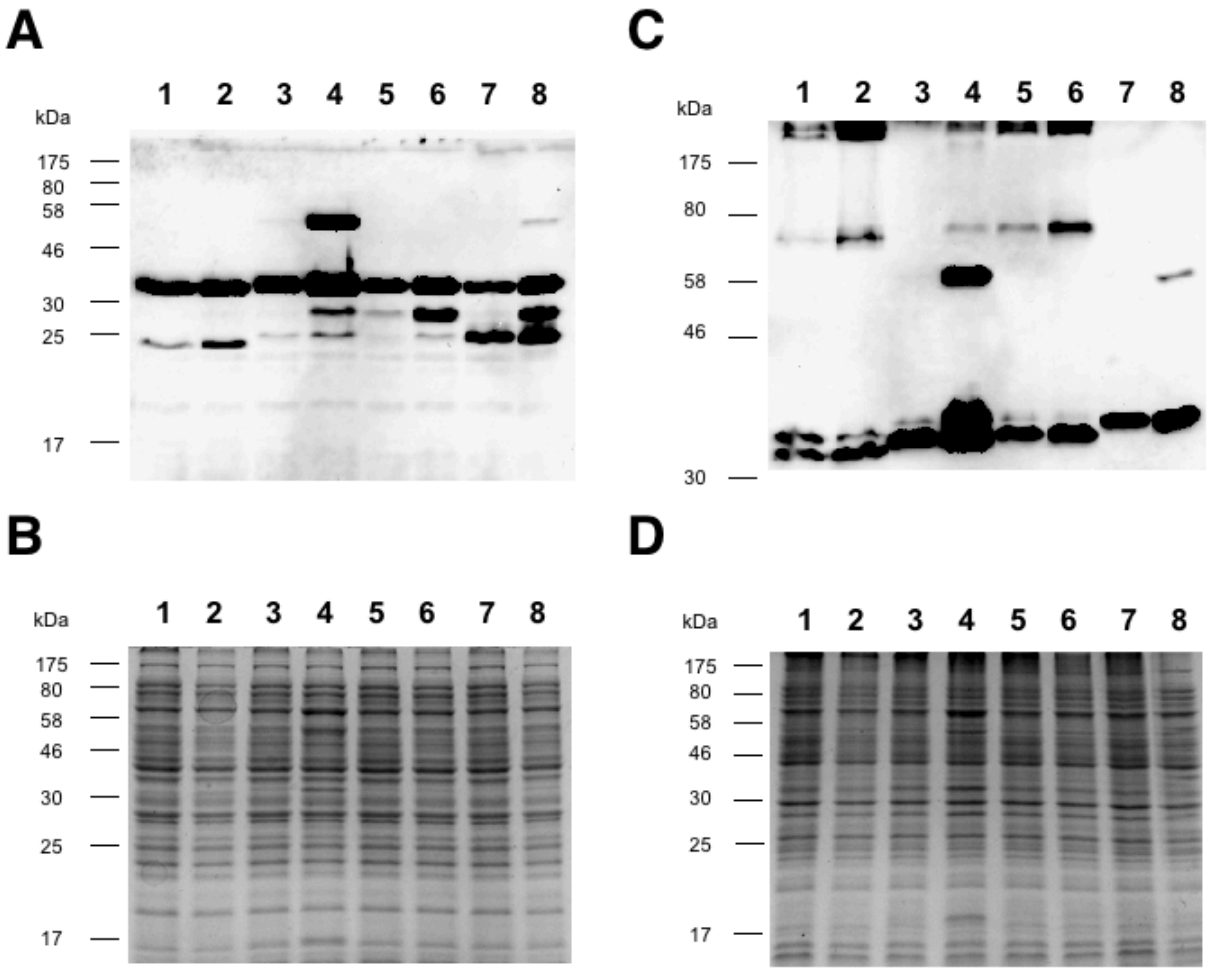

Figure 9: Immunoblot and Kang staining of pFLAGtoxR, pFLAGtoxRS, pFLAGtoxRS(Δ264) and pFLAGtoxR^{CC}S in an *E. coli* background. Cells were grown in LB and induced with either 0.005 mM or 0.05 mM IPTG at a starting OD_{600} of 0.4 and incubated at 37˚C for 60 min. Whole cell extracts of samples were prepared either under reducing (+ β-mercaptoethanol; panels **A** and **B**) or non-reducing conditions (- β-mercaptoethanol; panels **C** and **D**). Samples were loaded on 15% SDS gels and either transferred onto nitrocellulose membranes for immunoblotting (panels **A** and **C**) or stained with Coomassie brilliant blue after Kang et al. (panels **B** and **D**). 1: pFLAGtoxR 0.005 mM IPTG; 2: pFLAGtoxR 0.05 mM IPTG; 3: pFLAGtoxRS 0.005 mM IPTG; 4: pFLAGtoxRS 0.05 mM IPTG; 5: pFLAGtoxRS(Δ264) 0.005 mM IPTG; 6: pFLAGtoxRS(Δ264) 0.05 mM IPTG; 7: pFLAGtoxR^{CC}S 0.005 mM IPTG; 8: pFLAGtoxR^{CC}S 0.05 mM IPTG.

Oxidized monomeric ToxR builds a more compact conformation compared to reduced ToxR and therefore runs faster on SDS gels. Homodimeric ToxR has a size of about 70 kDa, whereas oligomeric ToxR runs close to the interface between stacking and separating gel.
In order to define if ToxS alters ToxR conformations, ToxR and ToxS were first coexpressed in *E. coli* XL-1 Blue. Whole cell extracts were prepared and either resuspended in sample buffer with or without the reducing agent β-mercaptoethanol. Samples were loaded on 15% SDS gels and subsequently transferred onto nitrocellulose membranes for immunoblot analysis. FLAG-tagged ToxR was detected with a FLAG specific antibody.

Panel A in **Fig. 9** shows the immunoblot of pFLAGtoxR, pFLAGtoxRS, pFLAGtoxRS(Δ264) and pFLAGtoxR^{CC}S in *E. coli* derived under reducing conditions, panel C those derived under non-reducing conditions. Each strain was incubated under low (0.005 mM IPTG) and under high (0.05 mM IPTG) inducing conditions in order to emphasize the importance of ToxRS expression levels on ToxR regulation. In the presence of reducing agent ToxR runs at around 35 kDa (panel A) in its monomeric reduced conformation. In the absence of reducing agent the additional conformations of ToxR can be observed. Oxidized monomeric ToxR runs below the reduced form, whereas homodimers and oligomers run at around 70 kDa and >175 kDa, respectively (pFLAGtoxR in lanes 1 and 2). For ToxR^{CC} (lanes 7 and 8) only the reduced monomeric form is visible due to the lack of cysteine residues and the resulting deficiency in disulfide bond formation. The shift detectable between ToxR monomers expressed from pFLAGtoxR to ToxR monomers expressed from pFLAGtoxRS, pFLAGtoxRS(Δ264) and pFLAGtoxR^{CC}S results from a 1 kDa difference of ToxR molecules due to the different restrictions sites used for construction of the vectors (HindIII and KpnI for pFLAGtoxR, KpnI and BglII for the other pFLAG-MAC™ vectors).
In those samples where ToxR and ToxS were coexpressed under high inducing conditions an additional cross reacting band at around 55 kDa appeared (lane 4), regardless of the presence or absence of β-mercaptoethanol. Furthermore, this band was SDS and heat resistant, since it remained unchanged when exposed to different temperatures and when boiled for varying time periods (40˚C, 65˚C and 100˚C for 1, 10 and 30 min; data not shown). Since ToxS has a molecular weight of about 20 kDa, a heterodimer with ToxR would result in a predicted 55 kDa complex. Ottemann and Mekalanos also observed an additional, but weak ToxR conformation at around 55 kDa in crosslinking experiments, which was absent in cells that did not express ToxS. Also we saw, that in samples where the major part of the periplasmic domain of ToxS was deleted (pFLAGtoxRS(Δ264), lanes 5 and 6) the 55 kDa band disappeared. These findings

suggest that the cross reacting band is indeed due to heterodimerization of ToxR and ToxS. Similar effects could be observed for ToxR^{CC}, even though heterodimerization appeared to be considerably weaker (lane 8). This might be a consequence of e.g. poorer recognition of ToxR^{CC} by ToxS resulting from improper folding, different susceptibility to SDS or degradation.
An additional ToxS dependent observation is the decrease of high molecular ToxR species in ToxRS coexpressed samples (panel C, lanes 3 and 4) in comparison to samples that lack ToxS (panel C, lanes 1 and 2) or express deficient ToxS (panel C, lanes 5 and 6).

In order to verify that same protein amounts were loaded onto the SDS gels for immunoblot analysis, SDS gels with the samples used for immunoblot analysis were stained with Coomassie brilliant blue after Kang in parallel. As can be seen in **Fig. 9** panel B and D approximately the same protein amounts were used.

2.3 No heterodimer visible in a *V. cholerae* background

Plasmids carrying ToxR and ToxS were then expressed in *V. cholerae* SP27459 Δ*toxRS* to test whether ToxS had the same influence on ToxR conformations in its native strain background. Experimental procedures were identical to the ones described above (see chapter 2.2). However, surprisingly, the ToxR/ToxS and ToxR^{CC}/ToxS heterodimers seen in *E. coli* backgrounds could not be detected in *V. cholerae,* neither under low (0.005 mM IPTG) nor under high (0.05 mM IPTG) expression conditions (**Fig. 10** lanes 1, 2 and 5, 6). Yet, the decrease in high molecular and especially in homodimeric ToxR species can still be observed in the presence of ToxS (**Fig. 10** panel C lanes 1 and 2) compared to ToxR in the absence of ToxS (**Fig. 10** panel C lanes 3 and 4), which indicates that ToxS is expressed and impedes formation of these ToxR molecules.

Kang staining of the samples that were run on SDS gels in parallel to immunoblot analysis again showed that approximately the same amounts of protein were applied (**Fig. 10** panel B and D).

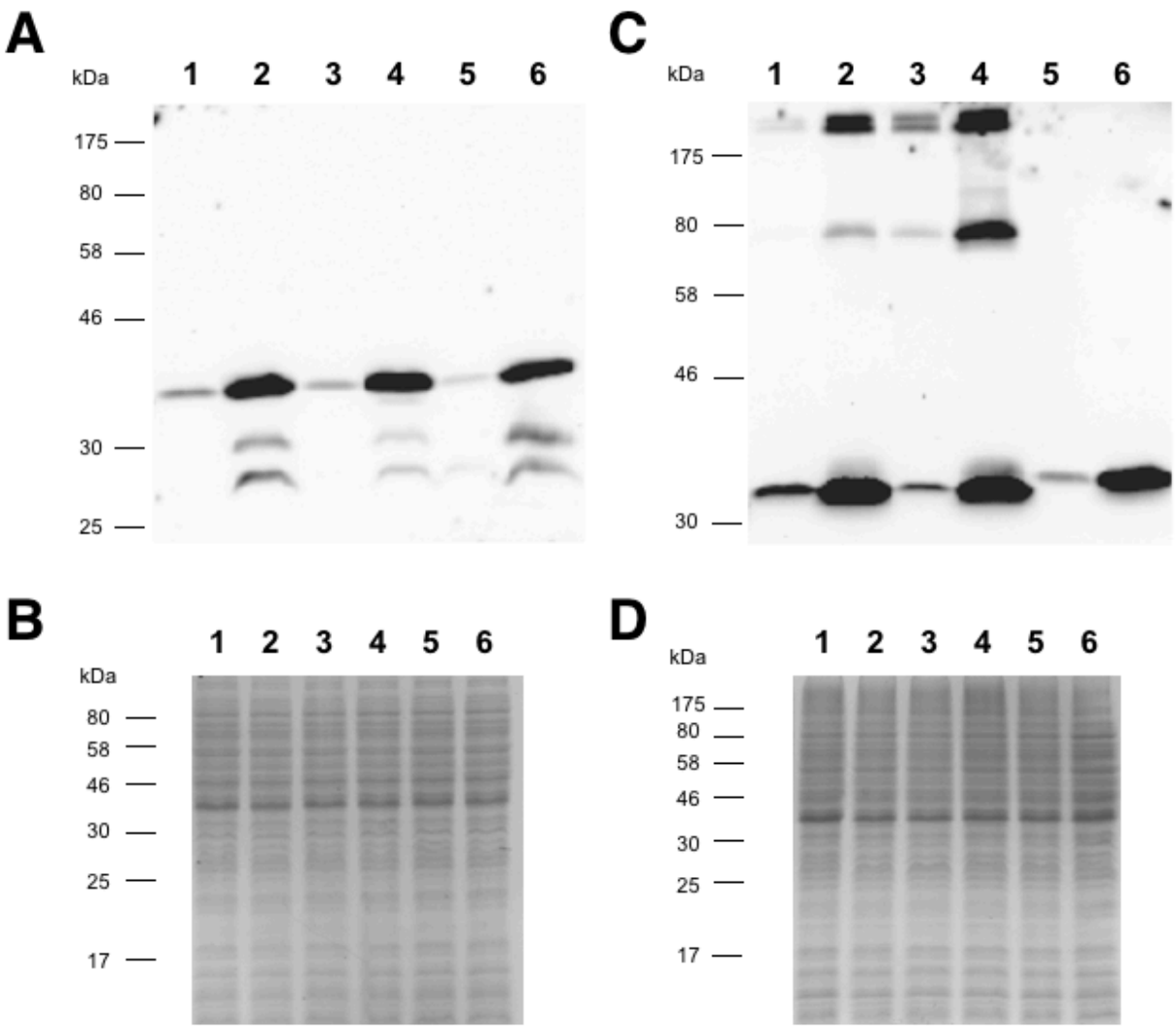

Figure 10: Immunoblot and Kang staining of pFLAGtoxRS, pFLAGtoxRS(Δ264) and pFLAGtoxR^{CC}S in a *V. cholerae* Δ*toxRS* background. Cells were grown in LB and induced with either 0.005 mM or 0.05 mM IPTG at a starting OD_{600} of 0.4 and incubated at 37˚C for 60 min. Whole cell extracts of samples were prepared either under reducing (+ β-mercaptoethanol; panels **A** and **B**) or non-reducing conditions (- β-mercaptoethanol; panels **C** and **D**). Samples were loaded on 15% SDS gels and either transferred onto nitrocellulose membranes for immunoblotting (panels **A** and **C**) or stained with Coomassie brilliant blue after Kang et al. (panels **B** and **D**). 1: pFLAGtoxRS 0.005 mM IPTG; 2: pFLAGtoxRS 0.05 mM IPTG; 3: pFLAGtoxRS(Δ264) 0.005 mM IPTG; 4: pFLAGtoxRS(Δ264) 0.05 mM IPTG; 5: pFLAGtoxR^{CC}S 0.005 mM IPTG; 6: pFLAGtoxR^{CC}S 0.05 mM IPTG.

2.4 ToxRS heterodimerization and ToxR operator binding act competitively

The lack of detectable heterodimerization in *V. cholerae* let the question of differences between *E. coli* and *V. cholerae* as strain backgrounds for ToxRS expression arise. A possible difference is the lack of ToxR operators in *E. coli*. In order to verify whether this was the reason for the undetectable heterodimerization in *V. cholerae*, ToxR operators were inserted into the pFLAG-MAC™ expression plasmids that already carried *toxRS*. This was done by amplifying known ToxR operator sites for *ompU* and *toxT* [Crawford et al., 1998; Krukonis et al., 2000] from *V. cholerae* SP27459 chromosome by PCR. This generated an about 370 bp fragment from -475 to -104 upstream of the transcription start of *ompU* and an about 220 bp fragment from -240 to -23 upstream of the transcription start of *toxT*, each flanked by BamHI restriction sites. These PCR fragments as well as pFLAGtoxRS and pFLAGtoxR^{CC}S were digested with BamHI, subsequently ligated and amplified in *E. coli* XL-1 Blue. The resulting pFLAGtoxRS_ompU and pFLAGtoxRS_toxT plasmids were further digested with AccI to generate pFLAGtoxRS(Δ264) _ompU and pFLAGtoxRS(Δ264)_toxT. Immunoblot analysis of ToxR expressed from these plasmids in *E. coli* was done as described above.

As can be seen in **Fig**. **11**, presence of ToxR operators indeed lead to a decrease in ToxRS heterodimerization (compare panel A and C lane 4 to lanes 5 and 6). A similar tendency could be observed for ToxR^{CC} (compare panel A and C lane 7 to lanes 8 and 9). Since in *V. cholerae* ToxR operators exist in abundance, a competitiveness between ToxRS heterodimerization and ToxR operator binding might be an explanation why no heterodimers could be detected in *V. cholerae* backgrounds.

Surprisingly, even though less heterodimers were present in those cells that additionally expressed ToxR operators, formation of high molecular ToxR species was still suppressed compared to ToxR without ToxS (panel C lanes 1-3). In addition, oxidized monomeric ToxR was left as the dominant form in presence of ToxR operators. These results suggest a model in which inter-chain disulfide bonded ToxR homodimers and high molecular conformations are not the active ToxR species for activation of gene transcription, whereas intra-chain disulfide bonded monomeric ToxR might be.

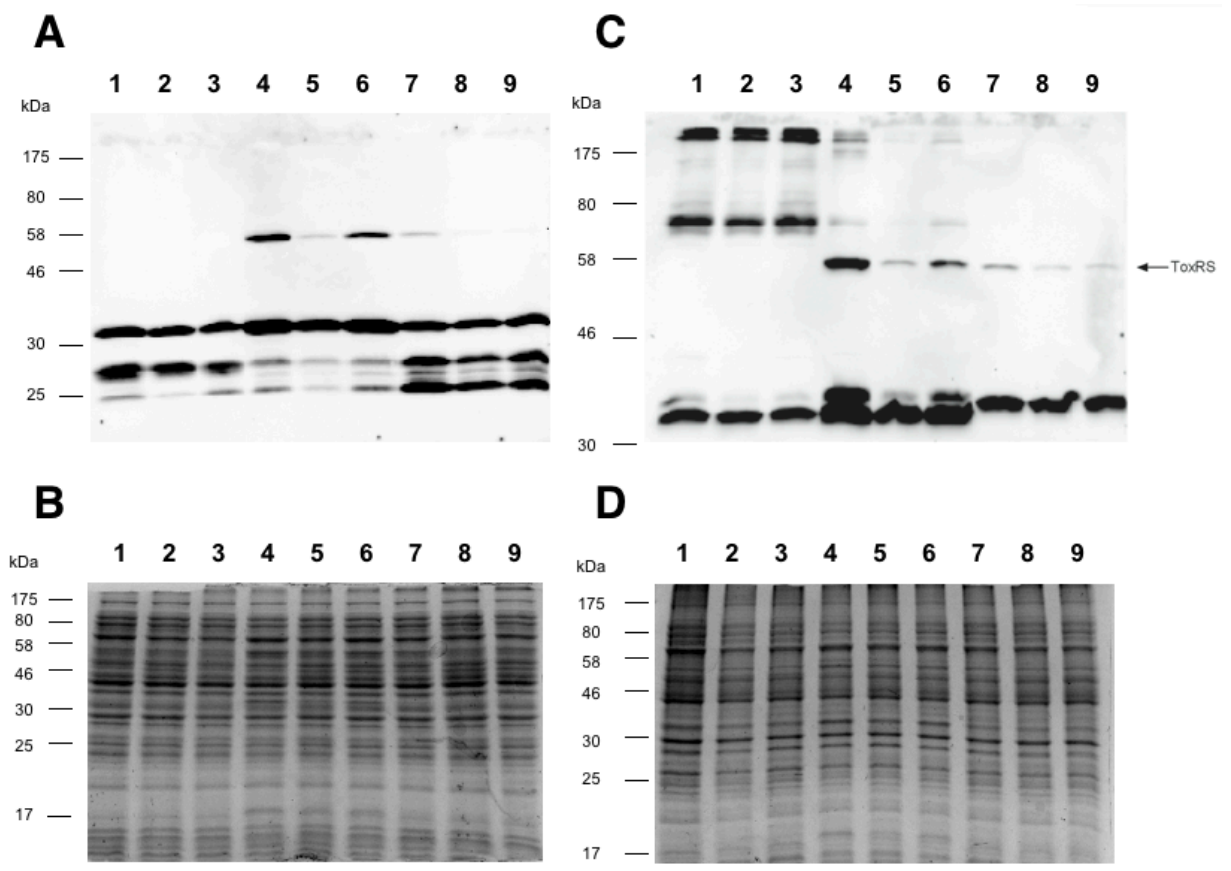

Figure 11: Immunoblot and Kang staining of pFLAGtoxRS(Δ264), pFLAGtoxRS and pFLAGtoxR^{CC}S with and without ToxR operator sites in an *E. coli* background. Cells were grown in LB and induced with 0.05 mM IPTG at a starting OD_{600} of 0.4 and incubated at 37˚C for 90 min. Whole cell extracts of samples were prepared either under reducing (+ β-mercaptoethanol; panels **A** and **B**) or non-reducing conditions (- β-mercaptoethanol; panels **C** and **D**). Samples were loaded on 15% SDS gels and either transferred onto nitrocellulose membranes for immunoblotting (panels **A** and **C**) or stained with Coomassie brilliant blue after Kang et al. (panels **B** and **D**). 1: pFLAGtoxRS(Δ264); 2: pFLAGtoxRS (Δ264)_ompU_operator; 3: pFLAGtoxRS(Δ264)_toxT_operator; 4: pFLAGtoxRS; 5: pFLAGtoxRS_ompU_operator; 6: pFLAGtoxRS_toxT_operator; 7: pFLAGtoxR^{CC}S; 8: pFLAGtoxR^{CC}S_ompU_operator; 9: pFLAGtoxR^{CC}S_toxT_operator.

Again, approximately the same amounts of proteins were applied for immunoblotting, as can be seen on the Kang stained gels in **Fig. 11** panels B and D.

In order to see whether the pFLAG-MAC™ constructs were also transcriptionally active, ToxR activity was analyzed by preparation of the outer membrane fraction of the strains and further separation of the proteins on 15% SDS gels. Strains carrying the expression plasmids were induced and incubated like the strains used for immunoblotting. In **Fig. 12** it can be seen that each plasmid (lanes 3 to 7, for details see figure legend) complemented the *toxRS* deletion mutants phenotype (lane 2) to *ompU* levels detected in wildtype (lane 1). Thus, the plasmids used for the conformation studies of ToxR carry functional ToxR proteins, which are able to regulate gene transcription in an overexpression state.

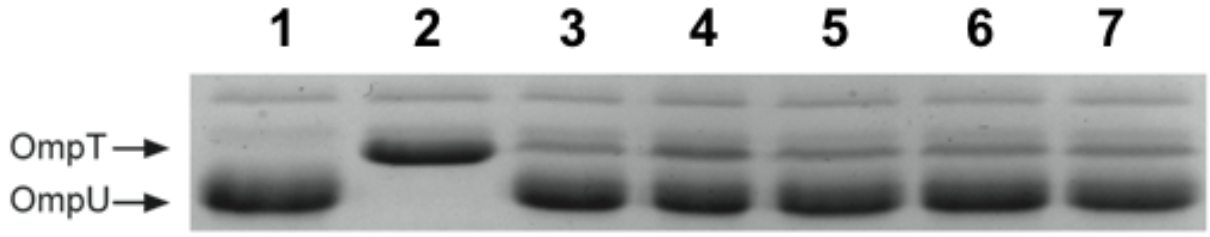

Figure 12: Outer membrane profile of *V. cholerae* wildtype and Δ*toxRS* with different complementation plasmids. Cells were grown in LB and induced with 0.05 mM IPTG at a starting OD_{600} of 0.4 and incubated at 37˚C for 90 min. Outer membrane extracts of samples were prepared and loaded on a 15% SDS gel. 1: SP27459; 2: SP27459 Δ*toxRS* pFLAG-MAC; 3: SP27459 Δ*toxRS* pFLAGtoxRS; 4: SP27459 Δ*toxRS* pFLAGtoxRS(Δ264); 5: SP27459 Δ*toxRS* pFLAGtoxR; 6: SP27459 Δ*toxRS* pFLAGtoxR^{CC}S; 7: SP27459 Δ*toxRS* pFLAGtoxR^{CC}.

3 ToxS influence on ToxR stability

To determine how stable ToxR and ToxR^{CC} were in a *toxS*$^{+}$ and *toxS*$^{-}$ background, degradation assays were performed. For this chloramphenicol (cm) was administered to the *toxRS* deletion mutants carrying either pFLAGtoxRS, pFLAGtoxR, pFLAGtoxR^{CC}S or pFLAGtoxR^{CC} after induction of the plasmids and incubation at 37˚C for 30 min. Cm inhibits protein translation by binding to the 50S subunit of the ribosome. Consequently, proteins are no longer produced as soon as cm is added to the cultures and stability of ToxR/ToxR^{CC} could be observed over a time period of 210 min. As a control cultures were also incubated without cm. Whole cell extracts were prepared and ToxR levels detected by immunoblot analysis as described in Materials and

Methods. As **Fig. 13** shows, wildtype ToxR in the presence of ToxS was constantly stable over the whole time period of the experiment (panel A), whereas ToxR was gradually degraded when ToxS was not present (panel B). A smaller proteolytic product of ToxR, which is stable in cells expressing ToxR and ToxS, also diminishes in the absence of ToxS. For $ToxR^{CC}$ without ToxS similar effects could be observed as for wildtype ToxR lacking ToxS (panel D). $ToxR^{CC}$ levels gradually decreased over time with the difference that the proteolytic product remained stable. Hence, ToxS seems to have a stabilizing effect on ToxR.

However, surprisingly in $ToxR^{CC}$ mutants when ToxS was coexpressed, $ToxR^{CC}$ was degraded even faster than without ToxS, even in the control without cm (panel C), raising the question whether ToxS could also act as a proteolytic targeting factor. Interestingly, a proteolytic product of about 29 kDa, which was not detectable in samples without ToxS, accumulated in the -cm control of $ToxR^{CC}$. This proteolytic product or the protease that generates this cleavage product seems to be short-lived, since it did not appear in samples that were treated with cm. Another observation that can be made, is that while $ToxR^{CC}$ levels in cells that expressed ToxS started to decrease only after 90 min post cm administration and had stayed stable until that point, degradation of $ToxR^{CC}$ in cells lacking ToxS was already visible at t_{45}, yet then proceeded at a slower pace.

These results suggest that ToxS can have two distinct roles: one is to stabilize wildtype ToxR and protect it from degradation, hence act as a chaperon, and the other is to promote degradation of mutant ToxR, hence act as a proteolytic targeting factor.

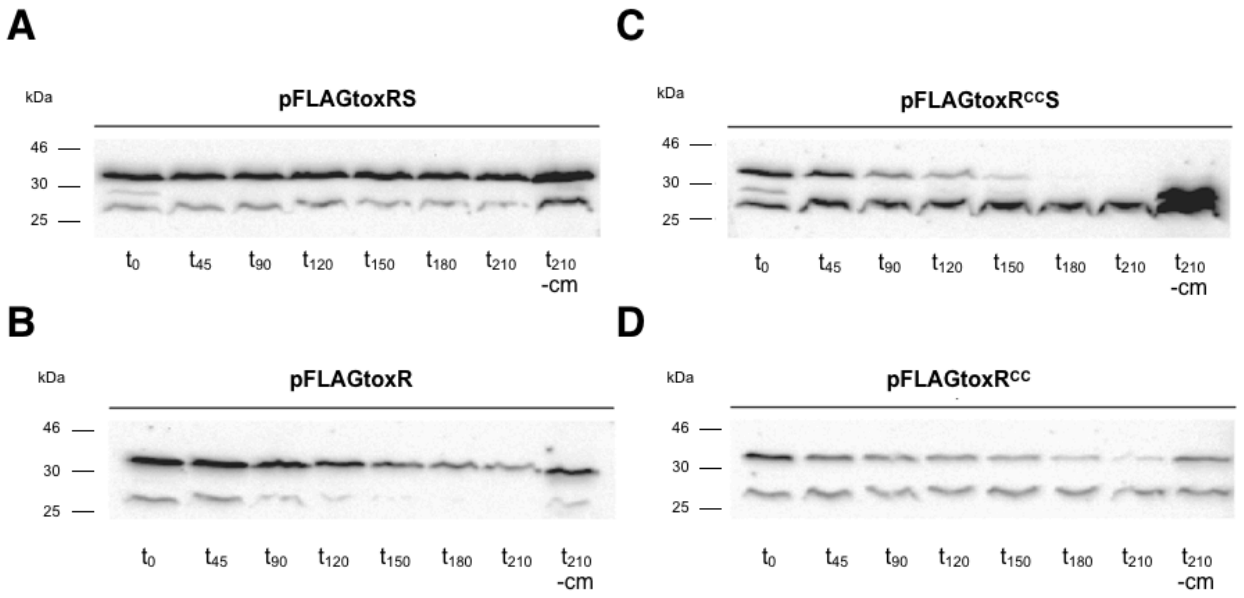

Figure 13: Degradation assay of pFLAGtoxRS, pFLAGtoxR, pFLAGtoxR^{CC}S and pFLAGtoxR^{CC} in a *V. cholerae* Δ*toxRS* background. Cells were grown in LB and induced with 0.05 mM IPTG at a starting OD_{600} of 0.4 and incubated at 37˚C for 30 min. 100 µg/ml chloramphenicol (cm) was added to cultures and samples taken at indicated time points (in min). Cultures grown without cm (-cm) served as a control. Whole cell extracts were prepared, loaded on 15% SDS gels and transferred onto nitrocellulose membranes for immunoblot analysis. **A**: SP27459 Δ*toxRS* pFLAGtoxRS; **B**: SP27459 Δ*toxRS* pFLAGtoxR; **C**: SP27459 Δ*toxRS* pFLAGtoxR^{CC}S; **D**: SP27459 Δ*toxRS* pFLAGtoxR^{CC}.

To verify that same amounts of proteins were applied for immunoblot analysis, samples were loaded on SDS gels for Kang staining in parallel. Approximately the same protein amounts were used as can be seen in **Fig. 14**.

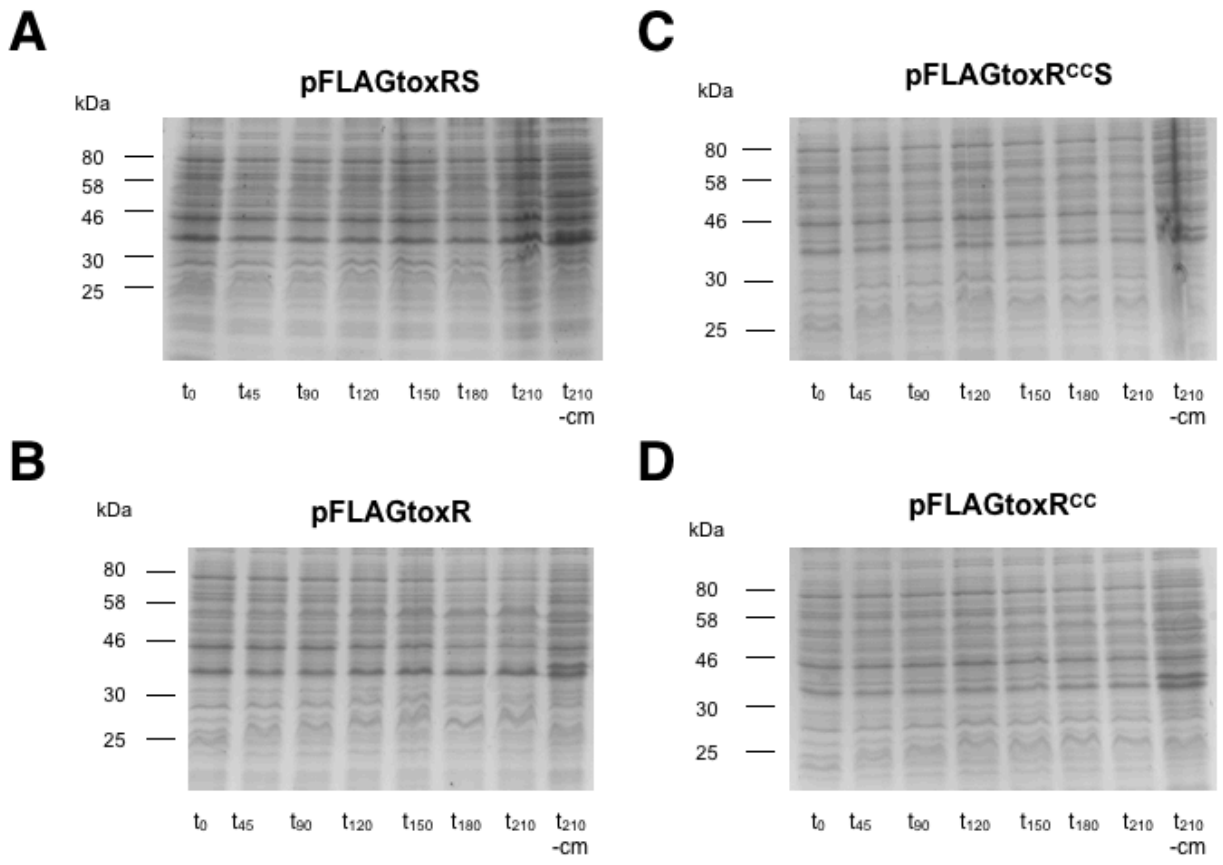

Figure 14: SDS gels of pFLAGtoxRS, pFLAGtoxR, pFLAGtoxR^{CC}S and pFLAGtoxR^{CC} in a *V. cholerae* Δ*toxRS* background. Whole cell extracts used for the degradation assay (see Fig. 13) were loaded on 15% SDS gels and stained with Coomassie brilliant blue after Kang et al. **A**: SP27459 Δ*toxRS* pFLAGtoxRS; **B**: SP27459 Δ*toxRS* pFLAGtoxR; **C**: SP27459 Δ*toxRS* pFLAGtoxR^{CC}S; **D**: SP27459 Δ*toxRS* pFLAGtoxR^{CC}.

V Discussion

The transcription factor ToxR is involved in virulence gene regulation [Miller and Mekalanos, 1984], as well as in regulation of multiple other genes, including the porins OmpU and OmpT [Miller et al., 1988]. While ToxR needs the second transcription factor TcpP only for activation of virulence genes [Häse and Mekalanos, 1998], the membrane bound and coexpressed factor ToxS is needed for proper ToxR transcriptional activity in general [Miller et al. 1989; DiRita and Mekalanos, 1991; Pfau and Taylor, 1998]. Yet, so far only little is known about the role of ToxS on ToxR activity. Several vague hypotheses on the function of ToxS were proposed, including a chaperone-like activity, even though studies on protection of ToxR from proteolysis gave inconsistent results [Beck at al, 2004; DiRita and Mekalanos, 1991; Dziejman and Mekalanos, 1994; Xu et al., 2010]. In this work ToxRS interaction with regard to cysteine dependent ToxR conformations and ToxR activity was analyzed.

Since ToxR is a positive activator of OmpU and a repressor of OmpT, these two porins are well suited reporters for ToxR activity [Miller et al., 1988]. Outer membrane preparations of *toxS* deletion mutants clearly display decreased ToxR activity on porin regulation, which confirms previous data that showed reduced ToxR activity on porin regulation [Mey et al., 2012] and CT expression in cells lacking ToxS [Miller et al. 1989; DiRita and Mekalanos, 1991; Pfau and Taylor, 1998]. Complementation of this phenotype with an arabinose inducible plasmid carrying *toxS* only partially restored ToxR activity for *ompT* repression, indicating that exact ratios of ToxR and ToxS are indispensable for proper gene regulation. Transcriptional control of ToxR regulated genes is highly sensitive, thus gene expression is activated by only low levels of ToxR. This makes the relative proportion of the co-transcribed proteins ToxR and ToxS even more critical. Our reasoning that the partial complementation for *ompT* with ptoxS was due to the fact that ToxR and ToxS were not co-transcribed, but expressed from different loci and regulated by different promoters, and thus could be overcome by coexpressing ToxR and ToxS from the same pBAD18 plasmid, proved to be a wrong assumption (data not shown).

A similarly reduced ToxR activity as in *toxS* deletion mutants could be observed in cells expressing ToxR that lacked the two periplasmic cysteine residues at positions 236 and 293. Biochemical analysis showed that these cysteine residues were capable of forming intra- and inter-chain disulfide bonds, resulting in oxidized ToxR monomers or disulfide bonded

homodimers, as well as in high order complexes with a molecular weight >175 kDa, which is in line with earlier findings [Ottemann and Mekalanos, 1996; Fengler et al., unpublished data]. Thus, activity of ToxR double cysteine mutants is limited to a reduced conformation only. While regulation of porin expression seems to be dependent on ToxR cysteines, as can be seen for ToxR^{CC} at the protein level by an altered porin profile, as well as at a transcriptional level measured by qRT-PCR [Fengler et al., unpublished data], regulation of virulence genes seems to be unaffected by the lack of cysteines. However, if cells encoding ToxR^{CC} additionally are ToxS deficient, ToxR activity for regulation of both, porins and virulence genes, drops significantly *in vitro*. This is in line with results published by Ottemann and Mekalanos, who saw that classical strains carrying a ToxR single cysteine mutant, where only the cysteine residue at position 236 was replaced by serine, and which, as it turned out later, displayed an additional polar effect on ToxS expression [Dziejman et al., 1999] were significantly impaired for CT production [Ottemann and Mekalanos, 1996]. Complementation with ptoxS could be achieved for porin regulation, however, only partially for CT expression. This might again be due to the necessity of exact ratios of ToxR and ToxS for proper transcriptional activity, which could not be obtained with the conditions used in this study, despite evaluation of several setups, including different concentrations of the inducing agent arabinose and varying length of induction. Surprisingly, *in vivo* colonization data of Δ*toxR*::FLAG*toxR*^{CC} Δ*toxS* did not display the pronounced decrease of virulence gene expression as seen in the *in vitro* experiments. On the contrary, the 2.4 fold attenuation of virulence *in vivo* is far from the about 100 fold attenuation for CT and TCP production *in vitro*. This discrepancy might result from an interference of the FLAG-tag of ToxR with DNA binding, since FLAG-tagged ToxR generally yielded slightly higher expression levels of ToxR regulated genes than untagged ToxR. This assumption is supported by the observation that a *toxS* deletion mutant carrying a FLAG-tagged ToxR differed about 12 fold in *in vivo* colonization compared to *toxS* deletion mutants expressing untagged ToxR (data not shown). Due to this probable interference with gene regulation, e.g. by stabilization of ToxR-DNA complexes, *in vivo* studies should not be performed anymore with strains carrying FLAG-tagged proteins.

Results of the *in vitro* studies of Δ*toxR*::FLAG*toxR*^{CC} Δ*toxS* suggest that ToxS does not modulate ToxR activity in a cysteine dependent way, since lack of ToxS in ToxR^{CC} mutants results in an additive defect on transcriptional activity. As was proposed earlier, ToxS might stabilize spontaneously forming transcriptionally active conformations of ToxR, which reduces the concentration of ToxR that is needed for transcriptional activity. Overexpression of ToxR

renders ToxS unnecessary [Miller and Mekalanos, 1989; Dziejman and Mekalanos, 1994; 1999], which is confirmed by our data showing that ToxR and ToxR^{CC} overexpressed from the pFLAG-MAC plasmids obviates the need for ToxS, resulting in OmpU levels comparable to wildtype. This again argues for the necessity of ToxS for ToxR under low protein concentrations, as encountered under physiological expression conditions.
Since promoter regions of ToxR regulated genes possess several ToxR binding sites, it was suggested that multiple ToxR proteins bind the promoters in order to activate gene expression [Crawford et al., 1998; Morgan et al., 2011; Krukonis et al., 2000]. ToxS might contribute to recruiting several ToxR molecules into close proximity to each other at the promoter sites, resulting in facilitated formation of transcriptionally active complexes. ToxR compared to ToxR^{CC} might have a higher probability of forming active complexes *per se*, and thus, the effect of lacking ToxS is not as pronounced as for ToxR^{CC}.

Results of the ToxR/ToxS coexpression studies seem to be in conflict with the hypothesis that ToxR activates gene transcription as a homodimer or higher order complex, as presence of heterologously expressed ToxS suppresses formation of these cysteine dependent high molecular ToxR species in favor of ToxRS heterodimerization in *E. coli* backgrounds. These findings are consistent with previous work by Ottemann and Mekalanos, who also observed an additional, albeit weak ToxR conformation at around 55 kDa in crosslinking experiments, which was absent in cells that did not express ToxS. Also they found that formation of ToxR homodimers was suppressed in the presence of ToxS [Ottemann and Mekalanos, 1996]. However, no further studies on the topic of cysteine dependent ToxR conformations in dependence of ToxS, as well as ToxRS heterodimerization were attempted thereafter. In comparison to the weak heterodimers that were obtained by Ottemann and Mekalanos only through chemical crosslinking, the heterodimers in this study were SDS and heat resistant. This results from a tight contact between ToxR and ToxS that restricts access of SDS to the interaction sites, like for example also seen for beta-glycosidase from a hyperthermophilic *Sulfolobus species* [Gentile et al., 2002], the ubiquitin-like protein HUB1 [Lueders et al., 2003] or spike proteins from Semliki Forest virus [Klimjack et al., 1994]. Given this stable ToxRS heterodimerization in *E. coli*, it was surprising that no heterodimer could be detected in *V. cholerae*, neither under the same expression conditions nor with various crosslinkers, such as DMA or SDA (data not shown). Assuming that the lack of ToxR operator sites in *E. coli* accounts for the differences in heterodimerization, introduction of ToxR binding sites into the expression systems that also encoded *toxRS* indeed resulted in decreased amounts of heterodimers in

E. coli, yet at the same time in no increase of cysteine dependent high order complexes of ToxR. The fact that ToxR is involved in regulation of about 150 genes [Bina et al., 2003] implies that multiple ToxR binding sites are present in *V. cholerae*, and thus adds to the observation that heterodimerization and ToxR DNA binding might not take place at the same time. Ergo, this finding constitutes an interesting novel detail of the function and activation mechanism of ToxR by ToxS and sheds light on when and how an interaction between these two proteins occurs.

ToxS is not needed for DNA binding of plasmid encoded ToxR [Pfau and Taylor, 1998], however, was found to reduce ToxR protein concentrations that are required for promoter binding in DNA gel mobility shift assays [Ottemann et al., 1992]. Thus, ToxS is not necessarily needed for, but still facilitates and possibly quickens ToxR promoter binding and is in either case crucial for activation of transcription. ToxR might therefore be able to bind DNA also in a conformation that fails to activate transcription, e.g. by displaying a conformation that is unable to interact with RNA-polymerase. ToxS might thereby display a chaperon-like function by modifying ToxR to adopt a transcriptionally active complex and/or stabilizing this conformation. Yet upon ToxR DNA binding, ToxS detaches from the ToxR-DNA complex. These transcriptionally active ToxR complexes either do not consist of covalently linked ToxR molecules, since no high molecular ToxR conformations were observed in this study, or they do, but we failed to detect them with the methods used. An intriguing observation is that in a *V. cholerae* background, as well as in *E. coli* in the presence of operators, the intra-chain disulfide bonded ToxR monomer is the dominant conformation, and thus it seems tempting to assume that positioning of several ToxR oxidized monomers in close proximity to each other at the DNA binding sites, facilitated by ToxS, promotes gene transcription. However, thereby the question arises why $ToxR^{CC}$, which fails to form oxidized monomers, is still able to activate gene transcription. This conflict cannot be explained with the data obtained so far, yet, as it seems reduced ToxR monomers are not precluded from transcriptional activity as long as ToxS is present. ToxS might perform a similar task for $ToxR^{CC}$ as for ToxR by recruiting several $ToxR^{CC}$ molecules to the DNA into close proximity to each other and thereby causing a conformational shift that leads to activation. Thus, ToxS still recognizes potentially misfolded ToxR molecules, even though an interaction seems to be less stable, given the weaker heterodimerization observable through immunoblot analysis. This might result e.g. from a conformational change due to the lacking cysteine residues and thus poorer binding affinity by ToxS, higher accessibility to interaction sites by SDS or degradation of $ToxR^{CC}$. In fact, ToxT unlike OmpU expression seems to be cysteine independent at all in the presence of ToxS, since no difference in production of CT and TCP could be

observed between ToxR and ToxR^{CC}, whereas porin expression was significantly altered for ToxR^{CC}. This finding of differences in ToxR gene regulation is not exceptional, as it was shown previously that ToxR displays different activation mechanisms at different promoters. Several mutants that also show a defective activation of OmpU expression, yet intermediate change for ToxT were identified and mutations mapped to the alpha-loop of the wHTH region, which is conserved among the OmpR family of transcription factors, with ToxR-V71A showing the most pronounced defect. ToxR-F69A was deficient for activation of OmpU expression, but was still able to bind the *ompU* promoter, which demonstrates that DNA binding and promoter activation are accomplished by separate ToxR residues [Morgan et al., 2011]. Also ToxS was found to affect ToxR activity rather than ToxR DNA binding [Pfau and Taylor, 1998]. So, while the *ompU* promoter region is recognized and activated by certain residues of the alpha-loop of ToxR, the *toxT* promoter is bound by residues of the wing domain of ToxR, which is also involved in interaction with TcpP. Thus even slight changes in the orientation of ToxR on the DNA might affect activation of OmpU expression or interaction with TcpP and subsequent transcription of *toxT* [Morgan et al., 2011]. If ToxS has a stabilizing and conformation modifying influence on ToxR, it might thereby remodel ToxR in a way that allows for ideal gene activation. Additionally, a cytoplasmic linker domain of ToxR, which is located between the HTH motif and the transmembrane segment within the conserved region that is shared by OmpR-like transcription regulators, was found to influence ToxRS interaction. Substitutions in this region resulted in ToxS-blind mutants that showed a ToxS^{-} phenotype even in the presence of ToxS. Thus this linker domain might be involved in passing on the signal from the periplasmic domain upon ToxRS interaction, resulting in an activating conformational shift in the cytoplasmic structure [DiRita and Mekalanos, 1991]. Moreover, this linker region was postulated to have an inhibitory effect on ToxR activity [Crawford et al., 2003], hence ToxS might assist in overcoming this inhibitory function. Similar effects of cytoplasmic mutations leading to failure to respond to periplasmic signals were found for the transmembrane regulator BvgS of *Bordetella pertussis* or for the membrane bound signal transmitter of chemotactic stimuli Tsr of *E. coli* [DiRita, 1992; Miller et al., 1992; Ames and Parkinson, 1988].

Still, the question why periplasmic truncations of ToxR are still able to activate gene transcription - either if fused to a dimerizing heterologous protein structure or even if only membrane anchored at all in case of *toxT* activation [Dziejman et al., 1999; Crawford et al., 2003; Miller and Mekalanos, 1995] - given that already two point mutations in the periplasmic structure result in decreased ToxR activity (ToxR^{CC}), which is even enhanced when ToxS is not present, remains unresolved and requires further experimentation. One explanation could be

that replacement of the cysteine residues, which are important stability- and structure-giving components of proteins [Heitmann, 1968; Moroder et al., 1996; Claiborne et al., 1993] alters the conformation in such a way that ToxR transcriptional activity is affected. Another explanation is that gene activation is dependent on a certain ToxRS threshold, so that upon a particular level of ToxR, be it wildtype ToxR, chimera or ToxR truncations, the probability of several ToxR molecules to e.g. randomly find each other at the promoter sites is much higher and independent of the cysteine residues or the periplasmic domain *per se*. This hypothesis is supported by our data showing that overexpressed $ToxR^{CC}$ mutants display a comparable OmpU expression profile as wildtype. Additionally, when NRES amino acids were added to cells grown in minimal medium, where they generally show an altered porin profile with more OmpT than OmpU expression even for wildtype, porin expression was reversed again. This was explained by a 2.5 fold increase in ToxR transcription [Mey et al., 2012]. The same effect was also seen for $ToxR^{CC}$ in minimal medium supplemented with NRES, as well as in AKI medium, which is used to induce virulence genes in El Tor strains. Therefore it was suggested that upon reaching a certain ToxRS expression threshold, which is more rapidly achieved in amino acid enriched media, such as AKI or within the human intestines due to the elevated ToxR levels, gene transcription becomes thiol-independent [Fengler et al., unpublished data] and possibly this threshold is higher in absence of ToxS. A similar effect was observed for the transcriptional regulators PsaEF of *Yersinia pestis*, which show homologies to ToxRS. Overexpression of these two proteins led to higher expression of the genetic product *psaA,* the major pilus protein, and made transcription insensitive to various growth conditions [Yang and Isberg, 1997].

Another aspect of ToxS influence on ToxR is its ability to protect ToxR from degradation. However, the constitutively expressed ToxR generally seems to be relatively stable, since even in the absence of ToxS proteolysis of ToxR could only be observed after 150 min and only proceeded at a slow pace. Even 210 min after cm administration, the time point at which the experiment was stopped, a fair amount of protein was present. Still, this reduced number of ToxR might contribute to the decrease in ToxR activity if ToxR threshold levels actually have an impact on activation of transcription. It has been shown before that ToxS has a stabilizing effect on ToxR, since a ToxR-PhoA chimera, where most part of the periplasmic domain of ToxR was still present, was proteolyzed in *toxS*$^{-}$ backgrounds [DiRita and Mekalanos, 1991]. Interestingly, the stabilizing influence of ToxS on ToxR is particularly effective in stationary phase conditions, whereas in mid-log cultures ToxR was not degraded in *toxS*$^{-}$ backgrounds [Xu et al., 2010; Beck et al., 2004]. In contrast to lack of ToxS, absence of TcpH results in rapid and complete

degradation of TcpP [Beck et al., 2004], indicating that protection of ToxR from proteolysis might be a side effect of the role of ToxS and not its sole function. However, for $ToxR^{CC}$ the role of ToxS in terms of stability seems to be completely different. While $ToxR^{CC}$ in the absence of ToxS follows the same pattern as ToxR without ToxS by showing a gradual degradation, coexpression of ToxS leads to a rapid partial proteolysis of $ToxR^{CC}$ even without administration of the translation inhibiting agent chloramphenicol. This raises the question whether ToxS, displaying chaperone-like features, could at the same time act as a proteolytic targeting factor, which discards misfolded ToxR, as was seen e.g. for the chaperone and ATPase component of the ClpAP protease, ClpA [Wickner et al., 1994]. A point mutation in the periplasmic domain of ToxS, L33S, lead to degradation of ToxR into a stable proteolytic product, which was about 7 kDa smaller than full-length ToxR. This proteolyzed product was found to still be capable of binding DNA, yet unable to activate gene transcription of a *ctx-lacZ* construct [Pfau and Taylor, 1998]. Also, $ToxR^{CC}$ coexpressed with ToxS accumulated a proteolytic product, which was about 6 kDa smaller than full-length ToxR and which was not present in cells that lacked ToxS. However, this cleavage product or the protease that generates it seems to be short-lived, since it did not appear in samples treated with cm. The increased susceptibility of ToxR to proteolysis in the presence ToxS L33S adds to the hypothesis that ToxS might also have proteolytic targeting properties, which might be inhibited by at least this one residue in the periplasmic domain of ToxS. Yet, more work needs to be done in order to support this assumption.
Interestingly, it is $ToxR^{CC}$ in the presence of ToxS, so the $ToxR^{CC}$ species that completely lacks full-length protein, which still functions properly for activation of virulence genes, but lack of ToxS results in complete loss of activity. It cannot be excluded that the proteolytic product that accumulates in $ToxR^{CC}$ coexpressed with ToxS is also transcriptionally active, thus further investigation needs to be directed this way. Moreover, immunoblot analysis of chromosomally encoded $ToxR^{CC}$ did not show any difference between *toxS*$^{+}$ and *toxS*$^{-}$ background (Fengler V., data not shown). So, since the expression systems that were used for this study produce the genes they encode in excess, it cannot be ruled out that the effect we see is somewhat artificial.

Recently it was found that ToxS could promote oxidation of the cysteine residues of ToxR in a *dsbA*$^{-}$ background. DsbA is a disulfide oxidoreductase that catalyzes thiol-redox reactions and was shown to be responsible for formation of the intra-chain disulfide bond of ToxR. While only reduced ToxR monomer was observed in a *dsbA toxS* deletion mutant, presence of ToxS in a *dsbA*$^{-}$ background lead to formation of oxidized ToxR monomers. Since ToxS does not possess any cysteine residues itself, it is unlikely that ToxS has a redox activity *per se*, but rather

functions in bringing the cysteine residues of ToxR into such close proximity of each other that DsbA or small oxidizing agents are able to catalyze the reaction [Fengler et al, unpublished data], which again argues for a chaperone-like activity of ToxS.

In conclusion, the results obtained in this study support the hypothesis that ToxS acts as a chaperone. As could be shown ToxR and ToxS interact with each other and this interaction is competitively influenced by the presence of ToxR DNA binding sites. By interacting with ToxR, ToxS prevents formation of disulfide bonded ToxR homodimers and oligomers, which might not represent appropriate activation complexes and by doing so, ToxS might augment the chance for transcriptionally competent ToxR conformations to bind to DNA promoter sites and thus increase the proportional representation of active ToxR for gene transcription. In line with previous studies [DiRita and Mekalanos, 1991; Xu et al., 2010] it could be shown that ToxS protects ToxR from proteolysis in stationary phase cultures. Moreover, it was observed that ToxS could also initiate degradation of potentially misfolded ToxR species, as no full-length $ToxR^{CC}$ could be detected anymore in stationary phase cultures when ToxS was present. ToxS could therefore possess two distinct roles: one is to stabilize properly folded ToxR, protect it from degradation and keep it active until operator binding, the other is to promote proteolysis of mutant ToxR and thus be also involved in scavenger activities in order to remove misfolded, transcriptionally inactive ToxR species.

VI References

Abuaita, B.H. and Withey, J.H. (2009). Bicarbonate induces *Vibrio cholerae* virulence gene expression by enhancing ToxT activity. Infect Immun 77, 4111-20.

Abuaita, B.H. and Withey, J.H. (2011). Termination of *Vibrio cholerae* virulence gene expression is mediated by proteolysis of the major virulence activator, ToxT. Mol Microbiol 81, 1640-53.

Akiyama, A. and Ito, K. (1988). Folding and assembly of bacterial alkaline phosphatase *in vitro* and *in vivo*. J Biol Chem 268, 8146-50.

Ames, P. and Parkinson, J.S. (1988). Transmembrane signaling by bacterial chemoreceptors: *E. coli* transducers with locked signal output. Cell 55, 817-26.

Banwell, J.G., Pierce, N.F., Mitra, R.C., Brigham, K.L., Caranasos, G.J., Keimowitz, R.I., Fedson, D.S., Thomas, J., Gorbach, S.L., Sack, R.B., and Mondal, A. (1970). Intestinal fluid and electrolyte transport in human cholera. J Clin Invest 49, 183-95.

Beck, N.A., Krukonis, E.S., and DiRita, V.J. (2004). TcpH influences virulence gene expression in *Vibrio cholerae* by inhibiting degradation of the transcription activator TcpP. J Bacteriol 186, 8309-16.

Bentivoglio, M. and Pacini, P. (1995). Filippo Pacini: A Determined Observer. Brain Res Bull 38, 161-5.

Bik, E.M., Bunschoten, A.E., Gouw, R.D., and Mooi, F.R. (1995). Genesis of the novel epidemic *Vibrio cholerae* O139 strain: evidence for horizontal transfer of genes involved in polysaccharide synthesis. EMBO J 14, 209-16.

Bina, J., Zhu, J., Dziejman, M., Farugue, S., Calderwood, S., and Mekalanos, J.J. (2003). ToxR regulon of *Vibrio cholerae* and its expression in vibrios shed by cholera patients. Proc Natl Acad Sci USA 100, 2801-6.

Birnboim, H.C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7, 1513-23.

Brown, R.C. and Taylor, R.K. (1995). Organization of *tcp*, *acf* and *toxT* genes within a ToxT-dependent operon. Mol Microbiol 16, 425-39.

Calvin, N.M. and Hanawalt, P.C. (1988). High-efficiency transformation of bacterial cells by electroporation. J Bacteriol 170, 2796-801.

Carlone, G.M., Thomas, M.L., Rumschlag, H.S., and Sottnek, F.O. (1986). Rapid microprocedure for isolating detergent-insoluble outer membrane proteins from *Haemophilus species*. J Clin Microbiol 24, 330-2.

Carroll, P.A., Tashima, K.T., Rogers, M.B., DiRita, V.J., and Calderwood, S.B. (1997). Phase variation in *tcpH* modulates expression of the ToxR regulon in *Vibrio cholerae*. Mol Microbiol 25, 1099-111.

Champion, G.A., Neely, M.N., Brennan, M.A., and DiRita, V.J. (1997). A branch in the ToxR regulatory cascade of *Vibrio cholerae* revealed by characterization of *toxT* mutant strains. Mol Microbiol 23, 323-31.

Childers, M.B., Cao, X., Weber, G.G., Demeler, B., Hart, P.J., and Klose, K.E. (2011). N-terminal residues of the *Vibrio cholerae* virulence regulatory protein ToxT involved in dimerization and modulation by fatty acids. J Biol Chem 286, 28644-55.

Childers, M.B. and Klose, K.E. (2007). Regulation of virulence in *Vibrio cholerae:* the ToxR regulon. Future Microbiol 2, 335-44.

Claiborne, A., Miller H., Parsonage, D., and Ross, R.P. (1993). Protein-sulfenic acid stabilization and function in enzyme catalysis and gene regulation. FASEB J 7, 1483-90.

Clemens, J.D. (2011). Vaccines in the time of cholera. Proc Natl Acad Sci USA 108, 8529-30.

Crawford, J.A., Kaper, J.B., and DiRita, V.J. (1998). Analysis of ToxR-dependent transcription activation of *ompU*, the gene encoding a major envelope protein in *Vibrio cholerae*. Mol Microbiol 29, 235-46.

Crawford, J.A., Krukonis, E.S., and DiRita, V.J. (2003). Membrane localization of the ToxR winged-helix domain is required for TcpP-mediated virulence gene activation in *Vibrio cholerae*. Mol Microbiol 47, 1459-73.

Davis, B.M. and Waldor, M.K. (2003). Filamentous phages linked to virulence of *Vibrio cholerae*. Curr Opin Microbiol 6, 35-42.

DiRita, V.J. (1992). Co-ordinate expression of virulence genes by ToxR in *Vibrio cholerae*. Mol Microbiol 6, 451-8.

DiRita, V.J. and Mekalanos, J.J. (1991). Periplasmic interaction between two membrane regulatory proteins, ToxR and ToxS, results in signal transduction and transcriptional activation. Cell 64, 29-37.

DiRita, V.J, Neely, M., Taylor, R.K., and Bruss, P.M. (1996). Differential expression of the ToxR regulon in classical and El Tor biotypes of *Vibrio cholerae* is due to biotype-specific control over *toxT* expression. Proc Natl Acad Sci USA 93, 7991-5.

DiRita, V.J., Parsot, C., Jander, G., and Mekalanos, J.J. (1991). Regulatory cascade controls virulence in *Vibrio cholerae.* Proc Natl Acad Sci USA 88, 5403-7.

Dziejman, M., Balon, E., Boyd, D., Fraser, C.M., Heidelberg, J.F., and Mekalanos, J.J. (2002). Comparative genomic analysis of *Vibrio cholerae*: genes that correlate with cholera endemic and pandemic disease. Proc Natl Acad Sci USA 99, 1556-61.

Dziejman, M., Kolmar, H., Fritz, H.J., and Mekalanos, J.J. (1999). ToxR co-operative interactions are not modulated by environmental conditions or periplasmic domain conformation. Mol Microbiol 31, 305-17.

Dziejman, M. and Mekalanos, J.J. (1994). Analysis of membrane protein interaction: ToxR can dimerize the amino terminus of phage lambda repressor. Mol Microbiol 13, 485-94.

Faruque, S.M., Albert, M.J., and Mekalanos, J.J. (1998). Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae*. Microbiol Mol Biol Rev 62, 1301-14.

Faruque, S.M., Biswas, K., Udden, S.M., Ahmad, Q., S., Sack, D.A., Nair, G.B., and Mekalanos, J.J. (2006). Transmissibility of cholera: in vivo-formed biofilms and their relationship to infectivity and persistence in the environment. Proc Natl Acad Sci USA 103, 6350-5.

Fengler, V.H.I., Boritsch, E.C., Tutz, S., Seper, A., Ebner, H., Schild, S., and Reidl, J. Is ToxR a thiol-switch based regulator in *Vibrio cholerae*? unpublished data.

Field, M., Fromm, D., al-Awqati, Q., Greenough, W.B. III (1972). Effect of cholera enterotoxin on ion transport across isolated ileal mucosa. J Clin Invest 51, 796-804.

Finkelstein, R.A. and LoSpalluto, J.J. (1969). Pathogenesis of experimental cholera. Preparation and isolation of choleragen and choleragenoid. J Exp Med 130, 185-202.

Freter, R. and Jones, G. W. (1976). Adhesive properties of *Vibrio cholerae*: Nature of the interaction with intact mucosal surfaces. Infect Immun 14, 246–56.

Gentile, F., Amodeo, P., Febbraio, F., Picaro, F., Motta, A., Formisano, S., and Nucci, R. (2002). SDS-resistant active and thermostable dimers are obtained from the dissociation of homotetrameric beta-glycosidase from hyperthermophilic *Sulfolobus solfataricus* in SDS. Stabilizing role of the A-C intermonomeric interface. J. Biol. Chem. 277, 44050-60.

Goss, T.J., Seaborn, C.P., Gray, M.D., and Krukonis, E.S. (2010). Identification of the TcpP-binding site in the *toxT* promoter of *Vibrio cholerae* and the role of ToxR in TcpP-mediated activation. Infect Immun 78, 4122-33.

Grimberg, J., Maguire, S., and Belluscio, L. (1989). A simple method for the preparation of plasmid and chromosomal *E. coli* DNA. Nucleic Acids Res 17, 8893.

Guo, Y., Guettouche, T., Fenna, M.., Boellmann, F., Pratt, W.B., Toft, D.O., Smith, D.F., Voellmy, R. (2001). Evidence for a mechanism of repression of heat shock factor 1 transcriptional activity by a multichaperone complex. J Biol Chem 276, 45791-9.

Gupta, S. and Chowdhury, R. (1997). Bile affects production of virulence factors and motility of *Vibrio cholerae*. Infect Immun 65, 1131-4.

Guzman, L.M., Belin, D., Carson, M.J., and Beckwith, J. (1995). Tight regulation, modulation, and high-level expression by vectors containing the arabinose pBAD promoter. J Bacteriol 177, 4121-30.

Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. J Mol Bio 166, 557-80.

Häse, C.C. and Mekalanos, J.J. (1998). TcpP protein is a positive regulator of virulence gene expression in *Vibrio cholerae*. Proc Natl Acad Sci USA 95, 730-4.

Heitmann, P. (1968). A Model for Sulfhydryl Groups in Proteins. Hydrophobic Interactions of the Cysteine Side Chain in Micelles. Eur J Biochem 3, 346–50.

Herrington, D.A., Hall, R.H., Losonsky, G., Mekalanos, J.J., Taylor, R.K., and Levine, M.M. (1988). Toxin, toxin-coregulated pili and the *toxR* regulation are essential for *Vibrio cholerae* pathogenesis in humans. J Exp Med 168, 1487-92.

Heyningen, S. Van (1974). Cholera toxin: interaction of subunits with ganglioside GM1. Science 183, 656-7.

Higgins, D. E. and DiRita, V. J. (1994). Transcriptional control of *toxT*, a regulatory gene in the ToxR regulon of *Vibrio* cholerae. Mol Microbiol, 14, 17–29.

Higgins, D.E., Nazareno, E., and DiRita, V.J. (1992). The virulence gene activator ToxT from *Vibrio cholerae* is a member of the AraC family of transcriptional activators. J Bacteriol 174, 6974-80.

Hogan, D.L., Ainsworth, M.A., and Isenberg, J.I. (1994). Review article: gastroduodenal bicarbonate secretion. Aliment Pharmacol Ther 8, 475-88.

Hopp, T. P., Prickett, K.S., Price, V.L., Libby, R.T., March, C.J., Cerretti, D.P., Urdal, D.L., and Conlon, P.J. (1988). A Short Polypeptide Marker Sequence Useful for Recombinant Protein Identification and Purification. Nature Biotechnology 6, 1204-10.

Howard-Jones, N. (1984). Robert Koch and the cholera vibrio: a centenary. Br Med J (Clin Res Ed) 288, 379-81.

Iwanaga, M. and Yamamoto, K. (1985). New medium for the production of cholera toxin by *Vibrio cholerae* O1 biotype El Tor. J Clin Microbiol 22, 405-8.

Kamal, A.M. (1974). The seventh pandemic of cholera, p1-14, in: Cholera. The W.B. Saunders Co., Philadelphia, Pa.

Kang D., Gho Y.S., Suh, M., and Kang, C. (2002). Highly sensitive and fast protein detection with Coomassie Brilliant Blue in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. BKCS 23, 11.

Kaper, J.B., Morris, J.G. Jr, and Levine, M.M. (1995). Cholera. Clin Microbiol Rev 8, 48-86.

Karaolis, D.K.R., Johnson, J.A., Bailey, C.C., Boedeker, E.C., Kaper, J.B., and Reeves, P.R. (1998). A *Vibrio cholerae* pathogenicity island associated with epidemic and pandemic strains. Proc Natl Acad Sci USA 95, 3134-9.

King, C.A. and van Heyningen, W.E. (1973). Deactivation of cholera toxin by a sialidase-resistant monosialosylganglioside. J Infect Dis 127, 639-47.

Kirn, T.J., Lafferty, M.J., Sandoe, C.M., and Taylor, R.K. (2000). Delineation of pilin domains required for bacterial association into microcolonies and intestinal colonization by *Vibrio cholerae*. Mol Microbiol 35, 896-910.

Klimjack, M.R., Jeffrey, S., and Kielian M. (1994). Membrane and protein interactions of a soluble form of the Semliki Forest virus fusion protein. J Virol 68, 6940-6.

Klose, K.E. (2000). The suckling mouse model of cholera. Trends Microbiol 8, 189-91.

Klose, K.E. (2001). Regulation of virulence in *Vibrio cholerae*. Int J Med Microbiol 291, 81-88.

Koch, R. (1884). An address on cholera and its bacillus. Br Med J 1884, 402-7.

Kolmar, H., Hennecke, F., Gotze, K., Janzer, B., Vogt, B., Mayer, F., and Fritz, H.J. (1995). Membrane insertion of the bacterial signal transduction protein ToxR and requirements of transcription activation studied by modular replacement of different protein substructures. EMBO J 14, 3895-904.

Kovach, M.E., Shaffer, M.D., and Peterson, K.M. (1996). A putative integrase gene defines the distal end of a large cluster of ToxR-regulated colonization genes in *Vibrio cholerae*. Microbiol 142, 2165-74.

Kovacikova, G., Lin, W., and Skorupski, K. (2004). *Vibrio cholerae* AphA uses a novel mechanism for virulence gene activation that involves interaction with the LysR-type regulator AphB at the *tcpPH* promoter. Mol Microbiol 53, 129-42.

Kovacikova, G. and Skorupski, K. (1999). A *Vibrio cholerae* LysR homolog, AphB, cooperates with AphA at the *tcpPH* promoter to activate expression of the ToxR virulence cascade. J Bacteriol 181, 4250-6.

Kovacikova, G. and Skorupski, K. (2000). Differential activation of the *tcpPH* promoter by AphB determines biotype specificity of virulence gene expression in *Vibrio cholerae*. J Bacteriol 182, 3228-38.

Kovacikova, G. and Skorupski, K. (2002). Regulation of virulence gene expression in *Vibrio cholerae* by quorum sensing: HapR functions at the *aphA* promoter. Mol Microbiol 46, 1135-47.

Krukonis, E.S. and DiRita, V.J. (2003). DNA binding and ToxR responsiveness by the wing domain of TcpP, an activator of virulence gene expression in *Vibrio cholerae*. Mol Cell 12, 157-65.

Krukonis, E.S., Yu, R.R., and DiRita, V.J. (2000). The *Vibrio cholerae* ToxR/TcpP/ToxT virulence cascade: distinct roles for two membrane-localized transcription activators on a single promoter. Mol Microbiol 38, 67-84.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-5.

Li, C.C., Crawford, J.A., DiRita, V.J., and Kaper, J.B. (2000). Molecular cloning and transcriptional regulation of *ompT*, a ToxR-repressed gene in *Vibrio cholerae*. Mol Microbiol 35, 189-203.

Li, C.C., Merrell, D.S., Camilli, A., and Kaper, J.B. (2002). ToxR interferes with CRP-dependent transcriptional activation of *ompT* in *Vibrio cholerae*. Mol Microbiol 43, 1577-89.

Liu, Z., Yang, M., Paterfreund, G.L., Tsou, A.M., Selamoglu, N., Daldal, F., Zhong, Z., Kan, B., and Zhu, J. (2011). *Vibrio cholerae* anaerobic induction of virulence gene expression is controlled by thiol-based switches of virulence regulator AphB. Proc Natl Acad Sci 108, 810-5.

Lönnroth, I. and Holmgren, J. (1973). Subunit structure of cholera toxin. J Gen Microbiol 76, 417-27.

López-Gigosos, R.M., Plaza, E., Díez-Díaz, R.M., and Calvo, M.J. (2011). Vaccination strategies to combat an infectious globe: oral cholera vaccines. J Glob Infect Dis 3, 56-62.

Lüders, J., Pyrowolakis, G., and Jentsch, S. (2003). The ubiquitin-like protein HUB1 forms SDS-resistant complexes with cellular proteins in the absence of ATP. EMBO Rep. 4, 1169-74.

Martinez-Hackert, E. and Stock, A.M. (1997). Structural relationships in the OmpR family of winged-helix transcription factors. J Mol Biol 269, 301-12.

Matson, J.S. and DiRita, V.J. (2005). Degradation of the membrane-localized virulence activator TcpP by the YaeL protease in *Vibrio cholerae*. Proc Natl Acad Sci USA 102, 16403-8.

Matson, J.S., Withey, H.H., and DiRita, V.J. (2007). Regulatory networks controlling *Vibrio cholerae* virulence gene expression. Infect Immun 75, 5542-9.

Merrell, D.S., Butler, S.M., Qadri, F., Dolganov, N.A., Alam, A., Cohen, M.B., Calderwood, S.B., Schoolnik, G.K., and Camilli, A. (2002). Host-induced epidemic spread of the cholera bacterium. Nature 417, 642–5.

Mey, A.R., Craig, S.A., and Payne, S.M. (2012). Effects of amino acid supplementation on porin expression and ToxR levels in *Vibrio cholerae*. Infect Immun 80, 518-28.

Miller, J.F., Johnson, S.A., Black, W.J., Beattie, D.T., Mekalanos, J.J., and Falkow, S. (1992). Constitutive sensory transduction mutations in the *Bordetella pertussis bvgS* gene. J Bacteriol 174, 970-9.

Miller V.L., DiRita, V.J., and Mekalanos, J.J. (1989). Identification of *toxS*, a regulatory gene whose product enhances ToxR-mediated activation of the cholera toxin promoter. J Bacteriol 171, 1288-93.

Miller, V.L. and Mekalanos, J.J. (1984). Synthesis of cholera toxin is positively regulated at the transcriptional level by *toxR*. Proc Natl Acad Sci USA 81, 3471-5.

Miller, V.L. and Mekalanos, J.J. (1985). Genetic analysis of the cholera toxin-positive regulatory gene *toxR*. J Bacteriol 163, 580-5.

Miller, V.L. and Mekalanos, J.J. (1988). A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J Bacteriol 170, 2575-83.

Miller, V.L., Taylor, R.K., and Mekalanos, J.J. (1987). Cholera toxin transcriptional activator *toxR* is a transmembrane DNA binding protein. Cell 48, 271-9.

Moisi, M., Jenul, C., Butler, S.M., New, A., Tutz, S., Reidl, J., Klose, K.E., Camilli, A., and Schild, S. (2009). A novel regulatory protein involved in motility of *Vibrio cholerae*. J Bacteriol 191, 7027-38.

Morales, V.M., Backman, A., and Bagdasarian, M. (1991). A series of wide-range low-copy-number vectors that allow direct screening for recombinants. Gene 97, 38-47.

Morgan, S.J., Felek, S., Gadwal, S., Koropatkin, N.M., Perry, J.W., Bryson, A.B., and Krukonis, E.S. (2011). The two faces of ToxR: activator of *ompU*, co-regulator of *toxT* in *Vibrio cholerae*. Mol Microbiol 81, 113-28.

Moroder, L., Besse, D., Musiol, H.J., Rudolph-Böhner, S., Siedler, F. (1996). Oxidative folding of cystine-rich peptides vs regioselective cysteine pairing strategies. Biopolymers 40, 207-34.

Moss, J. and Vaughan, M. (1977). Mechanism of action of choleragen. Evidence for ADP-ribosyltransferase activity with arginine as an acceptor. J Biol Chem 252, 2455-7.

Murley, Y., M., Carroll, P.A., Skorupski, K., Taylor, R.K., and Calderwood, S.B. (1999). Differential transcription of the *tcpPH* operon confers biotype-specific control of the *Vibrio cholerae* ToxR virulence regulon. Infect Immun 67, 5117-23.

Ottemann, K.M., DiRita, V.J., and Mekalanos, J.J. (1992). ToxR proteins with substitutions in residues conserved with OmpR fail to activate transcription from the cholera toxin promoter. J Bacteriol 174, 6807-14.

Ottemann, K.M., DiRita, V.J., and Mekalanos, J.J. (1995). Analysis of *Vibrio cholerae* ToxR function by construction of novel fusion proteins. Mol Microbiol 15, 719-31.

Ottemann, K.M. and Mekalanos, J.J. (1996). The ToxR protein of *Vibrio cholerae* forms homodimers and heterodimers. J Bacteriol 178, 156-62.

Parsot, C., Mekalanos, J.J. (1990). Expression of ToxR, the transcriptional activator of the virulence factors in *Vibrio cholerae*, is modulated by the heat shock response. Proc Natl Acad Sci USA 87, 9898-902.

Pearson, G.D.N., Woods, A., Chiang, S.L., and Mekalanos, J.J. (1993). CTX genetic element encodes a site-specific recombination system and an intestinal colonization factor. Proc Natl Acad Sci 90, 3750- 4.

Peterson, K.M. and Mekalanos, J.J. (1988). Characterization of the *Vibrio cholerae* ToxR regulon: identification of novel genes involved in intestinal colonization. Infect Immun 56, 2822-9.

Peterson, K.M. (2002). Expression of *Vibrio cholerae* virulence genes in response to environmental signals. Curr Issues Intest Microbiol 3, 29-38.

Pfau, J.D. and Taylor, R.K. (1998). Mutations in *toxR* and *toxS* that separate transcriptional activation from DNA binding at the cholera toxin gene promoter. J Bacteriol 180, 4724-33.

Provenzano, D. and Klose, K.E. (2000). Altered expression of the ToxR-regulated porins OmpU and OmpT diminishes *Vibrio cholerae* bile resistance, virulence factor expression, and intestinal colonization. Proc Natl Acad Sci USA 97, 10220-4.

Provenzano, D., Lauriano, C.M., and Klose, K.E. (2001). Characterization of the role of the ToxR-modulated outer membrane porins OmpU and OmpT in *Vibrio cholerae* virulence. J Bacteriol 183, 3652-62.

Provenzano, D., Schuhmacher, D.A., Barker, J.L., and Klose, K.E. (2000). The virulence regulatory protein ToxR mediates enhanced bile resistance in *Vibrio cholerae* and other pathogenic *Vibrio species*. Infect Immun 68, 1491-7.

Safa, A., Nair, G.B., and Kong, R.Y. (2010). Evolution of new variants of *Vibrio cholerae* O1. Trends Microbiol 18, 46-54.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74, 5463-7.

Schild, S., Tamayo, R., Nelson, E.J., Qadri, F., Calderwood, S.B., and Camilli, A. (2007). Genes induced late in infection increase fitness of *Vibrio cholerae* after release into the environment. Cell Host Microbe 2, 264–77.

Schwarz, S., Kehrenberg, C., Doublet, B., Cloeckaert, A. (2004). Molecular basis of bacterial resistance to chloramphenicol and florfenicol. FEMS Microbiol Rev 28, 519-42.

Skorupski, K. and Taylor, R.K. (1999). A new level in the *Vibrio cholerae* ToxR virulence cascade: AphA is required for transcriptional activation of the *tcpPH* operon. Mol Microbiol 31, 763-71.

Svennerholm, A.M., and Holmgren, J. (1978). Identification of *Escherichia coli* heat-labile enterotoxin by means of a ganglioside immunosorbent assay (GM1-ELISA) procedure. Curr Microbiol 1, 19-23.

Taylor, R.K., Miller, V.L., Furlong, D.B., and Mekalanos, J.J. (1987). Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc Natl Acad Sci USA 84, 2833-7.

Wachsmuth, K.I., Blake, P.A., and Olsvik, O. (1994). *Vibrio cholerae* and cholera: Molecular to global perspectives. ASM Press, Washington, D.C.

Waldor, M.K. and Mekalanos, J.J. (1996). Lysogenic conversion by filamentous phage encoding cholera toxin. Science 272, 1910-4.

Warburg, O. and Christian, W. **(1941).** Isolierung und Kristallisation des Gaerungsferments Enolase. Naturwissenschaften 29, 589-90.

Watson, N., Dunyak, D.S., Rosey, E.L., Slonczewski, J.L., and Olson, E.R. (1992). Identification of elements involved in transcriptional regulation of the *Escherichia coli cad* operon by external pH. J Bacteriol 174, 530-40.

Weber, G.G. and Klose, K.E. (2011). The complexity of ToxT-dependent transcription in *Vibrio cholerae*. Indian J Med Res 133, 201-6.

WHO (2008). WHO position paper on prevention and control of cholera outbreaks: WHO policy and recommendations.

WHO (2010). Oral cholera vaccines in mass immunization campaigns: guidance for planning and use.

WHO (2011). Cholera, 2010. Weekly epidemiological record 86, 325-40.

Wickner, S., Gottesman, S., Skowyra, D., Hoskins, J., McKenney, K., and Maurizi, M.R. (1994). A molecular chaperone, ClpA, functions like DnaK and DnaJ. Proc Natl Acad Sci USA 91, 12218-22.

Withey, J.H. and DiRita, V.J. (2006). The toxbox: specific DNA sequence requirements for activation of *Vibrio cholerae* virulence genes by ToxT. Mol Microbiol 59, 1779-89.

Xu, X., Stern, A.M., Liu, Z., Kan, B., and Zhu, J. (2010). Virulence regulator AphB enhances *toxR* transcription in *Vibrio cholerae*. BMC Microbiol. 10:3.

Yang, Y. and Isberg, R.R. (1997). Transcriptional regulation of the *Yersinia pseudotuberculosis* pH6 antigen adhesin by two envelope-associated components. Mol Microbiol 24, 499-510.

Yu, R.R. and DiRita, V.J. (1999). Analysis of an autoregulatory loop controlling ToxT, cholera toxin, and toxin-coregulated pilus production in *Vibrio cholerae*. J Bacteriol 181, 2584-92.

VII Appendix

1 Abbreviations

˚C	degree Celsius
A	Ampere
aa	amino acid
ACF	accessory colonization factor
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ap	ampicillin
ap^r	ampicillin resistant
APS	ammonium persulfate
ara	arabinose
ATP	adenosine triphosphate
bp	base pair
cAMP	cyclic AMP
CAPS	(3-[(3-cholamidopropyl)-dimethylamino]-propan sulfate
CI	competitive index
cm	chloramphenicol
cm^r	chloramphenicol resistant
CT	cholera toxin
CTXϕ	cholera toxin encoding bacteriophage
ddH_2O	double deionized water
dH_2O	deionized water
DNA	desoxyribonucleic acid
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
e.g.	for example (lat. *exempli gratia*)
EtOH	ethanol
Fig	figure
g	gramm
g	times gravity
GM	monosial ganglioside
h	hour
HEPES	2-(4-(2-hydroxyethyl)-1-piperazinyl)-ethansulfonic acid

i.e.	that is (lat. *id est*)
IM	inner membrane
IPTG	isopropyl-β-thiogalactopyranosid
kB	kilo base pair
kDa	kilo Dalton
km	kanamycin
km^r	kanamycin resistant
l	liter
LB	Luria-Bertani
LPS	lipopolysaccharide
m	meter
m	milli (10^{-3}) (prefix)
M	mole per liter
MeOH	methanol
min	minute
µ	micro (10^{-6}) (prefix)
n	nano (10^{-9}) (prefix)
NC	negative control
NEB	New England Biolabs
nt	nucleotide
NTP	nucleoside triphosphate
OD_{600}	optical density at 600 nm
OM	outer membrane
OMP	outer membrane preparation
ON	over night
ONC	overnight culture
PAA	polyacrylamide
PAGE	polyacrylamide gel electrophoresis
PC	positive control
PCR	polymerase chain reaction
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulfate
sec	second
sm	streptomycin

sm^r	streptomycin resistant
std	standard
t	time
TCP	toxin-coregulated pilus
TEMED	N,N,N,',N'-tetramethyl-ethylenediamine
$ToxR^{CC}$	ToxR double cysteine mutant
Tris	Tris(hydroxymethyl)-aminomethane
U	Units
V	Volt
VPI	Vibrio pathogenicity island
WHO	World Health Organization
wHTH	winged helix-turn-helix motif
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside