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# The Molecular Regulation of the Proteolysis of ToxR, a Main Virulence Factor in *Vibrio cholerae*

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# Index

# Abstract

*Vibrio cholerae* is a Gram-negative, comma shaped, highly motile enteric bacterial pathogen that causes the severe waterborne diarrhoeal disease cholera. Vomiting of clear fluid and profuse diarrhoea, leading to life-threatening water loss, dehydration and massive electrolyte imbalances are characteristic for the bacterial infection. The bacterium is an aquatic organism that persists in coastal areas, where it can be found as a free planktonic cell or attached to biotic or abiotic surfaces. Risk factors for contagion comprise poverty, poor sanitation and insufficient drinking water supplies. Cholera is still a global public health problem, since it is endemic in Southeast Asia, South America and Africa.

*V. cholerae* enters its human host by oral ingestion of contaminated food or water and colonizes the upper small intestine. To ensure colonization and to evoke the disease, the pathogen produces a number of virulence factors. The two most predominant among of them are the cholera enterotoxin and the toxin co-regulated pilus. The expression of these virulence genes is controlled by a regulatory cascade, the ToxR regulon. This network includes several transcriptional activators: AphAB, ToxRS, TcpPH and ToxT. The activity of the mentioned regulators can be affected by various extracellular factors, like osmolarity, temperature, pH, nutrient availability, iron limitation, antimicrobial peptides, growth inhibitory substances such as bile acids, factors of the innate immune system or other organic acids.

ToxR, one of the main virulence factors in *V. cholerae*, is embedded in the inner membrane and can be classified into three defined domains: an N-terminal cytoplasmic domain with a winged helix-turn-helix DNA binding motif, a hydrophobic α-helical transmembrane domain and a periplasmic domain, which comprises two cysteine residues. Beside of regulating the expression of virulence genes, ToxR is the transcriptional regulator of the two major outer membrane porin genes *ompU* and *ompT*. ToxR stability and transcriptional activity is enhanced by periplasmic interactions with another integral membrane protein, termed ToxS. The coding sequences of *toxR* and *toxS* are transcribed as an operon by the proximal *toxR* promoter. ToxRS together with TcpPH initiate the transcription of the master regulator, *toxT*. In turn, ToxT directly interact with the promoters of *ctx* and *tcp* gene loci. Thereby cholera toxin and toxin co-regulated pilus production is activated.

In this work, the proteolysis of ToxR is addressed, involving the impact of periplasmic cysteines and the effect of ToxS on ToxR stability. Former studies demonstrate that the cysteine residues of the periplasmic portion of TcpP are critical for protein stability. To investigate the function of the two periplasmic cysteines for ToxR stability, a ToxR double cysteine substitution to serine residues was used. We show that FLAGToxR$^{CC}$ undergoes increased proteolysis in presence of ToxS and that the addition of sublethal bile acid concentrations inhibit FLAGToxR$^{CC}$ degradation, indicating that cysteine residues are decisive for ToxS mediated proteolysis.

Corresponding studies already revealed that a ToxS variant affects ToxR protein half-life in classical *V. cholerae* strains. For this purpose, plasmid encoded FLAG-tagged ToxR which was coexpressed with such a ToxS point mutant was constructed and transformed into an O1 El Tor *V. cholerae* strain. The dynamics of FLAGToxR degradation in a *toxS*$^{L33S}$ background is demonstrated. It is shown that the exchange of a single amino acid in ToxS results in increased M9 maltose FLAGToxR proteolysis in a DegS dependent manner *in vitro*. Through the supplementation of sublethal concentrations of DTT we reveal that FLAGToxR in its reduced form is even more instable in presence of altered ToxS. Through further exploiting such assays, we demonstrate that the addition of sublethal bile salt (sodium deoxycholate) concentrations no longer have a stabilizing effect on FLAGToxR under the tested conditions. This indicates an active induction of ToxR degradation by an increased interaction of the altered periplasmic domain of ToxS.

This work further focuses on the activation and stability of chromosomally expressed *toxR*.

New finding show that ToxR levels in classical *V. cholerae* strains are reduced, once the cell finds itself under nutrient limitation and alkaline pH. We display that WT ToxR in *V. cholerae* O1 El Tor is not the substrate of regulated intramembrane proteolysis (RIP) under conditions mentioned above in a long term experiment. Moreover, we demonstrate that sublethal bile salt (sodium deoxycholate) concentrations positively influence ToxR stability in a Δ*dsbA* background and that FLAGToxR$^{CC}$ still allows bile dependent ToxR activity. These findings state that bile salts have two effects on ToxR – a long term and a short term effect. The long term effect comprises the direct correlation between ToxR stability and levels of bile salt concentrations. The short term effect is described by the dependence of ToxR activity and bile salt availability.

Since it was reported that ToxR undergoes regulated intramembrane proteolysis after entry in a dormant state at alkaline pH and nutrient limitation which involves the site-2 protease RseP in a RpoE dependent manner and the finding that DegS is responsible for ToxR degradation in presence of ToxS$^{L33S}$ under normal growth conditions, it is clear that the two proteases, DegS and RseP have at least two functions in *V. cholerae* – the regulated intramembrane proteolysis of the anti-$\sigma^E$ factor RseA and ToxR. Based on these facts, we elucidate a possible connection between the $\sigma^E$ mediated envelope stress response and the proteolytic degradation of ToxR.

In summary, novel insight into the molecular regulation of proteolysis and activation mechanisms of ToxR are described. The regulatory cascade of which the virulence factor ToxR is a part of, is influenced by a diversified spectrum of extracellular stimuli, which affect the outer membrane of the pathogen. These external stimuli signalize *V. cholerae* the proper time point for virulence gene expression. The redox state of the membrane bound transcriptional activator is critical to sense external signals and for ToxS mediated proteolysis. So far, there is no evidence for a linkage between ToxR proteolysis and $\sigma^E$ stress response, which makes ToxR an exciting future research target of great potential.

# Zusammenfassung

*Vibrio cholerae* ist ein Gram-negativer, kommaförmig gebogener, hoch motiler, bakterieller Darmerreger, der die durch Wasser übertragbare lebensbedrohliche Durchfallerkrankung Cholera verursacht. Erbrechen klarer Flüssigkeit und starke Durchfälle, die zu gefährlichen Wasserverlust, Dehydrierung und massiven Elektrolytstörungen führen, sind charakteristisch für diese bakterielle Infektion. Das Bakterium zählt zu den aquatischen Organismen, das in Küstengebieten als freie planktonische Zelle oder an biotische oder abiotische Oberflächen geheftet, zu finden ist. Risikofaktoren für eine Ansteckung umfassen Armut, schlechte Hygienebedingungen und unzureichende Trinkwasserversorgung. Cholera ist nach wie vor ein globales Gesundheitsproblem, da die Erkrankung in Südostasien, Südamerika und Afrika endemisch ist.

*V. cholerae* gelangt durch die orale Aufnahme von kontaminierten Lebensmitteln oder Wasser in den menschlichen Wirt und kolonisiert den oberen Dünndarm. Um eine Kolonisierung sicherzustellen und die Erkrankung hervorzurufen, produziert der Erreger eine Reihe von Virulenzfaktoren. Die beiden vorherrschenden unter diesen sind das Cholera-Enterotoxin und der Toxin-koregulierte Pilus. Die Expression dieser Virulenzgene wird durch eine Regulationskaskade, dem sogenannten ToxR Regulon, gesteuert. Zu diesem Netzwerk zählen mehrere Transkriptionsaktivatoren, wie AphAB, ToxRS, TcpPH und ToxT. Die Aktivität der genannten Regulatoren kann durch zahlreiche extrazelluläre Faktoren beeinflusst werden, wie Osmolarität, Temperatur, pH, Nährstoff- und Eisenverfügbarkeit, antimikrobielle Peptide, wachstumshemmende Substanzen, wie Gallensäuren, Faktoren des angeborenen Immunsystems und andere organische Säuren.

ToxR, einer der wichtigsten Virulenzfaktoren in *V. cholerae*, ist in der inneren Membran eingebettet und kann in drei definierte Domänen eingeteilt werden: eine N-terminale cytoplasmatische Domäne mit einem Helix-Turn-Helix DNA-Bindemotiv, einer hydrophoben, α-helikalen Transmembrandomäne und einer periplasmatischen Domäne, die zwei Cysteinreste aufweist. Neben der Regulation der Expression von Virulenzgenen, stellt ToxR den Transktiptionsregulator der beiden Hauptaußenmembranporingene *ompU* und *ompT* dar. Die Stabilität von ToxR und seine Transkriptionsaktivität wird durch periplasmatische Interaktionen mit einem anderen integralen Membranprotein, namens ToxS, verstärkt. Die kodierenden Sequenzen für *toxR* und *toxS* werden als Operon ausgehend vom proximalen *toxR* Promotor transkribiert. ToxRS initiieren gemeinsam mit TcpPH die Transkription des Masterregulators *toxT*. ToxT tritt wiederum direkt mit den Promotoren der Gencluster *ctx* und *tcp* in Wechselwirkung. Dadurch wird die Produktion von Choleratoxin und Toxin-koregulierten Pilus aktiviert.

Diese Arbeit befasst sich mit der Proteolyse von ToxR, wobei die Auswirkungen der periplasmatischen Cysteine und der Effekt von ToxS auf die Stabilität von ToxR näher betrachtet werden.

Frühere Studien zeigen, dass die Cysteinreste des periplasmatischen Teils von TcpP für seine Proteinstabilität entscheiden sind. Zur Aufklärung der Funktionen der beiden periplasmatischen Cysteinreste in Bezug auf Stabilität von ToxR, wurde eine ToxR Cystein Punktmutante verwendet. *In vitro* kann gezeigt werden, dass FLAGToxR$^{CC}$ in M9 Maltose und in Gegenwart von ToxS ein erhöhtes Proteolyseverhalten aufweist und dass durch die Zugabe von subletalen Konzentrationen an Gallensalzen (Natriumdeoxycholat) der Abbau von FLAGToxR$^{CC}$ inhibiert wird, was darauf hinweist, dass die Cysteinreste eine entscheidende Rolle in der durch ToxS vermittelten Proteolyse aufweisen.
Vorhergehende Studien zeigten bereits, dass eine veränderte ToxS Variante die Proteinhalbwertszeit von ToxR in klassischen *V. cholerae* Stämmen stark beeinflusst. Hierzu wurde ein Plasmid konstruiert, das für FLAGToxR und einer solchen Punktmutante in ToxS kodierte und in einem O1 El Tor *V. cholerae* Stamm transformiert. Die Dynamik des Abbaus von FLAGToxR in einem *toxS*$^{L33S}$ Hintergrund wird demonstriert. Es wird gezeigt, dass *in vitro* der Austausch einer einzigen Aminosäure in ToxS zu einer erhöhten Proteolyse von FLAGToxR in M9 Maltose in einem DegS abhängigen Weg führt. Durch Hinzufügen von subletalen Konzentrationen an DTT zeigen wir, dass FLAGToxR in seiner reduzierten Form, in Anwesenheit des veränderten ToxS, instabil ist. Die weiteren Auswertungen der Experimente zeigt auch, dass die Zugabe von subletalen Konzentrationen an Gallensalzen (Natriumdeoxycholat) zu den Kulturen, zu keiner stabilisierenden Wirkung auf FLAGToxR unter den getesteten Bedingungen führt. Das deutet auf eine aktive Induktion des proteolytischen Abbaus von ToxR hin, der durch eine erhöhte Interaktion mit der veränderten periplasmatischen Domäne von ToxS ausgelöst wird.
Neben der Analyse des proteolytischen Abbaus von überexprimierten FLAGToxR, konzentriert sich diese Arbeit auch auf die Aktivierung und Stabilität von chromosomal exprimierten *toxR*. Neue Erkenntnisse zeigen, dass die ToxR Mengen in klassischen *V. cholerae* Stämmen erniedrigt sind, wenn sich die Zellen unter Nährstoffmangelbedingungen und alkalischen pH befinden. Wir zeigen, dass WT ToxR in *V. cholerae* O1 El Tor unter den oben beschriebenen Bedingungen, in einem Langzeitexperiment, kein Substrat für die regulierte intramembrane Proteolyse darstellt. Darüber hinaus können wir beweisen, dass subletale Gallensalzkonzentrationen (Natriumdeoxycholat) einen positiven Einfluss auf die ToxR Stabilität in einem Δ*dsbA* Stamm haben. Außerdem belegen wir, dass FLAGToxR$^{CC}$ nach wie vor eine Gallensalz-abhängige ToxR Aktivierung ermöglicht. Diese Ergebnisse legen fest, dass Gallensalze zwei Effekte auf ToxR haben – eine lang- und eine kurzfristige Wirkung. Die Langzeitwirkung umfasst die direkte Korrelation zwischen ToxR Stabilität und Höhe an Gallensalzkonzentrationen. Die aktivierende Wirkung beschreibt die Abhängigkeit der ToxR Aktivität von der Verfügbarkeit an Gallensalzen.
Da postuliert wurde, dass die Zellen aufgrund von Nährstoffmangel und alkalischen pH in einen Ruhezustand übergehen, dass unter diesen Umständen ToxR durch regulierte intramembrane Proteolyse durch die Seite-2 Protease RseP in einem RpoE abhängigen Weg abgebaut wird und dass die Seite-1 Protease DegS u. a. für den ToxR Abbau in Anwesenheit von ToxS$^{L33S}$ unter normalen

Wachstumsbedingungen verantwortlich ist, ist bekannt, dass die beiden Proteasen DegS und RseP mindestens zwei Funktionen in *V. cholerae* übernehmen – die regulierte Proteolyse des anti-$\sigma^E$ Faktors RseA und die von ToxR. Basierend auf diesen Tatsachen verdeutlichen wir eine mögliche Verbindung zwischen der $\sigma^E$ abhängigen Außenmembranstressantwort und dem proteolytischen Abbau von ToxR. Zusammenfassend beschreibt diese Arbeit neue Einblicke in die molekulare Regulierung der Proteolyse und Aktivierungsmechanismen von ToxR. Das regulatorische Netzwerk, von dem auch ToxR einen Anteil ausmacht, wird von einem breit gefächerten Spektrum an extrazellulären Stimuli beeinflusst, das die äußere Membran des Erregers beeinträchtigt. Diese externen Impulse signalisieren *V. cholerae* den richtigen Zeitpunkt für die Expression der Virulenzgene. Der Redoxstatus des Membran gebundenen Transkriptionsfaktors ist für die Erfassung und Weiterverarbeitung der Signale, sowie für die ToxS vermittelte Proteolyse von ToxR wesentlich. Bis jetzt gibt es keine Hinweise auf eine Verbindung zwischen der Proteolyse von ToxR und der $\sigma^E$ abhängigen Stressantwort. Dadurch bleibt ToxR in Zukunft ein spannendes Forschungsziel mit großem Potential.

# I Introduction

## 1 *Vibrio cholerae*

### 1.1 Pathogenesis - The cholera disease

The facultative human pathogen *Vibrio cholerae* is a Gram-negative, highly motile bacterium and the causative agent of the devastating diarrhoeal disease cholera (Greek kholera from kholé "bile"). In 2014, 190,549 cholera cases with 2,231 deaths were reported to the World Health Organization (WHO) by 42 countries. But the organization estimates that there are 1.4 to 4.3 million infectious cases, of which 28,000 to 142,000 result in death due to cholera every year. Cholera especially affect children. Diarrhoeal diseases are the second cause of death for children under the age of 5 years worldwide (WHO, 2015a and 2015b).

Between 1817 and 1923 six pandemic outbreaks of cholera emerged. The seventh pandemic started to spread in 1961 and was caused by a new biotype, *V. cholerae* O1 El Tor. Today, the diarrhoeal disease is endemic in parts of Africa, South America and in the southeast of Asia, especially in India and Bangladesh [Blake, 1994; Faruque et al., 1998; Harris et al., 2012].

Cholera is contracted by contaminated food or water and leads to profuse diarrhea, also described as "rice water stool" and vomiting. These primary symptoms are strongly associated with massive dehydration, hypotensive shock and death resulting from organ failure. If the patients' symptoms remain untreated, death rates can exceed 70% [Lindenbaum et al., 1967; Sack et al., 2004]. An infectious dose of $10^8$ to $10^{11}$ colony forming units is needed to cause cholera, because of the acid sensitivity of the pathogens which have to face a low pH entering the gastrointestinal tract [Hornick et al., 1971; Cash et al., 1974]. After passing through the acid stomach, they colonise the upper small intestine by penetrating the mucus lining and adhering to intestinal epithelial cells. The virulence genes are expressed, eventually cholera toxin (CT) is secreted and cholera symtomps occur. The exotoxin causes massive water loss into the intestinal lumen due to an efflux of electrolytes. The diarrhoeal fluid consists of chloride, sodium, potassium, bicarbonate and mucus, but little of blood or proteins [Molla et al., 1981]. Great amounts of highly infectious and viable cells are shed by the stool of cholera patients (up to $10^8$ bacteria per g). Excreted cells can disseminate an environmental reservoir by contaminating food and water. Through the production of cholera toxin, the reinfection and spreading of the pathogen are ensured [Sack et al., 2004].

To reduce the number of cholera outbreaks and fatalities, the general access to safe drinking water and adequate sanitation is necessary. Cholera can be cured by effective treatment with rehydration solutions, electrolytes and perhaps antibiotics, which are administered orally or intravenously to

restore circulating blood volume. If an adequate treatment is promptly available, nearly all cases of death can be avoided [Nalin et al., 1986; Guerrant et al., 2003; Sack et al., 2004].

Two oral cholera vaccines (OCVs), pre-qualified by WHO, are currently available – Dukoral® and Shanchol®. Both are administered orally and comprise dead *V. cholerae* cells. Dukoral® serves protection for 4 to 6 months, but is not licensed for children younger than 2 years, whereas Shanchol® confers immunization against clinically significant *V. cholerae* O1 in endemic areas between 2 to 5 years (WHO, 2015a).

## 1.2 Biology and life cycle

*V. cholerae* belongs to the family of *Vibrionaceae* and is a facultative anaerobic, comma-shaped and halophilic bacterium with a single flagellum, for which the pathogen is highly motile. The *Vibrionaceae* are oxidase positive contrary to the closely related *Enterobacteriaceae* [Wachsmuth et al., 1994; Mandal et al., 2001]. Up to now, more than 200 different serogroups, based on differences in the somatic O-antigen structure of lipopolysaccharide (LPS), are known. Only two serogroups, O1 and O139, are toxigenic and can cause endemic or pandemic cholera. The O1 serogroup can be further subdivided into two defined biotypes, classical and El Tor. These two biotypes differ in phenotypes, biochemical properties and susceptibility of bacteriophages [Faruque et al., 1998; Sack et al., 2004]. Each of these biotypes can be separated into three serotypes by variations in methylation of the polysaccharide component of the O antigen: Ogawa, Inaba and Hikojima. The Hikojima serotype often goes through serotype switching and thereby cross reacts with all three known antisera [Shears 1995; Mandal et al., 2014]. In comparison to the O1 classical biotype, El Tor causes a milder form of disease and is reputed to survive better in both niches, the human host and aquatic environments. El Tor strains indicate higher haemolytic activities, express a mannose sensitive type IV pilus (MHSA) and are more resistant to polymyxin B than the O1 classical biotype [Wachsmuth et al., 1994]. These advantages of O1 El Tor led to a repression of classical strains in the last pandemic [Safa et al., 2010]. In late 1992, a new non-O1 strain arose in the Indian subcontinent, *V. cholerae* O139 (Bengal). The genomes of O139 Bengal and O1 El Tor are almost identical, except for gene loci encoding for LPS structure and capsule. Therefore, it is likely that Bengal originated from O1 El Tor by horizontal gene transfer acquiring genes encoding the O139 antigen. By that, *V. cholerae* O139 constitutes another causative pathogen of cholera [Johnson et al., 1994; Albert et al., 1997].

The genome of *V. cholerae* consists of two circular chromosomes of 2.9 Mb and 1.1 Mb that encode for 3,885 open reading frames. Most genes required for mechanisms and functions essential for growth and viability, like replication, transcription and translation are encoded on chromosome 1. A single copy of cholera toxin (CT) genes, *ctxAB* is also located on the larger chromosome. *ctxAB* is integrated within the genome of the temperate filamentous phage CTXΦ. The required receptor for

the transduction of CTXΦ phage is the toxin co-regulated pilus (TCP). Gene clusters for TCP assembly which is encoded within the *Vibrio* pathogenicity island (VPI) also reside on the first chromosome. Furthermore, the regulatory gene *toxR* and other genes involved in the ToxR regulatory cascade located within the VPI reside on the same chromosome, like the effector genes *ctxAB* and *tcpA* [Waldor and Mekalanos, 1996; Heidelberg et al., 2000].

Apart from being a facultative human pathogen, *V. cholerae* is a natural inhabitant of aquatic environments, like brackish and coastal-water areas, where it can be found as free planktonic cells or associated to biotic or abiotic surfaces. In this surrounding *V. cholerae* has to resist various chemical, physical and biological stresses, including extreme temperatures, nutrient limitation, oxidative stress, different pH levels and bacteriophage attacks [Faruque and Nair, 2002]. To protect themselves from these harsh environmental conditions, the bacteria can build up multicellular structures, so called biofilms, adhered to the exoskeleton of copepods, zooplankton, phytoplankton, algae or Chironomid egg masses. The attachment to chitinous surfaces is enabled by the MSHA pilus and existence is ensured by secretion of chitinases to provide a carbon and nitrogen source from the host [Huq et al., 1983; Chiavelli et al., 2001; Berg et al., 2007].

The transmission of cholera is caused by the ingestion of contaminated food or water. After passing through the gastric acid barrier, the acid sensitive pathogens penetrate the mucus lining and adhere to intestinal epithelial cells to colonize the small intestine. In the human host the cells are exposed to completely different challenges. Thus, bacterial growth and multiplication are not impaired, although acidity, elevated osmolarity, iron limitation, antimicrobial peptides, growth inhibitory agents, like bile acids and factors of the innate immune system (complement factors), intermittent nutrient privation and higher temperature are predominant in the human gut. Eventually *V. cholerae* can grow to high titers and start to produce CT causing secretory diarrhea. By that, highly infectious and viable cells are shed with the stool. The cycle begins again, when *V. cholerae* return to the water, which is associated with the entry into a viable but non culturable state [Reidl and Klose, 2002; Louis and O'Byrne, 2010; Mey et al., 2012].

## 1.3 Virulence factors

The pathogenicity of *V. cholerae* is based on the ability to colonize the intestinal epithelial cells and to cause the acute diarrhoeal symptoms. Two virulence factors, the CT, a potent enterotoxin that causes cholera disease and a pilus, an important colonization factor referred to as the toxin co-regulated pilus (TCP), are the most prominent.

The critical intestinal colonization factor of *V. cholerae* is the toxin co-regulated pilus. TCP is a thin, filamentous, flexible, homopolymeric and self-aggregating bundle forming type IV pilus on the surface of the pathogen. The 15 predicted genes encoding for TCP are organized in the *tcp* operon

(*tcpABCDEFGHIJNQRST*) which expression is strictly regulated and located on the 41 kb VPI [Brown and Taylor, 1995; Manning, 1997]. The first gene of the *tcp* operon *tcpA* encodes for the 20 kDa pilin subunit and is essential for successful colonization [Herrigton et al., 1988]. The TcpA subunit facilitates the formation of microcolonies and serves a receptor for the CTXΦ phage [Waldor and Mekalanos, 1996; Kirn et al., 2000].

The genes encoding the CT subunits *ctxA* and *ctxB* appear as an operon within the prophage state of the filamentous phage CTXΦ [Waldor and Mekalanos, 1996]. CT is a heterohexameric exotoxin consisting of one active A- and 5 similar B-subunits ($AB_5$; A=27 kDa; B=11.6 kDa) which is secreted across the bacterial outer membrane by a type II secretion system as a holotoxin. The idea that cholera is associated with a toxic agent had already been postulated by Robert Koch in the 1800s [Koch and Carter, 1987]. The interaction of CT with cell membranes of the host is established by the non-toxic homooligomeric $B_5$ subunit and $GM_1$ gangliosides exposed from the surface of intestinal epithelial cells [King and van Heyningen, 1973]. Subsequently, the A subunit is translocated into the host cell, where it is transported retrogradely through the trans-Golgi apparatus to the endoplasmic reticulum. Catalytic activity of the toxin requires the reduction of the disulphide bond that links A1 to A2 by cytosolic disulphide oxidoreductases. The intact A subunit and the holotoxin are inactive ADP-ribosyltransferases [Gill and Rappaport, 1979; Mekalanos et al., 1979; Moss et al., 1980]. By the enzymatic activity of the nicked $A_1$ subunit the G-protein $G_{s\alpha}$, a regulatory protein for adenylate cyclase is ADP-ribosylated. By that, adenylate cyclase is permanently active, resulting in high intracellular cAMP levels. In turn, cAMP increases chloride and bicarbonate secretion in crypt cells and decreases sodium chloride absorption in villus cells. This osmotic imbalance leads to passive water efflux into the intestinal lumen, which exceeds the absorption capacity of enterocytes [Wernick et al., 2010].

Another critical colonization factor of *V. cholerae* is the correct formation of LPS and O-antigen. Mutations in genes encoding for LPS biosynthesis reduce intestinal colonization. It is important for the pathogen to have an intact outer membrane to prevent the entry of harmful antibacterial agents and to withstand recognition by host compounds [Waldor et al., 1994; Iredell et al., 1998]. A further issue regarding an intact outer membrane constitute the outer membrane porins OmpT and OmpU. OmpU serves a higher protection against the noxious effects of anionic detergents, like bile salts [Provenzano et al., 2001]. A mutant strain harbouring an *ompU* deletion and in which *ompT* is set under the control of the *ompU* promoter, has a 100-fold lower colonization efficiency in infant mice [Provenzano et al., 2000; Klose, 2001]. Furthermore, the accessory colonization factor *acf* genes (*acfA*, *acfB*, *acfC* and *acfD*) also located on the VPI, are required for enhanced colonization [Peterson and Mekalanos, 1988]. The single polar flagellum of *V. cholerae*, driven by the sodium-motive force across the inner membrane is indispensable for chemotaxis. This controllable movement away from unfavourable and towards favourable environments is also a great concern for pathogenicity. The bacteria penetrate the

mucus lining and move by chemotaxis to the intestinal crypts, where they adhere to the epithelial cells of enterocytes and start to express the virulence genes, indicating that initiation of ToxR dependent virulence gene expression and the deactivation of motility are connected [Freter and O'Brien, 1981; Gardel and Mekalanos, 1996; Häse and Mekalanos, 1999; Kojima et al., 1999].

# 2 Virulence gene regulation in *V. cholerae*

## 2.1 The ToxR regulon

The expression of the main virulence genes, *ctxAB* and the *tcp* operon are under control of a regulatory network, the so called ToxR regulon (Figure 1). By this cascade, survival within the human host, colonization and CT secretion take place in response to environmental stimuli, such as temperature, osmolarity, pH, oxygen, bile acids and bicarbonate [Gupta and Chowdhury, 1997; Schuhmacher and Klose, 1999]. The ToxR regulon ensures proper timing of expression of the virulence factors and is composed of four transcription activators: AphAB, TcpPH, ToxRS and ToxT [Higgins et al., 1992; Hase and Mekalanos, 1998; Kovacikova and Skorupski, 1999; Crawford et al., 2003].

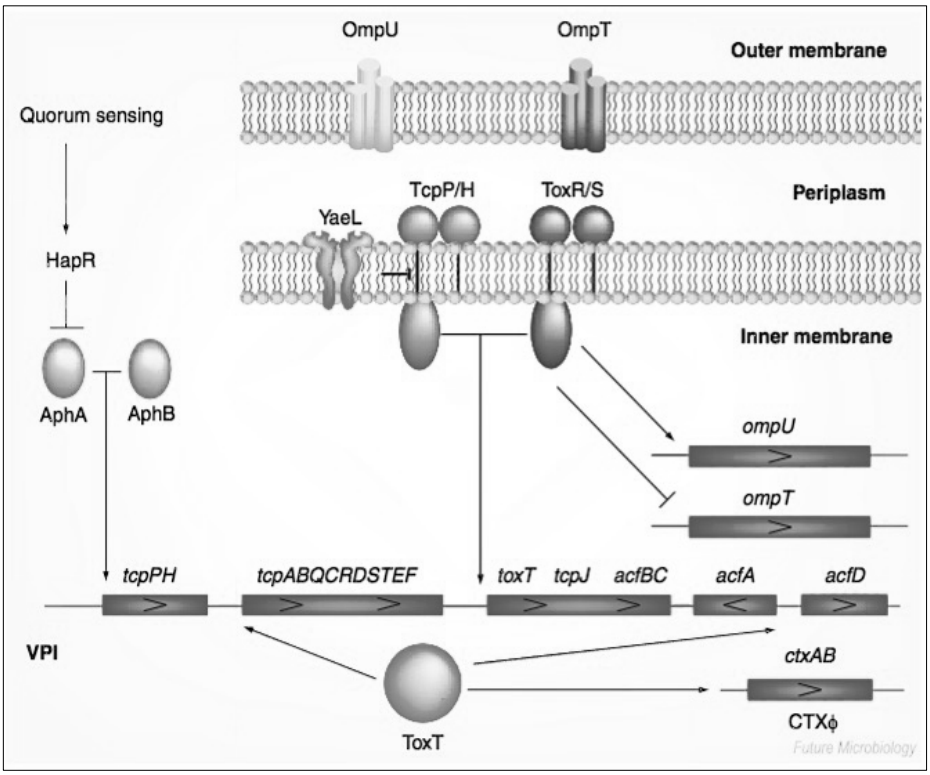


**Figure 1: The ToxR regulon – a cascade that controls the expression of the main virulence genes in *Vibrio cholerae* (Childers and Klose, 2007).** Environmental signals, such as quorum sensing modulate the induction of virulence gene expression. If low cell densities occur, the mRNA of the virulence regulator HapR is degraded. By that transcription of *aphA* is increased. AphAB activate transcription of *tcpPH* encoding for a transmembrane transcription activator located on the VPI, that in turn induce transcription of *toxT* in cooperation of ToxRS. ToxT, the master virulence regulator enables expression of *ctxAB* and the *tcp* operon. Additionally, ToxR by itself, regulates the expression of the outer membrane porins *ompU* and *ompT*.

The virulence cascade starts with the activation of the transcription regulators AphAB under anaerobic conditions, which stimulate the initiation of transcription at the promoter of *tcpPH* on the VPI. *aphA* is controlled by a quorum sensing system, which expression is repressed at high cell densities, whereas AphB activity is achieved by a switch of a cysteine to sense alterations in the redox potential in the human intestine and leads to DNA binding. An AphA dimer binds cooperatively with the LysR-like activator AphB and OhrR at the *tcpPH* promoter and promote gene expression at the *tcpPH* locus [Zhu et al., 2002; Kovacikova et al., 2010; Liu et al., 2011].

The initial induction of *toxT* transcription is obtained by cooperative binding of two integral proteins of the inner membrane, TcpP and ToxR [Miller and Mekalanos, 1985; Häse and Mekalanos, 1998; Krukonis et al., 2000]. Both regulators harbour cytoplasmic domains with winged helix-turn-helix (wHTH) DNA binding motifs, that exhibit homology with OmpR-like transcription factors. TcpP and ToxR bind with different affinities to the *toxT* promoter. Indicating that ToxR serves an enhancer for TcpP binding to DNA regions adjacent to the RNA polymerase binding site [Miller et al., 1987; Krukonis et al., 2000; Krukonis and DiRita, 2003; Haas et al., 2015]. TcpP and ToxR additionally comprise a transmembrane and a periplasmic domain, which is unlikely for the OmpR-like transcription factor family. Whereby the periplasmic domains are suggested to sense environmental signals to distinguish between the aquatic habitat and the human intestine. The TcpPH complex is activated by bile acids through formations of intramolecular disulphide bonds between thiol groups of periplasmic cysteines of TcpP [Yang et al., 2013]. The state of these periplasmic cysteines of TcpP and the presence of the membrane protein TcpH are also a deciding factors for protein degradation and therefore virulence gene expression [Morgan et al., 2016]. The periplasmic domain of TcpH contains one single cysteine residue. TcpH assist the formation of a disulphide bond in the periplasmic domain of TcpP with this single cysteine, thereby defending TcpP from degradation by intermembrane proteases, like RseP (YaeL) [Matson and DiRita, 2005; Morgan et al., 2016]. In contrast to *tcpPH*, *toxR* together with *toxS* are encoded in the ancestral chromosome and are constitutively expressed. By appropriate binding of the two protein complexes TcpPH and ToxRS to the promoter region located within the VPI, the *toxT* transcription is activated.

ToxT, the master regulator of virulence gene expression directly activates the transcription of *ctxAB*, the *tcp* operon and *acf* genes by binding to respective promoters. ToxT belongs to AraC-like transcriptional activators and is located in the cytoplasm [Higgins et al., 1992; Bina et al., 2003]. ToxT consists of two domains, an N-terminal domain, which can dimerize upon extracellular signals and a C-terminal domain, which shares homology to AraC-like transcriptional activators and is capable of DNA binding [Higgins et al., 1992]. 13 bp AT rich sequences are characteristic for promoters that are under control of ToxT. Such toxbox sequences exist in several orientations located upstream from all ToxT regulated genes and differ in their number [Withey and DiRita, 2006]. The transcription

promoting activity of ToxT depends on the presence and binding of co-factors, such as fatty acids and bicarbonate, which are found in the human intestine [Childers et al., 2011; Thomson and Withey, 2014]. On the other hand there is evidence that bile negatively regulates the transcriptional activation of ToxT [Schuhmacher and Klose, 1999]. Other findings revealed an RNA thermometer motif in the 5'UTR of *toxT*, which covers the Shine-Dalgarno element and therefore inhibits ribosomal binding at low temperatures [Weber et al., 2014]. A mechanism for deactivation of virulence factor expression is the proteolytic degradation of ToxT and TcpP in late phases of infection prior to escape from the host [Abuaita and Withey, 2011]. *V. cholerae* strains with a Δ*toxR*, Δ*tcpP* or Δ*toxT* background neither secrete CT or produce TCP and are highly attenuated for virulence [Champion et al., 1997].

## 2.2 ToxR, a transmembrane transcription activator

The expression of the two main virulence factors, CT and TCP encoded by the *ctx* and *tcp* operons, is positively and negatively, but strictly regulated at the transcriptional level in response to environmental signals. ToxR and its participation as a positive regulator of genes involved in pathogenesis in *V. cholerae* was first described in 1984 by Miller and Mekalanos. ToxR was the first identified activator for *ctxAB* and *toxT* in the regulatory cascade, termed ToxR regulon [Miller and Mekalanos, 1984; DiRita et al., 1991]. Apart from that, ToxR regulates independently of TcpP, the expression of more than 150 other genes involved in metabolism and physiology of *V. cholerae* [Peterson and Mekalanos, 1988; Bina et al., 2003]. In contrast to other transcriptional regulators involved in the ToxR regulon, *toxR* is encoded in the ancestral genome (chromosome 1). The *toxR* locus also exists in genomes of other *Vibrio* subspecies, indicating that ToxR functions evolved in *V. cholerae* by the uptake of the horizontally transferred elements VPI and CTXΦ [Heidelberg et al., 2000; Provenzano et al., 2000].

ToxR is a 32.5 kDa transmembrane protein incorporated in the inner membrane. Overall it consists of 294 amino acids, which can be classified in three different domains: an N-terminal cytoplasmic domain harbouring a winged helix-turn-helix (wHTH) DNA binding motif (180 aa), a single hydrophobic transmembrane domain (16 aa) and a C-terminal periplasmic domain (100 aa). On the basis of the ability to bind DNA and the assumption that the periplasmic domain sense extracellular signals, ToxR acts as an one component signal transduction system, with a periplasmic sensor and a cytoplasmic response regulator [Miller et al., 1987; DiRita et al., 1991; Ottemann et al., 1992; Kolmar et al., 1995]. ToxR can be classified as a member of the OmpR like transcription factors, due to homology of ToxR cytoplasmic DNA binding section and the characteristic wHTH DNA binding motif. DNA binding is facilitated by conserved amino acids of this portion [Ottemann et al., 1992; Martínez-Hackert and Stock, 1997]. ToxR recognizes a direct repeat, 5'-TNAAA-$N_5$-TNAAA-3', located upstream of several ToxR dependent promoters, such as *toxT*, *ompU*, *ompT* and *ctxA*. Although the ToxR binding site in the

*toxT* operator region is orientated oppositely from other ToxR regulated promoters, that means ToxR prefers an inverted orientation when it supports the *toxT* promoter activation [Goss et al., 2013].

ToxR is embedded into the inner membrane of *V. cholerae* by its α-helical and hydrophobic transmembrane domain. Different findings revealed a contradictory discussion about the necessity of the obliged conjunction to the inner membrane. On the one hand, the transmembrane domain is required for cooperative activation of the *toxT* promoter with TcpP, but on the other hand, ToxR association to the inner membrane is not necessary for activating the *ompU* and repressing the *ompT* promoter [Crawford et al., 2003]. However, ToxR transmembrane domain is needed for the ToxR mediated bile resistance. Bile salts intercalate, amongst others into the inner membrane, where ToxR may interact with the emulsifying agents and by that directly induce *ctxAB* expression independent of ToxT [Crawford et al., 2003; Hung and Mekalanos, 2005]. Furthermore, the transmembrane domain harbours a proline residue at position 192 that introduces a turn into the α-helix by bending other local amino acids [Cordes et al., 2002]. All in all, very little is known about the function of the ToxR transmembrane domain.

The C-terminal portion of ToxR is exposed to the periplasm, is thought to perceive environmental signals and thereby initiate virulence gene expression. The periplasmic domain consists of two cysteine residues at amino acid positions 236 and 293, which are possible objects for disulphide bond formation. By that, ToxR can remain in two different monomeric forms, one with reduced cysteine residues and one with an intramolecular disulphide bond. Additionally, by building up intermolecular disulphide bonds high molecular ToxR forms, like homodimer or oligomer forms are also possible [Ottemann and Mekalanos, 1996]. Recently, Fengler et al. described that ToxR activity is influenced by the redox state of its periplasmic cysteine residues (C236 and C293) and by that identified the oxidized form of ToxR to be active and necessary for the regulation of the outer membrane porins *ompT* and *ompU*, but not for initiation of *toxT* transcription. Mutations in *dsbAB* and substitutions of the cysteines to serine residues in ToxR do not affect the virulence gene expression but disrupt the porin production pattern [Fengler et al., 2012]. Up to now, external stimuli, modifying ToxR activity still remain unclear. Environmental signals, like bicarbonate or anaerobiosis, were characterized to have an impact on TcpP, AphB and ToxT [Abuaita and Whithey, 2009; Liu et al., 2011]. The only extracellular stimuli leading to increased ToxR activity was achieved by addition of four amino acids NRES to minimal medium, which resulted in enhanced *toxR* transcription [Mey et al., 2012]. Remarkably, other key players of the virulence cascade such as OhrR, AphB and TcpP consist of cysteines. The regulators use thiol-based switches to sense the anoxic intestinal environment and activate transcription of *tcp* by binding to the *tcpPH* promoter. The modification of sulfhydryl groups of OhrR and AphB to their reduced state is required for successful colonization and induction of virulence gene expression [Liu et al., 2011; Fan et al., 2014; Liu et al., 2016]. The formation of intramolecular disulphide bonds in the periplasmic domain

of TcpP is decisive for the protein's stability and activity, the activation of transcription of *toxT*. Interestingly, the stability of TcpP also relies on the coexpressed protein TcpH, which harbours a single periplasmic cysteine residue, facilitating proper TcpP folding [Morgan et al., 2016]. Furthermore, the stability of ToxR molecules without periplasmic cysteines depends on the presence of ToxS. In presence of ToxS the proteolytic breakdown of such ToxR molecules is increased, compared to Δ*toxS* mutant strains [Lembke, 2016].

## 2.3 The transmembrane protein ToxS

As described above, ToxR activity and stability are influenced by another membrane located protein, ToxS. ToxS is, similar to ToxR a transmembrane protein embedded in the inner membrane of *V. cholerae*. *toxS* is located downstream of *toxR* comprising one operon and are as well co transcribed with the proximal *toxR* promoter [Miller et al., 1989]. ToxS is a 19 kDa protein with 173 aa periplasmic domain and a short 6-8 aa comprising cytoplasmic portion, which are linked to each other by a single transmembrane α-helical and hydrophobic domain (20 aa) [DiRita and Mekalanos, 1991]. Although missing a periplasmic cysteine residue, ToxS can be compared to TcpH [Häse and Mekalanos, 1998]. ToxS has no influence on *toxR* transcription, but rather enhances ToxR activity by direct protein-protein interactions between the periplasmic portions, leading to heterodimers, whereas ToxR homodimer formation is suppressed [Ottemann and Mekalanos, 1996]. Other findings reveal that deletion of a portion of ToxR periplasmic domain abrogated the interaction with ToxS. A periplasmic ToxR-PhoA fusion protein is dependent on ToxS in accordance with ToxR activity, suggesting that ToxS protects this fusion protein from proteolytic degradation by periplasmic interaction [DiRita and Mekalanos, 1991]. More recent studies give evidence that ToxS is necessary for ToxR mediated *omp* gene expression by addition of the NRES mix to minimal medium in El Tor strains, but not for the increase of ToxR levels in response to the supplemented amino acids. These findings suggest a chaperon function of ToxS [Mey et al., 2012]. Fengler et al. clarified the presence of ToxRS heterodimers in *E. coli* but not in *V. cholerae*. The presence of ToxR binding sites (e.g. *ompU* operator) in *E. coli* diminishes ToxRS heterodimerization. Because of the abundant presence of ToxR operators in the genome of *V. cholerae*, the ToxRS heterodimer cannot be observed [Fengler et al., 2012]. However, ToxR is capable of activating virulence gene expression in the absence of ToxS, if *toxR* is overexpressed. In contrast, at chromosomally expressed ToxR levels, ToxS is required for ToxR activity [Miller et al., 1989]. It has been demonstrated that ToxS lowers the effective amounts of ToxR which are required to activate the *ctx* promoter and that ToxR is stable bound to the promoter region *in vitro*, but *in vivo* full transcriptional activation by ToxR can only be obtained in presence of ToxS [Ottemann et al., 1992; Pfau and Taylor, 1998]. Moreover, in *Salmonella typhimurium* a ToxS variant in which the periplasmic leucine at aa position 33 is replaced by a serine results in susceptibility of ToxR to proteolytic

degradation. Leading to the suggestion that the function of ToxS is to build up complexes and interact with ToxR. ToxS enhance proper assembly and stability of the ToxR periplasmic domain. This function of ToxS is thought to be needed to establish a transcriptional activation complex, but not a ToxR-DNA complex and to protect the transcriptional regulator from premature proteolysis [Pfau and Taylor, 1998]. The $ToxS^{L33S}$ periplasmic mutant also leads towards an increased ToxR proteolysis in O1 classical *V. cholerae* strains. Based on this enhanced proteolysis of ToxR, four putative site-1 proteases are identified: DegS, DegP, VesC and TapA, whereby the corresponding site-2 protease is characterized to be RseP [Almagro-Moreno et al., 2015a; Almagro-Moreno et al., 2015b]. Altogether, very little is known about the function of ToxS, how ToxS can modulate ToxR activity and stability or how ToxS function can be influenced by environmental signals to beware *V. cholerae* of expressing genes (e.g. *ctxAB* or *tcp* operon) in niches in which their action is not required.

## 2.4 The outer membrane porins OmpT and OmpU

The transmembrane transcription factor ToxR regulates the expression of more than 150 genes in *V. cholerae* [Bina et al., 2003]. Two among of them are *ompT* and *ompU*, which encode for two outer membrane porins. ToxR directly activates the expression of *ompU* and by that simultaneously represses the transcription of *ompT*, which is the only known gene under negative regulation of ToxR [Li et al., 2000]. The consensus sequence 5'-TNAAA-$N_5$TNAAA-3' or the complemented version for ToxR specific binding are located within the promoter region of ToxR regulated genes, such as *ompU* and *ompT*. Whereby the *ompT* promoter contain two ToxR binding sites and the *ompU* promoter three [Goss et al., 2013]. ToxR disrupts the DNA binding site of cAMP receptor protein (CRP) by interacting with its ToxR boxes and additionally extinguish the basal transcription of *ompT* [Li et al., 2002]. Interestingly, Fur positively influences the transcription of *ompT*. Fur usually represents a repressor in *V. cholerae* and regulates gene expression in response to the availability of iron. Thereby, iron also supports the expression of *ompT* [Craig et al., 2001]. OmpT is the predominant outer membrane porin under nutrient limiting conditions, like growth in the late stationary phase or in minimal media, whereby OmpU is the primary OMP under nutrient rich conditions, such as growth in full media [Miller and Mekalanos, 1988; Li et al., 2002]. The addition of bile acids [Provenzano et al., 2000] or the supplementation of the NRES mix to minimal medium can change the OMP profile by switching nearly solely exclusively to OmpU [Mey et al., 2012].

OmpT and OmpU are the two major pore-forming proteins of the outer membrane. They are organized as homotrimer hydrophilic barrel channels, whereby predominately β-sheets are shaping the porins with different pore sizes. The 38 kDa protein OmpU has an effective radius of 0.55 nm, while OmpT has a molecular weight of 40 kDa and an effective radius of 0.43 nm. Furthermore, a monomeric unit of OmpU contains a β-sheet which is associated to three to four $Ca^{2+}$ ions. Obviation of these cations

irreversibly disrupt the tertiary structure of OmpU [Chakrabarti et al., 1996; Crawford et al., 1998; Duret and Delcour, 2010]. Correct secretion and assembly of the two oppositely regulated porins into the outer membrane requires the type II extracellular protein secretion system, which is also involved in CT secretion [Sandkvist et al., 1997].

The trimeric β-barrels allow the transport of hydrophilic solutes and the passive diffusion of other small molecules between the periplasm and the extracellular space [Chakrabarti et al., 1996]. OmpT is a much less cation-selective channel and additionally the overall permeability is greater in comparison to OmpU [Simonet et al., 2003]. Although OmpU exhibits a larger pore size than OmpT, it displays a greater specificity for negatively charged agents, like bile salts. The exclusive presence of OmpT in the outer membrane leads to higher and sever sensitivity to bile salts and antimicrobial peptides in the human intestine. Therefore, the precise regulation of the expression of outer membrane porins is essential in both niches, the aquatic environment and the human host. Besides, as already mentioned above, a mutant strain harbouring an *ompU* deletion and in which *ompT* is set under the control of the *ompU* promoter, has a 100-fold lower colonization efficiency in infant mice and express less CT and TCP. These findings reveal that porin adaptation is critical for pathogenesis, including intestinal colonization, bile resistance and virulence factor expression [Provenzano and Klose, 2000; Klose, 2001; Provenzano et al., 2001]. However, neither OmpU nor OmpT is essential for colonization [Provenzano et al., 2001].

## 3 SigmaE regulated envelope stress response in Gram-negative bacteria

The priority for the regulation of gene expression in bacteria is the point of transcription initiation. The regulatory key players of this step are sigma (σ) factors. σ factors comprise a homologous family of proteins which control the global genetic changes by enabling the initiation of transcription of specific genes. σ factors associate with the catalytically relevant core RNA polymerase and lead the resulting holoenzyme to a certain subset of promoter sites [Burgess et al., 1969]. The homology of the σ factor family is due to defined regions of highly conserved amino acid sequences for proper DNA binding [Lonetto et al., 1992; Gruber and Bryant, 1997]. Group 1 σ factors, such as the housekeeping factor, $\sigma^{70}$, are essential for survival and viability and control an extensive variety of genes connected to normal cell growth [Gruber and Gross, 2003]. Most bacterial species harbour alternative σ factors which compete in binding to a limited pool of RNA polymerase core enzymes. The alternative σ factor direct transcription of specific regulons to provide a respond to changing environmental or developing stimuli, mediated by redirection of transcription and access to the shared expression machinery. Such regulons include specified genes, which are implicated in motility, iron transport, nutrient deprivation, high cell density, stress response to temperature and chemical stresses and other adaptive pathways [Helmann and Chamberlin, 1988; Gross et al., 1992; Wosten, 1998]. Under conditions that do not

require these specialized functions, most alternative σ factors are negatively controlled by anti-σ factors [Hughes and Mathee, 1998].

Generally, bacterial stress is a term for a condition that results in protein denaturation in a compartment specific fashion. The alternative σ factor, $\sigma^E$ in *E. coli* is encoded by *rpoE* and responds to extracytoplasmic stress in the periplasmic compartment by activating the expression of genes that alleviate the harmful effects of unfolded proteins in the periplasmic compartment [De Las Peñas et al., 1997a; Missiakas et al., 1997]. The release and activity of $\sigma^E$ is caused by stresses such as heat shock, ethanol, overexpression of outer membrane proteins, misfolding of periplasmic proteins or other extracellular stresses leading to unfolded envelope proteins [Mecsas et al., 1995; Rouvière et al., 1995; Hayden et al., 2008].

*E. coli* $\sigma^E$ factor belongs to the biggest family of alternative σ factors which predominately regulate extracytoplasmic functions [Lonetto et al., 1994; Missiakas and Raina, 1998; Raivio and Silhavy, 2001; Helmann, 2002], which are also found in a great variety of bacterial genomes, including many pathogens like *V. cholerae* which encode for putative orthologues [Helmann, 2002] (*rpoE*: VC0395_A2045; *rseA*: VCM66_2389; *rseB*: VCM66_2388; *degS*: VC0565; *rseP*: EN18_14360). In *E. coli*, *rpoE* is encoded within an operon with its regulatory genes *rseA*, *rseB* and *rseC* [De Las Peñas et al., 1997b; Missiakas et al., 1997].

The extracellular stress stimulus is transmitted from the periplasm to the cytoplasmic transcription machinery by a signal transduction pathway relieving inhibition of $\sigma^E$ by its anti-σ factor RseA to the cytoplasm, termed $\sigma^E$ pathway or $\sigma^E$ stress response [Connolly et al., 1997; Danese and Silhavy, 1997]. Under stress conditions, the sequentially proteolytic breakdown of the anti-σ factor by two membrane proteases DegS and RseP to liberate $\sigma^E$ is termed regulated intramembrane proteolysis (RIP) [De Las Peñas et al., 1997; Ehrmann and Clausen, 2004]. RseA is a transmembrane protein of the inner membrane. The N-terminal domain tightly sequesters $\sigma^E$ to the cytoplasmic side of the inner membrane and thereby blocks the transcription of $\sigma^E$ dependent genes. The C-terminal domain is exposed to the periplasm and binds a supportive negative regulator RseB [De Las Peñas et al., 1997b; Missiakas et al., 1997]. RseB suppresses the proteolytic activity of DegS for RseA cleavage [Grigorova et al., 2004; Chaba et al., 2007]. Whether in complete absence of *rseB* or in RseB derivates which lower the protein interaction with RseA, a faster degradation of RseA and therefore activation of transcription of $\sigma^E$ dependent genes is observed [Collinet et al., 2000; Grigorova et al., 2004]. RseB covers the DegS cleavage site of RseA (Val148-Ser149) and thereby rendering RseA more resistant to DegS cleavage [Cezairliyan and Sauer, 2007; Kim et al., 2010].

Misfolded C-terminal portions of periplasmic proteins are recognized by the PDZ domain of DegS, a protease embedded in the inner membrane of Gram-negative bacteria. DegS is a member of the DegP/HtrA family of serine proteases [Waller and Sauer, 1996; Pallen and Wren, 1997] and is required

for RseA cleavage [Ades et al., 1999; Alba et al., 2001]. Through the interaction between unfolded proteins and the PDZ domain, a conformational change is induced that activates DegS [Walsh et al., 2003; Wilken et al. 2004], which attacks RseA at its periplasmic portion by competing with RseB, whereby the $\sigma^E$ signalling pathway is induced (Figure 2).

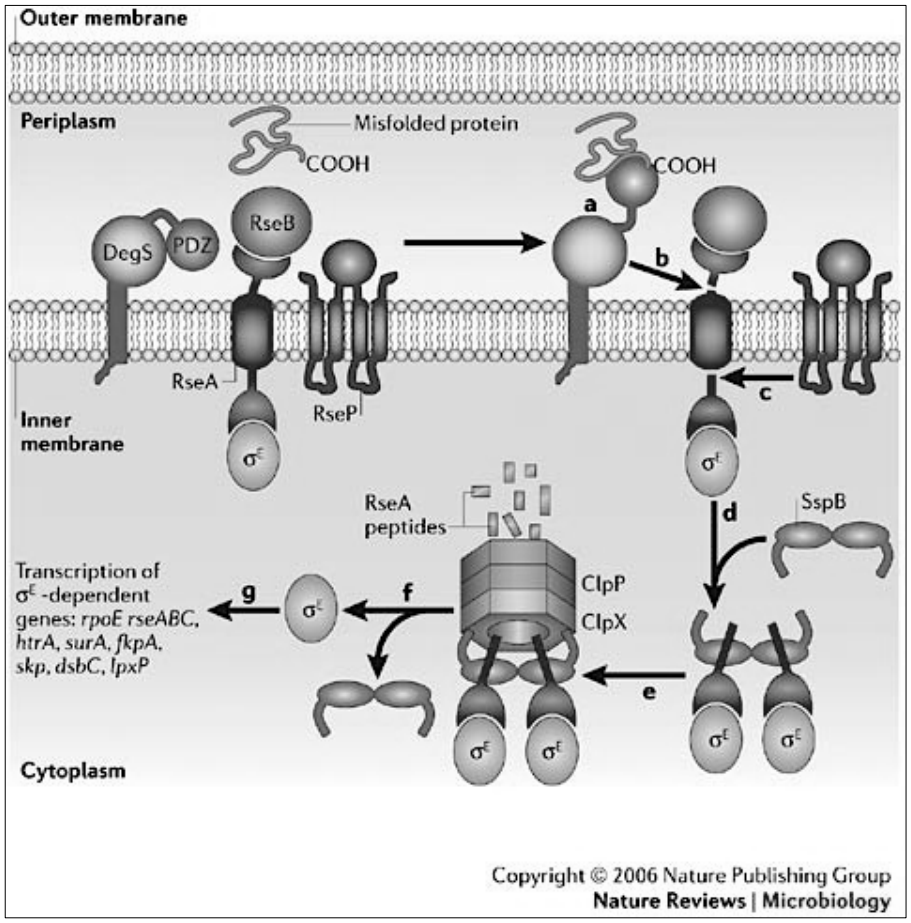


**Figure 2: Regulation of $\sigma^E$ activity by regulated intramembrane proteolysis in *Escherichia coli* (Rowley et al., 2006).** Extracellular stresses which affect the correct maintenance of envelope proteins induce a pathway which is known as the SigmaE ($\sigma^E$) stress response. The main mechanism for $\sigma^E$ release and activation is the degradation of RseA by the consecutive action of two membrane proteases DegS and RseP. In the absence of misfolded proteins in the periplasm, the PDZ domain of DegS prevents a premature cleavage of RseA. If misfolded proteins occur, the PDZ domain of DegS recognizes a carboxyterminal motif of outer membrane proteins (OMPs) (**a**). This results in DegS activation and cleavage of RseA at its periplasmic portion (**b**), which in turn triggers the attack by RseP protease at the cytoplasmic cleavage site of RseA (**c**). $\sigma^E$-RseA inhibitory complex is released to the cytoplasm, where it is bound to SspB (**d**), which leads the complex to ClpXP (**e**). The protease complex ClpXP selectively degrades the RseA portion which results in release of $\sigma^E$ (**f**). Free $\sigma^E$ protein binds to core RNA polymerase and leads the transcription apparatus to promoters of $\sigma^E$ dependent genes.

The inhibition enhancing protein RseB is also released to the periplasmic space due to proteolytic degradation of RseA [De Las Peñas et al., 1997b; Missiakas et al., 1997; Kim et al., 2010]. In turn, a second cleavage in the cytoplasmic domain of RseA mediated by RseP, releases a $\sigma^E$-RseA complex from the cytoplasmic side of the inner membrane [Ades et al., 1999; Alba et al. 2002; Kanehara et al.

2002]. RseP is an inner membrane $Zn^{2+}$ metalloprotease and essential for viability [Dartigalongue et al., 2001; Kanehara et al., 2001]. The two mentioned membrane bound proteases DegS and RseP are also responsible for the regulated degradation of the virulence gene regulator ToxR [Almagro-Moreno et al., 2015a; Almagro-Moreno et al., 2015b].

The $\sigma^E$-RseA complex binds to SspB in the cytoplasm, which directly delivers the proteins to the AAA+ protease ClpXP for degradation. ClpXP selectively cleaves the remaining RseA portion, which results in free cytoplasmic $\sigma^E$ [Ades et al., 1999; Flynn, 2004]. The release of $\sigma^E$ allows interaction with the core RNA polymerase and redirect the RNA polymerase holoenzyme to $\sigma^E$ dependent promoters with the consensus sequence 5'-ggAACtt-$N_{16}$-TCnaA-3' [Rezuchova et al., 2003; Rhodius et al., 2005]. Such a promoter is located upstream from the *rpoE rseABC* operon [Rouviere et al., 1995]. Increased expression from this promoter ensues increased levels of $\sigma^E$ and the anti-σ factor RseA, that operate homeostatically to deactivate the $\sigma^E$ stress response once the activating stimulus is gone. Other $\sigma^E$ dependent genes encode for periplasmic foldases, outer membrane chaperones, enzymes that are involved in fatty acid, oligosaccharide or LPS synthesis and assembly, but also cytoplasmic genes which are involved in replication, transcription and translation [Dartigalongue et al., 2001; Rezuchova et al., 2003; Rhodius et al., 2005]. Additionally, in a *V. cholerae hfq* deletion mutant, the activity and expression of the alternative sigma factor RpoE are notably increased. According to transcriptome analysis of a Δ*hfq* mutant strain compared to that of a Δ*rseA* mutant, it was observed, that half of the genes in *V. cholerae* are upregulated in a Δ*hfq* background. *dsbA*, but not *degS*, is found to be upregulated in such a deletion circumstance. Therefore, the *dsbA* expression is under control of $\sigma^E$ [Ding et al., 2004]. In *V. cholerae* the $\sigma^E$ cascade seems to be similar as it is illustrated for *E. coli*, whereat a peptide emerging from the misfolded and by ToxR regulated OmpU is the initiating signal for $\sigma^E$ release [Kovacikova and Skorupski, 2002; Mathur et al., 2007; Davis and Waldor, 2009]. Furthermore, $\sigma^E$ can also be induced by growth phase conditional guanosine 3',5' bispyrophosphate in reaction to nutrient availability [Costanzo and Ades, 2006].

In summary, the function of the alternative sigma factor $\sigma^E$ is situated in envelope stress response, preserving membrane integrity and preventing fatal cell membrane stress or disruption.

## 3.1 Dsb system dependent oxidative protein folding in the periplasmic space of Gram-negative bacteria

In Gram-negative bacteria, the formation of disulphide bonds between cysteine residues of proteins in the periplasmic compartment is an enzyme dependent process, which is catalysed by the Dsb proteins. Correct structure and folding of proteins are important issues to sense and respond to environmental and physiological signals. Cysteine residues are the preeminent substrates of redox associated protein folding and regulation. Disulphide bridges promote a substantial role in the tertiary

structure of a protein and are commonly decisive for protein stability. The altered redox state of a protein can also influence the catalytic or regulating properties [Sevier and Kaiser, 2002; Klomsiri et al., 2011]. Therefore, the state of cysteine residues and the introduction of disulphide bonds in the reductive compartment are crucial events. The formation of disulphide bonds between two thiol groups (-SH) is derived by two electrons. This reaction is catalysed by the thiol-disulphide oxidoreductases DsbAB and DsbCD, belonging to the thioredoxin superfamily. DsbAB are necessary for the establishment of disulphide bonds in unfolded *de novo* synthesized polypeptide chains, which are exported from the cytoplasm to the periplasmic space. Whereas DsbCD rearrange non-native disulphide bonds and are responsible for isomerization [Bardwell et al., 1991; Collet and Bardwell, 2002; Nakamoto and Bardwell, 2004]. Figure 3 displays the Dsb system in the *E. coli* periplasm.

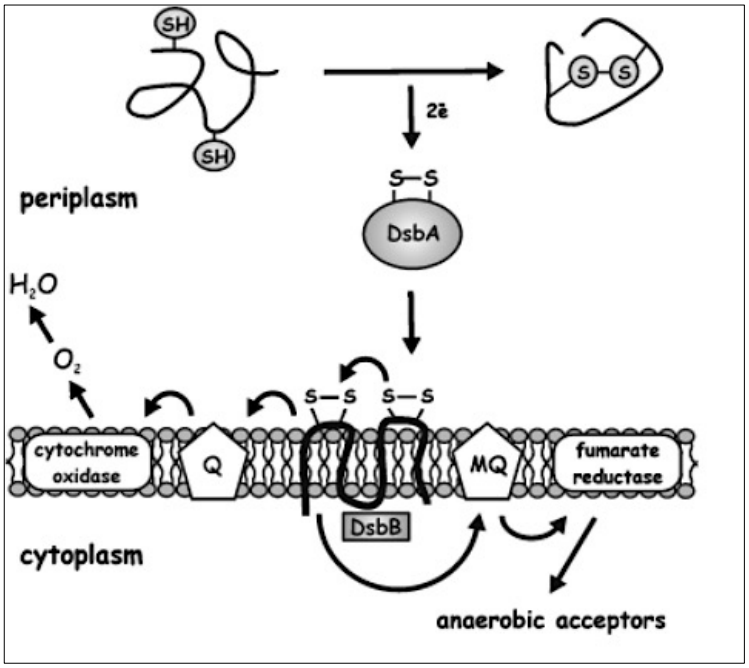


**Figure 3: Oxidative protein folding in the periplasmic space of Gram-negative bacteria by the Dsb system [Lasica and Jagusztyn-Krynicka, 2007].** DsbA introduce disulphide bonds into *de novo* synthesized polypeptides in the periplasm. By that, DsbA is released in its reduced form and needs to be reoxidized to start a new oxidation. This reoxidation is catalysed by the integral quinone reductase DsbB. Electron flow continues from DsbB to ubiquinones (Q) and terminal oxidases under aerobic conditions. The cytochrome oxidases transfer electrons to molecular oxygen in a reaction associated with $H^+$ resulting in $H_2O$ production. In the absence of oxygen, DsbB transmits the received electrons to menaquinone (MQ). In turn, anaerobic reductases, like nitrate reductase or fumerate reductase, ensure the reoxidation of menaquinone. The electron flow is displayed by black arrows.

DsbA is a periplasmic soluble protein with a molecular weight of 21 kDa comprising a thioredoxin domain, which harbours the active site and the essential CXXC motif [Bradwell et al., 1991]. DsbA catalyses the oxidation of cysteine residues of unfolded proteins that are designated for periplasmic location. The enzyme implies high oxidative power, because of the CXXC motif structure and the low $pK_a$ value of the crucial cysteine at amino acid position 30. DsbA forms an intermolecular disulphide bond between a cysteine of the substrate protein and Cys-30. The disulphide bond is transferred to

the substrate protein and reduced DsbA is released [Zapun et al., 1993]. To catalyse the next oxidation, DsbA has to be reoxidized by DsbB [Missiakas et al., 1993]. The 21 kDa DsbB protein is integrated into the inner membrane and comprises two essential pairs of cysteines exposed to the periplasm, also organized in CXXC motifs [Collet and Bardwell, 2002]. To actively catalyse the oxidation of DsbA, DsbB has to appear in its oxidized state as well. Under aerobic conditions, DsbB oxidation is attained by passing the electrons to quinones and cytochrome oxidase, which finally reduces molecular oxygen. In the absence of oxygen, the electrons are passed to menaquinone to nitrate reductase or fumerate reductase [Bader et al., 1999].

*V. cholerae* also encodes for periplasmic disulphide bond forming and correcting proteins. DsbAB and DsbCD homologues exist [Yu et al., 1992; Segatori et al., 2006]. As already mentioned above, in *V. cholerae* the transcriptional initiation of *dsbAB* and *dsbCD* is dependent on the alternative sigma factor $\sigma^E$ [Ding et al., 2004; Ruiz and Silhavy, 2005]. A *dsbA* deletion strain causes a colonization defect in infant mice, indicating an essential role in pathogenesis [Peterson and Mekalanos, 1988; Peek and Taylor, 1992]. This phenotype can be explained by the possibly general involvement of DsbA in the formation of disulphide bridges of cysteine residues containing virulence associated proteins, such as TCP or CT [Peek and Taylor, 1992; Yu et al., 1992]. Moreover, a *dsbA* or *dsbB*, but not a *dsbC* deletion strain affects ToxR dependent OMP regulation [Fengler et al., 2012].

## 4 Aim of this study

ToxR is a central transcriptional regulator in *V. cholerae*, controlling the virulence gene expression from two horizontally obtained elements, VPI and CTXΦ, as well as 150 other physiological and metabolical genes in response to environmental changes [Miller et al., 1987; Provenzano et al., 2000; Peterson 2002; Bina et al., 2003]. The expression of virulence genes is organized by a regulatory network, the ToxR regulon, comprising the transcriptional regulators AphAB, TcpPH, ToxRS and ToxT [Miller et al., 1984; Skorupski and Taylor, 1999; Childers and Klose, 2007].

The global regulator ToxR is illustrated to function as a one component signal transduction system with a periplasmic sensor and a cytoplasmic response regulator [Miller et al., 1987; Kolmar et al., 1995]. The regulatory protein is encoded by *toxR* in the ancestral genome and is co-transcribed with *toxS*, encoding for a protein embedded in the inner membrane and harbouring a periplasmic domain [Miller et al., 1989; DiRita and Mekalanos, 1991]. ToxS is thought to protect ToxR from premature proteolysis and to act like a chaperon to ensure properly transcriptionally active ToxR conformations [Ottemann et al., 1992; Pfau and Taylor, 1998;Mey et al., 2012]. So far, many questions concerning ToxR stability, activation and ToxR dependent gene expression remain to be answered. Furthermore, the influence of environmental stimuli and the function of ToxS on ToxR are also poorly understood. Consequently,

this study focuses on the interaction of ToxR and ToxS, ToxR activity, stability and the ability to sense environmental signals.

A ToxS variant in which the periplasmic leucine at aa position 33 is exchanged by a serine residue was first published in *Salmonella typhimurium* [Pfau and Taylor, 1998]. This ToxS$^{L33S}$ periplasmic mutant leads to susceptibility of ToxR to proteolytic degradation in O1 classical *V. cholerae* strains [Almagro-Moreno et al., 2015b]. Consequently, the question arises whether ToxS$^{L33S}$ also challenges ToxR stability in El Tor *V. cholerae* strains. To investigate the proteolytic half-life of ToxR and to understand the dynamics of ToxR degradation in a *toxS*$^{L33S}$ background, a Δ*toxRS* strain carrying the plasmid pFLAGtoxRS$^{L33S}$ was used and protein stability assays were carried out.

Due to this enhanced proteolysis of ToxR in classical *V. cholerae* strains in presence of ToxS$^{L33S}$, four putative site-1 proteases for ToxR degradation are identified [Almagro-Moreno et al., 2015b]. Additionally it was already known that two distinct proteolytic ToxR fragments appear, indicating that at least two proteases are responsible for ToxR degradation [Lembke, 2016]. In dependence on these facts, the impact of DegS was determined by using a Δ*degS* deletion strain harbouring pFLAGtoxRS$^{L33S}$ and protein stability assays were performed.

Moreover, Lembke found out that bile salts have a stabilizing effect on ToxR variants that usually undergo increased proteolysis. Based on this, *toxRS* deficient strains harbouring pFLAGtoxRS$^{L33S}$ were grown under conditions, which are known to affect the redox status of proteins and which are suspected to have a stabilizing impact on protein half-life. Thus, the effect of DTT and bile salts (sodium deoxycholate) was investigated by performing chloramphenicol experiments.

Recent publications revealed that ToxR activity is tightly linked to the redox state of the periplasmic cysteines. Substitutions of ToxR cysteines to serine residues in a Δ*toxS* mutant strain severely affect the *ctx* and *tcp* gene expression compared to unaltered ToxR [Fengler et al., 2012]. Other findings showed that cysteine residues in TcpP and TcpH are crucial for protein stability [Morgan et al., 2016]. In virtue of this, *toxRS* deficient strains harbouring pFLAGtoxR$^{C236SC293S}$_toxS were grown in absence or presence of bile salts (sodium deoxycholate) to determine their effect on ToxR$^{CC}$ stability in protein degradation assays.

Lately, RseP was identified to be the site-2 protease, which is responsible for ToxR degradation during the late stationary phase. It was postulated that ToxR undergoes regulated intramembrane proteolysis under nutrient limitation and alkaline pH conditions after the cells entered a dormant state. [Almagro-Moreno et al., 2015a]. So far, it is not understood what environmental stimuli *in vivo* cause the signal for preventing the activity and triggering the degradation of ToxR. To further describe the attributes of conditions under which ToxR is resistant to proteolytic decomposition and remains in its active form,

long term experiments of chromosomally expressed *toxR* or FLAG*toxR*$^{C236SC293S}$, also in a Δ*dsbA* mutant strain, using media supplemented with bile salts were carried out.

Since it is known from literature that ToxR undergoes RIP after entry in a dormant state at alkaline pH and nutrient limitation which involves the site-2 protease RseP in a RpoE dependent manner [Almagro-Moreno et al., 2015a] and the finding of four putative site-1 proteases, including DegS, which are responsible for ToxR degradation in presence of ToxS$^{L33S}$ under normal growth conditions [Almagro-Moreno et al., 2015b], it is clear that DegS and RseP have at least two functions in *V. cholerae* – the RIP of RseA [Ding et al., 2004; Hayden et al. 2004] and ToxR. Furthermore, it was reported that in *V. cholerae* the inducing signal for $\sigma^E$ release is the exposure of a carboxy-terminal peptide of the ToxR regulated outer membrane porin OmpU [Kovacikova and Skorupski, 2002; Mathur et al., 2007; Davis and Waldor, 2009]. Moreover, in *V. cholerae* the transcriptional initiation of *dsbAB* and *dsbCD* is dependent on the alternative sigma factor $\sigma^E$ [Ding et al., 2004; Ruiz and Silhavy, 2005]. A *dsbA* or *dsbB*, but not a *dsbC* deletion strain affects ToxR dependent OMP regulation [Fengler et al, 2012]. On the strength of this, we propose the hypothesis whether there is a possible connection between the $\sigma^E$ regulated stress response and the protein degradation of ToxR. To answer these issues, we wanted to elucidate how the expression of *degS* and *dsbA* is regulated and whether bile salts, DTT, polymyxin B or the synthetic tripeptide YDF, mimicking envelope stress, are capable to activate the transcription of *degS* or *dsbA*. Therefore, chromosomal *degSphoA* and *dsbAphoA* fusion strains were used to carry out PhoA assays under respective conditions. Additionally, we also investigated whether ToxR is a target of the site-1 protease DegS under membrane stress mimicking conditions [Walsh et al., 2003; Mathur et al., 2007; Almagro-Moreno et al. 2015b; Lembke, 2016]. Therefore, a Δ*toxRS* strain carrying the plasmid pFLAGtoxRS was grown in presence of the synthetic tripeptide YDF and protein stability assays were carried out.

# II Materials and Methods

## 1 Materials

### 1.1 Chemicals and equipment

All chemicals used in this work were acquired from BD Biosciences (Schwechat, Austria), Bio-Rad Laboratories, Inc. (Vienna, Austria), Carl Roth GmbH + Co. KG (Karlsruhe, Germany), Centic Biotec (Heidelberg, Germany), Fermentas (St. Leon-Rot, Germany), Fluka BioChemika (Buchs, Switzerland), Merck KGaA (Darmstadt, Germany), neoLab Migge Laborbedarf-Vertriebs GmbH (Heidelberg, Germany), PEQLAB Biotechnologie GmbH (Erlangen, Germany), Serva GmbH (Heidelberg, Germany), Sigma-Aldrich, Inc. (Taufkirchen, Germany), Thermo Fisher Scientific GmbH (Vienna, Austria) and QIAGEN (Hilden, Germany).

Plastic materials were purchased from BD Falcon (Schwechat, Austria), Eppendorf AG (Hamburg, Germany), Greiner Bio-One GmbH (Solingen-Wald, Germany), Sarstedt AG + Co (Wiener Neudorf, Austria), Scherf Praezision Europa GmbH (Meiningen, Germany), Sterilin Limited (Cambridge, Great Britain) and VWR International GmbH (Vienna, Austria).

Distilled water was produced with a FONTANAVAPOR distillery (Büchi Labortechnik AG, Flawil, Switzerland). Ultra-pure RNase free water ($ddH_2O$), which was used for enzymatic reactions like PCR, was purchased from Fresenius Kabi GmbH (Graz, Austria). Instruments and devices used in this study are described in Table 1.

**Table 1:** Technical equipment

| Technical equipment | Manufacturer |
|---|---|
| Agarose-gel system for ethidium bromide stained gels, Bio-Vision CN-3000-WL | Vilber Lourmat (Eberhardzell, Germany) |
| Agarose gel unit, Wide Mini-Sub Cell GT | Bio-Rad Laboratories, Inc. |
| Analytical balance, BP121S | Sartorius AG (Göttingen, Germany) |
| Autoclave, Systec V-150 | Systec GmbH (Wettenberg, Germany) |
| Balance, GP3202 | Sartorius AG |
| Bunsen burner, Teclu-Safety Burner | Juchheim Laborgeräte (Bernkastel Kues, Germany) |
| Centrifuge, 5810R | Eppendorf AG |
| CertoClav, Multicontrol 1020 | CertoClav Sterilizer GmbH (Traun, Austria) |
| Clean Bench, Type EF/SB | Clean Air Techniek (Ja Woerden, Netherlands) |
| Electroporation cuvette, 100 µl liquid capacity, 1mm gap width | Eppendorf AG |
| Electroporator, 2510 | Eppendorf AG |

| | |
|---|---|
| Fiber-optic ultra-micro measuring cell, TrayCell® | Hellma Analytics (Müllheim, Germany) |
| Freezer (-20°C), UMKS 3600 | Allectric (Vienna, Austria) |
| Freezer (-70°C), 958 | Thermo Fisher, Inc. |
| Heating Block, Digital Heatblock | VWR International |
| Ice machine, MF 30 | Scotsman Ice (Milan, Italy) |
| Incubator, BD 115 | BINDER GmbH (Tuttlingen, Germany) |
| Magnetic stirrer, MR Hei-Mix S | Heidolph Instruments (Schwabach, Germany) |
| Microcentrifuge, 5415 R | Eppendorf AG |
| Molecular imager ChemiDoc XRS System | Bio-Rad Laboratories, Inc. |
| Multichannel pipette, Research | Eppendorf AG |
| Multi flask shaker, VKS 75 A control | Edmund Bühler GmbH (Hechingen, Germany) |
| pH-Meter, Metrohm 632 | Metrohm AG (Herisau, Switzerland) |
| Pipettes, adjustable | Gilson, Inc. (Middleton, USA)<br>Eppendorf, AG |
| Pipettor, Accu-jet | BRAND (Wertheim, Germany) |
| Power supply, PowerPac Basic | Bio-Rad Laboratories, Inc. |
| PowerLyzer®24 | MO BIO Laboratories, Inc. |
| Refrigerated table centrifuge, 5810R | Eppendorf AG |
| Refrigerated table microcentrifuge, 5415R | Eppendorf AG |
| Rocker (4°C), Promax 2020 | Heidolph Instruments |
| Rocker (RT), Stuart Gyro-Rocker SSL3 | Bibby Scientific |
| SDS PAGE electrophoresis unit, Mini-PROTEAN Tetra cell | Bio-Rad Laboratories, Inc. |
| Shaker Multitron Standard Infors HT | Infors AG (Bottmingen, Switzerland) |
| Spectral photometer, Life Science UV/Vis - Spectrophotometer DU 730 | Beckman Coulter, Inc. |
| Table microcentrifuge, 5415D | Eppendorf AG |
| Thermal cycler, C1000 | Bio-Rad Laboratories, Inc. |
| Thermo mixer, ThermoStat plus | Eppendorf AG |
| Touch mixer, Vortex-Genie 1 Touch Mixer | Scientific Industries, Inc. (New York, USA) |
| Transfer tank electrophoresis unit, TE22 | Hoefer, Inc. (Holliston, USA) |
| UV chamber, GS Gene Linker | Bio-Rad Laboratories, Inc. |
| UV transilluminator, TFX-20M | Vilber-Lourmat |
| Water bath, 1003 | GFL (Burgwedel, Germany) |

## 1.2 Bacterial strains, plasmids and oligonucleotides

The strains (Table 2), plasmids (Table 3) and oligonucleotides (Table 4) used in this study are listed and described below. For construction of gene fusion mutants, the vector pGP704phoA was used and maintained in *E. coli* DH5αλpir. Transformation of plasmids in *E. coli* and *V. cholerae* was done by electroporation. For maintenance and enrichment of expression and cloning vectors *E. coli* DH5αλpir was used. *E. coli* SM10λpir was used to introduce plasmids into *V. cholerae* by conjugation. The spontaneous streptomycin resistant mutant of *V. cholerae* O1 Inaba El Tor clinical isolate, SP27459 was used as a wild type (WT) strain in all experiments [Pearson et al., 1993]. All *E. coli* strains were grown in lysogeny broth (LB) at 37°C and 180 rpm. Unless indicated otherwise, *V. cholerae* strains were either grown in LB or in M9 minimal medium supplemented with 0.2% maltose as a carbon source at 37°C and 180 rpm. All strains were sustained in LB medium containing 20% glycerol at -70°C.

**Table 2:** Bacterial strains

| Strain | Relevant characteristics | Reference |
|---|---|---|
| ***E. coli strains*** | | |
| DH5αλpir | F$^-$ Φ80*dlacZ*Δ*M15*Δ(*argF lac*)*U169 deoR recA1 endA1 hsdR17* ($r_K^- m_K^+$) *supE44 thi-1 gyrA69 relA1 λ recA*::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] |
| XL1-Blue | F`::Tn10 *proA*$^+$*B*$^+$ *lac*$^q$ Δ(*lacZ*)M151 *recA1 endA1 gyrA46* (Nal$^r$) *thi hsdR17* ($r_K^- m_K^+$) *supE44 relA1 lac* | NEB |
| ***V. cholerae strains*** | | |
| SP27459 | O1 Inaba, El Tor, clinical isolate, Bangladesh 1976, spontaneous sm$^r$ | [Pearson et al., 1993] |
| SP27459 Δ*toxR* | Deletion in *toxR*, sm$^r$ | [Fengler et al., 2012] |
| SP27459 Δ*toxRS* | Deletion in *toxR* and *toxS*, sm$^r$ | [Fengler et al. 2012] |
| SP27459 Δ*toxR*::FLAG*toxR*$^{C236SC293S}$ | *toxR* replaced by FLAG*toxR*$^{C236SC293S}$, sm$^r$ | [Fengler et al. 2012] |
| SP27459 Δ*dsbA*::*km* Δ*toxR*::FLAG*toxR* | *dsbA* replaced by *kan* cassette, *toxR* replaced by FLAG*toxR*, sm$^r$, km$^r$ | [Fengler et al. 2012] |
| SP27459 Δ*degS*::*cm* Δ*toxR*::FLAG*toxR*$^{C236SC293S}$ | *degS* replaced by *cat* cassette, *toxR* replaced by FLAG*toxR*$^{C236SC293S}$, sm$^r$, cm$^r$ | Tutz, S. |
| SP27459 Δ*toxRS* Δ*degS*::*cm* | Deletion in *toxR* and *toxS*, *degS* replaced by *cat* cassette, sm$^r$, cm$^r$ | Tutz, S. |
| SP27459 pGP704dsbAphoA | Chromosomal *phoA* fusion to *dsbA*, sm$^r$, ap$^r$ | Tutz, S. |

| | | |
|---|---|---|
| SP27459 pGP704degSphoA | Chromosomal *phoA* fusion to *degS*, $sm^r$, $ap^r$ | This study |
| SP27459 pGPphoAompU | Chromosomal *phoA* fusion to *ompU*, $sm^r$, $ap^r$ | - |
| SP27459 *celAB*::phoArecpGP704 | *chsR* replaced by phoArecpGP704, $sm^r$ | [Berg et al., 2007] |

**Table 3:** Plasmids

| **Plasmid** | **Description** | **Reference** |
|---|---|---|
| pFLAG-MAC™ | Expression vector with N-terminal FLAG-Tag, IPTG inducible, $ap^r$ | Sigma-Aldrich |
| pFLAGtoxRS | *toxR* and *toxS* of SP27459 in pFLAG-MAC™, $ap^r$ | [Fengler et al., 2012] |
| pFLAGtoxR$^{C236SC293S}$_toxS | *toxR*$^{C236SC293S}$ point mutant and *toxS* of SP27459 in pFLAG- MAC™, $ap^r$ | [Fengler et al., 2012] |
| pFLAGtoxRS$^{L33S}$ | *toxR* and *toxS* L33S point mutant of SP27459 in pFLAG-MAC™, $ap^r$ | This study |
| pGP704degSphoA | *degS* of SP27459 in pGP704phoA | This study |

**Table 4:** Oligonucleotide primers

| **Oligonucleotide** | **Sequence (5' – 3')** [a, b] |
|---|---|
| ToxR_N-terminaler-FLAG_*Kpn*I_fw | ATTGGTACCCATGTTCGGATTAGGACACAACTCA |
| *Bgl*II_toxRS_3'_FLAG | TTAAGATCTTTAAGAATTACTGAACAGTACGGT |
| FORWARD TOXS/L | GGGAGTGACTTCAAGCTTGAGCAAGTGT**C**AACCTCTCGAGAA |
| REVERSE TOXS/L | CATTTTGGACTGCCATTCTCGAGAGGTT**G**ACACTTGCTCAAG |
| degS_fw_*Sac*I | TTGAGCTCACAACCACGCAACATCGG |
| degS_rv_*Kpn*I | TTGGTACCGAATCACACTCGTCTAATC |

[a] Restriction sites are underlined.
[b] Bold letters indicate nucleotides changed to obtain amino acid substitutions.

## 1.3 Growth media and buffers

### Media

| | |
|---|---|
| LB medium | 10 g/l tryptone<br>5 g/l yeast extract<br>10 g/l NaCl |
| LB agar | 10 g/l tryptone<br>5 g/l yeast extract<br>10 g/l NaCl<br>16 g/l Agar |
| M9 minimal medium | 6 g/l $Na_2PO_4 \cdot H_2O$<br>3 g/l $KH_2PO_4$<br>0.5 g/l NaCl<br>1 g/l $NH_4Cl$<br>0.1 mM $CaCl_2$<br>2 mM $MgSO_4$<br>0.2 % maltose |

If required, following sterile filtered supplements were added to media after autoclaving:

| | |
|---|---|
| Ampicillin | 100 mg/ml in $ddH_2O$ (stock solution; storage -20°C)<br>End concentration: 100 µg/ml or 50 µg/ml in combination with other antibiotics |
| Chloramphenicol | 30 mg/ml in 96% EtOH (stock solution; storage 4°C)<br>End concentration: 2 µg/ml for *V. cholerae* |
| Kanamycin | 50 mg/ml in $ddH_2O$ (stock solution; storage 4°C)<br>End concentration: 50 µg/ml |
| Streptomycin | 100 mg/ml in $ddH_2O$ (stock solution; storage 4°C)<br>End concentration: 100 µg/ml |
| Maltose | 20% in $ddH_2O$ (stock solution; storage RT)<br>End concentration: 0.2% |
| IPTG<br>(Isopropyl-β-galactopyranosid) | 1 M in $ddH_2O$ (stock solution; storage -20°C)<br>end concentration: 0.05 mM |

| | |
|---|---|
| DTT<br>(1,4-Dithiothreitol) | 1 M in sterile $ddH_2O$ (stock solution; no storage possible, always freshly prepared)<br>end concentration: 3 mM |
| Bile<br>(Sodium deoxycholate) | 0.8% or 8% in sterile $ddH_2O$ (stock solution; no storage possible always freshly prepared)<br>end concentration: 0.01% - 0.1% |
| Polymyxin B | 20 mg/ml in sterile $ddH_2O$ (stock solution; no storage possible, always freshly prepared)<br>end concentration: 2 µg/ml |

## Buffers

**DNA agarose gel electrophoresis**

| | |
|---|---|
| 50x TAE buffer | 242 g/l Tris<br>5.71% glacial acetic acid<br>0.05 mM EDTA (pH 8) |
| 6x loading dye | 10 mM Tris/HCl (pH 7.6)<br>60 mM EDTA (pH 8)<br>60% glycerol<br>0.03% xylene cyanol FF<br>0.03% bromphenol blue |

**Plasmid DNA preparation**

| | |
|---|---|
| S1 buffer | 50 mM Tris/HCl (pH 8)<br>10 mM EDTA (pH 8)<br>100 µg/ml RNase A |
| S2 buffer | 200 mM NaOH<br>1% SDS |
| S3 buffer | 2.8 M KAc (pH 5.1) |

**SDS-PAGE**

| | |
|---|---|
| Stacking gel (stock solution) | 0.5 M Tris/HCl (pH 6.8)<br>0.4% SDS |

| | |
|---|---|
| Separating gel (stock solution) | 1.5 M Tris/HCl (pH 8.8) |
| | 0.4% SDS |
| Stacking gel (4%) | 3.75 ml stacking gel (stock solution) |
| for 6 gels | 1.95 ml acrylamide (30%) – bisacrylamide (0.8%) |
| | 9.21 ml $ddH_2O$ |
| | 75 µl 10% APS |
| | 45 µl 99% TEMED |
| Separating gel (15%) | 7.5 ml separating gel (stock solution) |
| for 6 gels | 15 ml acrylamide (30%) – bisacrylamide (0.8%) |
| | 7.35 ml $ddH_2O$ |
| | 158 µl 10% APS |
| | 45 µl 99% TEMED |
| 5x SDS-PAGE running buffer | 15.1 g/l Tris |
| | 94 g/l glycine |
| | 0.5% SDS |
| | pH 8.3 |
| 5x Laemmli buffer | 55 g/l SDS |
| | 20.5 g/l EDTA |
| | 8.5 g/l $Na_2H_2PO_4 \cdot 2H_2O$ |
| | pH 7.2 |
| | 25% glycerol |
| | 0.1% bromphenol blue |
| | 55 ml/l β-mercaptoethanol (optional) |
| Staining solution after Kang | 5% aluminiumsulfat-hexadecahydrat |
| | 10% EtOH |
| | 0.02% coomassie blue CBB G-250 |
| | 2% ortho-phosphoric acid |

**Immunoblot analysis**

| | |
|---|---|
| CAPS buffer | 10 mM CAPS |
| | 10% MeOH |
| | pH 11 |

| | |
|---|---|
| TBS | 20 mM Tris/HCl (pH 7.5)<br>150 mM NaCl |
| TBS tween triton | 20 mM Tris/HCl (pH 7.5)<br>250 mM NaCl<br>0.05% tween 20<br>0.2% triton X-100 |
| Blocking buffer | 10% skim milk<br>1x TBS |

**Phosphatase assay**

| | |
|---|---|
| P1 buffer | 10 mM Tris/HCl (pH 8)<br>150 mM NaCl |
| P2 buffer | 10 mM Tris/HCl (pH 8)<br>0.1 mM $ZnCl_2$ |
| p-Nitrophenylphosphate | 0.4% in $ddH_2O$ |
| Stop solution | 2.5 M $K_2HPO_4$ |

# 2 Methods

## 2.1 Preparation of electrocompetent cells and transformation

Electrocompetent cells were prepared based on the protocol of Calvin and Hanawalt [Calvin and Hanawalt, 1988]. For an overnight culture (ONC) the required bacteria were grown in 10 ml LB medium with the optional antibiotics at 37°C and 180 rpm. Using the ONC, 0.2 up to 0.8 l LB medium were inoculated to an $OD_{600}$ of 0.1. Cells were grown until an $OD_{600}$ of 0.8 at 37°C and 180 rpm. Subsequently, the culture was cooled down on ice for 30 min. The cells were harvested (15 min, 4000 rpm, 4°C) and washed three times: once using the whole culture volume, once using half of it and once the quarter of the volume was used. For *E. coli*, ice cold and sterile $ddH_2O$ was used, whereas *V. cholerae* was washed and resuspended in sterile 2 mM $CaCl_2$. Finally, the bacteria were washed with glycerol (15%) for *E. coli* or glycerol (15%) with $CaCl_2$ (2 mM) for *V. cholerae* using 0.2 times of the culture volume. After the washing steps the supernatant was discarded carefully. The rest of the liquid was used to resuspend the pellet. Aliquots of 100 – 120 µl were stored at -70°C or directly utilized for transformation.

Aliquoted suspensions were defrosted on ice before transformation. Plasmid DNA (2-8 µl) was mixed with competent cells, incubated for 10 min on ice and transferred to a precooled electroporation cuvette. *E. coli* was electroporated with 2.5 kV and *V. cholerae* with 1.8 kV. Right after, the cells were absorbed in 1 ml preheated (37°C) LB medium to recover at 37°C for 15 min without shaking and at 37°C, 180 rpm for 1 h. In the next step, aliquots of 10 µl, 100 µl and the rest were plated on LB plates in presence of selective antibiotics and incubated at 37°C overnight. The grown colonies were patched on plates with selective antibiotics and incubated at 37°C overnight. On the next day, positive clones were tested by colony PCR.

## 2.2 Recombinant DNA techniques and construction of expression and cloning vectors

Purification of DNA fragments from PCR samples or from agarose gels and plasmid DNA preparations were performed using QIAquick® PCR purification, QIAquick® gel extraction and QIAprep® Spin Miniprep Kits (QIAgen, Germany) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed in the Thermal cycler C1000 (Bio-Rad) using Q5® high fidelity DNA polymerase for subcloning and sequencing and *Taq* polymerase for verification of received constructs by colony PCR. PCR components (Table 5) and PCR protocols are listed and described below. All polymerases, restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs (Frankfurt, Germany). Oligonucleotide primers used in this study are listed in Table 4 and were purchased from Integrated DNA Technologies (IDT®, Iowa). DNA sequencing was performed by dideoxynucleotide chain termination method by Sanger [Sanger et al., 1977] with an automated DNA sequencer, according to LGC Genomics (Berlin, Germany).

**Table 5:** PCR components

| **PCR using Q5®DNA polymerase** | **50 µl** | **PCR using *Taq* DNA polymerase** | **25 µl** |
|---|---|---|---|
| Q5 Buffer (5x) | 10 µl | Thermo Pol Buffer (10x) | 2.5 µl |
| Forward primer [1 µg/ml] | 2.5 µl | Forward primer [1 µg/ml] | 1 µl |
| Reverse primer [1 µg/ml] | 2.5 µl | Reverse primer [1 µg/ml] | 1 µl |
| dNTPs [10 mM] | 1 µl | dNTPs [10 mM] | 1 µl |
| Q5® DNA polymerase [2 U/µl] | 0.5 µl | *Taq* DNA polymerase [5 U/µl] | 0.2 µl |
| Template DNA | x µl | Template DNA | x µl |
| $ddH_2O$ | x µl | $ddH_2O$ | x µl |

Denaturation temperature of PCR was 98°C for Q5®polymerase and 94°C for *Taq* polymerase. The annealing temperature was calculated with the sequence of the oligonucleotide primers and the following formula: $T_m$ °C = 2 · (A + T) + 4 · (G +C) -5. Unless the melting temperatures between the forward and reverse primer deviated, the lower temperature was used. Elongation was carried out at 72°C between 30 sec and 1 min or longer, depending on the length of the generated fragment and polymerase. The overall amount of cycles for each PCR was 30.
To verify correct cloning, colony PCR was performed. One colony was resuspended in 20 µl SDS (0.2%) and boiled at 100°C for 10 min. In the next step the suspension was centrifuged for 1 min. The supernatant was diluted in dd$H_2O$ 1:5. 2 µl of this solution was applied as a template in the PCR using the *Taq* polymerase program.

The L33S mutation in *toxS* was generated by SOE PCR (splicing by overlap extension PCR) [Ho et al., 1989]. The first part of SOE PCR was the generation of two overlapping fragments, using pFLAGtoxRS as a template together with oligonucleotide pairs ToxR_N-terminaler-FLAG_*Kpn*I_fw and REVERSE TOXS/L as well as FORWARD TOXS/L and *Bgl*II_toxRS_3'_FLAG, respectively. The oligonucleotide primers FORWARD TOXS/L and REVERSE TOXS/L carried the overlap sequence and the point mutation, that resulted in an amino acid substitution at position 33: a leucine was exchanged by a serine residue. The two overlapping PCR products were used as templates in the second PCR. The oligonucleotide primer pair ToxR_N-terminaler-FLAG_*Kpn*I_fw and *Bgl*II_toxRS_3'_FLAG were added to the reaction tubes after 10 cycles. The resulting PCR fragment was digested with the respective restriction endonucleases and ligated into a similarly digested pFLAG-MAC™ for directional cloning. The resulting derivates were transformed into *E. coli* DH5αλpir followed by the transformation into *V. cholerae* Δ*toxRS*. The point mutation was confirmed by sequencing (data not shown).

Chromosomal *phoA* fusions to *degS* were constructed by using PCR, conjugation and homologous recombination. The oligonucleotide primers were chosen in a way, that a 600 bp fragment was generated by PCR which is equivalent to *degS*, additionally encoded the stop codon of *degS* and it was ensured that *phoA* was inserted downstream of *degS*. The fragments were generated by PCR using chromosomal DNA of *V. cholerae* SP27459 and the oligonucleotide primer pair *degS*_fw_*Sac*I and *degS*_rv_*Kpn*I. The resulting PCR product was digested with *Sac*I and *Kpn*I and ligated into pGP704phoA that had been digested similarly to enable directional cloning. The ligation product was transformed into *E. coli* DH5αλpir, followed by the transformation into *E. coli* SM10λpir and was further transferred into *V. cholerae* SP27459 by conjugation. *V. cholerae* integrated the plasmid into its chromosome via homologous recombination because of the presence of selective antibiotics. This event resulted in a mutant strain having a *degSphoA* fusion in the *degS* locus. The correct integration was confirmed by colony PCR (data not shown).

## 2.3 Preparation of whole cell extracts

To prepare whole cell extracts (WCE) of bacterial strains, cultures were grown in LB medium under selective conditions at 37°C and 180 rpm overnight. Suspension of the ONC was used to inoculate the main culture, LB or M9 minimal medium, to an $OD_{600}$ of 0.1. Before inoculating M9 minimal medium, cells were washed with M9 minimal medium (5 min, 5000 rpm, RT). Cultures with or without additional agents like bile, DTT or YDF were incubated at 37°C and 180 rpm up to an $OD_{600}$ of 0.4 to 0.6. For strains carrying the vector pFLAG-MAC™, gene expression was induced with 0.05 mM IPTG for 1 h at the late-log phase. Afterwards, equal amounts of cells (1 ml of $OD_{600}$ 1.5) were harvested by centrifugation (5 min, 5000 rpm, RT). Cell pellets were resuspended in 60 µl 5x Laemmli sample buffer [Laemmli, 1970], boiled at 100°C for 30 min and stored at -20°C. Before separation in an SDS-PAGE, samples were additionally boiled at 100°C for 10 min.

## 2.4 SDS-PAGE

Protein samples were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in 15% mini-PROTEAN acrylamide gels. Electrophoresis was performed in 1x SDS running buffer at RT using 80 V or 13 mA per gel. For detection of proteins 2 – 10 µl of whole cell extract solutions were loaded. Gels were washed twice in $dH_2O$ for 10 min and stained with Coomassie brilliant blue overnight [Kang et al., 2002] or transferred to a nitrocellulose membrane using a tank blotting unit. Protein sizes were specified by comparing the migration of the protein bands to defined bands of a protein marker (Prestained Protein Marker, Broad Range, 7 – 175 kDa, New England Biolabs). Pictures were taken with the ChemiDoc™ XRS System.

## 2.5 Immunoblot analysis

Immunoblot analysis was performed according to Schild et al. [Schild, et al., 2008] using a tank-blotting unit. After SDS-PAGE the proteins were transferred to a Hybond™-C nitrocellulose membrane (Amersham Biosciences, Freiburg) in CAPS buffer at RT using 220 mA for 90 min. Afterwards the membrane was air dried, washed twice in Tris buffered saline (TBS) and blocked by incubation with 10% skim milk in TBS at 4°C by gentle shaking. After blocking, the membrane was washed again as described above and then it was incubated with the primary antibody (Table 6) at RT for 2 h. The exposure with the primary antibody was followed by two washing steps in TBS-TT and one in TBS for 10 min each. Then, the respective secondary antibody (Table 6), conjugated to horseradish peroxidase, was added to the membrane at RT for 1h. The nitrocellulose membrane was washed four times in TBS-TT and once in TBS for 10 min, respectively. Chemiluminescence was detected by using the substrate Immune-Star™ WesternC™ Kit (Bio-Rad) and visualized by the Molecular Imager

ChemiDoc™ XRS System. In parallel, the same amount of protein samples was loaded and separated by SDS-PAGE to ensure equal loading on the blotted gels.

**Table 6:** Primary and secondary antibodies

| Antibody | Dilution | Reference |
|---|---|---|
| **Primary antibody** | | |
| Mouse monoclonal anti-FLAG M2 antibody | 1:2000 in 10% skim milk TBS | Sigma-Aldrich |
| Mouse polyclonal anti-OmpU antibody | 1:500 in 10% skim milk TBS | Leitner, D. |
| Rabbit polyclonal α-ToxR antibody | 1:1000 in 10% skim milk TBS supplemented with 0.2% sodium azide | Zhu, J. |
| **Secondary antibody** | | |
| Horseradish peroxidase conjugated goat anti mouse antibody | 1:7500 in 10% skim milk TBS | Dianova GmbH, Hamburg |
| Horseradish peroxidase conjugated goat anti rabbit antibody | 1:10000 in 10% skim milk TBS | Dianova GmbH, Hamburg |

## 2.6 Preparation of pre-absorbed α-ToxR-antibodies

For preparation of pre-absorbed α-ToxR antibodies, proteins in whole cell extracts of SP27459 Δ*toxR* were separated by SDS-PAGE and blotted on a nitrocellulose membrane. The membrane was air dried and washed twice in TBS for 5 min each. In the next step, it was blocked by incubation with 10% skim milk TBS at RT for 2 h by gentle shaking. The nitrocellulose membrane was washed again as described above and exposed to α-ToxR antibody (1:1000 dilution in 10% skim milk TBS) at 4°C overnight. The pre-absorbed α-ToxR antibody was collected and 0.2% sodium azide was added. Non-specific antibodies were clung to the nitrocellulose membrane. The α-ToxR antibody was stored at 4°C.

## 2.7 Protein stability assay (chloramphenicol experiment)

Chloramphenicol experiments were performed to determine the protein stability. 40 ml of M9 minimal medium 0.2% maltose, if required with selective antibiotics, were inoculated to a start $OD_{600}$ of 0.1 with a *V. cholerae* ONC. Cultures without or with an additional compound like bile (0.1% sodium deoxycholate, Sigma Aldrich) were incubated at 37°C and 180 rpm until they reached an $OD_{600}$ of 0.4 to 0.6. As they reached the exponential phase, the expression of genes, cloned into the vector pFLAG-MAC™, was induced by adding 0.05 mM IPTG for 1 h at 37°C and 180 rpm. Additionally, the

reducing substance DTT (3 mM, Roth) was also added at this point of growth. For testing agents, like the tripeptide YDF (10µg/ml, Centic Biotec), the substance was added to the culture after 1 h expression of ToxRS from the plasmid. Subsequently, 5 ml of culture were transferred to a pre-heated culture flask and incubated at 37°C and 180 rpm for the remaining time period (210 min) of the assay. This aliquot served as a –cm control. Chloramphenicol (100 µg/ml) was added to the rest of the culture. Whole cell extracts were prepared from the with chloramphenicol supplemented culture at defined time points. Samples were separated by SDS-PAGE in mini-PROTEAN acrylamide gels (15%) for further immunoblot analysis with antibodies against FLAG-Tags.

## 2.8 Phosphatase assay

To determine the activity of promoters and the transcriptional levels of genes like *degS*, *dsbA* or *ompU*, the phosphatase activities in strains carrying respective *phoA* fusions were detected [Manoil, 1991]. Strains were either cultivated in LB or M9 minimal medium 0.2% maltose with selective antibiotics. Main cultures were inoculated with suspension of an ONC (LB medium with selective antibiotics) to an $OD_{600}$ of 0.1 and incubated at 37°C and 180 rpm until they reached an $OD_{600}$ of 0.4 to 0.6. At the exponential phase, the cultures were supplemented without or with additional agents, like bile, YDF, DTT or polymyxin B and incubated at 37°C and 180 rpm for a certain period of time. 2 ml of the cultures were harvested by centrifugation (5000 rpm, 5 min, RT). The cell pellet was washed and resuspended with 1 ml P1 buffer (5000 rpm, 5 min, RT). 100 µl of this suspension were used to measure the $OD_{600}$ and another 100 µl were added to 900 µl P2 buffer. 25 µl chloroform and SDS (0.1%) were added, mixed and incubated at RT for 5 min. The enzymatic reaction was started by adding 100 µl of *p*-nitrophenylphosphate (*p*-NPP 0.4%). After the suspension turned yellow, but no longer than 30 min, the enzymatic reaction was stopped with 125 µl $K_2HPO_4$ (2.5 M). The negative control of the assay contained all chemicals except culture. After centrifugation (20000 rpm, 5 min, RT) 600 µl were used to determine the $OD_{405}$. The phosphatase activity was calculated using the formula according to Miller:

$$\text{Specific Activity} \quad \left[\frac{\mu mol}{min \cdot l}\right] = \frac{\Delta E_{405} \cdot V_t}{t \cdot \varepsilon \cdot V_b \cdot d \cdot OD_{600}}$$

$\Delta E_{405}$ = extinction at 405 nm

$V_t$ = final volume of the suspension

$OD_{600}$ = extinction at 600 nm (amount of bacteria)

ε = extinction coefficient of p-nitrophenol (9600 l/mol · cm)

$V_b$ = sample volume

d = coat thickness of the cuvette

t = reaction time (min)

# III Results

## 1 ToxS$^{L33S}$ and its impact on protein stability of ToxR

The capability of bacterial pathogens to regulate the expression of virulence genes is necessary for their survival when they switch between different environments. Therefore, it is also decisive to appropriately modify the availability of virulence regulators, such as ToxR by controlled proteolysis, to ensure not to waste energy. ToxS is thought to enhance dimerization of ToxR, facilitate its transcriptional activation or have a stabilizing effect on ToxR [Miller et al., 1989; DiRita and Mekalanos, 1991; Pfau and Taylor, 1998]. Nevertheless, the actual function of ToxS still remains unclear.

By introducing a mutation into the periplasmic domain of ToxS, the overall amounts of *V. cholerae* full length ToxR expressed in *S. typhimurium* are extremely reduced [Pfau and Taylor, 1998]. Due to the possible alteration of ToxR by this ToxS variant, ToxS$^{L33S}$, the working group led by Taylor, R. K. identified four putative site-1 proteases: DegS, DegP, VesC and TapA in a classical strain, which cleave the periplasmic domain of ToxR [Almagro-Moreno et al., 2015b]. To understand the dynamics of ToxR degradation in a *toxS$^{L33S}$* background and to illustrate the potential of this mutation in El Tor strains, the leucine at position 33 was replaced by a serine as described in chapter II.2.2.

### 1.1 ToxS$^{L33S}$ induces proteolysis of ToxR in a DegS dependent manner

To determine, whether the exchange of the leucine at position 33 to a serine residue in ToxS also results in a destabilization of ToxR in El Tor strains, protein stability assays using chloramphenicol were performed. Chloramphenicol belongs to the bacteriostatic antibiotics, which directly interacts with the compound of the 50S ribosomal subunit, that has peptidyl transferase activity. By that, it prevents protein chain elongation, because the peptide bond formation is blocked [Jardetzky, 1963; Wolfe and Hahn, 1965].

Figure 4 presents the protein stability assays of plasmid encoded FLAGToxRS$^{L33S}$ in a Δ*toxRS* (Figure 4, panel A) or Δ*degS*::*cm* Δ*toxRS* (Figure 4, panel B) background. *toxR* was cloned into pFLAG-MAC™ together with *toxS$^{L33S}$* under control of a *lac* operator and a *tac* promoter to guarantee high expression levels. Strains were cultivated in M9 minimal medium with 0.2% maltose to prevent metabolic repression, which can occur while induction of the *tac* promoter of pFLAG-MAC™, if glucose is present. Cultures were incubated until they reached an $OD_{600}$ of 0.4 to 0.6. During exponential phase, gene expression was induced with 0.05 mM IPTG for 1 h, followed by the supplement of 100 µg/ml chloramphenicol (cm) to inhibit protein biosynthesis. Samples were taken at defined time points, WCE were prepared and separated by SDS-PAGE (15%) followed by an immunoblot analysis with monoclonal anti-FLAG M2 serum.

Additionally, bacterial growth should have been inhibited by the addition of chloramphenicol, due to the fact that it is a bacteriostatic agent. However, it was observed that the corresponding strains had no growth defect during all performed chloramphenicol experiments (data not shown).

Panel A of Figure 4 displays that FLAGToxR undergoes enhanced proteolytic degradation in the presence of ToxS$^{L33S}$. This increased proteolysis of FLAGToxR becomes visible by the extinction of full length FLAGToxR (35 kDa) and the enrichment of two smaller proteolytic fragments (28 kDa and 32 kDa). This result suggests that ToxS$^{L33S}$ induces actively the proteolysis of FLAGToxR during in which two distinct proteolytic fragments occur, that are the outcome of at least two proteases.
Panel B of Figure 4Figure 4 demonstrates the protein degradation assay of SP27459 Δ*degS*::*cm* Δ*toxRS* pFLAGtoxRS$^{L33S}$. In a *degS* deficient strain FLAGToxR is no longer forced to undergo proteolysis triggered by the presence of ToxS$^{L33S}$ and is more stable compared to conditions under which the site-1 protease is still present. The proteolytic fragments of FLAGToxR are hardly visible, indicating that the serine protease DegS plays a crucial role in the induction of ToxR proteolysis, depending on the presence of ToxS$^{L33S}$.

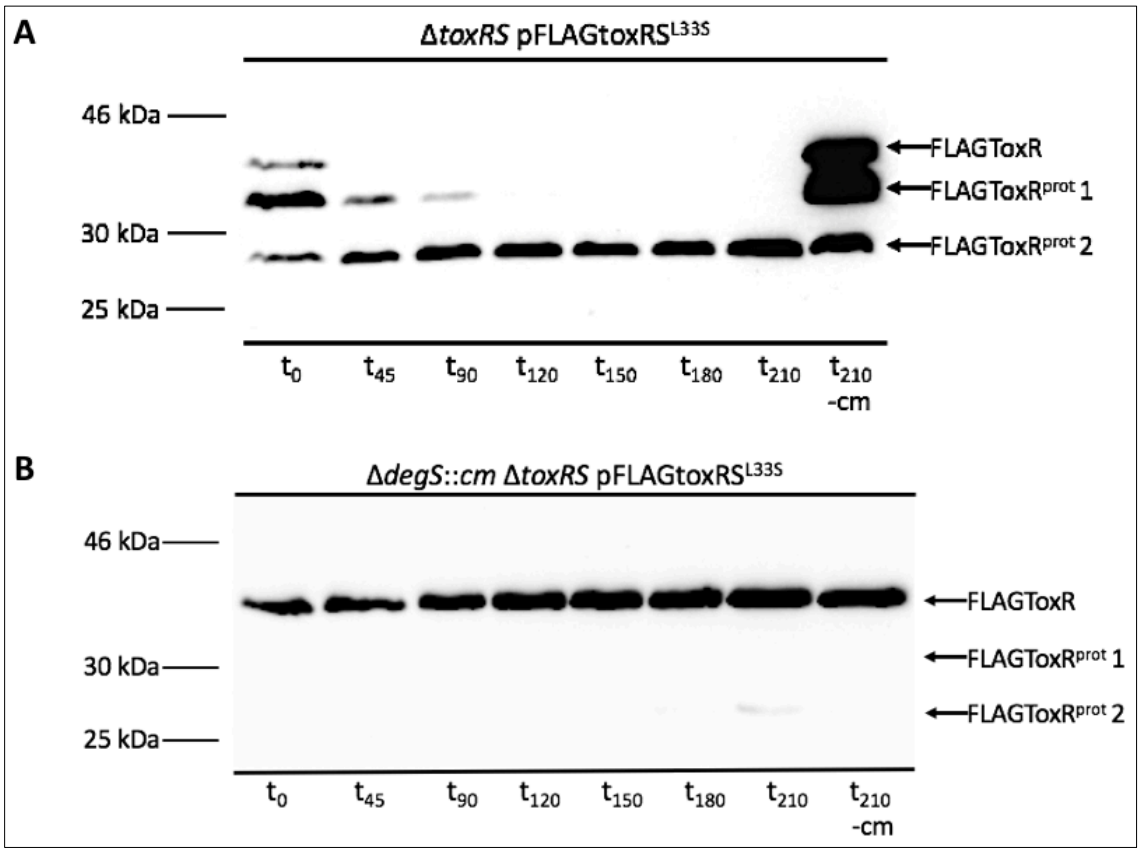


**Figure 4: Protein stability assays of plasmid encoded FLAGToxR coexpressed with ToxS$^{L33S}$ in SP27459 Δ*toxRS* or in SP27459 Δ*degS*::*cm* Δ*toxRS*.** Cells were grown in 40 ml M9 minimal medium 0.2% maltose with adequate antibiotics until they reached an $OD_{600}$ of 0.4 to 0.6. In the exponential phase, gene expression from the plasmid was induced with 0.05 mM IPTG for 1 h. Subsequently, protein biosynthesis was blocked with 100 µg/ml chloramphenicol (cm). Samples were taken at indicated time points (min). Cultures grown without chloramphenicol (-cm) served as a control. Equal protein amounts of WCE were separated by SDS-PAGE (15%) and visualized by immunoblot analysis using anti-FLAG M2 serum.

## 1.2 ToxS$^{L33S}$ prevents ToxR stabilization by bile salts

To be a successful intestinal pathogen, *V. cholerae* must be able to sense the environment for changes of various factors. One challenging factor in the small intestine is bile. Bile acids major function is to digest fats in the intestine by emulsifying and solubilizing lipids. Beside of digestion, bile also has great effects on cell membranes and therefore antimicrobial properties [Begley et al., 2005]. Because of the ability of intercalating into lipid membranes, bile can also have effects on transmembrane proteins, such as ToxRS [Yang et al., 2013]. Thus, it can be possible, that bile salts also have a promoting effect on the expression of virulence genes in *V. cholerae*. For that, ToxR could be stabilized by bile salts. To directly test whether FLAGToxR is a substrate of proteolysis in presence of bile salts and ToxS$^{L33S}$, a protein degradation assay was performed. SP27459 Δ*toxRS* pFLAGtoxRS$^{L33S}$ was grown in M9 minimal medium 0.2% maltose supplemented with 0.1% sodium deoxycholate. Cultures were incubated until they reached an $OD_{600}$ between 0.4 to 0.6. During the exponential phase, gene expression from the plasmid was induced with 0.05 mM IPTG for 1 h, followed by the supplement of 100 µg/ml cm. Samples were taken at defined time points, WCE were prepared, separated by SDS-PAGE (15%) and visualized by immunoblot analysis with monoclonal anti-FLAG M2 serum.

The promoting effect towards FLAGToxR degradation in presence of ToxS$^{L33S}$ and sublethal concentrations of sodium deoxycholate is shown in Figure 5. Full length and the first proteolytic fragment of FLAGToxR are barely detectable at the beginning of the experiment. This indicates, that bile may enhance the proteolysis of FLAGToxR under coexistence of ToxS$^{L33S}$.

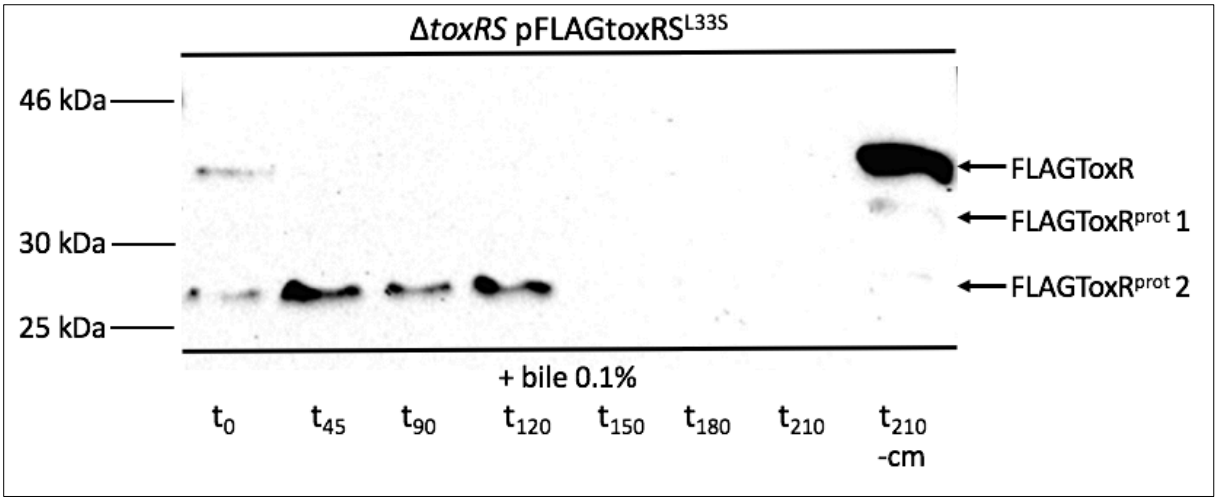


**Figure 5: Protein stability assay of plasmid encoded FLAGToxR coexpressed with ToxS$^{L33S}$ in SP27459 Δ*toxRS* in presence of bile.** Cells were grown in 40 ml M9 minimal medium 0.2% maltose with adequate antibiotics and sodium deoxycholate (0.1%) until they reached an $OD_{600}$ of 0.4 to 0.6. In the exponential phase, gene expression from the plasmid was induced with 0.05 mM IPTG for 1 h. Subsequently, protein biosynthesis was blocked with 100 µg/ml chloramphenicol (cm). Samples were taken at indicated time points (min). Cultures grown without chloramphenicol (-cm) served as a control. Equal protein amounts of WCE were separated by SDS-PAGE (15%) and visualized by immunoblot analysis using anti-FLAG M2 serum.

## 1.3 DTT accelerates ToxR degradation in presence of ToxS$^{L33S}$

It was reported that the two cysteine residues located in the periplasmic domain of ToxR play an important role for ToxR stability in the presence or absence of ToxS. For that, the cysteine residues C236 and C293 were exchanged to serine residues [Fengler et al., 2012]. In presence of ToxS, proteolysis of FLAGToxR$^{C236SC293S}$ (FLAGToxR$^{CC}$) is increased, whereas in absence of ToxS, FLAGToxR$^{CC}$ remains fairly stable [Lembke, 2016].

To create comparable conditions to these observations, sublethal concentrations of 1,4-dithiothreitol (DTT, 3 mM) were added to the cultures to investigate the influence of DTT on FLAGToxR stability in presence of ToxS$^{L33S}$. DTT is a reducing substance, that can change protein functions by reducing disulphide bonds [Cleland, 1964]. SP27459 Δ*toxRS* pFLAGtoxRS$^{L33S}$ was grown in M9 minimal medium 0.2% maltose until the culture reached an $OD_{600}$ of 0.4 to 0.6. During the exponential phase, gene expression from the plasmid was induced with 0.05 mM IPTG for 1 h and DTT was added. Subsequently, 100 µg/ml cm was added. Samples were taken at defined time points, WCE were prepared, separated by SDS-PAGE (15%) and visualized by immunoblot analysis with monoclonal anti-FLAG M2 serum.

The degradation assay in Figure 6 demonstrates that FLAGToxR is more instable in presence of ToxS$^{L33S}$ if the periplasmic cysteine residues appear exclusively in their reduced form than under non reducing conditions (Figure 4, panel A). Full length FLAGToxR is not detectable at all, neither in the –cm control. The first proteolytic fragment of FLAGToxR is hardly visible at time point $t_0$ of the assay and in the –cm control. In contrast to that, the second proteolytic fragment of FLAGToxR accumulates, indicating that the activity of the first protease is highly induced under reducing conditions.

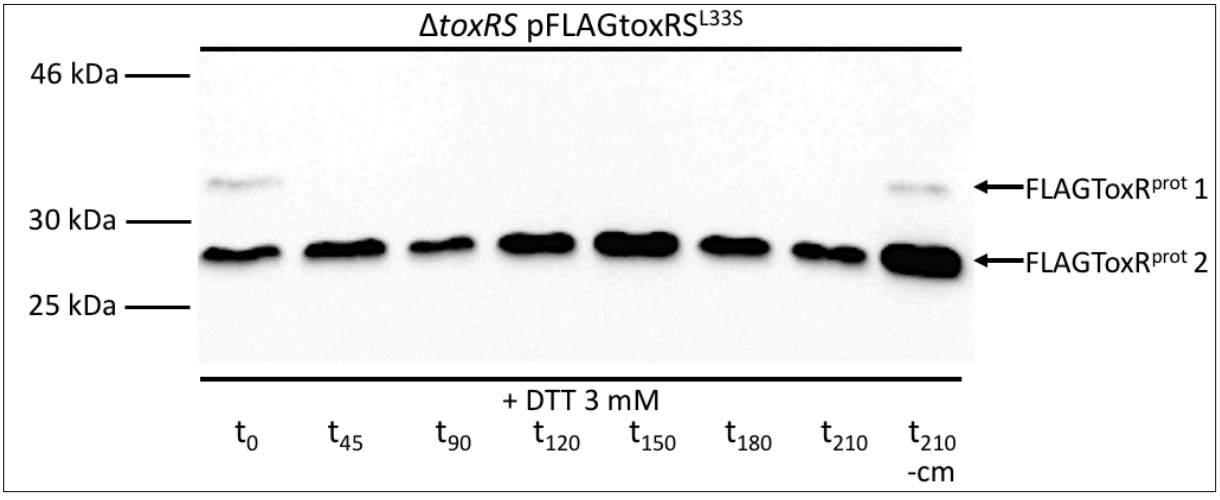


**Figure 6: Protein stability assay of plasmid encoded FLAGToxR coexpressed with ToxS$^{L33S}$ in SP27459 Δ*toxRS* in presence of DTT.** Cells were grown in 40 ml M9 minimal medium 0.2% maltose with adequate antibiotics until they reached an $OD_{600}$ of 0.4 to 0.6. In the exponential phase, gene expression from the plasmid was induced with 0.05 mM IPTG for 1 h and DTT (3 mM) was supplemented. Subsequently, protein biosynthesis was blocked with 100 µg/ml chloramphenicol (cm). Samples were taken at indicated time points (min). Cultures grown without chloramphenicol (-cm) served as a control. Equal protein amounts of WCE were separated by SDS-PAGE (15%) and visualized by immunoblot analysis using anti-FLAG M2 serum.

## 2 The periplasmic cysteine residues of ToxR and their effect on its stability

The cysteine residues in the periplasmic domain of TcpP (C207 and C218) can form intramolecular disulphide bonds. By replacing these cysteine residues and therefore lacking the capability of building up disulphide bonds, it was demonstrated that the redox state of the periplasmic domain of TcpP is decisive for TcpP steadiness and virulence gene expression. TcpH possesses one cysteine (C114) in its periplasmic domain, which may help to build up disulphide bond formation in TcpP by interacting with C207 and C218 of TcpP [Morgan et al., 2016].

As described by Fengler et al. ToxR activity is influenced by the redox state of its periplasmic cysteine residues (C236 and C293) and by the interaction between ToxR and ToxS. A substitution of cysteines to serine residues in ToxR (ToxR$^{CC}$) did not affect the virulence gene expression or disrupt the porin production pattern under virulence inducing conditions. Furthermore, the stability of ToxR$^{CC}$, if highly expressed from pFLAG-MAC™, depends on the presence of ToxS. In Δ*toxS* mutant strains, the protein steadiness of FLAGToxR$^{CC}$ is bigger than in strains encoding for *toxS* [Lembke, 2016].

For these reasons, the hypothesis came up, whether the cysteine residues of ToxR are critical for the protein's resistance of degradation. To verify this hypothesis, protein degradation assay of FLAGToxR$^{C236SC293S}$ (FLAGToxR$^{CC}$) was performed. SP27459 Δ*toxRS* pFLAGtoxR$^{C236SC293S}$_toxS was grown in M9 minimal medium 0.2% maltose without and with sublethal concentrations of sodium deoxycholate (0.1%) until they reached an $OD_{600}$ of 0.4 to 0.6. During the exponential phase, gene expression from the plasmid was induced with 0.05 mM IPTG for 1 h. Subsequently, 100 µg/ml cm was added. Samples were taken at defined time points, WCE were prepared, separated by SDS-PAGE (15%) and visualized by immunoblot analysis with monoclonal anti-FLAG M2 serum.

Panel A of Figure 7 shows that FLAGToxR$^{CC}$ is under increased proteolysis. Full length FLAGToxR$^{CC}$ is not detectable anymore after time point $t_{90}$ of the assay. No proteolytic fragments of FLAGToxR$^{CC}$ are visual, which is due to blotting effects. This indicates that the substitution of the two periplasmic cysteine residues of ToxR causes increased protein degradation.

Impressively, the degradation of FLAGToxR$^{CC}$ is reversed by the addition of 0.1% sodium deoxycholate (Figure 7, panel B) . FLAGToxR$^{CC}$ can be rescued by bile salts.

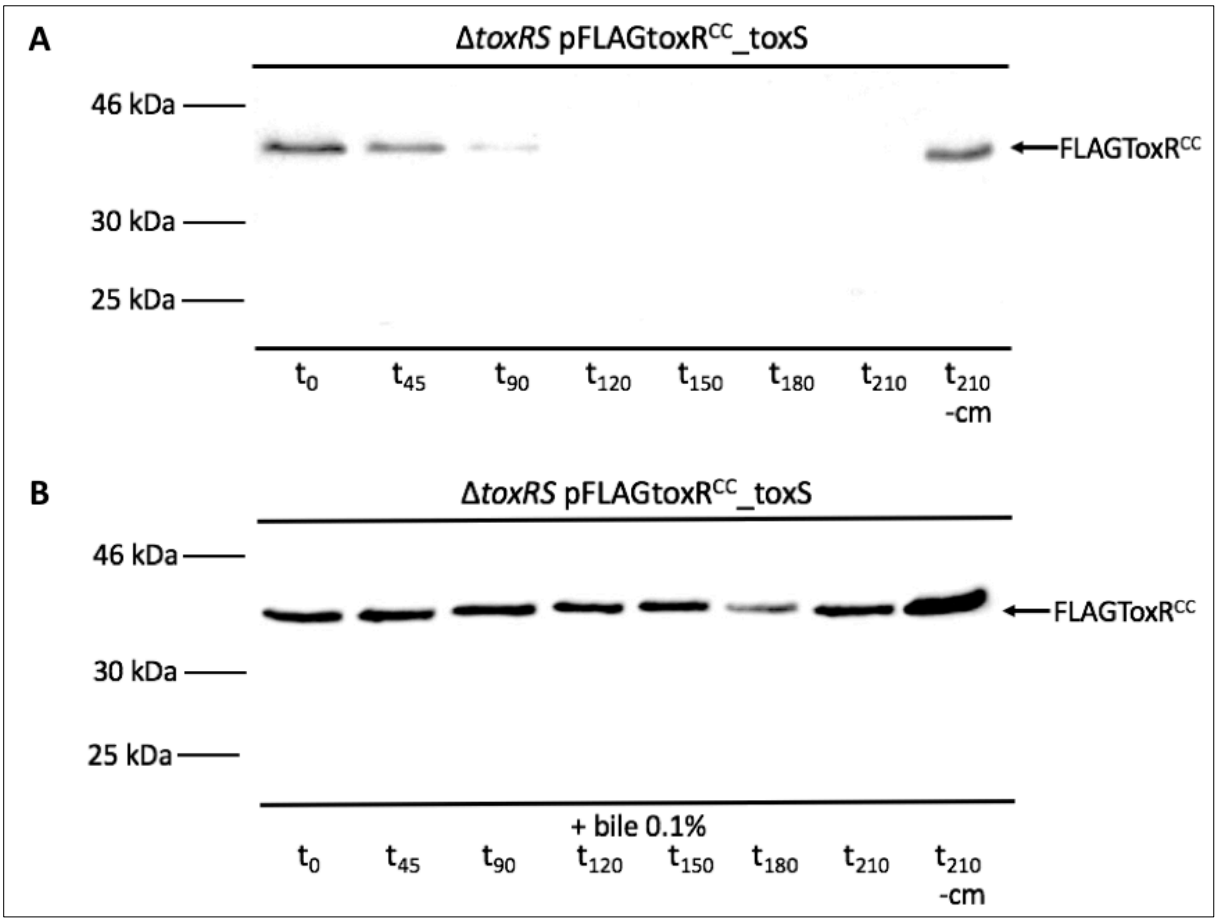


**Figure 7: Protein stability assay of plasmid encoded FLAGToxR$^{C236SC293S}$ (ToxR$^{CC}$) coexpressed with ToxS in SP27459 Δ*toxRS* in absence or presence bile.** Cells were grown in 40 ml M9 minimal medium 0.2% maltose with adequate antibiotics and, if necessary with 0.1% sodium deoxycholate, until they reached an $OD_{600}$ of 0.4 to 0.6. In the exponential phase, gene expression from the plasmid was induced with 0.05 mM IPTG for 1 h. Subsequently, protein biosynthesis was blocked with 100 µg/ml chloramphenicol (cm). Samples were taken at indicated time points (min). Cultures grown without chloramphenicol (-cm) served as a control. Equal protein amounts of WCE were separated by SDS-PAGE (15%) and visualized by immunoblot analysis using anti-FLAG M2 serum.

## 3 Analysis of stability and activation of chromosomally expressed *toxR*

Lately, RseP was identified to be the site-2 protease, which is responsible for ToxR degradation during the late stationary phase. It was postulated that ToxR undergoes regulated intermembrane proteolysis under nutrient limitation and alkaline pH conditions after the cells entered a dormant state. [Almagro-Moreno et al., 2015a]. So far, it is not understood what environmental stimuli *in vivo* cause the signal for preventing the activity and triggering the degradation of ToxR. To further describe the attributes of conditions under which ToxR is resistant to proteolytic decomposition and remains in its active form, long term experiments of chromosomally expressed *toxR* or FLAG*toxR*$^{C236SC293S}$, also in a Δ*dsbA* mutant strain, using media supplemented with bile salts were carried out.

## 3.1 ToxR remains stable under nutrient limitation and alkaline pH

New findings show that ToxR levels in the cells decrease drastically, once the culture obtained nutrient limitation and alkaline pH [Almagro-Moreno et al., 2015a]. Primarily, *V. cholerae* O395 (O1 classical) was used in this study, but there were also experiments described in which *V. cholerae* N16961 (O1 El Tor) was used. Reduced levels of ToxR in the El Tor Biotype were observed in the late stationary phase. It took 72 hours for ToxR to be vanished completely in LB pH 9.3. Based on these results, we questioned whether chromosomally produced ToxR of *V. cholerae* SP27459 is also substrate of proteolysis under nutrient limitation. Additionally, we addressed whether in this case, ToxR proteolysis can be inhibited by bile salts.

The wild type was incubated in M9 minimal medium 0.2% maltose without or with sodium deoxycholate (0.1%) for up to 72 hours. Samples were taken at indicated time points. Equal protein amounts of WCE were separated by SDS-PAGE (15%) and visualized by immunoblot analysis using polyclonal α-ToxR serum.

The influence of bile salts on the stability of chromosomally produced WT ToxR (~32 kDa) in a long term assay is displayed in Figure 8. For the whole time span of the experiment, full length ToxR is detectable. By that, it is not observable whether bile salts have a stabilizing effect on ToxR or not. The unequal intensities are explainable by loading mistakes. Furthermore, the starting pH of M9 minimal medium 0.2% maltose was 7.0. After 24 h of incubation, the pH value of the supernatant of a culture without bile salts was pH 6.5. Thereby, ToxR stability was tested only under starving conditions, but not under alkaline pH. Nevertheless, there is evidence provided for ToxR to be stable for at least 96 h in both, LB and M9 minimal medium with 0.2% maltose incubated aerobically or anaerobically. Regardless of the availability of oxygen or media, ToxR remains stable for 96 hours (data not shown).

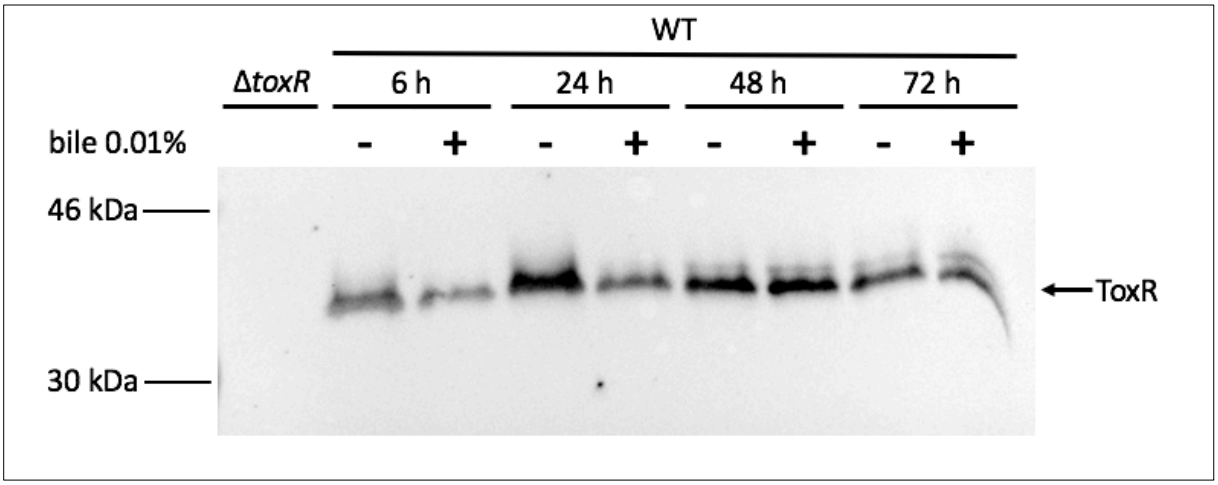


**Figure 8: Immunoblot analysis of SP27459 Δ*toxR* and the WT in a long term assay.** Cells were grown in M9 minimal medium 0.2% maltose with adequate antibiotics and, if necessary with 0.01% sodium deoxycholate. Cultures were incubated for up to 72 hours. Samples were taken at indicated time points (hours). Equal protein amounts of WCE were separated by SDS-PAGE (15%) and visualized by immunoblot analysis using α-ToxR serum.

Based on the fact that the pH of M9 minimal medium 0.2% maltose post inoculation shift from pH 7.0 to pH 6.5, we also tested whether the levels of chromosomally produced ToxR start decreasing at alkaline pH and nutrient limitation in the late stationary phase according to Almagro-Moreno et al., 2015a. To determine this, overnight cultures of the WT grown in LB at 37°C and 180 rpm were pelleted and resuspended in sterile phosphate buffered saline (PBS) at pH 7.4 and pH 9.5 for 48 hours. Samples were taken at indicated time points. Equal protein amounts of WCE were separated by SDS-PAGE (15%) and visualized by immunoblot analysis using polyclonal α-ToxR serum.

As shown in Figure 9 ToxR levels remain stable in response to nutrient limitation and alkaline pH in the late stationary phase. The overall amount of ToxR is lower after 24 hours at both analysed pH values. The findings of Almagro-Moreno et al., 2015a show decreased ToxR levels in PBS pH 9.3 after only 3 hours. In comparison with these results, chromosomally expressed *toxR* by *V. cholerae* O1 Inaba El Tor point towards a resistance of degradation under nutrient limitation and alkaline pH in the late stationary phase.

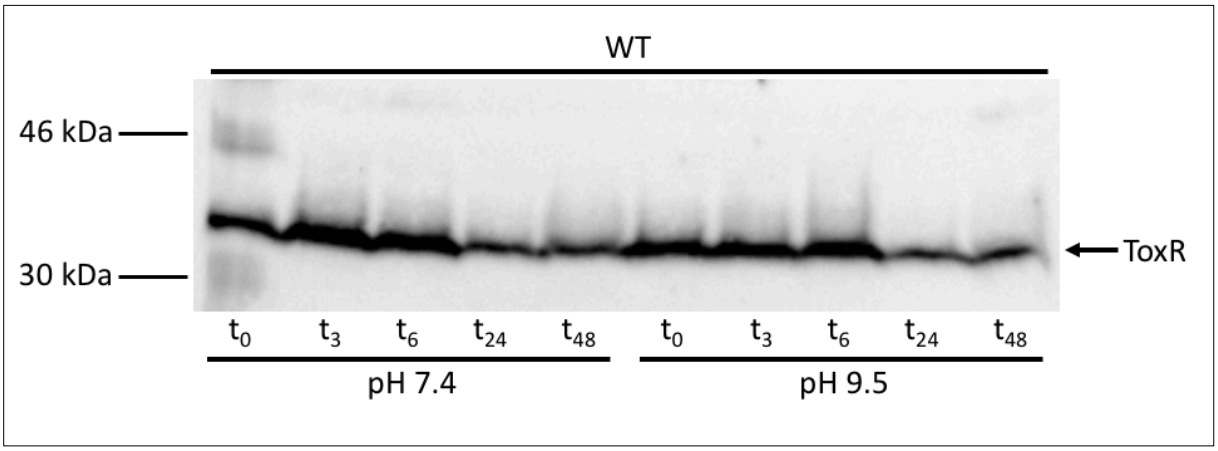


**Figure 9: Immunoblot analysis of the WT to test the stability of ToxR at different pH levels and nutrition limitation.** WT was grown in LB medium with adequate antibiotics overnight. Cells were harvested by centrifugation (4000 rpm, 20 min, RT). The pellets were resuspended in sterile 1x phosphate buffered saline (PBS) pH 7.4 or pH 9.5, respectively. Cultures were incubated at 37°C for up to 48 hours. Samples were taken at indicated time points (hours). Equal protein amounts of WCE were separated by SDS-PAGE (15%) and visualized by immunoblot analysis using α-ToxR serum.

## 3.2 Bile salts positively influence ToxR stability in a Δ*dsbA* background

DsbA is a thiol-disulphide oxidoreductase belonging to the thioredoxin superfamily. DsbA is located in the periplasm of *V. cholerae* and other Gram-negative bacteria and catalyses the introduction of disulphide bonds into periplasmic proteins. The active site of DsbA in *V. cholerae* contains a typical **C**PH**C** motif. To build up disulphide bonds appropriately, it is necessary, that the cysteines of DsbA's active site are in their oxidized form [Kadokura and Beckwith, 2010]. On account of the fact that ToxR

harbours two periplasmic cysteine residues (C236 and C293), it represents a potential substrate for DsbA, whereas FLAGToxR$^{C236SC293S}$ (FLAGToxR$^{CC}$) can no longer be transformed by DsbA [Ottemann and Mekalanos, 1996; Fengler et al., 2012]. As shown above (chapter III.2), FLAGToxR$^{CC}$, over produced from a plasmid, has a low protein half-life, which can be extended by bile salts. Based on these facts, the question arose, how chromosomally produced FLAGToxR$^{CC}$ and ToxR in a Δ*dsbA* mutant strain react to proteolysis and bile salts.

SP27459 Δ*toxR*, the WT, SP27459 Δ*toxR*::*FLAGtoxR*$^{C236SC293S}$ and SP27459 Δ*dsbA*::*km* were grown in M9 minimal medium 0.2% maltose without and with sodium deoxycholate (0.01% or 0.1%) for up to 24 hours. Samples were taken at indicated time points. Equal protein amounts of WCE were separated by SDS-PAGE (15%) and visualized by immunoblot analysis using polyclonal α-ToxR serum.

Panel A of Figure 10 presents the ToxR levels derived from samples of the WT, SP27459 Δ*toxR*::*FLAGtoxR*$^{C236SC293S}$ and SP27459 Δ*dsbA*::*km* treated without or with 0.01% bile at time point 3 h. ToxR is detectable in every lane, except the one, which corresponds to Δ*toxR*. The overall amount of ToxR in SP27459 Δ*toxR*::*FLAGtoxR*$^{C236SC293S}$ is decreased. This indicates an increased proteolysis of FLAGToxR$^{CC}$ in this strain. Apart from that, there is no difference between ToxR levels derived from samples treated with bile or not.

Panel B of Figure 10 demonstrates ToxR levels gained from samples as described above at time point 24 hours. There is no difference of ToxR levels of the WT. FLAGToxR$^{CC}$ is not detectable anymore, regardless of the presence or absence of bile. In the Δ*dsbA* mutant strain is a contrast between the untreated and treated sample observable. Interestingly, a stabilizing impact on ToxR is accompanied by the addition of bile.

Panel C of Figure 10 shows ToxR levels of samples in the same order as depicted above at time point 24 hours using 10-fold higher bile concentrations (0.1%). In lanes reflecting samples of Δ*toxR* and SP27459 Δ*toxR*::*FLAGtoxR*$^{C236SC293S}$ without bile, background bands (~34 kDa) are visible. The intensity of ToxR bands of the WT remain the same. FLAGToxR$^{CC}$ levels are triflingly increased by the addition of 0.1% bile. The proteolysis inhibiting effect of bile on ToxR in a *dsbA*$^{-}$ background is even more intense due to the 10-fold higher bile concentration. Consequently, a positive influence of bile salts on ToxR in strains lacking *dsbA* is observed. This indicates that bile is still capable of stabilizing ToxR as long as the cysteines exist and there is the possibility that ToxR may change its redox status.

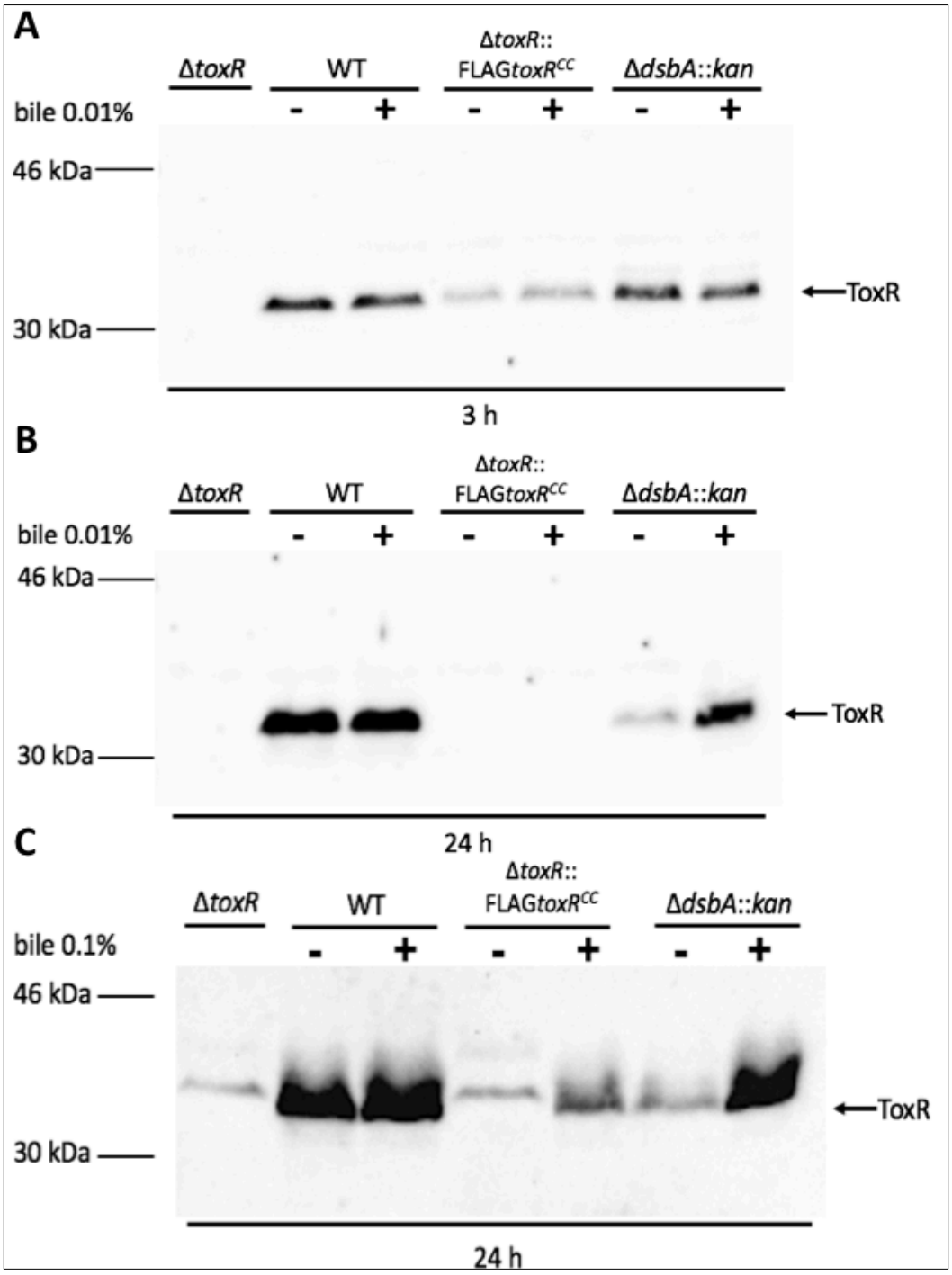


**Figure 10: Immunoblot analysis of SP27459 Δ*toxR*, the WT, SP27459 Δ*toxR*::*FLAGtoxR*$^{C236SC293S}$ (Δ*toxR*::*FLAGtoxR*$^{CC}$) and SP27459 Δ*dsbA*::*kan*.** Cells were grown in M9 minimal medium 0.2% maltose with adequate antibiotics and, if necessary with 0.01% (panel A and B) or 0.1% (panel C) sodium deoxycholate. Cultures were incubated for up to 24 hours. Samples were taken at indicated time points (hours). Equal protein amounts of WCE were separated by SDS-PAGE (15%) and visualized by immunoblot analysis using α-ToxR serum. Panel A displays the immunoblot of respective strains treated with 0.01% sodium deoxycholate at time point 3 h (exposure time 308 sec.), whereas panel B demonstrates the ToxR levels derived from samples treated with the same bile concentration at time point 24 h (exposure time 308 sec.). Panel C shows the detected ToxR levels gained from samples treated with 0.1% sodium deoxycholate at time point 24 h (exposure time 385 sec.).

## 3.3 ToxR$^{CC}$ still allows a bile dependent ToxR activity

A strategy of *V. cholerae* to react quickly to environmental changes and to be able to survive in two completely different niches, is to modify the compounds of the outer membrane. Two major porins of the outer membrane in *V. cholerae* are OmpT (40 kDa) and OmpU (38 kDa), which are regulated reciprocally by ToxR [Provenzano and Klose, 2000]. ToxR is responsible for the direct activation of *ompU* [Crawford et al., 1998] and for the repression of *ompT* [Li et al., 2000]. OmpU is the predominant outer membrane porin during an infection of the human host [LaRocque et al., 2008]. *V. cholerae* is able to sense bile acids in the human intestine and react to this environmental stimulus by altering the expression of its outer membrane porins to build up a resistance. OmpU is less permeable for negatively charged agents, like bile acids and prevents bile to enter the cell [Duret and Delcour, 2010; Mathur and Waldor, 2004; Provenzano and Klose, 2000].

*In vitro*, OmpT represents the prevalent porin in minimal media while OmpU is present predominately in rich media [Miller and Mekalanos, 1988; Li et al., 2002]. The addition of the four amino acids asparagine, arginine, glutamate and serine (NRES mix) as well as the supplementation with bile acids of minimal medium leads to an increase of OmpU production and OmpT repression and therefore to an activation of ToxR [Miller and Mekalanos, 1988; Provenzano et al., 2000]. It was shown that *toxR* mRNA and ToxR protein amounts increase drastically in response to cultivation in minimal media supplemented with the NRES mix, which is not connected with growth expansion. In contrast to that, no alteration in ToxR levels are noticed if bile was present in minimal media [Mey et al. 2012].

The conformation of the periplasmic domain of ToxR is proposed to be important to sense environmental signals. As already mentioned, the transcriptional factor ToxR in *V. cholerae* possesses two cysteines (C236 and C293) located in the periplasmic domain of the protein [Ottemann and Mekalanos, 1996]. The exchange of these two cysteines to serine residues as described by Fengler et al., results in a ToxR variant, which cannot form intramolecular disulphide bonds and thereby is unstable. The diminished protein half-life of ToxR can be elongated by the addition of sublethal concentrations of bile acids. But this stabilizing effect is mainly observable if *toxR*$^{CC}$ is overexpressed from a plasmid (Figure 7), not if *toxR*$^{CC}$ is expressed at chromosomal levels (Figure 10). Furthermore, there is evidence, that OmpU production is reduced in a Δ*dsbA* mutant strain grown under non ToxR activating conditions (M9 minimal medium 0.4% glycerol), although the transcription of *toxR* is not altered [Fengler et al., 2012]. Apart from that, there are results suggesting that the ToxR transmembrane domain is involved in bile mediated ToxR activation. The replacement of the transmembrane domain by a single transmembrane domain of the permease LacY leads to a decreased ToxR activity in response to bile acids [Hung and Mekalanos, 2005]. Hence, we asked, whether ToxR$^{CC}$ can be still activated by bile, although the overall levels of chromosomally produced ToxR$^{CC}$ are diminished.

SP27459 Δ*toxR*, the WT and SP27459 Δ*toxR*::*FLAGtoxR*$^{C236SC293S}$ were grown in M9 minimal medium 0.2% maltose until they reached an $OD_{600}$ of 0.8. The cultures were divided into two aliquots, of which one was supplemented with 0.1% sodium deoxycholate. Samples were taken at indicated time points (20 min and 2 h). Equal protein amounts were separated by SDS-PAGE (15%) and visualized by immunoblot analysis using either polyclonal α-ToxR serum or polyclonal anti-OmpU serum.

Panel A of Figure 11 presents ToxR or FLAGToxR$^{CC}$ levels in presence or absence of bile after 20 min or 2 h, respectively. The presence of bile acids does not increase ToxR or FLAGToxR$^{CC}$ levels. Although the overall amount of FLAGToxR$^{CC}$ is reduced, which is consistent with the findings displayed in Figure 10. Panel B of Figure 11 shows the OmpU levels corresponding to bile availability. No OmpU is produced in the Δ*toxR* mutant strain. OmpU levels are increased by the addition of bile salts in the WT already after 20 min. The FLAG*toxR*$^{CC}$ expressing strain shows a delayed ToxR mediated OmpU production in response to bile salts. After 20 min of exposure to bile salts, decreased OmpU levels are visible compared to the WT. But yet, OmpU amounts in SP27459 Δ*toxR*::*FLAGtoxR*$^{CC}$ reach WT levels after 2 h incubation with bile salts. We suggest that this delayed *ompU* expression is connected with the lower available amount of FLAGToxR$^{CC}$. Meaning if FLAGToxR$^{CC}$ levels would be as high as WT ToxR, the OmpU levels in response to bile at time point 20 min would also be as high as in the WT. To sum up, a ToxR variant missing cysteines and by that lacking the availability to form intramolecular disulphide bonds, still allows a bile dependent ToxR activation.

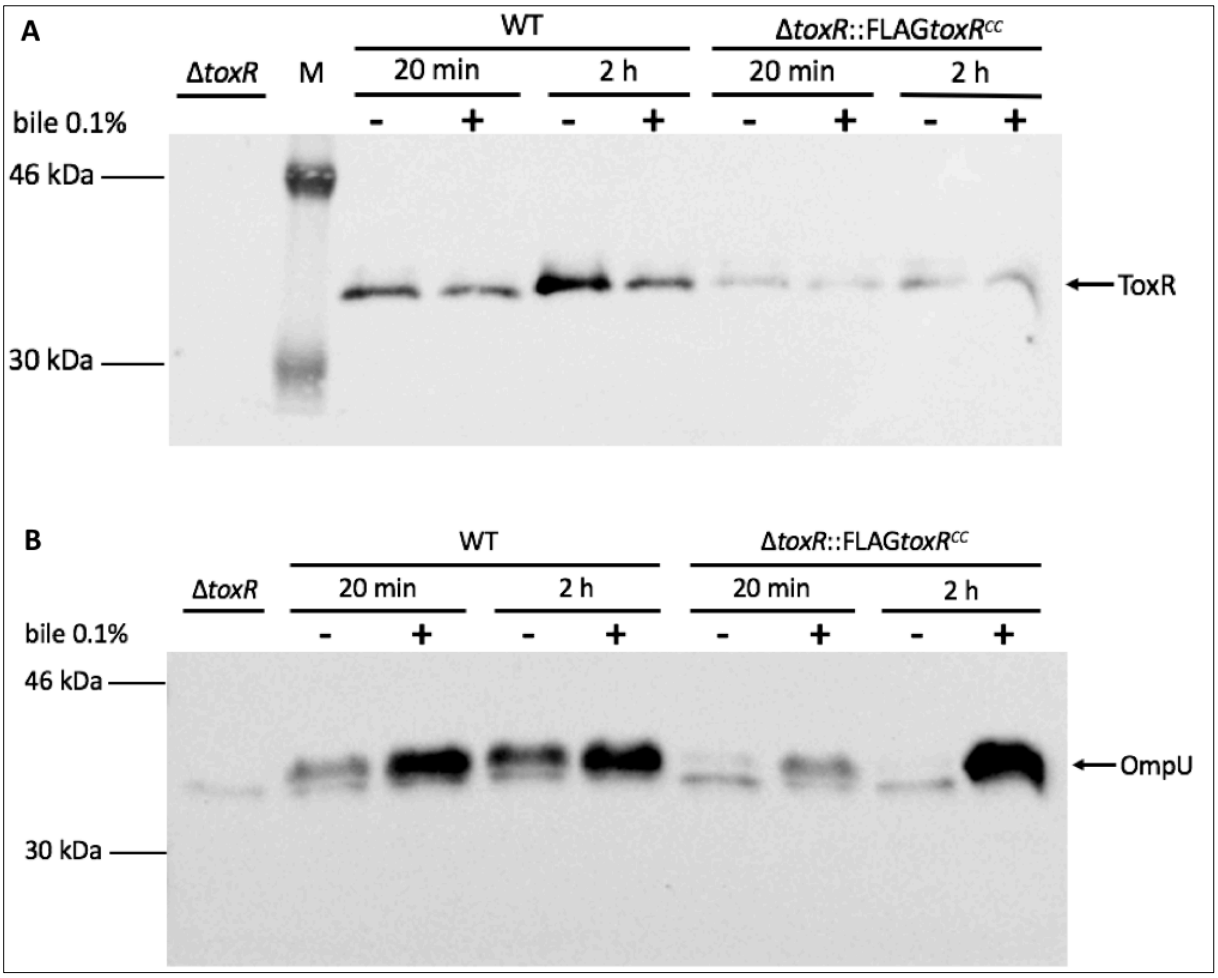


**Figure 11: Immunoblot analysis of SP27459 Δ*toxR*, the WT and SP27459 Δ*toxR*::*FLAGtoxR*$^{C236SC293S}$ (Δ*toxR*::*FLAGtoxR*$^{CC}$).** Cells were grown in M9 minimal medium 0.2% maltose with adequate antibiotics, until they reached an $OD_{600}$ of 0.8. The cultures were divided into two aliquots, of which one was supplemented with 0.1% sodium deoxycholate. Samples were taken at indicated time points (min or hours). Equal protein amounts of WCE were separated by SDS-PAGE (15%) and visualized by immunoblot analysis using either α-ToxR serum (panel A) or anti-OmpU serum (panel B).

# 4 Analysis of the SigmaE pathway in *Vibrio cholerae*

The surface of *V. cholerae* is the first contact point with its host. Therefore, it constitutes a major target for the antibacterial defence of the host [Rowley et al., 2006]. Such host defences cause stresses that damage components of the cell envelope, like periplasmic and outer membrane proteins [Ruiz et al., 2006]. To prevent a severe loss of membrane integrity, enteric pathogens evolved a resistance to antimicrobial agents, which is associated with the extracytoplasmic stress response controlled by the alternative sigma factor $\sigma^E$ (RpoE). Misfolded proteins in the periplasm are thought to trigger the induction of the extracytoplasmic stress response [Mecasa et al., 1993]. The $\sigma^E$ pathway and the genetic organization of $\sigma^E$ dependent genes in *V. cholerae* contain parallels to the RpoE dependent stress response in *E. coli* (see Introduction). Although in *V. cholerae* the inducing signal for $\sigma^E$ release

is the exposure of a carboxy-terminal peptide of OmpU [Kovacikova and Skorupski, 2002; Mathur et al., 2007; Davis and Waldor, 2009]. Since it was reported that ToxR undergoes RIP after entry in a dormant state at alkaline pH and nutrient limitation which involves the site-2 protease RseP in a RpoE dependent manner [Almagro-Moreno et al., 2015a] and the finding of four putative site-1 proteases, including DegS, which are responsible for ToxR degradation in presence of $ToxS^{L33S}$ under normal growth conditions [Almagro-Moreno et al., 2015b], it is clear that DegS and RseP have at least two functions in *V. cholerae* – the RIP of RseA [Ding et al., 2004; Hayden et al. 2004] and ToxR.

## 4.1 Transcription of *degSphoA* cannot be induced by bile salts, DTT or polymyxin B

Based on the facts described above, we wanted to elucidate how the expression of *degS* is regulated and whether bile salts, DTT or polymyxin B are capable to activate the transcription of *degS*.
Bile salts intercalate into membranes and can disturb the membrane integrity [Alabalak et al., 1996] by dissolving membrane lipids and causing dissociation of membrane proteins [Coleman et al. 1980; Vyvoda et al. 1977]. DTT is a reducing agent and is able to change the structure, folding, and function of a protein by changing the redox state of the protein [Cleland, 1964]. The pentacationic, cyclic lipodecapeptide polymyxin B can interact with components of the bacterial cell envelope of Gram-negative bacteria, because of its electrostatic and hydrophobic properties. Though, these interactions with LPS and phospholipids, the permeability of the outer membrane can be negatively influenced [Mathur and Waldor, 2004; Bilecen et al., 2015;]. Based on the similarities of the properties of the listed agents, they all have the potential to modulate the expression and activity of the extracytoplasmic stress regulator $\sigma^E$ due to affecting phospholipids and proteins of the outer membrane [Mathur et al., 2007; Haines-Menges et al., 2014]. To determine the activation of *degS* transcription, PhoA assays were carried out. Strains, encoding for different *phoA* reporter gene fusions, like SP27459 pGP704degSphoA, SP27459 *celAB*::phoArecpGP704 (V385) and SP27459 pGPphoAompU were grown in M9 minimal medium 0.2% maltose until they reached an $OD_{600}$ between 0.4 to 0.6. In the exponential phase, the cultures were supplemented with different agents, like bile (0.01% or 0.1%), DTT (3 mM) or polymyxin B (2 µg/ml), respectively. Samples were taken after 1 hour or overnight and PhoA activity, more precisely the *degS* or *ompU* promoter activity was measured in Miller units.

Panel A (1 h) and B (overnight) of Figure 12 displays the measured Miller units, which represent the *degS* promoter activity under the tested conditions, normalized to WT PhoA activities. At first, comparing the two time points of the untreated culture, a decrease in PhoA activities is observable. A trend towards activation of *degS* transcription in cultures supplemented with 0.1% bile after 1 h will be discussed. But compared with the appropriate sample after incubation overnight, a massive loss of PhoA activity is detected. We suggest, this loss is due to the increased membrane permeability by the addition of bile and thereby the alkaline phosphatase PhoA cannot be longer stored in the periplasmic

space. Although it is known, that the alkaline phosphatase PhoA is a homodimeric protein, which contains two intramolecular disulphide bonds, that are formed after the export to the periplasmic space [Sone et al., 1997], we decided, it is worth a try, to test DTT. After 1 hour DTT treatment, the number of Miller units is lower compared to the untreated sample. This suggests, that PhoA homodimers cannot be formed properly, because of DTT action. However, a trend towards an increase in Miller units of DTT treated cultures is detected, even though the overall amount of PhoA activities is diminished over time. Additionally, it is important to mention, that DTT is not longer than 4 h stable at 37°C and therefore not a good marker for the used experimental setup.

To determine the amount of PhoA activity which is lost overnight, because of cell lysis, the strain SP27459 *celAB*::phoArecpGP704 (V385) [Berg et al., 2007] was applied in PhoA assays. V385 harbours a chromosomal *phoA* fusion within the locus encoding for the chitin uptake system. The *phoA* expression of V385 should always be constitutively active under the tested conditions.

Panel C (1 h) and D (overnight) of Figure 12 shows the measured Miller units in the respective strain normalized to WT PhoA activities. The *phoA* transcription in V385 is not altered by the addition of 0.01% sodium deoxycholate or sublethal concentrations of polymyxin B (2 µg/ml), but a loss of a fourth in PhoA activity is noticed overnight. This displays that the storage of alkaline phosphatase in the periplasm is limited by cell lysis. Apart from a decrease in PhoA activity overnight, there is no significant activation of *degS* promoters, due to bile salts or polymyxin B, detectable. Investigations with SP27459 pGP704dsbAphoA under the same conditions reveal similar results (data not shown).

As described above, ToxR is supposed to react to environmental stimuli with its two periplasmic cysteines [Ottemann and Mekalanos, 1996; Fengler et al., 2012]. However, ToxR also sense bile salts and reacts with changes in gene expression to adapt to new environmental conditions [Provenzano and Klose, 2000]. In presence of 0.01% sodium deoxycholate for 1 h, the *ompU* promoter experiences a 3-fold increase in activity. Overnight, the *ompU* transcription doubled. Furthermore, the activity of the untreated SP27459 pGPphoAompU rises overnight. This indicates, that the 10-fold lower bile concentration of 0.01% results in a lower membrane permeability or damage.

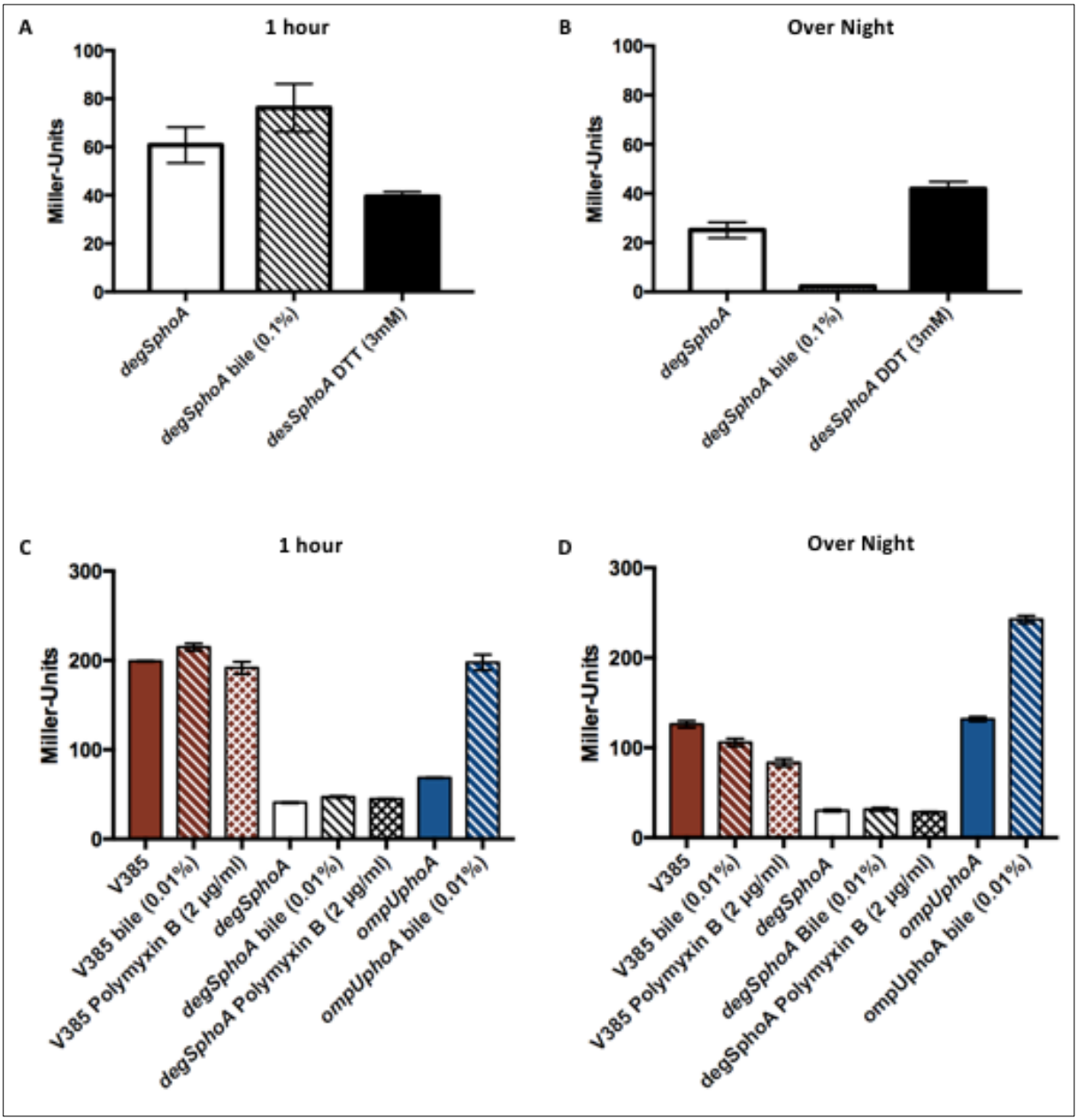


**Figure 12: Measurement of *degS* and *ompU* transcription by *phoA* reporter gene fusions in WT.** Shown are activities of the alkaline phosphatase PhoA, which was under the control of the natural *degS* or *ompU* promoter. Therefore, SP27459 *celAB*::phoArecpGP704 (V385), SP27459 pGP704degSphoA and SP27459 pGPphoAompU were grown in M9 minimal medium 0.2% maltose with adequate antibiotics until they reached an $OD_{600}$ of 0.4 to 0.6. In the exponential phase, the cultures were supplemented with different agents, like bile (0.01% or 0.1%), DTT (3 mM) or polymyxin B (2 µg/ml), respectively. After 1 hour (panels A and C) or overnight (panels B and D) cells were harvested and PhoA assays were performed. Miller units were measured 2 times independently.

## 4.2 Transcriptional levels of *dsbAphoA* are not altered in presence of the tripeptide YDF

In a *V. cholerae hfq* deletion mutant, the activity and expression of the alternative sigma factor RpoE are notably increased. According to transcriptome analysis of a Δ*hfq* mutant strain compared to that of a Δ*rseA* mutant, it was observed, that half of the genes in *V. cholerae* are upregulated in a Δ*hfq* background. *dsbA*, but not *degS*, is found to be upregulated in such a deletion circumstance. Therefore, the *dsbA* expression is under control of $\sigma^E$ [Ding et al., 2004].

Misfolding of specific outer membrane proteins (OMPs) are thought to result in an exposure of C-terminal motifs (tyrosine – X - phenylalanine) that are usually hidden. The aromatic amino acid residues of this motif bind selectively to residues in the periplasmic PDZ domain of the site-1 protease DegS embedded in the inner membrane. This conformational changing induces the regulated intramembrane proteolysis (RIP) of the anti-sigma factor RseA, that eventually results in the release of the alternative sigma factor $\sigma^E$ to the cytoplasm [Walsh et al. 2003; Wilken et al. 2004]. $\sigma^E$ positively regulates the production of RpoE and also upregulates the expression of periplasmic chaperones genes and genes encoding for membrane repairing responses [Dartigalongue et al., 2001; Rezuchova et al., 2003; Ding et al., 2004]. It was reported that OmpU in *V. cholerae* contribute to antimicrobial peptide resistance by modifying the activity of $\sigma^E$ which results in an OmpU-dependent signalling pathway leading in an upregulation of the *V. cholerae* RpoE stress response [Mathur et al., 2007].

Since it was published that OmpU is relevant to build up a resistance to SDS and bile [Provenzano and Klose, 2000], we hypothesized whether the $\sigma^E$ dependent regulon can be artificially induced by the synthetic tripeptide tyrosine – aspartic acid – phenylalanine (YDF). Therefore, the influence of YDF on the $\sigma^E$ pathway was investigated by using chromosomal *dsbAphoA* fusion stains as a read out. The control strain SP27459 *celAB*::phoArecpGP704 (V385) and SP27459 pGPdsbAphoA were incubated either in M9 minimal medium 0.2% maltose or LB medium until reached they reached an $OD_{600}$ of 0.5 to 0.6. During the exponential phase 10 µg/ml YDF were added to respective cultures for 60 min. Subsequently, the PhoA activity, more precisely the *dsbA* promoter activation was measured in Miller units.

Figure 13 displays that the synthetic tripeptide YDF has no influence on the transcription of *dsbA*. No significant differences in PhoA activities of the different cultures were detectable. This indicates that the signalling cascade that results in upregulation of $\sigma^E$ regulon, cannot be induced artificially. At least for incubation in minimal medium, the possible metabolization of the supplemented tripeptide by the cells, could be an explanation for this result. But according to $OD_{600}$ measurements during the incubation phase, no benefit for the supplemented cultures was observable.

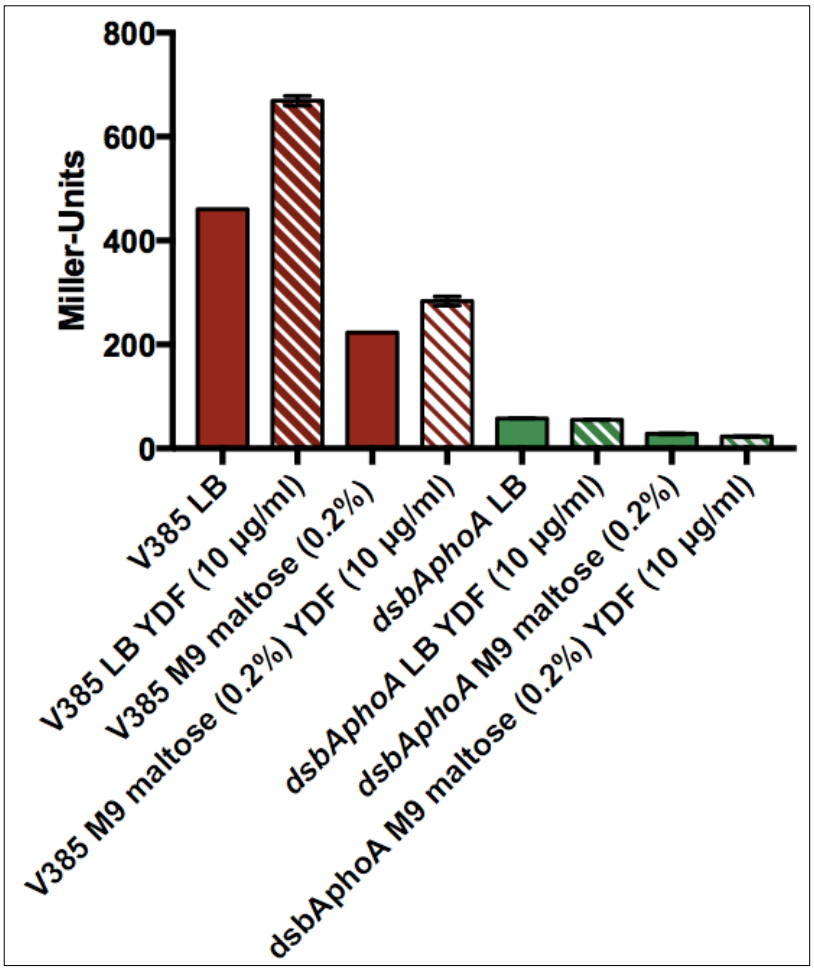


**Figure 13: Measurement of *dsbA* transcription by *phoA* reporter gene fusion in WT.** Shown are activities of the alkaline phosphatase PhoA, which was under the control of the natural *dsbA* promoter. Therefore, SP 27459 *celAB*::phoArecpGP704 (V385) and SP27459 pGP704dsbAphoA were grown in M9 minimal medium 0.2% maltose with adequate antibiotics until they reached an $OD_{600}$ of 0.4 – 0.6. In the exponential phase, the cultures were supplemented with the tripeptide YDF (10 µg/ml) and incubated for 60 min. Subsequently, cells were harvested and PhoA assays were performed. Miller units were measured two times independently.

## 4.3 ToxR is no target of the site-1 protease DegS under conditions mimicking membrane stress

There is evidence that ToxR stability depends on the presence of site-1 protease DegS [Lembke, 2016; Almagro-Moreno et al. 2015b] also shown in Figure 4 panel B. Based on the facts, that DegS has at least two different substrates (RseA and ToxR) in *V. cholerae* and that the protease is activated by interactions between an exposed C-terminal motif YDF of OMP and its PDZ domain [Walsh et al., 2003; Mathur et al., 2007], we asked whether there is a connection to ToxR breakdown. To verify this hypothesis, protein degradation assay of FLAGToxR in presence of ToxS and YDF was performed. SP27459 Δ*toxRS* pFLAGtoxRS was grown in M9 minimal medium 0.2% maltose until they reached an

$OD_{600}$ of 0.4 to 0.6. During the exponential phase, gene expression from the plasmid was induced with 0.05 mM IPTG for 1 h. Subsequently, right before the addition of 100 µg/ml cm to block biosynthesis, 10 µg/ml YDF was added, to prevent a possibly premature metabolization, but assuming to trigger DegS activity, rather than *degS* expression. Samples were taken at defined time points, WCE were prepared, separated by SDS-PAGE (15%) and visualized by immunoblot analysis with monoclonal anti-FLAG M2 serum.

Panel A and B of Figure 14 demonstrate the protein stability of FLAGToxR in absence or presence of YDF. No destabilizing effect of FLAGToxR is visible derived from cultures treated with the synthetic peptide YDF. Full length FLAGToxR is detectable at every time point of the assay. Proteolytic fragments of FLAGToxR are not observed. This data indicates that ToxR is no substrate of DegS under conditions mimicking membrane stress.

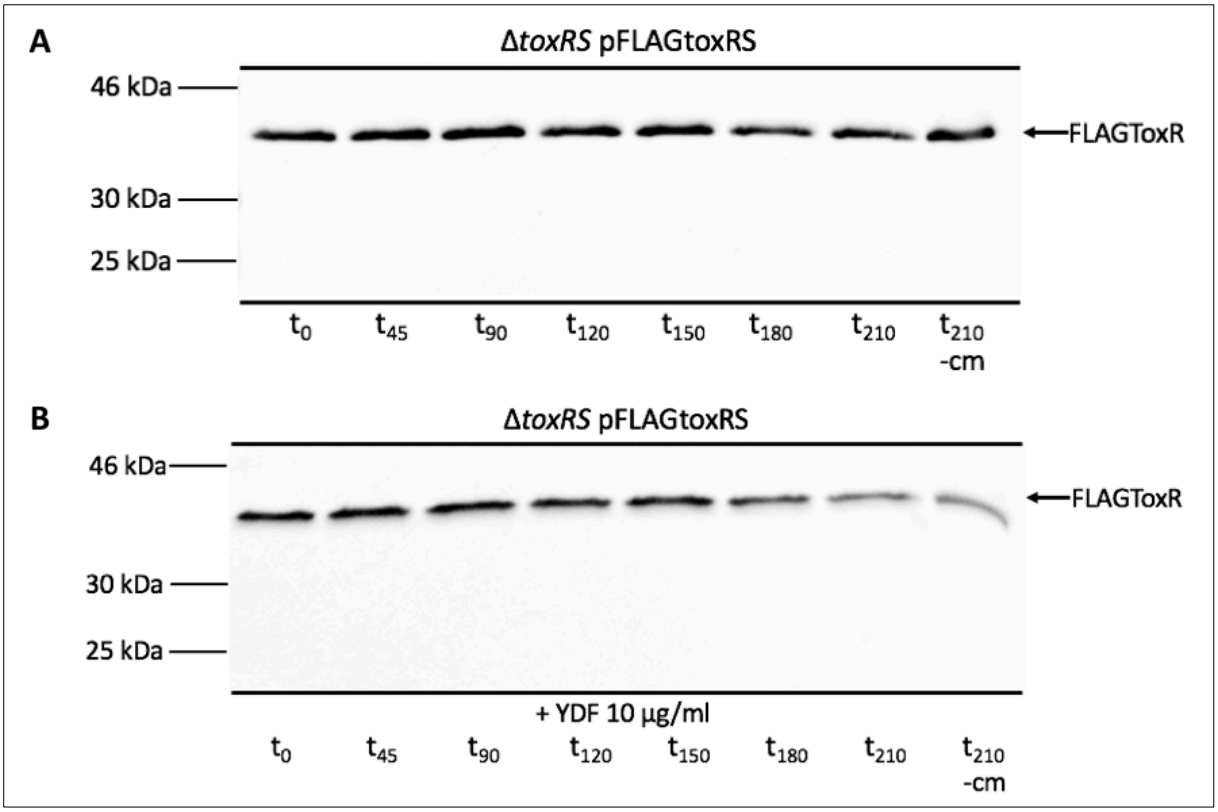


**Figure 14: Protein stability assay of plasmid encoded FLAGToxR coexpressed with ToxS in SP27459 Δ*toxRS* in absence or presence of the tripeptide YDF.** Cells were grown in 40 ml M9 minimal medium 0.2% maltose with adequate antibiotics until they reached an $OD_{600}$ of 0.4 to 0.6. In the exponential phase, gene expression from the plasmid was induced with 0.05 mM IPTG for 1 h. Subsequently, right before the addition of 100 µg/ml chloramphenicol (cm) to block protein biosynthesis, 10 µg/ml YDF (tyrosine - aspartic acid – phenylalanine) was added, to prevent a possibly premature metabolization. Samples were taken at indicated time points (min). Cultures grown without chloramphenicol (-cm) served as a control. Equal protein amounts of WCE were separated by SDS PAGE (15%) and visualized by immunoblot analysis using anti-FLAG M2 serum.

# IV Discussion

During its life cycle, *Vibrio cholerae*, the microorganism responsible for the devastating diarrhoeal disease cholera, has to withstand several chemical, biological and physical stresses. Pathogen persistence in an aquatic environment is linked to exposure to completely different environmental stimuli as if compared to the human gut [Miller and Mekalanos, 1988; Faruque and Nair, 2002; Mey et al., 2012]. Thus, the bacterium has to be adapted to both disparate niches, for which it exhibits a tightly regulated responsive gene regulation system. The adaption and resistance to these ecological factors have vital importance for the survival of the pathogen. One of the main virulence regulators in *V. cholerae*, the transcriptional activator ToxR, recognizes the presence of bile salts, variations in osmosis and oxygen availability. ToxR conveys the resistance or response to these extracellular signals [Miller et al., 1987; Miller and Mekalanos, 1988; Provenzano and Klose, 2000; Fan et al., 2004]. Until now, the mechanisms of how ToxR respond to ecological factors and how these signals influence ToxR stability and activity have not been defined.

It is known from literature, that ToxR interacts with another integral membrane protein ToxS and that ToxRS can build heterodimers [Ottemann and Mekalanos, 1996; Fengler et al., 2012]. It is believed that ToxS acts as a chaperon for ToxR, since it is necessary for ToxR to activate virulence gene expression and prevents the premature proteolysis of the transcriptional activator [Miller et al., 1989; DiRita and Mekalanos, 1991; Ottemann et al., 1992; Pfau and Taylor, 1998; Mey et al., 2012]. All in all, the molecular function of ToxS is unknown, but functional similarities with the transmembrane protein TcpH are predicted. The two proteins TcpP and TcpH are embedded in the inner membrane, like ToxRS. TcpP alone is capable of activating the transcription of *toxT*, although the activation is enhanced when TcpH is present [Häse and Mekalanos, 1998]. TcpH comprises one periplasmic cysteine residue by which the protein facilitates the intramolecular disulphide bond formation in TcpP, which is decisive for protein stability and activity [Beck et al., 2004; Morgan et al., 2016]. This protein conformation protects TcpP from a premature degradation by YaeL (RseP), which plays another major role in the release of the alternative sigma factor, $\sigma^E$ [Alba et al., 2002; Matson and DiRita, 2005].

Within this study a detailed analysis of proteolytic degradation and activation of ToxR and the connection between ToxR proteolysis and the $\sigma^E$ mediated stress response are addressed.

## 1 Analysis of the proteolytic degradation of ToxR

### 1.1 ToxS$^{L33S}$, a pitiless fate for ToxR that requires high activity of the site-1 protease DegS

Early on, a point mutation in the periplasmic domain of ToxS was identified in *S. typhimurium*. This ToxS variant caused the proteolysis of *V. cholerae* ToxR expressed in *Salmonella* [Pfau and Taylor,

1998]. Due to the possible structural alteration of ToxR by ToxS$^{L33S}$, four putative site-1 proteases were identified in a classical strain: DegS, DegP, VesC and TapA [Almagro-Moreno et al., 2015b]. To investigate the dynamics of ToxR degradation in a *toxS*$^{L33S}$ background and to illustrate the potential of this mutation in herein used *V. cholerae* El Tor strains, the leucine at aa position 33 was replaced by a serine residue. To test our hypothesis, we generated pFLAG-MAC™ encoding for *toxRS*$^{L33S}$. Overexpression of pFLAGToxRS$^{L33S}$ in a Δ*toxRS* strain results in enhanced proteolytic degradation of FLAGToxR (Figure 4, panel A). By comparing the results of protein stability assays of pFLAGtoxR and pFLAGtoxRS$^{L33S}$ in Δ*toxRS* mutant strains, it can be shown that FLAGToxR is degraded more slowly in presence of an incomplete ToxS (264 bp internal deletion in *toxS*) [Boritsch, 2012] than in coexistence of ToxS$^{L33S}$. Meaning that the exchange of one single amino acid in ToxS periplasmic domain did not result in dysfunctional ToxS or in a knock out scenario for *toxS*. Based on these facts, it seems that the protein-protein interaction between the periplasmic portion of ToxS$^{L33S}$ and FLAGToxR are tighter. By that, structural changes and the disclosure of cleavage sites of FLAGToxR periplasmic domain activate a proteolytic cascade which results in at least two proteolytic fragments (Figure 4, panel A).

As generally known, proteolytic fragments arise by the action of endoproteases. The proteases responsible for the appearance of two distinct fragments of 28 kDa and 32 kDa as displayed in Figure 4, needed to be identified. As already stated, several site-1 proteases involved in ToxR degradation under normal growth conditions and the presence of ToxS$^{L33S}$ in classical *V. cholerae* strains were identified. Another report revealed the identification of the site-2 protease RseP and degradation of ToxR under starving conditions at alkaline pH after the entry into a dormant state [Almagro-Moreno, et al., 2015a; Almagro-Moreno, et al., 2015b]. Pursuant to these findings, we have the evidence that ToxR stability depends in particular on the site-1 serine protease DegS. The protein stability assay of SP27459 Δ*degS*::*cm* Δ*toxRS* pFLAGtoxRS$^{L33S}$ displays that FLAGToxR is no longer enforced to undergo proteolysis (Figure 4, panel B). Interestingly, by the conducted amino acid substitution in ToxS$^{L33S}$, bioinformatic analysis reveals an additional DegS cleavage site (VS). Thereby, it would be also possible that the serine protease DegS is attracted by the unnatural valine-serine motif of ToxS$^{L33S}$. It is conceivable that the once active protease also yields FLAGToxR susceptible to proteolysis. ToxR comprises a VS motif at aa positions 273 and 274, exactly at two-thirds of the sequence section which is located between the two periplasmic cysteine residues. However, it has already been proven by Lembke that FLAGToxR$^{CC}$ or FLAGToxR (incubated with 3 mM DTT) stability depends on the protease DegS (Lembke, 2016). Nevertheless, also in this ToxR$^{CC}$ variant, the replacement of the cysteines by serine residues results in two additionally artificial VS motifs. Thereby, the sensitivity of a ToxR variant in which the cysteines were replaced by other amino acids, avoiding artificial DegS cleavage sites, needs to be controlled. In addition, the calculated theoretical molecular weight for complete FLAGToxR

is 33.5 kDa, the Mw of the FLAG-Tag and ToxR up to aa V273 is 31.2 kDa [ExPASy Bioinformatics Resource Portal, Compute pI/Mw tool; http://web.expasy.org/compute_pi/].

Furthermore, to be a successful intestinal pathogen, *V. cholerae* must be able to sense the environment for changes of various factors. Since the liver secretes up to 750 ml bile into the duodenum per day [Boyer and Bloomer, 1974], exposure to bile also features a considerable challenge for *V. cholerae*. Beside of digestion of fatty acids, bile also has great effects on cell membranes and therefore antimicrobial properties [Begley et al., 2005]. Beside of intercalating into lipid membranes, bile also has an impact on transmembrane proteins, like ToxRS [Hung and Mekalanos, 2005; Yang et al., 2013]. Bile acids (sodium deoxycholate) positively influence the activity of the virulence regulator ToxR [Provenzano and Klose, 2000]. Since it is known that bile acids inhibit FLAGToxR$^{CC}$ proteolysis in absence or presence of ToxS [Lembke, 2016], we observed that bile salts have no stabilizing effect on FLAGToxR if ToxS$^{L33S}$ is present (Figure 5). Both, full length FLAGToxR and the first proteolytic fragment were hardy visible. Therefore, it is tempting to surmise that bile salts cumulatively induce the proteolysis of FLAGToxR in presence of ToxS$^{L33S}$ and that sodium deoxycholate have an increased activating effect on the site-1 protease, e.g. DegS.

The importance of the two periplasmic cysteines of ToxR on the protein's stability has been proven by Fengler et al. and by Lembke [Fengler et al., 2012; Lembke, 2016]. SP27459 Δ*toxRS* pFLAGToxRS$^{L33S}$ was incubated with 3 mM DTT, a reducing agent, which can alter protein folding by reducing disulphide bonds [Cleland, 1964]. If the periplasmic cysteine residues appear exclusively in their reduced form, FLAGToxR more rapidly undergoes proteolysis in coexistence of ToxS$^{L33S}$ (Figure 6) than under non-reducing conditions (Figure 4).

To sum up, the most supported model suggests ToxS to enhance stability and correct assembly of ToxR, but ToxS is for sure not necessary for ToxR DNA binding [Ottemann et al.,1992; Pfau and Taylor, 1998; Almagro-Moreno et al., 2015b]. In accordance to that, it is possible that ToxS$^{L33S}$ constitute a variant which is no longer capable to differentiate between diverse ToxR conformations. Thus, these findings suggest that ToxS$^{L33S}$ actively trigger the proteolytic degradation of FLAGToxR, regardless of the redox state of FLAGToxR or the presence of protein stabilizing substances. To further elucidate the functions of ToxS and the mechanisms of ToxR proteolysis in coexistence of ToxS$^{L33S}$, degradation assays should be conducted using strains with chromosomal start to stop deletions in *toxS* and the chromosomal *toxS*$^{L33S}$ point mutation in the *toxS* locus. By that, the genes *toxRS* would be expressed by their natural promoter at lower levels, which would allow insights into unaffected reactions of the proteins under different conditions, e.g. treatment with DTT or sodium deoxycholate.

### 1.2 Relevance of ToxR periplasmic cysteine residues regarding proteolysis

The persistence of *V. cholerae* to survive in both niches, the aquatic environment and the human intestine, requires highly adapted responses to withstand the changing environments [Reidl and Klose, 2002]. The capability comprises inter alia the sensing of different oxygen concentrations [Marrero et al., 2009; Liu et al., 2011]. The main virulence regulator ToxR comprises two periplasmic cysteine residues at aa positions C236 and C293 [Fengler et al., 2012]. Bacterial pathogens react appropriately to altering oxygen levels, which is commonly necessary for successful colonization and virulence gene regulation. It has already been reported that the cysteine residues of TcpP in its periplasmic domain are decisive for protein stability [Morgan et al., 2016]. It still remains unknown what environmental signals are recognized by the cell and which can thereby mediate ToxR activity or stability by changing the redox status of ToxR by influencing the intramolecular disulphide bond formation [Liu et al., 2011]. As it has been recently demonstrated by Lembke, the proteolysis of FLAGToxR$^{CC}$ depends on the presence of ToxS. A mutant strain missing *toxS* reveals an increased FLAGToxR$^{CC}$ stability [Lembke, 2016]. The ToxR periplasmic cysteine residues were exchanged to serines as described by Fengler et al. to further explain their role in protein half-life time [Fengler et al., 2012].

It can be demonstrated that the levels of overexpressed FLAGToxR$^{CC}$ decreases over time. This indicates that a ToxR variant, which is no longer able to build up intramolecular disulphide bonds is substrate of increased proteolysis (Figure 7, panel A). In contrast to the result of the chloramphenicol experiment of SP27459 Δ*toxRS* pFLAGtoxRS$^{L33S}$ (Figure 5), the proteolytic degradation of FLAGToxR$^{CC}$ can be reversed by the addition of 0.1% sodium deoxycholate (Figure 7, panel B). In this context, ToxR cysteine residues play a main role to specify ToxR stability. The reduced and more disordered form of ToxR seem to trigger ToxR degradation in a ToxS dependent way [Lembke, 2016]. The responsible molecular mechanism by which this proteolytic breakdown is achieved is still not known.

Additionally as apparent in Figure 7, panel A, no proteolytic fragments as in Figure 4 were detectable. This result can be explained by a worse expression of pFLAGtoxR$^{CC}$S or blotting effects, which mean that this experiment needs to be repeated.

## 2 Analysis of stability and activation of chromosomally expressed *toxR*

### 2.1 Nutrient limitation and alkaline pH – conditions with no effect on ToxR stability in *V. cholerae* O1 El Tor

Recently, the zinc metalloprotease RseP was characterized to be involved in the regulated intramembrane proteolysis of ToxR in a RpoE dependent manner and in response to nutrient limitation and alkaline pH during late stationary phase [Almagro-Moreno et al., 2015a]. The research group of Taylor, R.K. discovers reduced ToxR levels in classical biotype strain O395 after incubation of 48 hours

in LB medium pH 9.3. Moreover, they also performed the same experiment with *V. cholerae* N16961, biotype El Tor, where they find ToxR to be vanished completely after 72 hours in LB medium pH 9.3. We prove here that chromosomally produced WT ToxR in the herein used *V. cholerae* O1 El Tor in M9 minimal medium remains stable over a time period of 72 hours under nutrient limitation. Furthermore we observed that bile salts (sodium deoxycholate) have no detectable stabilizing impact on WT ToxR in a long term experiment since ToxR remains stable (Figure 8). Additionally, it has to be mentioned that the pH value of the supernatant of a M9 0.2% maltose culture after 24 hours of incubation was 6.5. Hence, the ToxR stability was only observed under nutrient limitation and not under alkaline pH. Because of that, this experiment needs to be repeated to gain comparable conditions and results. Nevertheless, there is evidence provided that WT ToxR remains stable for at least 96 hours in both, LB and M9 minimal medium 0.2% maltose after anaerobic or aerobic incubation (data not shown). Unfortunately, the pH values of these cultures were not observed. But these results clearly show that ToxR is stabilized regardless of the availability of oxygen or media for 96 hours.

Analogous to the experimental setup of Almagro-Moreno et al. to examine ToxR levels in late stationary phase and nutrient limitation, an ONC of the WT was pelleted and resuspended in PBS pH 7.4 and pH 9.5. They postulate that ToxR levels decrease in cultures exposed to nutrient limitation and pH 9.3 after only 3 hours, whereas ToxR remains stable in PBS pH 7.0 in O395 classical strain. In contrast to that we demonstrate that ToxR levels in *V. cholerae* O1 El Tor remain stable in response to nutrient limitation, regardless to neutral or alkaline pH for at least 48 hours (Figure 9). These results suggest chromosomally expressed *toxR* by *V. cholerae* O1 Inaba El Tor to be resistant against degradation under nutrient limitation and alkaline pH in late stationary phase.

All in all, these findings probably point into a direction that the downregulation of virulence and the escape mechanisms from the host in classical and El Tor strains are completely differently regulated. Since the O1 El Tor biotype is presumed to survive better in both niches, the aquatic environment and the human intestine [Wachsmuth et al., 1994] and it is known that ToxR controls more than 150 other genes [Peterson and Mekalanos, 1988; Bina et al., 2003], one can assume that ToxR is maybe more stable in El Tor strains to bridge the transition between the two different niches of its life cycle better than classical biotypes. Perhaps this significantly higher stability of the transcriptional activator will fit in the list of advantages of O1 El Tor in the future.

## 2.2 Bile salts affect ToxR stability and activity regardless of the redox state of ToxR periplasmic cysteine residues

In *V. cholerae dsbAB* homologues exist, which encode for thiol-disulphide oxidoreductases that catalyse sulfhydryl oxidation of periplasmic proteins [Yu et al., 1992]. Deletion strains with a Δ*dsbAB* background reveal defects in folding periplasmic proteins accurately, whereby the two virulence

factors CTX and TCP are also severely affected [Peek and Taylor, 1992]. To build up disulphide bonds appropriately, it is necessary, that the cysteine residues of DsbA's active site are in their oxidized form, which is catalysed by the integral enzyme DsbB [Kadokura and Beckwith, 2010]. On account of the fact, that ToxR comprises two cysteines in its periplasmic domain (C236 and C293), it constitutes a potential substrate for DsbA, whereas the double cysteine to serine mutant (FLAGToxR$^{C236SC293S}$) can no longer be remodelled by DsbA [Ottemann and Mekalanos, 1996; Fengler et al., 2012]. Apart from the stabilizing impact, bile salts also enhance the activity of the transcriptional activator [Provenzano and Klose, 2000; Lembke, 2016]. It is proposed that ToxR-mediated bile resistance was an early event in the evolution of the human pathogen and necessary to establish the bacterium's current life cycle [Provenzano and Klose, 2000]. The transmembrane domain of ToxR was identified to be a critical element in bile induced protein activation, but the exact activation mechanism is still unknown [Hung and Mekalanos, 2005]. Interestingly, ToxS has no periplasmic cysteine residue. Therefore, ToxS has no ability to assist with the introduction of disulphide bonds in the periplasmic domain of ToxR, like it is suggested for TcpPH. A mutant strain, harbouring a 264 bp internal deletion in *toxS*, resulting an incomplete ToxS protein and expressing *toxR* is attenuated for *ctx* and *tcp* virulence gene expression compared to the WT *in vivo* [Fengler et al., 2012]. By that, other ToxR activating mechanisms must exist that do not rely on ToxR disulphide bond formation or on the redox status of the integral protein. Thus, our new findings might give novel insights.

As it is displayed in Figure 7, FLAGToxR$^{CC}$, overproduced from a plasmid, undergoes rapid proteolysis and this degrading effect can be reversed by the supplementation of bile salts (sodium deoxycholate). At first, bile salts have no observable stabilizing impact on chromosomally produced WT ToxR levels (Figure 10, panel A,B and C). Compared to these, the overall amounts of FLAGToxR$^{CC}$ are diminished or even undetectable at any time point, which suggests an increased proteolytic degradation of the double cysteine mutant, regardless of the presence of bile (Figure 10, panel A,B and C). A protein stabilizing bile effect is observable in the Δ*dsbA* mutant strain at time point 24 hours, which is more profound at 10-fold higher bile concentrations (0.1%) (Figure 10, panel B and C).

Beside of the stabilizing effect of bile salts on ToxR, we have evidence that FLAGToxR$^{CC}$ still allows a bile dependent induction of ToxR activity (Figure 11, panel B). Although the overall amount of FLAGToxR$^{CC}$ is reduced compared to WT ToxR levels (Figure 11, panel A), which is consistent with the results displayed in Figure 10, FLAGToxR$^{CC}$ enables the production of OmpU corresponding to bile availability. After 20 min of exposure to bile salts, decreased OmpU levels are detectable compared to WT. But yet, OmpU amounts produced in SP27459 Δ*toxR*::*FLAGtoxR*$^{CC}$ achieve WT levels after 2 h of incubation with 0.1% bile (Figure 11, panel B). We suggest that this delayed increase of OmpU levels depends on the accessible reduced overall amount of FLAGToxR$^{CC}$ in the cells. Stating that under circumstances that would allow as high chromosomally produced FLAGToxR$^{CC}$ levels as WT ToxR

amounts, SP27459 Δ*toxR*::*FLAGtoxR*$^{CC}$ would also result in high OmpU levels after a short exposure to bile salts. For this scenario, a comparison with a classical saturation of an enzymatic reaction would be obvious in which the product (e.g. OmpU) concentration increases more slowly, because of the low availability of the enzyme (e.g. FLAGToxR$^{CC}$) and the consistent disposability of the substrate (e.g. ToxR specific DNA consensus sequences within the promoter region of *ompU* and *ompT*). In conclusion, the proteolysis of chromosomally produced ToxR or FLAGToxR$^{CC}$ should be further investigated in Δ*degS* mutant strains. Thereby a DegS activating mechanism by overexpression of *toxRS* from a plasmid can be excluded. Furthermore, the hypothesis arises if chromosomal levels of ToxR or FLAG ToxR$^{CC}$ are still capable of activating the expression of OmpU in presence of bile salts in such a *degS* deletion strain or even in a Δ*degS* Δ*dsbA* double knock out mutant.

Bile salts intercalate into the lipid membranes of *V. cholerae*, because of their amphipathic properties [Alabalak et al., 1996]. Our discoveries manifest that bile salts do not enhance stabilization of ToxR by the oxidation of the periplasmic cysteines, because FLAGToxR$^{CC}$ is devoid of these amino acids and the ToxR variant is only stabilized marginally at chromosomally produced levels. In line with that, WT ToxR experiences a stabilizing impact by bile salts in a Δ*dsbA* mutant strain, in which the oxidized form of ToxR is not completely excluded. Moreover, there is evidence that OmpU production is decreased in a Δ*dsbA* mutant strain incubated under non ToxR activating conditions (M9 minimal medium 0.4% glycerol), even though the transcription of *toxR* is not changed [Fengler et al., 2012]. By that, the presumption that bile salts have pro-oxidizing effects might have to be rejected. Indeed, bile can shift the ratio between reduced and oxidized ToxR, since the oxidized status of the transmembrane regulator is the favoured conformation for the activation of the regulation of outer membrane porins [Fengler et al., 2012]. Therefore it is valid that Hung and Mekalanos postulate that the transmembrane domain of ToxR is crucial for the activation of the transcriptional regulator [Hung and Mekalanos, 2005]. It is demonstrated in *E. coli* that bile salts destabilize the hydrophobic cores of proteins [Cremers et al., 2014]. This proves that bile effects the secondary structure of proteins by damaging the integrity of α-helices, like the one of ToxR. The transmembrane portion of ToxR comprises a single proline residue that implements a turn into the α-helix [Cordes et al., 2002]. Proline residues are recurrent in α-helices but with high preferences at the beginning or end of an α-helix introducing a hinge to link the next secondary portion [Kim and Kang, 1999]. Surprisingly, the specific proline (P192) of ToxR is located in the middle of the membrane spanning element. This unusual position decreases the stability of the integral portion as it disarranges the formation of hydrogen bonds within the α-helix. Based on these listed facts, one might presume bile salts stabilize the transmembrane domain of ToxR by intercalating into the lipid membrane and interacting with the amino acids, which results in conformational changes, increased stability and initiation of activity.

In summary, we show here that that bile salts have two effects on ToxR – a long term and a short term effect. The long term effect comprises the direct correlation between ToxR stability and levels of bile salt concentrations. The short term effect is described by the dependence of ToxR activity and bile salt availability.

## 3 Hypothetical connection between ToxR proteolysis and $\sigma^E$ mediated stress response

$\sigma^E$ mediated stress response pathway in Gram-negative bacteria is very well characterized. The genetic organization of $\sigma^E$ dependent genes and the $\sigma^E$ pathway in *V. cholerae* contains several homologies and parallels to the prototype RpoE response system in *E. coli*. The inducing signal for $\sigma^E$ release in *V. cholerae* is the exposure of a carboxy terminal tripeptide of OmpU to the periplasmic compartment and interaction of PDZ domain of DegS [Kovacikova and Skorupski, 2002; Mathur et al., 2007; Davis and Waldor, 2009]. Well known genes whose expression are under the control of $\sigma^E$ are e.g. *rpoE*, *rseABC*, *htrA* and *dsbAB* in *V. cholerae* [Ding et al., 2004; Ruiz and Silhavy, 2005]. It is reported that ToxR goes through RIP after entry a dormant state at alkaline pH and nutrient limitation which involves the site-2 protease RseP in a RpoE dependent manner [Almagro-Moreno et al., 2015a]. These findings are followed by the identification of four putative site-1 proteases, among of them DegS, which are responsible for ToxR proteolysis under normal growth conditions and the presence of $ToxS^{L33S}$ [Almagro-Moreno et al., 2015b]. Thus, the conclusion can be drawn that DegS and RseP have at least two roles in *V. cholerae*, the RIP of the anti-sigma factor RseA [Ding et al., 2004; Hayden et al., 2004] and the RIP of ToxR. Recently, Lembke proves that overproduction of FLAGToxR in presence of 3 mM DTT or $FLAGToxR^{CC}$ proteolysis foremost depends on the protease DegS. In absence of *degS*, ToxR stability is similar as determined in the presence of bile salts [Lembke, 2016]. In accordance with these findings we demonstrate that degradation of overproduced FLAGToxR caused by $ToxS^{L33S}$ is also dependent on DegS (Figure 4, panel B).

Substances like bile salts, polymyxin B and DTT are membrane damaging substances or have protein affecting properties, which might point into a direction suspecting a response by the $\sigma^E$ pathway and ToxRS. To elucidate this issue, we investigated the induction of *degS* transcription by the listed agents with PhoA assays using respective *phoA* fusion strains (Figure 12). Unfortunately, no assay displays a significant effect indicating that the *degS* promoter activity remains the same under the tested conditions. These results are consistent with the findings by Ding et al., who find *dsbA* but not *degS* upregulated in transcriptome analysis of $\sigma^E$ dependent genes [Ding et al., 2004]. As expected, the *ompU* transcription is activated by ToxR sensing bile salts in the medium at both time points 1 h and overnight. Whereby the *ompU* transcripts even rise more overnight. It is notably that the overall amount of PhoA activity is diminished over night by increased cell lysis. This is not true for the

untreated *ompUphoA* fusion strain, indicating that the 10-fold diminished bile concentration leads to a lower membrane permeability and cell damage (Figure 12, panel C and D).

The C-terminal aromatic amino acids of the tripeptide YDF of outer membrane proteins are believed to be exposed as a reaction to membrane damage, which in turn is recognized by the PDZ domain of the integral site-1 protease DegS that initiates the sequential degradation of the anti- $\sigma^E$ factor RseA [Mathur et al., 2007]. The measurement of the PhoA activity derived from *dsbAphoA* fusion strains exposed to 10 µg/ml YDF for 1 h, displayed no significant effect, indicating that the upregulation of the $\sigma^E$ regulon cannot be initiated artificially (Figure 13). Possibly the alkaline phosphatase PhoA is not the accurate reporter for membrane stress investigations, because the enzyme is also stored in the periplasmic space. The measurement by qRT-PCR would be a better and more precise opportunity for answering these issues.

To round the investigations of the hypothesis linking the activation of the $\sigma^E$ dependent envelope stress response and the proteolytic degradation of ToxR, we attempted to mimic DegS induction by supplementing YDF (10 µg/ml) and observed the protein half-life of ToxR in chloramphenicol experiments (Figure 14). There is a marginal difference of FLAGToxR levels observable between the untreated culture and the one exposed to the synthetic tripeptide. Although one can argue that the FLAGToxR levels decrease towards the end of the protein stability assay (Figure 14, panel B). Particularly the –cm control shows reduced FLAGToxR level, even though in this strain, the protein expression was not inhibited and the overall protein amount was extremely increased on the corresponding SDS-gel (data not shown). Perhaps, the time points for this experimental setup should be chosen differentially, to be able to examine the proteolysis of FLAGToxR under conditions mimicking membrane stress. Moreover, it would be interesting to investigate the molecular function of ToxS a Δ*toxS* strain in a DegS attracting situation.

# V References


**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*, 1640-1653.

**Ades, S. E., Connolly, L. E., Alba, B. M., and Gross, C. A. (1999)**. The *Escherichia coli* sigma(E)-dependent extracytoplasmic stress response is controlled by the regulated proteolysis of an anti-sigma factor. *Genes Dev., 13*, 2449-2461.

**Alabalak, A., Zeidel, M. L., Zucker, S. D., Jackson, A. A., and Donovan, J. M. (1996)**. Effects of submicellar bile salt concentrations on biological membrane permeability to low molecular weight non-ionic solutes. *Biochem., 35*, 7936-7945.

**Alba, B. M., Leeds, J. A., Onufryk, C., Lu, C. Z., and Gross, C. A. (2002)**. DegS and YaeL participate sequentially in the cleavage of RseA to activate the sigma(E)-dependent extracytoplasmic stress response. *Genes Dev., 16*, 2156-2168.

**Alba, B. M., Zhang, H. J., Pelayo, J. C., and Gross, C. A. (2001)**. *degS* (*hhoB*) is an essential *Escherichia coli* gene whose indispensable function is to provide $\sigma^E$ activity. *Mol. Microbiol., 40*, 1323-1333.

**Albert, M. J., Niere, M., and Motarjemi, Y. (1997)**. The role of food in the epidemiology of cholera. *World Health Stat. Q.,* (50), 111-118.

**Almagro-Moreno, S., Kim, T. K., Skorupski, K., and Taylor, R. K. (2015a)**. Proteolysis of virulence regulator ToxR is associated with entry of *Vibrio cholerae* into a dormant state. *PLoS Genet., 11*

**Almagro-Moreno, S., Root, M. Z., and Taylor, R. K. (2015b)**. Role of ToxS in the proteolytic cascade of virulence regulator ToxR in *Vibrio cholerae*. *Mol. Microbiol., 98*, 963-976.

**Bader, M. W., Xie, T., Yu, C. A., and Bardwell, J. C. (2000)**. Disulfide bonds are generated by quinone reduction. *J. Biol. Chem., 275*, 26082-26088.

**Bardwell, J. C., McGovern, K., and Beckwith, J. (1991)**. Identification of a protein required for disulfide bond formation *in vivo*. *Cell Rep., 67*, 581-589.

**Begley, M., Gahan, C. G. M., and Hill, C. (2005)**. The interaction between bacteria and bile. *FEMS Microbiol. Rev., 29*, 625-651.

**Berg, T., Schild, S., and Reidl, J. (2007)**. Regulation of the chitobiose-phosphotransferase system in *Vibrio cholerae*. *Arch. Microbiol., 187*, 433-439.

**Bilecen, K., Fong, J. C. N., Cheng, A., Jones, C. J., Zamorano-Sánchez, D., Yildiz, F. H., et al. (2015)**. Polymyxin B resistance and biofilm formation in *Vibrio cholerae* are controlled by the response regulator CarR. *Infect. Immun., 83*, 1199-1209.

**Bina, J., Zhu, J., Dziejman, M., Faruque, S., Calderwood, S., and Mekalanos, J. (2003)**. ToxR regulon of *Vibrio cholerae* and its expression in vibrios shed by cholera patients. *Proc. Natl. Acad. Sci. U.S.A., 100*, 2801-2806.

**Blake, P. A (1994)**. Historical perspectives on pandemic cholera. In *Vibrio cholerae* and cholera: Molecular to global perspectives. *American Society for Microbiology Press, Washington D.C.*, 293-295.

**Boritsch, E. C. (2012)**. Virulence gene regulation in *Vibrio cholerae*: Modulation of ToxR activity by ToxS. *Masterarbeit,* 49-52.

**Boyer, J.L., and Bloomer, J.R. (1974)**. Canalicular bile secretion in man. Studies utilizing the biliary implications. *Curr. Opin. Microbiol., 6,* 93-100.

**Brown, R. C., and Taylor, R. K. (1995)**. Organization of *tcp*, *acf* and *toxT* genes within a ToxT-dependent operon. *Mol. Microbiol., 16*, 425-439.

**Burgess, R. R., Travers, A. A., Dunn, J. J., and Bautz, E. K. F. (1969)**. Factor stimulating transcription by RNA polymerase. *Nature, 221*, 43-44.

**Calvin, N. M., and Hanawalt, P. C. (1988)**. High-efficiency transformation of bacterial cells by electroporation. *J. Bacteriol., 170*, 2796-2801.

**Campbell, E. A., Tupy, J. L., Gruber, T. M., Wang, S., Sharp, M. M., Gross, C. A., et al. (2003)**. Crystal structure of *Escherichia coli* sigmaE with the cytoplasmic domain of its anti-sigma RseA. *Mol. Cell, 11*, 1067-1078.

**Cash, R. A., Music, S. I., Libonati, J. P., Snyder, M. J. J., Wnzel, R. P., and Hornick, R. B. (1974)**. Response of man to infection with *Vibrio cholerae*. I. clinical, serological, and bacteriologic responses to knoen inoculum. *J. Infect. Dis., 129*, 45-52.

**Cezairliyan, B. O., and Sauer, R. T. (2007)**. Inhibition of regulated proteolysis by RseB. *Proc. Natl. Acad. Sci. U.S.A., 104*, 3771-3776.

**Chaba, R., Grigorova, I. L., Flynn, J. M., Baker, T. A., and Gross, C. A. (2007)**. Design principles of the proteolytic cascade governing the sigmaE-mediated envelope stress response in *Escherichia coli*: Keys to graded, buffered, and rapid signal transduction. *Genes Dev., 21*, 124-136.

**Chakrabarti, S. R., Chaudhuri, K., Sen, K., and Das, J. (1996)**. Porins of *Vibrio cholerae*: Purification and characterization of OmpU. *J. Bacteriol., 178*, 524-530.

**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*, 323-331.

**Chiavelli, D. A., Marsh, J. W., and Taylor, R. K. (2001)**. The mannose-sensitive hemaglutinin of *Vibrio cholerae* promotes addherence to zooplankton. *Appl. Environ. Microbiol., 67*, 3220-3225.

**Childers, B. M., and Klose, K. E. (2007)**. Regulation of virulence in *Vibrio cholerae*: The ToxR regulon. *Future Microbiol., 2*, 335-344.

**Childers, B. M., Cao, X., Weber, G. G., Demeler, B., Hart, P. J., and Klose, K. E. (2011)**. N-terminal redisued of *Vibrio cholerae* virulence regulatory protein ToxT involved in dimerization and modulation by fatty acids. *J. Biol. Chem., 286*, 28644-28655.

**Cleland, W. W. (1964)**. Dithiothreitol, a new protective reagent for SH groups. *Biochem., 3*, 480-482.

**Coleman, R., Lowe, P. J., and Billington, D. (1980)**. Memebrane lipid composition and susceptibility to bile salt damage. *BBA - Biomembranes, 599*, 294-300.

**Collet, J. F., and Bardwell, J. C. (2002)**. Oxidative protein folding in bacteria. *Mol Microbiol., 44*, 1-8.

**Collinet, B., Yukawa, H., Chen, T., Herrera, C., and Missiakas, D. (2000)**. RseB binding to the periplasmic domain of RseA modulates the RseA:SigmaE interaction in the cytoplasm and the availability of sigmaE RNA polymerase. *J. Biol. Chem., 275*, 33898-33904.

**Connolly, L., De Las Peñas, A., Alba, B. M., and Gross, C. A. (1997)**. The respond to extracytoplasmic stress in *Escherichia coli* is controlled by partially overlapping pathways. *Genes Dev., 11*, 2012-2021.

**Cordes, F. S., Bright, J. N., and Sansom, M. S. P. (2002)**. Proline-induced distortions of transmembrane helices. *J. Mol. Biol., 323*, 951-960.

**Costanzo, A., and Ades, S. E. (2006)**. Growth pase-dependent regulatio of the extracytoplasmic stress factor, E, by guanosine 3',5'-bispyrophosphate (ppGpp). *J. Bacteriol., 188*, 4627-4634.

**Craig, S. A., Carpenter, C. D., Mey, A. R., Wyckoff, E. E., and Payne, S. M. (2011)**. Positive regulation of the *Vibrio cholerae* porin OmpT by iron and fur. *J. Bacteriol., 193*, 6505-6511.

**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*, 235-246.

**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*, 1459-1473.

**Cremers, C.M., Knoefler, E.S., Vitvitsky, V., Banerjee, R., Jakob, U. (2014)**. Bile salts act as effective protein-unfolding agents and instigators of disulphide stress in vivo. *Proc. Natl. Acad. Sci. U.S.A. 111*, E1610-9.

**Danese, P. N., and Silhavy, T. J. (1997)**. The sigma (E) and the cpx signal transduction systems control the synthesis of periplasmic protein-folding enzymes in *Escherichia coli*. *Genes Dev., 11*, 1183-1193.

**Dartigalongue, C., Loferer, H., and Raina, S. (2001)**. EcfE, a new essential inner membrane protease: Its role in the regulation of heat shock response in *Escherichia coli*. *EMBO J., 20*, 5908-5918.

**Dartigalongue, C., Missiakas, D., and Raina, S. (2001)**. Characterization of the *Escherichia coli* sigma E regulon. *J. Biol. Chem., 276*, 20866-20875.

**Davis, B. M., and Waldor, M. K. (2009)**. High-throughput sequencing reveals suppressors of *Vibrio cholerae rpoE* mutations: One fewer porin is enough. *Nucleic Acids Res, 37*, 5757-5767.

**De Las Peñas, A., Connolly, L., and Gross, C. A. (1997a)**. $\sigma^E$ is an essential sigma factor in *Escherichia coli*. *J. Bacteriol., 179*, 6862-6864.

**De Las Peñas, A., Connolly, L., and Gross, C. A. (1997b)**. The sigmaE-mediated response to extracytoplasmic stress in *Escherichia coli* is transduced by RseA and RseB, two negative regulators of sigmaE. *Mol. Microbiol., 24*, 373-385.

**Ding, Y., Davis, B. M., and Waldor, M. K. (2004)**. Hfq is essential for *Vibrio cholerae* virulence and downregulates sigma expression. *Mol. Microbiol., 53*, 345-354.

**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., Parsot, C., Jander, G., and Mekalanos, J. J. (1991)**. Regulatory cascade controls virulence in *Vibrio cholerae*. *Proc. Natl. Acad. Sci. U.S.A., 88*, 5403-5407.

**Duret, G., and Delcour, A. H. (2010)**. Size and dynamics of the *Vibrio cholerae* porins OmpU and OmpT probed by polymer exclusion. *Biophys. J., 98*, 1820-1829.

**Ehrmann, M., and Clausen, T. (2004)**. Proteolysis as a regulatory mechanism. *Annu. Rev. Genet., 38*, 709-724.

**Fan, F., Jabeen, N., Birdwell, L. D., Zhu, J., and Kan, D. (2014)**. Enhanced interaction of *Vibrio cholerae* virulence regulators TcpP and ToxR under oxygen-limiting conditions. *Infect. Immun., 82*, 1676-1682.

**Faruque, S. M., Albert, M. J., and Mekalanos, J. J. (1998)**. Epidemiology, gentics, and ecology of toxigenic *Vibrio cholerae*. *Microbiol. Mol. Rev, 62*, 1301-1314.

**Faruque, S. M., and Nair, G. B. (2002)**. Molecular ecology of toxigenic *Vibrio cholerae*. *Microbiol. and Immun., 46*, 59-66.

**Fengler V.H.I., Boritsch, E. C., Tutz, S., Seper, A., Ebner, H., Roier, S., et al. (2012)**. Disulfide bond formation and ToxR activity in *Vibrio cholerae*. *Plos One, 7*, e47756.

**Flynn, J. M., Levchenko, I., Sauer, R. T., and Baker, T. A. (2004)**. Modulating substrate choice: The SspB adaptor delivers a regulator of the extracytoplasmic-stress response to the AAA+ protease ClpXP for degradation. *Genes Dev., 18*, 2292-2301.

**Frater, R., and O'Brien, P. C. (1981)**. Role of chemotaxis in the association of motile bacteria with intestinal mucosa: Fitness and virulence of nonchemotactic *Vibrio cholerae* mutants in infant mice. *Infect. Immun., 34*, 222-233.

**Gardel, C. L., and Mekalanos, J. J. (1996)**. Alterations in *Vibrio cholerae* motility phenotypes correlate with changes in virulence facter expression. *Infect. Immun., 64*, 2246-2255.

**Gill, D. M., and Rapport, R. S. (1979)**. Origin of the enzymatically active A1 fragment of cholera toxin. *J Infect. Dis., 139*, 674-680.

**Goss, T. J., Morgan, S. J., French, E. L., and Krukonis, E. S. (2013)**. ToxR recognizes a direct repeat element in the *toxT, ompU, ompT*, and *ctxA* promoters of *Vibrio cholerae* to regulate transcription. *Infect. Immun., 81*, 884-895.

**Grigorova, I. L., Chaba, R., Zhang, H. J., Alba, B. M., Rhodium, V., Herman, C., et al. (2004)**. Fine-tuning of the *Escherichia coli* sigmaE envelope stress response relies on multiple mechanisms to inhibit signal-dependent proteolysis of the transmembrane anti-sigma factor, RseA. *Genes Dev., 18*, 2686-2697.

**Gross, C. A., Lonetto, M., and Losick, R. (1992)**. Bacterial sigma factors. in transcriptional regulation yamamoto, K.; McKnight, S., eds. *Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press,* 129-176.

**Gruber, T. M., and Bryant, D. A. (1997)**. Molecular systematic studies of eubacteria, using sigma70-type sigma factors group 1 and group 2. *J. Bacteriol., 179*, 1734-1747.

**Gruber, T. M., and Gross, C. A. (2003)**. Multiple sigma subunits and the partitioning of bacterial transcription space. *Anna. Rev. Microbiol., 57*, 441-466.

**Guerrant, R. L., Carneiro-Filho, B. A., and Willingham, R. A. (2003)**. Cholera, diarrhea, and oral rehydration therapy: Triumph and indictment. *Clin. Infect. Dis., 37*, 398-405.

**Gupta, S., and Chowdhury, R. (1997)**. Bile affects production of virulence factors and motility of *Vibrio cholerae*. *Infect. Immun., 65*, 1131-1134.

**Haas, B. L., Matson, J. S., DiRita, V. J., and Biteen, J. S. (2015)**. Single-molecule tracking in live *Vibrio cholerae* reveals that ToxR recruits the membrane-bound virulence regulator TcpP to the *toxT* promoter. *Mol. Microbiol., 96*, 4-13.

**Haines-Menges, B., Whitaker, W. B., and Boyd, E. F. (2014)**. Alternative sigma factor RpoE is important for *Vibrio parahaemolyticus* cell envelope stress response and intestinal colonization. *Infect. Immun., 82*, 3667-3677.

**Hanahan, D. (1883)**. Studies of transformation of *Escherichia coli* with plasmids. *J. Mol. Bio., 166*, 557-580.

**Harris, J. B., LaRocque, R. C., Qadri, F., Ryan, E. T., and Calderwood, S. B. (2012)**. Cholera. *Lancet, 379*, 2466-2476.

**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. U.S.A., 95*, 730-734.

**Häse, C. C., and Mekalanos, J. J. (1999)**. Effects of changes in membrane sodium flux on virulence gene expression in *Vibrio cholerae*. *Proc. Natl. Acad. Sci. U.S.A., 96*, 3183-3187.

**Hayden, J. D., Ades, S. E., and Sandler, S. (2008)**. The extrcytoplasmic stress factor $\sigma^E$ is required to maintain cell envelope integrity in *Escherichia coli*. *Plos One, 3*, e1573.

**Heidelberg, J. F., Eisen, J. A., Nelson, W. C., Clayton, R., Gewinn, M. L., Dodson, R. J., et al. (2000)**. DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. *Nature, 406*, 477-483.

**Helmann, J. D., and Chamberlin, M. J. (1988)**. Structure and function of bacterial sigma factors. *Annu. Rev. Biochem., 57*, 839-872.

**Helmann, J. D. (2002)**. The extracytoplasmic function (ECF) sigma factors. *Adv. Microb. Physiol., 46*, 47-110.

**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 regulon are essential for *Vibrio cholerae* pathogenesis in humans. *J. Exp. Med., 168*, 1487-1492.

**Higgins, D. E., Nazareno, E., and DiRita, V. J. (1992)**. The virulence gene activator ToxT from *Vibrio cholerae* is a member of the AraC familiy of transcriptional activators. *J. Bacteriol., 174*, 6974-6980.

**Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989)**. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene, 77*, 51-59.

**Hornick, R. B., Music, S. I., and Wenzel, R. (1971)**. The broad street pump revisited: Response of volunteers to ingested cholera vibrios. *Bull. N. Y. Awad. Med., 47*, 1181-1191.

**Hughes, K. T., and Mathee, K. (1998)**. The anti-sigma factors. *Annu. Rev. Microbiol., 52*, 231-286.

**Hung, D. T., and Mekalanos, J. J. (2005)**. Bile acids induce cholera toxin expression in *Vibrio cholerae* in a ToxT-independent manner. *Proc. Natl. Acad. Sci. U.S.A., 102*, 3028-3033.

**Huq, A. e. a. (1983)**. Ecological relationships between *Vibrio cholerae* and planktonic crustacean codepods. *Appl. Environ. Microbiol., 45*, 275-283.

**Iredell, J. R., Stroher, U. H., Ward, H. M., and Manning, P. A. (1998)**. Lipopolysaccheride O-antigen expression and the effect of its absence on virulence in *rfb* mutants of *Vibrio cholerae* O1. *FEMS Immunol. Med. Microbiol., 20*, 45-54.

**Jardetzky, O. (1963)**. Studies on the mechanisms of action of chloramphenicol - the conformation of chloramphenicol in solution. *J. Biol. Chem., 238*, 2498-2508.

**Johnson, J. A., Salles, C. A., Panigraphi, P., Albert, M. J., Wright, A. C., Johnson, R. J., et al. (1994)**. *Vibrio cholerae* O139 synonym bengal is closely related to *Vibrio cholerae* el tor but has important differences. *Infect. Immun., 62*, 2108-2110.

**Kadokura, H., and Beckwith, J. (2010)**. Mechanisms of oxidative protein folding in the bacterial cell envelope. *Antioxidants Redox Signal., 13*, 1231-1246.

**Kanehara, K., Akiyama, A., and Ito, K. (2001)**. Characterization of the *yaeL* gene product and its S2P-protease motifs in *Escherichia coli*. *Gene, 281*, 71-79.

**Kanehara, K., Ito, K., and Akiyama, Y. (2002)**. YaeL (EcfE) activates the sigma(E) pathway of stress response through a site-2 cleavage of anti-sigma(E), RseA. *Genes Dev., 16*, 2147-2155.

**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. *Bull. Korean Chem. Soc., 11*, 1511-1512.

**Karaolis, D. K., 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. U.S.A., 95*, 3134-3139.

**Kim, D. Y., Kwon, E., Choi, J., Hwang, H. Y., and Kim, K. K. (2010)**. Structural basis for the negative regulation of bacterial stress response by RseB. *Protein Sci., 19*, 1258-1263.

**Kim, M.K., and Kang, Y.K. (1999)**. Positional preference of proline in α-helices. *Protein Sci., 8*, 1492-1499.

**King, C. A., and van Hennigen, W. E. (1973)**. Deactivation of cholera toxin by a sialidase resistant monosialosylganglioside. *J. Infect. Dis., 127*, 639-647.

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

**Klomsiri, C., Kaiplus, P. A., and Poole, L. B. (2011)**. Cysteine-based redox switches in enzymes. *Antioxidants Redox Signal., 14*, 1065-1077.

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

**Koch, R., and Carter, K. C. (1987)**. Essays of robert koch. *Greenwood Press, N.Y., U.S.A.,*

**Kojima, S., Yamamoto, K., Kawagishi, I., and Hommo, M. (1999)**. The polar flagellar motor of *Vibrio cholerae* is driven by an $Na^+$ motive force. *J. Bacteriol., 181*, 1927-1930.

**Kolmaar, H., Hennecke, F., Götze, K., Ganzer, B., Vogt, B., Mayer, F., et al. (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*, 3895-3904.

**Kovacikova, G., and Skorupski, K. (1999)**. A *Vibrio cholerae* LysR homolog, AphB cooperates with AphA at the *tcpPH* promotor to activate expression of the ToxR virulence cascade. *J. Bacteriol., 181*, 4250-4256.

**Kovacikova, G., and Skorupski, K. (2002)**. The alternative sigma factor sigma(E) plays important role in intestinal survival and virulence in *Vibrio cholerae*. *Infect. Immun., 70*, 5355-5362.

**Kovacikova, G., Lin, W., and Skorupski, K. (2010)**. The LysR-type virulence activator AphB regulates the expression of genes in *Vibrio cholerae* in response to low pH and anaerobiosis. *J. Bacteriol., 192*, 4181-4191.

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

**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*, 157-165.

**Laemmli, U. K. (1970)**. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 15*, 680-685.

**LaRocque, R. C., Krastins, B., Harris, J. B., Lebrun, L. M., Parker, K. C., Chase, M., et al. (2008)**. Proteomic analysis of *Vibrio cholerae* in human stool. *Infect. Immun., 76*, 4145-4151.

**Lasica, A. M., and Jagusztyn-Krynicka, E.K. (2007)**. The role of Dsb proteins of Gram-negative bacteria in the process of pathogenesis. *FEMS Microbiol. Rev., 31,* 626-636.

**Lembke, M. (2016)**. The activation mechanism of ToxR, a main virulence regulator in *Vibrio cholerae*. *Masterarbeit,* 52-60.

**Li, C. C., Craford, 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), 1589.

**Lindenbaum, J., Greenough, W. B., and Islam, M. R. (1967)**. Antibiotic therapy of cholera in children. *Bull World Health Organ., 37*, 529-538.

**Liu, Z., Yang, M., Meterfreund, G. L., Tost, A. M., Selamoglu, N., Daldal, F., et al. (2011)**. *Vibrio cholerae* anaerobic induction of virulence gene expression is controlled by thiol-based switches of virulence regulator AphB. *Proc. Natl. Acad. Sci. U.S.A., 108*, 810-815.

**Liu, Z., Wang, H., Zhou, Z., Nasser, N., Xiang, F., Kan, B., et al. (2016)**. Differential thiol-based switches jump-start *Vibrio cholerae* pathogenesis. *Cell Rep., 14*, 347-354.

**Lonetto, M., Gribskov, M., and Gross, C. A. (1992)**. The $\sigma^{70}$ family: Sequence conversation and evolutionary relationships. *J. Bacteriol., 174*, 3843-3849.

**Lonetto, M., Brown, K. L., Rudd, K. E., and Buttner, M. J. (1994)**. Analysis of the *streptomyces coelicolor sigE* gene reveals the existence of a subfamily of eubacterial RNA polymerase σ factors involved in the regulation of extracytoplasmic functions. *Proc. Natl. Acad. Sci. U.S.A., 91*, 7573-7577.

**Louis, P., and O'Byrne, C. P. (2010)**. Life in the gut: Microbial responses to stress in the gastrointestinal tract. *Sci. Progr., 93*, 7-36.

**Mandal, S., Mandal, M. D., and Pal, N. K. (2011)**. Cholera: A great global concern. *Asian Pacific Journal of Tropical Med.* , 573-580.

**Manning, P. A. (1997)**. The *tcp* gene cluster of *Vibrio cholerae*. *Gene, 192*, 63-70.

**Manoil, C (1991)**. Analysis of membrane protein topology using alkaline phosphatase and beta-galactosidase gene fusions. *Meth. in Cell Biol., 34*, 61-75.

**Marrero, K., Sánchez, A., Rodríguez-Ulloa, A., González, L.J., Castellanos-Srra, L., Paz-Lago, D., Campos, J., Rodríguez, B.L., Suzarte, E., Ledón, T., Padrón, G., and Fando, R. (2009)**. Anaerobic growth promotes synthesis of colonization factors encoded at the *Vibrio* pathogenicity island in *Vibrio cholerae* El Tor. *Res. Microbiol. 160,* 48-56.

**Martínez-Hackert, E., and Stock, A. M. (1997)**. Structural relationships in the OmpR family of winged-helix transcription factors. *J. Mol. Biol., 13*, 301-312.

**Mathur, J., and Waldor, M. K. (2004)**. The *Vibrio cholerae* ToxR-regulated porin OmpU confers resistance to antimicrobial peptides. *Infect. Immun., 72*, 3577-3583.

**Mathur, J., Davis, B. M., and Waldor, M. K. (2007)**. Antimicrobial peptides activate the *Vibrio cholerae* sigmaE regulon through an OmpU-depending signalling pathway. *Mol. Microbiol., 63*, 848-858.

**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. U.S.A., 102*, 16403-16408.

**Mecsas, J., Rouviere, P. E., Erickson, J. W., Donohue, T. J., and Gross, C. A. (1993)**. The activity of $\sigma^{E}$, an *Escherichia coli* heat-inducible σ-factor, is modulated by expression of outer membrane proteins. *Genes Dev., 7*, 2618-2628.

**Mescas, J., Welch, R., Ericsson, J. W., and Gross, C. A. (1995)**. Identification and characterization of an outer membrane protein, OmpX, in *Escherichia coli* that is homologous to a family of outer membrane proteins including ail of *yersinia enterocolitica*. *J. Bacteriol., 177*, 799-804.

**Mekalanos, J. J., Collier, R. J., and Roming, W. R. (1979)**. Enzymatic activity of cholera toxin. II. relationships to proteolytic processing, disulphide bond reduction, and subunit composition. *J. Biol. Chem., 254*, 5855-5861.

**Mey, A. R., Craig, S. A., and Payne, S. M. (2012)**. Effects of amino acid supplementation in porin expression and ToxR levels in *Vibrio cholerae*. *Infect. Immun., 80*, 518-528.

**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. U.S.A., 81*, 3471-3475.

**Miller, V. L., and Mekalanos, J. J. (1985)**. Genetic analysis of the cholera toxin-positive regulatory gene *toxR*. *J. Bacteriol., 163*, 580-585.

**Miller, V. L., Taylor, R. K., and Mekalanos, J. J. (1987)**. Cholera toxin transcriptional activator ToxR is a transmembrane DNA binding protein. *Cell, 48*, 271-279.

**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*, 2575-2783.

**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 promotor. *J. Bacteriol, 171*, 1288-1293.

**Miller, V. L., and Mekalanos, J. J. (1989)**. 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, 171*, 1288-1293.

**Missinkas, D., Georgopoulos, C., and Raina, S. (1993)**. Identification and characterization of the *Escherichia coli* gene *dsbB*, whose product is involved in the formation of disulfide bonds *in vivo*. *Proc. Natl. Acad. Sci. U.S.A., 90*, 7084-7088.

**Missiakas, D., Mayer, M. P., Lemaire, M., Georgopoulos, C., and Raina, S. (1997)**. Modulation of the *Escherichia coli* sigmaE (RpoE) heat-shock transcription-factor activity by the RseA, RseB and RseC proteins. *Mol. Microbiol., 24*, 355-371.

**Missiakas, D., and Raina, S. (1998)**. The extracytoplasmic function sigma factors: Role and regulation. *Mol. Microbiol., 28*

**Molla, A. M., Rahman, M., Sarker, S. A., Sack, D. A., and Molla, A. (1981)**. Stool electrolyte content and purging rates in diarrhea caused by rotavirus, enterotoxigenic *E. coli*, and *V. cholerae* in children. *J Pediatr., 98*, 835-838.

**Morgan, S. J., French, E. L., Thomson, J. L., Seaborn, C. P., Shivela, C. A., and Krukonis, E. S. (2015)**. Formation of an intramolecular periplasmic disulfide bond in TcpP protects TcpP and TcpH from degradation in *Vibrio cholerae*. *J. Bacteriol., 198*, 498-509.

**Moss, J., Stanley, S. J., Morin, J. E., and Dixon, J. E. (1980)**. Activation of choleragen by thiol: Protein disulfide oxidoreductase. *J. Biol. Chem., 255*, 11085-11087.

**Nakamoto, H., and Bardwell, J. C. (2004)**. Catalysis of disulfide bond formation and isomerization in the *Escherichia coli* periplasm. *Biochem. Biophys., 1694*, 111-119.

**Nalin, D. R., Cash, R. A., Islam, R., Molla, M., and Phillips, R. A. (1968)**. Oral maintenance therapy for cholera in adults. *Lancet, 2*, 370-373.

**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*, 6807-6814.

**Ottemann, K. M., and Mekalanos, J. J. (1996)**. The ToxR protein of *Vibrio cholerae* forms homodimers and herterodimers. *J. Bacteriol., 178*, 156-162.

**Pallen, M. J., and Wren, B. W. (1997)**. The HtrA family of serine proteases. *Mol. Microbiol., 26*, 209-221.

**Pearson, G. D., 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. U.S.A., 90*, 3750-3754.

**Peek, J. A., and Taylor, R. K. (1992)**. Characterization of a periplasmic thiol:Disulfide interchange protein required for the functional maturation of secreted virulence factors of *Vibrio cholerae*. *Proc. Natl. Acad. Sci. U.S.A., 89*, 6210-6214.

**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*, 2822-2829.

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

**Pfau, J. D., and Taylor, R. K. (1998)**. Mutations in *toxR* and *toxS* that separate transcriptional activation from DNA binding at cholera toxin gene promotor. *J. Bacteriol., 180*, 4724-4733.

**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. U.S.A., 97*, 10220-10224.

**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*, 1491-1497.

**Provenzano, D., Lauriano, C. M., and Klose, K. E. (2001)**. Characterization of the role of the ToxR-modulated outer membrane porins OmU and OmpT in *Vibrio cholerae* virulence. *J. Bacteriol., 183*, 3652-3662.

**Raivio, T. L., and Silhavy, T. J. (2001)**. Periplasmic stress and ECF sigma factors. *Annu. Rev. Microbiol., 55*, 591-624.

**Reidl, J., and Klose, K. E. (2002)**. *Vibrio cholerae* and cholera: Out of the water and into the host. *FEMS Microbiol. Rev., 26*, 125-139.

**Rezuchova, B., Miticka, H., Homerova, D., Roberts, M., and Kormanec, J. (2003)**. New member of the *Escherichia coli* sigmaE regulon idientified by a two-plasmid system. *FEMS Mirobiol. Lett., 225*, 1-7.

**Rhodius, V. A., Sah, W. C., Nonaka, G., West, J., and Gross, C. A. (2005)**. Conserved and variable functions of the $\sigma^E$ stress response related genomes. *PLoS Biol., 4*, e2.

**Rietsch, A., Belin, D., Martin, N., and Beckwith, J. (1996)**. An *in vivo* pathway for disulfide bond isomerization in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A., 93*, 13048-13053.

**Rouvière, P. E., De Las Peñas, A., Mecsas, J., Lu, C. Z., Rudd, K. E., and Gross, C. A. (1995)**. *rpoE*, the gene encoding the second heat-shock sigma factor, sigma E, in *Escherichia coli*. *EMBO. J., 14*, 1032-1042.

**Rowley, G., Spector, M., Kormanec, J., and Roberts, M. (2006)**. Pushing the envelope: Extracytoplasmic stress responses in bacterial pathogens. *Nature Rev. Microbiol., 4*, 383-394.

**Ruiz, N., and Silhavy, T. J. (2005)**. Sensing external stress: Watchdogs of the *Escherichia coli* cell envelope. *Curr. Opin. Microbiol., 8*, 122-126.

**Ruiz, N., Kahne, D., and Silhavy, T. J. (2006)**. Advances in understanding outer-membrane biogenesis. *Nature Rev. Microbiol., 4*, 57-66.

**Sack, D. A., Sack, R. B., Nair, G. B., and Siddique, A. K. (2004)**. Cholera. this review provides an overview of cholera, from the disease itself to its epidemiology and virulence gene regulation. *Lancet, 363*, 223-233.

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

**Sandkvist, M., Michel, L. O., Hough, L. P., Morales, V. M., Bagdasarian, M., Koomey, M., et al. (1997)**. Secretion and outer membrane biogenesis in *Vibrio choelrae*. *J. Bacteriol., 179*, 6994-7003.

**Sanger, F., Nicken, S., and Coulson, A. R. (1977)**. DNA sequencing with chain-termination inhibitors. *Proc. Natl. Acad. Sci. U.S.A., 74*, 5463-5467.

**Schild, S., Nelson, E. J., and Camilli, A. (2008)**. Immunization with *Vibrio cholerae* outer membrane vesicles induces protective immunity in mice. *Infect. Immun., 76*, 4554-4563.

**Schuhmacher, D. A., and Klose, K. E. (1999)**. Environmental signals modulate ToxT-dependent virulence factor expression in *Vibrio cholerae*. *J. Bacteriol., 181*, 1508-1514.

**Segatori, L., Murphy, L., Arredondo, S., Kadokura, H., Gilbert, H., Beckwith, J., et al. (2006)**. Conserved role of the linker alpha-helix of the bacterial disulfide isomerase DsbC in the avoidance of misoxidation by DsbB. *J. Biol. Chem., 281*, 4911-4919.

**Sevier, C. S., and Kaiser, C. A. (2002)**. Formation and transfer of disulphide bonds in living cells. *Nat. Rev. Mol. Cell Biol., 3*, 836-847.

**Shears, P. (1994)**. Cholera. *Ann. Trop. Med. Parasitol., 88*, 109-122.

**Simonet, V. C., Balsé, A., Klose, K. E., and Delcour, A. H. (2003)**. The *Vibrio cholerae* porins OmpU and OmpT have distincr channel properties. *J. Biol. Chem., 278*, 17539-17545.

**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*, 763-771.

**Sone, M., Kishigami, S., Yoshihisa, T., and Ito, K. (1997)**. Roles of disulfide bonds in bacterial alkaline phosphatase. *J. Biol. Chem., 272*, 6174-6178.

**Thomson, J. J., and Wither, J. H. (2014)**. Bicarbonate increases binding affinity of *Vibrio cholerae* ToxT to virulence gene promoters. *J. Bacteriol., 196*, 3872-3880.

**Vyvoda, O. S., Coleman, R., and Holdsworth, G. (1977)**. Effects of different bile salts upon the composition and morphology of a liver plasma membrane preparation. deoxycholare is more membrane damaging than cholate and its conjugates. *Biochem. Biophys. Acta, 465*, 68-76.

**Wachsmute, K. I., Blake, P. A., and Olsvik, Ø. (1994)**. *Vibrio cholerae* and cholera: Molecular to global perspectives. *ASM Press, Washington, DC,*

**Waldor, M. K., Colwell, R., and Mekalanos, J. J. (1994)**. The *Vibrio cholerae* O139 serogroup antigen includes an O-antigen capsule and lipopolysaccharide virulence determinants. *Proc. Natl. Acad. Sci. U.S.A., 91*, 11388-11392.

**Waldor, M. K., and Mekalanos, J. J. (1996)**. Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science, 272*, 1910-1914.

**Waller, P. R. H., and Sauer, R. T. (1996)**. Characterization of *degQ* and *degS*, *Escherichia coli* genes encoding homologs of the DegP protease. *J. Bacteriol., 178*, 1146-1153.

**Walsh, N. P., Alba, B. M., Bose, B., Gross, C. A., and Sauer, R. T. (2003)**. OMP peptide signals initiate the envelope-stress response by activating DegS protease via relief of inhibition mediated by its PDZ domain. *Cell, 113*, 61-71.

**Weber, G. G., Kortmann, J., Narberhaus, F., and Klose, K. E. (2014)**. RNA thermometer control temperature.dependent virulence factor expression in *Vibrio cholerae*. *Proc. Natl. Acad. Sci. U.S.A., 111*, 14241-14246.

**Wernicke, N. L. B., Chinnapen, D. J. -., Cho, J. A., and Lenker, W. I. (2010)**. Cholera toxin: An intracellular journey into the cytosol by way of endoplasmic reticulum. *Toxins, 2*(310), 325.

**WHO (2015a)**. Cholera. *Fact Sheet, 107*

**WHO (2015b)**. Weekly epidemiological record cholera 2014.*40*, 517-544.

**Wilken, C., Kitzing, K., Kurzbauer, R., Ehrmann, M., and Clausen, T. (2004)**. Crystal structure of the DegS stress sensor: How a PDZ domain recognizes misfolded protein and activates a protease. *Cell, 415*, 389-395.

**Witten, 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*, 1779-1789.

**Wolfe, A. D., and Hahn, F. E. (1965)**. Mode of action of chloramphenicol. IX. effects of chloramphenicol upon a ribosomal amino acid polymerization system and its binding to bacterial ribosome. *Biochem. Biophys. Acta, 95*, 146-155.

**Wosten, M. M. S. M. (1998)**. Eubacterial sigma factors. *FEMS Microbiology Reviews, 22*, 127-150.

**Yang, M., Liu, Z., Hughes, C., Ster, A. M., Wang, H., Zhong, Z., et al. (2013)**. Bile salt-induced intermolecular disulfide bond formation activates *Vibrio cholerae* virulence. *Proc. Natl. Acad. Sci. U.S.A., 110*, 2348-2353.

**Yu, J., Webb, H., and Hirst, T. R. (1992)**. A homologue of the *Escherichia coli* DsbA protein involved in disulphide bond formation is required for enterotoxin biogenesis in *Vibrio cholerae*. *Mol. Microbiol., 6*, 1949-1958.

**Zaun, A., Missiakas, D., Raina, S., and Creighton, T. E. (1995)**. Structural and functional characterization of DsbC, a protein involved in disulfide bond formation in *Escherichia coli*. *Biochem., 34*, 5075-5089.

**Zhu, J., Miller, M. B., Vance, R. E., Dziejman, M., Bassler, B. L., and Mekalanos, J. J. (2002)**. Quorum-sensing regulators control virulence gene expression in *Vibrio cholerae*. *Proc. Natl. Acad. Sci. U.S.A., 99*, 3129-3134.

# VI Abbreviations

| | |
|---|---|
| °C | degree Celsius |
| A | Ampere |
| aa | amino acid |
| ADP | adenosine diphosphate |
| ap | ampicillin |
| $ap^r$ | ampicillin resistant |
| APS | ammonium persulphate |
| bp | base pair |
| cAMP | cyclic adenosine monophosphate |
| CAPS | (3[(3-cholamidopropyl)-dimethylamino]-propansulphate |
| cfu | colony forming unit |
| cm | chloramphenicol |
| $cm^r$ | chloramphenicol resistant |
| CRP | cAMP receptor protein |
| CT | cholera toxin |
| CTXΦ | cholera toxin encoding bacteriophage |
| $ddH_2O$ | double deionized water |
| DNA | deoxyribonucleic acid |
| dNTP | deoxynucleoside triphosphate |
| Dsb | disulphide bond |
| DTT | 1,4 dithiothreitol |
| EDTA | ethylenediaminetetraacetic acid |
| e.g. | for example (lat. exempli gratia) |
| EtOH | ethanol |
| fw | forward |
| g | gramm |
| g | times gravity |
| h | hour |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| i.e. | that is (lat. id est) |
| IPTG | isopropyl-β-thiogalactopyranosid |
| KAc | potassium acetate |
| kb | kilo base pair |

| | |
|---|---|
| kDa | kilo Dalton |
| km | kanamycin |
| $km^r$ | kanamycin resistant |
| l | liter |
| LB | lysogeny broth |
| LPS | lipopolysaccharide |
| m | meter |
| m | milli ($10^{-3}$) prefix |
| M | mole per liter |
| MeOH | methanol |
| min | minute |
| mRNA | messenger RNA |
| MSHA | mannose sensitive type IV pilus |
| n | nano ($10^{-9}$) prefix |
| NRES | amino acids: asparagine, arginine, glutamate and serine |
| $OD_{600}$ | optical density at 600 nm |
| OMP | outer membrane protein |
| ONC | overnight culture |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| qRT-PCR | quantitative real time PCR |
| RNA | ribonucleic acid |
| rpm | revolutions per minute |
| rRNA | ribosomal RNA |
| RT | room temperature |
| rv | reverse |
| SDS | sodium dodecyl sulphate |
| sec | second |
| sm | streptomycin |
| $sm^r$ | streptomycin resistant |
| TBS | Tris-buffered saline |
| TCP | toxin co-regulated pilus |
| TEMED | tetramethylethylenediamine |
| TM | transmembrane |
| $ToxR^{CC}$ | $ToxR^{C236SC293S}$ (ToxR double-cysteine mutant) |

| | |
|---|---|
| $ToxS^{L33S}$ | ToxS leucine to serine substitution |
| Tris | 2-Amino-2-hydroxymethyl-propane-1,3-diol |
| UV | ultra-violette |
| V | volt |
| VPI | *Vibrio* pathogenicity island |
| VS | amino acids: valine and serine |
| WCE | whole cell extract |
| WHO | world health organization |
| wHTH | winged helix-turn-helix |
| WT | wild type |
| YDF | amino acids: tyrosine, aspartic acid and phenylalanine |
| µ | micro ($10^{-6}$) prefix |
| $\sigma^E$ | Sigma factor E |