The regulatory circuit of natural competence for transformation in the human pathogen *Vibrio cholerae*

THÈSE N^{O} 6078 (2014)

PRÉSENTÉE LE 7 FÉVRIER 2014

À LA FACULTÉ DES SCIENCES DE LA VIE
UNITÉ DE LA PROF. BLOKESCH
PROGRAMME DOCTORAL EN BIOTECHNOLOGIE ET GÉNIE BIOLOGIQUE

ÉCOLE POLYTECHNIQUE FÉDÉRALE DE LAUSANNE

POUR L'OBTENTION DU GRADE DE DOCTEUR ÈS SCIENCES

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Suisse
2014

To my Mom…

Acknowledgments

I would like to thank my supervisor Prof. Melanie Blokesch for her great advise and support during all the four years of my Ph.D studies. I admire and thank her for her grandiose ability to motivate me at anytime, and for transmitting me the passion for her job. My work experience under her guidance was extremely important to become a researcher, to make my future working decisions and to render my Ph.D. an important step of my life.

I would also like to thank all Blokesch research group, in particular Dr. Juliane Kühn, Patrick Seitz, Marciano Marisa Wynn, Allyn Knox, Sandrine Borgeaud, Dr. Charles Van der Henst , Dr. Lisa Metzger, Tiziana Scrignari and the former members Olga de Souza Silva and Gaia Suckow. It was a great pleasure to work and collaborate with them and to share enjoyable moments inside and outside the laboratory.

I am also very grateful to Prof. John McKinney and Prof. Stewart Cole. They have been very helpful mentors, they gave me important professional suggestions and supported and encouraged my future career plans.

I warmly thank all the jury members for having accepted to be part of my thesis committee and for having critically review my work.

I am very grateful too to the members of the neighbor laboratories for their collaborative and friendly working spirit; in particular I want to thank Dr. Claudia Sala and Dr. Jefferey Chen for very helpful discussions and for sharing knowledge and technologies.

A big thank to all my friends in Lausanne for all the amusing, enjoyable and uncountable times we spent together.

And finally I want to thank my loved ones for being always present in my living and for strongly supporting my decisions.

Abstract

The human pathogen *Vibrio cholerae* is the causative agent of the disease cholera, which is far from extinct. This Gram-negative bacterium is a normal member of aquatic environments where it lives in association with the chitinous exoskeleton of small zooplankton. During this association, the bacterium initiates the developmental program of natural competence for transformation. This developmental state represents a mechanism of horizontal gene transfer and consists of the ability to take up free DNA from the environment and to recombine it with the bacterial chromosome. The inducer of natural competence is chitin, the main component of the exoskeleton of zooplankton and the most abundant polymer in the aquatic environment. As natural competence for transformation could lead to the evolution of more pathogenic bacterial strains it is important to understand the molecular mechanisms behind its activation. The aims of this thesis were to investigate the expression pattern of the competence genes within the bacterial population, and to decipher how natural competence is regulated.

Here, we investigated the expression of the competence genes at the single cell level by using fluorescent transcriptional reporter fusions. We detected and quantified the gene expression by epifluorescence microscopy and flow cytometry, and demonstrated that the competence regulon is uniformly expressed within the population under homogeneous competence-inducing growth conditions. Interestingly, we observed a heterogeneous expression pattern when the bacteria were grown on chitin surfaces, likely due to the heterogeneity of the signaling molecules involved in the initiation of natural competence.

Apart from chitin-sensing and degradation two other pathways are responsible for the establishment of natural competence for transformation in *V. cholerae*: quorum sensing (QS) and carbon catabolite repression (CCR). We focused on QS and demonstrated that only a subset of the competence genes is co-regulated

by QS. We then identified a new transcriptional regulator, QstR, whose expression is dependent on the proteins HapR and TfoX, the master regulators of QS and transformation, respectively. And indeed, we could show that the HapR protein directly binds the promoter region of *qstR in vitro*. To conclude this part of the project we demonstrated that QstR is an intermediate regulator between HapR and the QS-dependent competence genes, which links the pathways of QS to chitin-sensing and degradation.

Finally we investigated the role of the third pathway involved in natural competence, CCR. The cAMP receptor protein (CRP) is the global regulator of this pathway and is necessary for natural competence to occur. It was suggested for another naturally competent bacterium, *Haemophilus influenzae,* that CRP activates the transcription of the competence genes by binding in conjunction with the transformation regulator Sxy (or TfoX in *V. cholerae*) to a competence-specific CRP binding site called CRP-S. Notably, we identified a putative CRP-S site within the promoter region of the competence gene *comEA*. By site-directed mutagenesis we showed that this motif plays a role in the expression of *comEA* and in natural competence for transformation.

Overall these findings allowed us to significantly elucidate the regulatory network driving natural competence for transformation in *V. cholerae*. We concluded this thesis with a new working model highlighting the crosstalk among all the three regulatory pathways involved in the regulation of this developmental program.

Keywords: *Vibrio cholerae*, natural competence, chitin, horizontal gene transfer, quorum sensing, carbon catabolite repression, cAMP receptor protein, CRP-S site.

Riassunto

Il patogeno umano *Vibrio cholerae* è l'agente del colera, malattia ben lontana dall'essere estinta. Questo batterio Gram-negativo è solito degli ambienti acquatici dove vive associato con l'esoscheletro chitinoso delle piccole specie di zooplancton. Durante quest'associazione, il batterio inizia il programma di sviluppo della competenza naturale per la trasformazione batterica. Questo stato di sviluppo rappresenta un meccanismo di trasferimento genico orizzontale e consiste nella capacità di acquisire DNA dall'ambiente esterno e di effettuare una ricombinazione tra quest'ultimo e il cromosoma batterico. L'induttore della competenza naturale è la chitina, il componente principale dell'esoscheletro dello zooplancton e il principale polimero degli ambienti acquatici. Dato che la competenza naturale per la trasformazione batterica potrebbe dirigere l'evoluzione di un maggior numero di ceppi batterici patogeni, è importante capire i meccanismi molecolari che risiedono dietro la sua attivazione. Gli scopi di questa tesi sono stati quelli di investigare il pattern di espressione dei geni della competenza all'interno della popolazione batterica, e di decifrare come la competenza naturale fosse regolata.

Dunque, abbiamo investigato l'espressione dei geni della competenza a livello delle singole cellule attraverso l'uso di costrutti trascrizionali fluorescenti. Abbiamo rivelato e quantificato l'espressione genica tramite microscopia a epifluorescenza e citofluorimetria, e dimostrato che l'unità genica regolativa della competenza è uniformemente espressa all'interno della popolazione in presenza di condizioni di crescita che inducano la competenza in modo omogeneo. Da notare, abbiamo osservato un pattern di espressione eterogeneo quando i batteri sono stati cresciuti su superfici di chitina, probabilmente dovuto all'eterogeneità delle molecole segnale coinvolte nell'avviamento della competenza naturale.

Oltre chitin-sensing e -degradation, altri due pathway sono responsabili dello stabilimento della competenza naturale per la trasformazione batterica in *V.*

cholerae: quorum sensing (QS) e carbon catabolite repression (CCR). Ci siamo focalizzati sul QS e abbiamo dimostrato che solo una parte dei geni della competenza è co-regolato da questo pathway. A seguire abbiamo identificato un nuovo regolatore trascrizionale, QstR, la cui espressione è dipendente dalle proteine HapR e TfoX, master regolatori rispettivamente del QS e della trasformazione batterica. E infatti, abbiamo potuto mostrare che la proteina HapR lega direttamente la regione promotore del gene *qstR in vitro*. Per concludere questa parte del progetto abbiamo dimostrato che la proteina QstR è un regolatore intermedio tra la proteina HapR e i geni della competenza dipendenti dal QS, e che QstR connette i pathway del QS e del chitin-sensing e -degradation.

Infine abbiamo investigato il ruolo del terzo pathway coinvolto nella competenza naturale, CCR. La proteina recettore del cAMP (CRP) è il regolatore globale di questo pathway ed è necessaria affinché la competenza naturale si verifichi. In un altro batterio naturalmente competente, *Haemophilus influenzae*, è stato proposto che la proteina CRP, assieme al regolatore della trasformazione batterica Sxy (o TfoX in *V. cholerae*), attiva la trascrizione dei geni della competenza tramite il legame di siti specifici per la competenza chiamati CRP-S. Da notare, abbiamo identificato un ipotetico sito CRP-S all'interno della regione promotore del gene della competenza *comEA*. Attraverso mutagenesi sito-diretta abbiamo mostrato che questo motivo ha un ruolo nell'espressione di *comEA* e nella competenza naturale per la trasformazione batterica.

Complessivamente queste scoperte ci hanno permesso di delucidare il network regolatore che guida la competenza naturale per la trasformazione batterica in *V. cholerae*. Abbiamo concluso questa tesi con un nuovo working model che mette in rilievo il crosstalk tra tutti e tre i pathway regolatori coinvolti in questo programma di sviluppo.

Parole chiave: *Vibrio cholerae*, competenza naturale, chitina, trasferimento genico orizzontale, quorum sensing, repressione da catabolita, proteina recettore del cAMP, siti CRP-S.

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Abbreviations

AC or CyaA	Adenylate Cyclase
AI-2	Autoinducer 2 (universal communication)
bp	Base Pair
cAMP	3',5'- cyclic adenosine monophosphate
CAP	Catabolite Activator Protein (synonym of CRP)
CBP	Chitin Binding Protein
CCR	Carbon Catabolite Repression
CRP	cAMP receptor protein
CRE	Competence Regulatory Element
CAI-1	Cholera Autoinducer 1 (intra-species communication)
CT	Cholera Toxin
EMSA	Electrophoretic Mobility Shift Assay
ER	Endoplasmatic Reticulum
GbpA	N-acetylglucosamine binding protein A
GlcNAc	N-acetylglucosamine
Glc	Glucose
HCD	High Cell Density
HGT	Horizontal Gene Transfer
LCD	Low Cell Density
LPS	Lipopolysaccharide
OD_{600}	Optical Density at 600nm
QS	Quorum Sensing
QstR	QS and TfoX-dependent regulator
RNAP	RNA polymerase
TCP	Toxin Co-regulated Pilus
VPI	*V. cholerae* Pathogenicity Island

Chapter 1.

General Introduction

1.1 Cholera and the etiological agent *Vibrio cholerae*

Cholera is a disease affecting the human intestine caused by the bacterium *Vibrio cholerae*. The dynamics of this disease are quite complex and in order to understand the etiological agent, epidemiological, ecological and evolutionary studies are required. This first section is dedicated to a short description of cholera and its pathogenesis in an effort to link the specific topic of this thesis with the disease.

First described in the nineteenth century, cholera most likely occurred in ancient civilizations and it is still a major public health problem. The disease is transmitted by ingestion of contaminated food and water and can spread quickly in the absence of good hygiene. One fact that contributes considerably to the propagation of the disease is the existence of many infected but asymptomatic individuals (McCormack *et al.*, 1969) that can disseminate the bacteria in the environment for several days after the onset of the unrecognized infection. Infection requires the ingestion of a large number of bacteria and studies conducted in healthy volunteers demonstrated that cholera infection results from an initial inoculum of up to 10^{11} bacteria; however this number drops when bacteria are protected by food or when the acidity of the stomach decreases (Kaper *et al.*, 1995; Cash *et al.*, 1974).

The disease manifests as acute, watery, painless diarrhoea often accompanied by vomiting, and the gravity of the symptoms varies greatly among individuals. In severe cases, the loss of fluid reaches 500 to 1000ml/h and leads to massive dehydration that can cause death in a few hours if patients are not treated promptly (Kaper *et al.*, 1995). The treatment of cholera is straightforward and consists of oral or intravenous infusion of water and electrolytes. The treatment is

successful up to 80% of the cases (WHO, 2012) and the global case-fatality rate is around 1% (1.2% in 2012; WHO, 2012; WHO, 2013). In cholera endemic area hospitalization rate is higher in young children (Glass *et al.*, 1982) and diarrhoeal diseases are estimated to be the second cause of death in children (Bryce *et al.*, 2005). Moreover the genetics of the host contribute to the severity of the illness, for example, individuals with the O blood group are more susceptible to develop severe symptoms even if they do not have increased risk of infection (Tacket *et al.*, 1995; Harris *et al.*, 2005).

According to World Health Organization (WHO) guidelines, severe cholera cases are treated by rehydration and antibiotic treatment to decrease the purging rate and the time of the illness. Following the Haitian cholera outbreak, it was suggested that antibiotic treatments should also be extended to the moderate infection cases to prevent a massive spread of the infection in this country (Nelson *et al.*, 2011), however this topic remains under debate due to the potential emergence of antibiotic resistant strains of *V. cholerae* as previously observed (Mwansa *et al.*, 2007).

Simple recommendations such as drinking clean water, eating cooked food and using proper sanitation are effective in preventing the contraction of the disease. However the socio-economic status of areas where cholera is endemic and epidemic results in poor hygiene and poverty, making it difficult to prevent the disease in developing countries (Waldman *et al.*, 2013).

Another tool available for the prevention and the control of the disease is vaccination. Despite the failure of the first attempts to produce a parental vaccine due to low efficacy and severe side effects, an oral vaccine consisting of killed whole cells is nowadays available. However, the two-dose administration and the relatively short-term protection do not allow this vaccine to be a conclusive and optimal solution for preventing the disease (Hill *et al.*, 2006; Clemens *et al.*, 2011; Pastor *et al.*, 2013).

1.1.1 Epidemiology

Cholera primarily affects the developing countries of Asia, Africa and South/Central America. In 2012, there were more than 200,000 reported infections and 3000 deaths (WHO, 2013; Figure 1); however epidemiological data usually underestimate the actual numbers because many cases are not reported. Indeed, some endemic areas, such as India and Bangladesh, are not included in the map (Figure 1) because in these countries, cholera cases are recorded as bacterial diarrhoeal disease. In other geographic regions, cholera is not endemic (yet) but was imported by potentially asymptomatic individuals coming from endemic areas. This was likely the case for the Haiti: no cholera cases had been reported in Haiti for more than a century until the onset of the devastating outbreak in October 2010, where 600,000 cases and more than 7000 deaths occurred in two years (Barzilay *et al.*, 2013). It is likely that cholera was introduced to Haiti by United Nations (UN) Security Forces coming from Nepal, a country affected by cholera outbreaks at that time. This is supported by the characteristics of the strains isolated during the Haiti outbreak, which were nearly identical to the dominant Asian strains and distinct from those of Latin American areas (Chin *et al.,* 2011; Hendriksen *et al.,* 2011).

Figure 1. Countries reporting cholera in 2012 (WHO, 2013).

1.1.2 *Vibrio cholerae* and the genetic diversity

V. cholerae is a Gram-negative, rod-shaped bacterium with a polar flagellum. The cholera agent was first described by Filippo Pacini in 1854 as the curved bacterium overcrowding the intestine of the cholera victims (Pacini F, 1854) and isolated by Robert Koch in 1884. Since then, many studies have focused on the genetic characterization of the bacterium and on the molecular mechanisms responsible for the disease (Kaper *et al.*, 2005).

V. cholerae is classified into serogroups based on variants of the O antigen that, as part of the lipopolysaccharide (LPS), protrudes from the cellular surface. More than 200 serogroups have been identified, but only serogroups O1 and O139 are associated with pandemic and epidemic cholera outbreaks. Nevertheless, not all strains in these serogroups are pathogenic and other serogroups are occasionally the cause of cholera outbreaks (Beltran *et al.*, 1999). An overview of the different *V. cholerae* serogroups is shown in Figure 2.

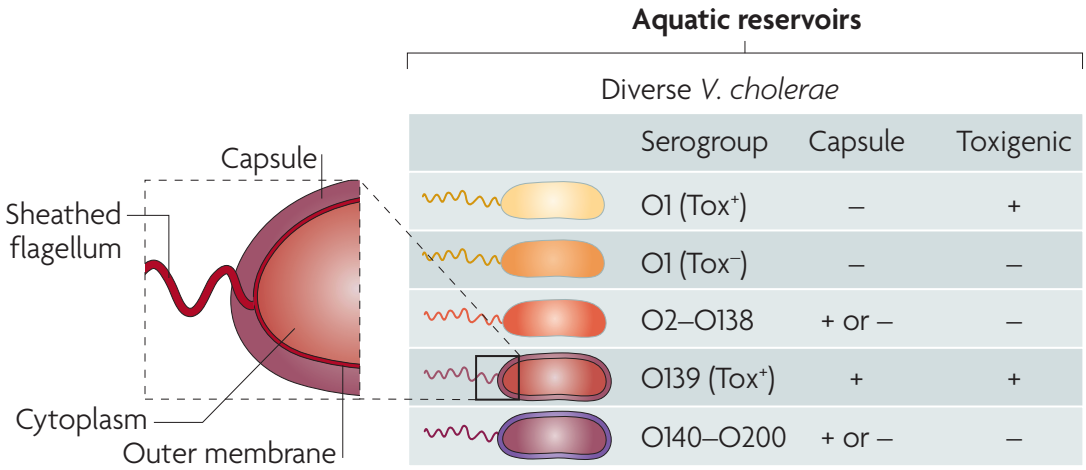

Figure 2. Schematic representation of the different *V. cholerae* serogroups (from Nelson *et al.*, 2009)

Since 1817, seven cholera pandemics have been described. The last, which started in 1961 in Indonesia, has spread to more than 30 countries and is still ongoing. The agent of the current pandemic is *V. cholerae* O1 El Tor biotype, which coexisted for some time with the O139 serogroup that appeared in 1992 and caused epidemics in India and in the neighbouring countries (Sack *et al.*, 2003; Ramamurthy

et al., 1993; Faruque *et al.*, 1998).

Even if O139 and O1 serogroups possess different O antigens, and despite the presence of an additional external capsule in O139, the two serogroups share many features and are responsible for infections yielding similar immune responses (Calia *et al.*, 1994; Albert, 1994; Quadri *et al.*, 1997). These findings suggest that the O139 serogroup most likely originated from an O1 strain through a process of homologous recombination that replaced the original O1-antigen biosynthesis gene cluster with that carried by O139 (Bik *et al.*, 1995; Mooi & Bik, 1997). Moreover, comparative genomic analysis showed that the O1 and O139 serogroups possess a high degree of genetic similarity but are quite different from non-O1 and non-O139 serogroups, suggesting that O1 and O139 belong to the same evolutionary lineage (Dziejman *et al.*, 2002; Dziejman *et al.*, 2005).

1.1.3 Pathogenesis and virulence factors

The genome of *V. cholerae* was sequenced in 2000 and consists of two circular chromosomes (Figure 3). Chromosome I contains genes necessary for the majority of cellular functions and pathogenicity while chromosome II, which was originally thought to be a megaplasmid, contains accessories genes and the integron island (Heidelberg *et al.*, 2000). However, as several essential genes are present on chromosome II, it is likely a real chromosome (Waldor & RayChaudhuri, 2000).

Two primary virulence factors are responsible for the pathogenicity of *V. cholerae*: toxin co-regulated pilus (TCP) and cholera toxin (CT). The TCP is a type IV pilus important for the first step of the infection, the colonization of the apical membrane of intestinal cells. The genes encoding for the TCP are part of a cluster of virulence-associated genes carried on the *V. cholerae* pathogenicity island 1 (VPI-1). The VPI-1 was suggested to have phage origin as it shares the following features with other pathogenicity islands: low G+C content, phage-like attachment (*att*) site at both extremities and presence of genes indispensable for DNA mobility, such as a transposase-like gene (*vpiT*) and a phage-like integrase gene (*int*) (Karaolis *et al.*, 1998); Karaolis *et al.* (1999) identified VPI-1 as the genome of a filamentous bacteriophage, VPIϕ, and showed that VPIϕ is transferred between *V. cholerae* strains (Karaolis *et al.*, 1999); however this finding remains controversial because a study conducted by Faruque *et al.* (2003) showed that *V. cholerae* strains carrying

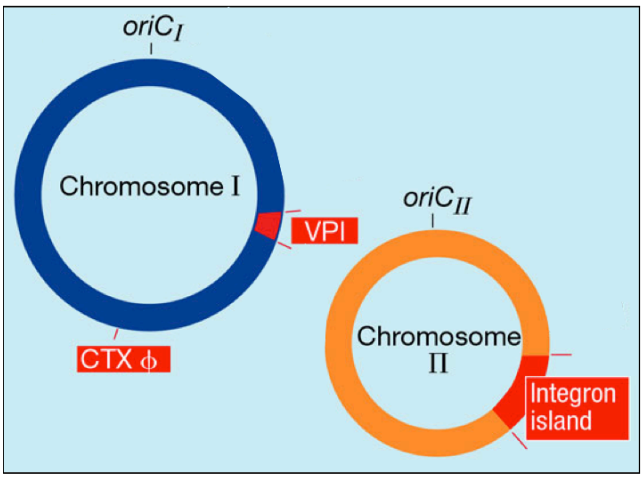

Figure 3. Scheme of the *Vibrio cholerae* chromosomes and localization of the main virulence factors / pathogenicity islands (modified from Waldor & RayChaudhuri, 2000).

VPI-1 are unable to produce VPIϕ particles (Faruque *et al.*, 2003).

The other major virulence factor is CT, which is composed of a single copy of the active subunit A and five copies of the binding subunit B. The genes coding for the CT subunits, *ctxA* and *ctxB*, are part of the CTX genetic element that is in fact the integrated genome (prophage) of the lysogenic filamentous bacteriophage CTXΦ, a virus that infects *V. cholerae*. Interestingly, TCP, which is indispensable to *V. cholerae* for colonizing the human intestine, is also the receptor that CTXΦ uses to enter bacterial cells. CTXΦ is secreted from *V. cholerae* without lysis of the cell through the bacterial membrane channel EpsD that is a component of the Eps type II secretion system (T2SS). The latter mediates the secretion of several proteins such as CT (Mcleod *et al.*, 2005).

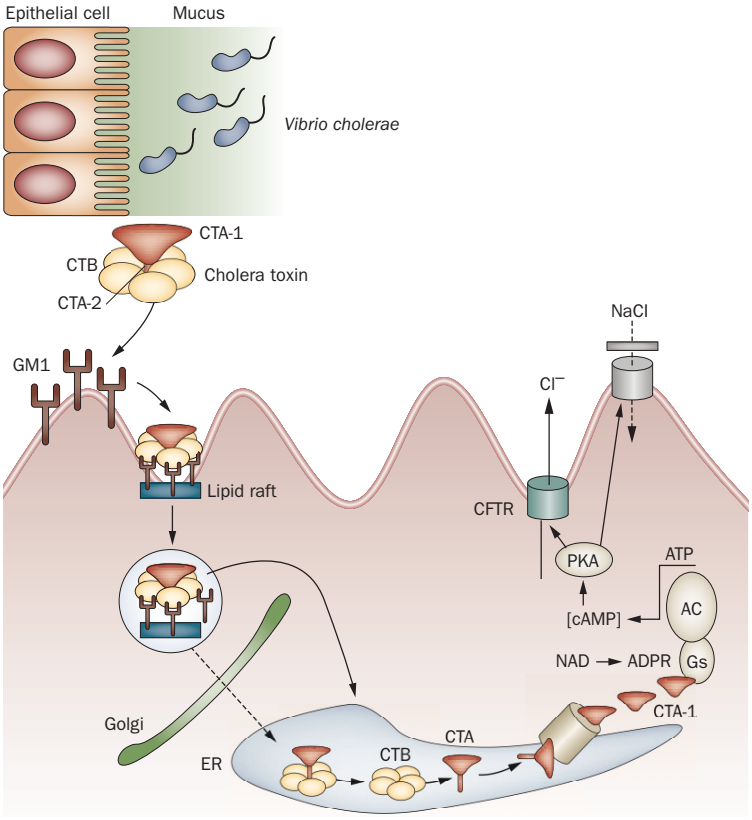

Figure 4. Intracellular pathway of cholera toxin. Upon CT binding to the GM1 ganglioside receptor of the epithelial cells, the toxin is internalized and translocated first to the Golgi apparatus and then to the endoplasmatic reticulum (ER). The CT-A1 subunit is separated from the other CT components and ADP-ribosylates the $G_{s\alpha}$ subunit of a regulatory small GTPase. This leads to a permanent activation of the adenylate cyclase (AC) that synthesizes the secondary messenger cAMP. As a consequence the main chloride channel (CFRT) is phosphorylated and the passage of electrolytes is deregulated (Clemens *et al.*, 2011).

The specific mechanism by which CT causes cholera is summarized in Figure 4. Overall, CT provokes a massive movement of electrolytes and water from the cytoplasm of the intestinal cells to the lumen, thus causing the diarrhoeal disease (Broeck *et al*., 2007).

Despite TCP and CT being the major virulence factors, many others are necessary for a successful infection. Moreover, *V. cholerae* strains which belong to non-epidemic and non-pandemic serogroups (non-O1 and non-O139 serogroups) that lack TCP and CT encoding genes also produce severe diarrhoeal diseases. This is probably due to the presence of alternative virulence factors such as a putative type III secretion system, a hemolysin-based secretion apparatus used by Gram-negative pathogens to inject virulence factors into eukaryotic cells (Dziejman *et al*., 2005).

1.2 *Vibrio cholerae* in the environment

The ecology of *V. cholerae* is extremely important to understanding the life cycle of this bacterium and to explain by which mechanisms the bacterium persists and evolves in the environment. Colwell and co-workers first suggested that *V. cholerae* and other *Vibrio* species are natural members of aquatic environments (Colwell *et al.*, 1977; Colwell *et al.*, 1981). Now it is fully accepted that *V. cholerae* is an autochthonous member of several marine habitats such as rivers, coastal water and estuaries. Moreover *V. cholerae* strains isolated from these habitats are mostly non-pathogenic and belong to non-O1 serogroups (Colwell & Huq, 1994; Tison *et al.*, 1986).

V. cholerae populates aquatic environments as free-living planktonic cells or as cells attached to abiotic and biotic surfaces and persists in a viable but non-culturable state under unfavourable environmental condition (Roszak & Colwell, 1987; Brayton *et al.*, 1987; Huq *et al.*, 1990). Viable but non-culturable state refers to a dormant form characterized by reduced size and coccoid shape that cannot be cultivated under laboratory conditions but is detectable by molecular or immunochemical methods (Huq *et al.*, 1990; Chalyanan *et al.*, 2007). Interestingly, cells in this dormant state can switch to a culturable state in the human intestine, by passage through an animal or by co-culture with eukaryotic cells (Colwell *et al.*, 1996; Alam *et al.*, 2007; Senoh *et al.*, 2010).

The attachment of *V. cholerae* to aquatic surfaces is thought to be important for the persistence of the bacterium in the environment (Huq *et al.*, 1983). Biotic surfaces represent a convenient microenvironment for bacterial growth and colonization but also guarantee the provision of nutrients. Known environmental reservoirs for *V. cholerae* are phytoplankton and zooplankton. *V. cholerae* is also found associated with many living marine organisms such as protozoa, marine bivalves and aquatic birds, though it is still unclear whether these organisms play a role in *V. cholerae* persistence and transmission (Ogg *et al.*, 1989; Tamplin *et al.*, 1990; Vezzulli *et al.*, 2010).

1.2.1 Life cycle of *Vibrio cholerae*

Transition of *V. cholerae* from the aquatic environment to the human host is mediated by the ingestion of contaminated water and/or food containing a high number of bacteria. These bacteria are most likely attached to biotic surfaces, since simple water filtration reduced cholera cases in Bangladesh (Colwell *et al.*, 2003). A schematic representation of the life cycle of *V. cholerae* is shown in Figure 5.

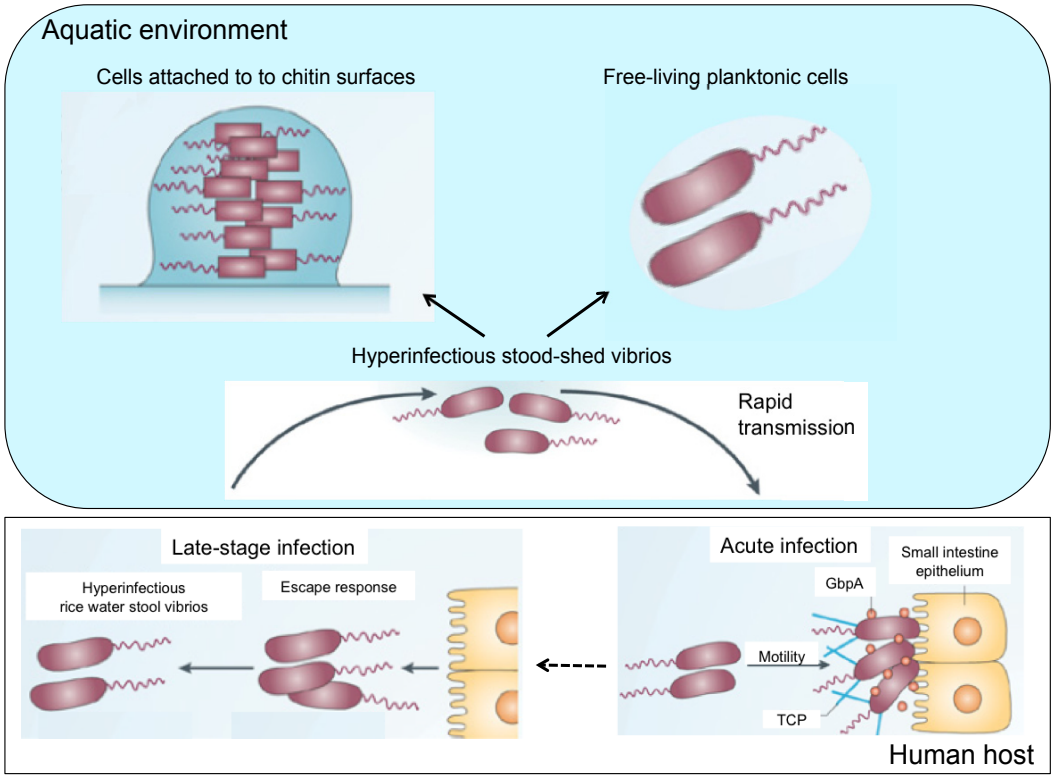

Figure 5. Life cycle of *V. cholerae* (adapted from Nelson *et al.*, 2009)

Once the bacteria are ingested and cross the acidic compartment of the human stomach, their motility is crucial to establishing an infection. The unique polar flagellum of *V. cholerae* allows the bacterium to pass through the mucus layer that covers the epithelial cells and to reach the crypts of the small intestine (Butler & Camilli, 2005).

Following this, the bacterium expresses its virulence factors, colonizes the human intestinal niche and establishes an acute infection. As previously mentioned, TCP is one of the virulence factors important for the colonization of the host but

other many factors contribute to this first step of the infection. Interestingly, GlcNAc-binding protein A (GbpA), one of the bacterial proteins involved in *V. cholerae* attachment to chitin surfaces, is also known to be an important colonization factor for the human intestine (Kirn *et al.*, 2005). The same domain of GbpA is able to bind certain types of chitin as well as glycosylated proteins that cover the surface of the intestinal epithelial cells, the mucins (Wong *et al.*, 2012).

During the late stage of the infection, *V. cholerae* cells detach from the intestine in a process known as 'mucosal escape response' (Nielsen *et al.*, 2006) and prior to leaving the human host, changes in bacterial gene expression occur to guarantee adaptation of *V. cholerae* to aquatic habitats (Schild *et al.*, 2007). Interestingly, the shed *V. cholerae* cells are in a hyperinfectious state, which despite lasting only for a few hours, seems to facilitate the human transmission of *V. cholerae* infection. Once *V. cholerae* is back in its natural habitat, the bacterium colonizes biotic surfaces or persists as free-swimming planktonic cell (Nelson *et al.*, 2009).

1.2.2 Link between aquatic environment and cholera outbreaks

Cholera is considered a paradigm of waterborne diseases. Many studies have tried to better understand this disease by focusing on the factors that influence the appearance of the outbreaks (Colwell, 2004).

Cholera is endemic to the coastal regions of India, Bangladesh and Latin America, which all share similar environmental and socio-economic conditions. In these regions, the disease never disappears and usually has one or several seasonal peaks that often correlate with an elevation in water temperature. In Bangladesh, the seasonality of cholera outbreaks has two peaks, the first after the monsoon in September and the second between March and May (Glass *et al.*, 1982; Sack *et al.*, 2003; Alam *et al.*, 2006). A link between oceans warming and the diffusion of pathogenic *Vibrios* has been advanced but this theory is still under debate (Vezzullli *et al.*, 2013).

Besides water temperature, many other abiotic and biotic factors favour the growth of *V. cholerae* in the environment as well the acquisition of virulence factors. Colwell and co-workers introduced a hierarchical model of factors that are directly and indirectly involved in the transmission of cholera (Figure 6). For example, pH and sunlight are important abiotic factors that influence *V. cholerae* cells as well as the induction of the CTX prophage that is thought to be important for the spreading of the CT virulence factor (Lipp *et al.*, 2002; Faruque & Mekalanos, 2003). Interestingly, seasonal cholera outbreaks inversely correlate with the presence of the bacteriophages that are serogroups-specific for pathogenic *V. cholerae* O1 and O139 strains (Faruque *et al.*, 2004).

Abiotic factors are also indirectly involved in cholera transmission because they influence the growth of phytoplankton and aquatic plants, both occupying the next step in the hierarchic model. Phytoplankton and aquatic plants are also ecological niches of *V. cholerae*, represent a nutrient source for both bacteria and zooplankton, and influence the pH of the environment with their respiration. Zooplankton and other crustaceans are at the top of the model (Lipp *et al.*, 2002). Huq and co-workers were the first to show the attachment of *V. cholerae* to the surface of copepods, small crustaceans that are part of zooplankton (Huq *et al.*,

1983). Abiotic factors may also play a role in this step of the hierarchy as, in artificial systems, the attachment of bacteria to live copepods was influenced by salinity, pH and temperature (Huq *et al.*, 1984). The seasonality of cholera outbreaks has also been linked to copepod blooms (egg production), which have a seasonal variation and a net increase twice a year, corresponding to cholera peaks in Bangladesh (Kiørboe & Nielsen, 1994).

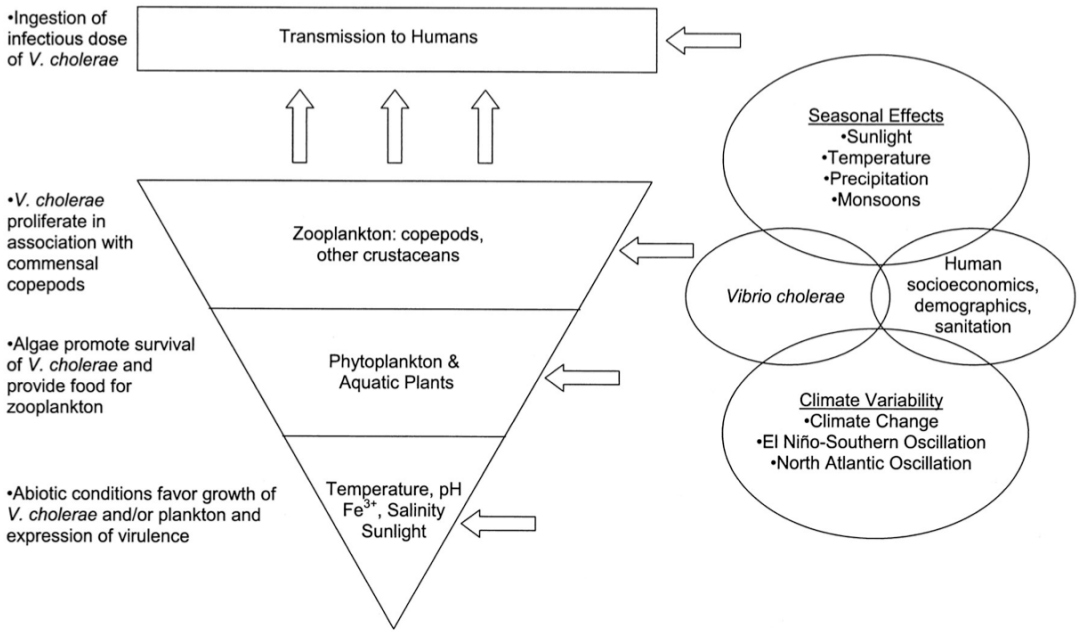

Figure 6. Hierarchical distribution of the factors influencing cholera transmission (Lipp *et al.*, 2002).

Zooplankton blooms promote the attachment of the free-living planktonic *V. cholerae* cells and favor bacterial multiplication on the their chitinous exoskeleton. Attachment of *V. cholerae* cells to the chitin surfaces was shown to increase bacterial survival under adverse conditions such as the acid environment of the stomach or low water temperatures indicating that chitinous surfaces of zooplankton influence the persistence of *V. cholerae* in the environment (Nalin *et al.*, 1979; Amako *et al.*, 1987). Moreover, chitin surfaces promote a successful infection of the human host by acting as a vehicle for a large number of protected bacteria (Lipp *et al.*, 2002; Pruzzo *et al.*, 2008; Nahar *et al.*, 2012).

1.3 Mechanisms involved in the evolution of *Vibrio cholerae*

The acquisition of new genetic traits results from vertically-transmitted mutations or the movement of genes by horizontal gene transfer (HGT). The role of HGT in the evolution of bacteria was recognized only a half-century ago with the massive spreading of multi-drug resistance (Davies & Davies, 2010). Comparative genomic studies of *V. cholerae* strains isolated from different sources allowed the identification of different *V. cholerae* lineages and suggested an evolutionary model that explains the wide genetic diversity of this bacterial species by mechanisms of HGT (Chun *et al.*, 2009).

Three mechanisms of HGT exist in bacteria: conjugation, transduction and transformation, and all are known to be involved in evolution (see Figure 7). Apart from the acquisition of antibiotic resistance genes, these mechanisms are quite important because they allow the transfer of genes encoding virulence factors and metabolic and catabolic pathways. Conjugation requires physical contact between donor and recipient cells and consists in transferring genetic material usually located on plasmids. Transduction is a virus-mediated HGT mechanism (Ochman *et al.*, 2000). In *V. cholerae*, transduction is responsible for the acquisition of the *ctx* genes, which encode one of the two main virulence factors (transmitted by the CTXΦ phage; Waldor & Mekalanos, 1996; Mcleod *et al.*, 2005).

The last mechanism of HGT known in prokaryotes is transformation, called natural transformation when it occurs naturally. Bacteria that are naturally competent are in a developmental state wherein they are able to take up extracellular DNA from the environment. Once this DNA is in the cytoplasm, it can recombine with the bacterial genome by homologous recombination thereby transforming the bacterium. Accordingly, the bacteria carrying the new genetic material are called transformants. This competence program is present in both Gram-positive and -negative bacteria, but the mechanisms of activation, regulation and DNA uptake vary greatly among different species (Chen & Dubnau, 2004; Seitz & Blokesch, 2013a).

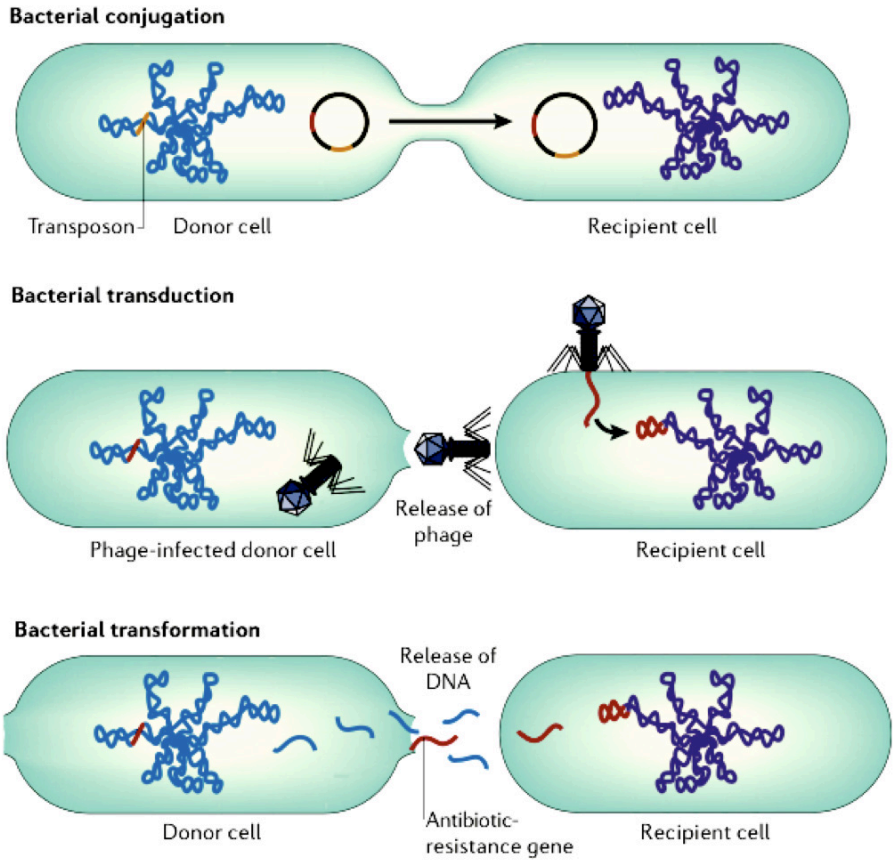

Figure 7. Mechanisms of horizontal gene transfer (adapted from Furuya & Lowy, 2006).

The emergency of new pathogenic strains is thought to originate from non-pathogenic ancestors that acquired the virulence genes (Mekalanos *et al.*, 1997; Faruque & Mekalanos, 2003). This is supported by the fact that some strains isolated from aquatic environments are not pathogenic but carry some virulence factors and therefore represent a reservoir of bacteria that can become pathogenic upon acquisition of additional toxigenic traits (Chakraborty *et al.*, 2000). Studies supporting the importance of natural competence in the evolution of *V. cholerae* are presented in the next sections together with a more detailed description of the development of natural competence.

1.3.1 Natural competence for transformation

V. cholerae initiates the process of natural competence for transformation in aquatic environments during its association with the zooplankton (e.g. copepods). Chitin, the main component of the exoskeleton of the small crustaceans, is the inducer of this developmental program (Meibom *et al*., 2005).

Three different models explain why bacteria take up DNA from the environment. The extracellular DNA is considered i) a source of carbon, nitrogen and phosphorus; ii) genetic material to be used for DNA repair; or iii) the source of new genes for bacterial evolution and diversity (Chen & Dubnau, 2004; Seitz & Blokesch, 2013b).

The prospective impact of natural competence for transformation in the evolution and pathogenesis of *V. cholerae* was first investigated in a study conducted by Blokesch and Schoolnik (2007). As previously mentioned, the pathogenic *V. cholerae* O139 serogroup only emerged in 1992 and coexists with the *V. cholerae* O1 serogroup, the strain responsible for the current cholera pandemic (section 1.1.2). The similarities between these strains indicate that the O139 serogroup might indeed be derived from an O1 ancestral strain through acquisition of the O139 O-antigen features. Blokesch and Schoolnik showed that natural competence for transformation *a)* can lead to the uptake of DNA fragments containing the entire O-antigen biosynthetic gene cluster (> 42 kbp for O139), *b*) that this can lead to serogroup conversion by homologous recombination, and *c)* that such serogroup conversion processes occur between a recipient O1 strain and a donor O139 strain when grown together on a chitin surface (Blokesch & Schoolnik, 2007).

Natural competence for transformation could also be the mechanism responsible for the appearance of hybrid O1 *V. cholerae* strains that belong to the current pandemic strain (El Tor biotype) but carry the variant CT from the CTXΦ prophage of the strain that dominated previous pandemics (Classical biotype). Indeed, co-cultivation of these strains carrying the two variants of the CTXΦ prophage on a chitin surface resulted in transformation-mediated emergence of such hybrid strains (Udden *et al*., 2008).

Overall these data together with recent studies describing that "*V. cholerae* undergoes extensive genetic recombination via lateral gene transfer" (Chun *et al*., 2009) strongly indicates that natural competence for transformation contributes to the genetic diversity of *V. cholerae* and to the origin of pathogenic strains. Thus, understanding the mechanism that controls this process is of prime importance.

Among the genes up-regulated by chitin (the inducer), there are those coding for the master regulator of transformation (TfoX), for several structural components of a type IV pilus (the *pil* genes), for proteins involved in DNA translocation across the periplasmic space and the inner membrane, and proteins protecting the single-stranded DNA and mediating the homologous recombination process (Meibom *et al*., 2004; Meibom *et al*., 2005; Seitz & Blokesch, 2013b). These genes are necessary for natural competence to occur and are considered part of the competence regulon of *V. cholerae*. The DNA uptake process is probably initiated by extension and subsequent retraction of the type IV pilus (Meibom *et al*., 2005; Seitz & Blokesch 2013b) encoded by nine *pil* genes, four of which identified by Fullner and Mekalanos (1999) (Fullner & Mekalanos, 1999); however, whether this pilus is also involved in the pathogenicity of *V. cholerae* is unknown.

Another chitin-regulated gene necessary for natural transformation is the *comEA* gene, which encodes a periplasmic DNA binding protein (Seitz *et al*., 2014). After the retraction of the pilus, the ComEA protein is likely involved in DNA binding and translocation within the periplasmic space and towards the cytoplasm (Provvedi & Dubnau, 1999; Meibom *et al*., 2005; Seitz *et al*., 2014).

The regulatory network of natural competence and transformation in *V. cholerae* was not well understood at the time this thesis project was initiated. However, there were indications that apart from chitin sensing and degradation, two other pathways, namely quorum sensing (QS) and carbon catabolite repression (CCR), were also involved in the regulation of natural competence. The following sections describe each of these three pathways and review the main findings that are linked to this thesis work.

1.3.1.1 Chitin sensing and degradation

Chitin is an insoluble polymer of N-acetyl-D-glucosamine, produced mainly in aquatic environments at an average annual production of 10^{10} to 10^{11} tons. Absent in prokaryotes, plants and vertebrates, this polymer is found in fungi, insects, crustaceans and other invertebrates. Chitin represents the second-most abundant polysaccharide in nature and therefore requires proper recycling (Gooday, 1990).

Chitin degradation is mainly due to marine bacteria that use chitin as a source of carbon and nitrogen. The degradation process can be divided into four steps: 1) chitin sensing, 2) attachment to the chitin surface, 3) expression of the genes involved in chitin catabolism, and 4) chitin degradation (Keyhani & Roseman, 1999). Motile bacteria swim towards the chitin source by chemotaxis, likely following a gradient of chemoattractants released by crustaceans and other organisms that contain chitin. Once the bacteria reach the chitin surface, proteins that specifically bind chitin allow their adhesion to the surface (Keyhani & Roseman, 1999). In *V. cholerae*, the GbpA protein binds a sugar exposed on both the chitin surface and the human epithelial cells (Kirn *et al*., 2005). However other factors are likely involved in the adhesion to the chitin surfaces such as the mannose-sensitive hemagglutinin (MSH) pilus that is a type IV pilus shown to participate in biofilm formation and promote adhesion to zooplankton and chitin beads (Watnick *et al*., 1999; Chiavelli *et al*., 2001; Meibom *et al*., 2004).

After bacterial attachment, chitin is degraded by the activity of extracellular enzymes called chitinases. The degradation products are mainly dimers of N-acetyl-D-glucosamine $(GlcNAc)_2$ which can be further hydrolyzed to monomers by the action of periplasmic β-N-acetylglucosaminidase (Keyhani & Roseman, 1999). The model proposed by Li and Roseman (2004) is shown in Figure 8. After chitin degradation, the resulting dimers $(GlcNAc)_2$ diffuse from the extracellular space to the periplasm through porins located in the outer membrane, and there bind a chitin binding protein (CBP). This binding provokes the dissociation of CBP from a two-component sensor kinase (ChiS) located in the inner membrane. As a consequence, ChiS is unlocked and can activate the regulatory cascade that induces the expression of the chitin-catabolic genes. At the same time, chitin dimers are translocated into

the cytoplasm by an ABC transporter and are finally catabolized (Li & Roseman, 2004).

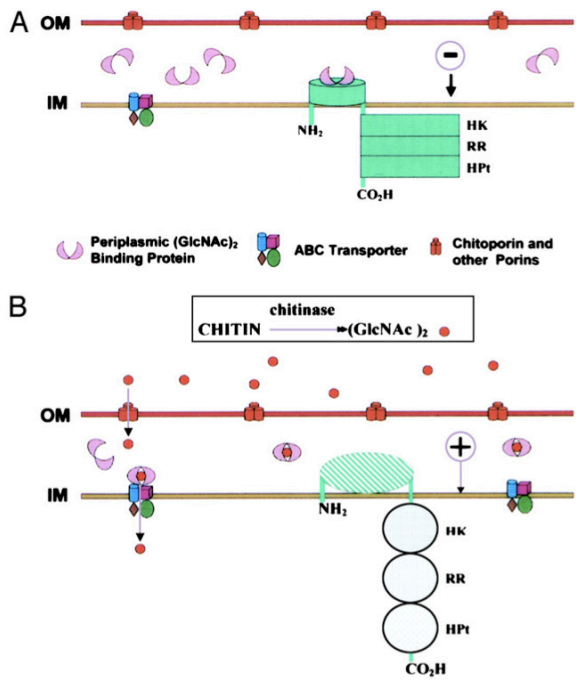

Figure 8. Model for the regulation of the chitin sensor. The chitin sensor (ChiS, shown in green) contains a periplasmic, a cytoplasmic and an inner membrane (IM) domain. The cytoplasmic domain consists of three subdomains: the histidine kinase/phosphatase domain (HK), the autophosphorylatable response regulator domain (RR) and the HPt domain, which contains a phosphorylatable histidine. **A.** In the absence of a chitin source, ChiS is in a locked conformation due to the binding of the periplasmic chitin binding protein (CBP) and chitinolytic genes are repressed. **B.** In the presence of a chitin source, chitin degradation products, mainly dimers of N-acetyl-glucosamine $(GlcNAc)_2$, diffuse into the periplasm through porins located in the outer membrane (OM). Through its binding to the $(GlcNAc)_2$ molecules CBP dissociates from ChiS, ChiS is thereby rendered into its active state and leads to the expression of the chitinolytic genes (from Li & Roseman, 2004).

The identification of the genes induced by chitin was accomplished by comparing the microarray expression profiles of *V. cholerae* strains grown in the presence and absence of a chitin source. These genes are mainly necessary for chitin degradation and translocation, e.g. genes encoding three extracellular chitinases (*chiA-1*, *chiA-2*, *VC0769*), a periplasmic chitodextrinase, a chitin-specific porin of the outer membrane and an ABC type transporter, and their expression is also dependent on the presence of the sensor ChiS (Meibom *et al*., 2004).

One important gene induced by chitin is *tfoX* (also called *sxy*), whose product is homologous to the master regulator of transformation in the naturally competent bacterium *Haemophilus influenzae* (Redfield, 1991). This suggested and then proved that *V. cholerae* acquires natural competence in the presence of chitin (Meibom *et al*., 2004; Meibom *et al*., 2005). As shown in Figure 9, chitin induces the expression of *tfoX*, which in turn induces the chitin-catabolic genes as well as the competence

genes. The exact regulatory cascade that allows chitin oligomers to induce the expression of *tfoX* is still unknown.

Yamamoto and co-workers showed that the $(GlcNAc)_2$ disaccharide not only positively regulates the transcription of *tfoX* but also its translation; in the absence of chitin, the translation of *tfoX* mRNA is inhibited by a base-pairing mechanism involving two regulatory elements located upstream and downstream the GTC start codon of the gene (Yamamoto *et al.*, 2010). Formation of the inhibition loop is suppressed by the action of a small regulatory RNA, TfoR, transcribed in the presence of $(GlcNAc)_2$. Indeed, a base pairing between the TfoR sRNA and the *tfoX* upstream regulatory region allows the translation of *tfoX* mRNA (Yamamoto *et al.*, 2011).

The chitin sensing and degradation pathway is conserved within the *Vibrionaceae* family and the presence of the *chiA* gene, encoding for the extracellular chitinase A, is a good indicator of the ability to use chitin as a carbon and nitrogen source (Hunt *et al.*, 2008). Moreover, the high similarity between TfoX and TfoR in *Vibrio* species suggests that natural competence and transformation is likely a conserved mechanism (Yamamoto *et al.*, 2011), which was experimentally confirmed for three other *Vibrio* species - *V. vulnificus*, *V. parahaemolyticus* and *V. fischeri* (Gulig *et al.*, 2009; Chen *et al.*, 2010; Pollak-Berti *et al.*, 2010).

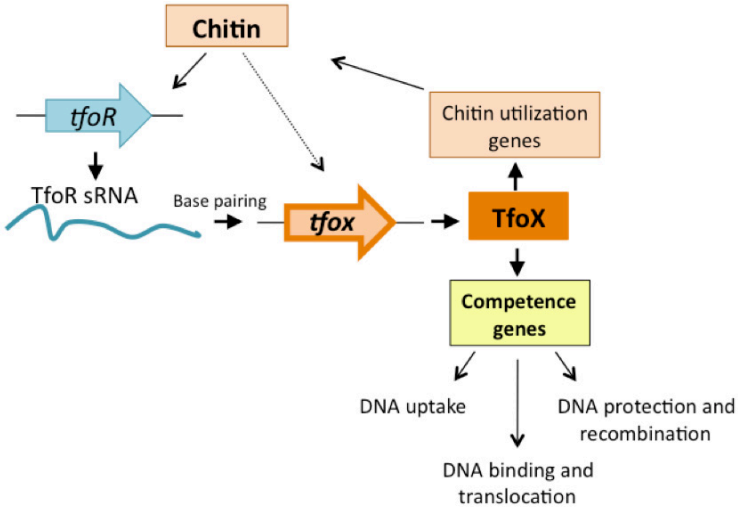

Figure 9. Scheme of the chitin regulatory pathway. Expression of the gene encoding the master regulator of transformation (TfoX) is induced by chitin. Chitin also mediates translational regulation of *tfoX* by the expression of the small RNA TfoR. TfoX is then responsible for the expression of the genes involved in chitin utilization as well as the competence genes.

1.3.1.2 Quorum sensing

Quorum sensing (QS) describes a phenomenon of cellular communication that allows bacteria to behave as unison within the population. First described by Greenberg and colleagues for *V. fischeri*, QS was then found to be a general mechanism in other *Vibrio species* (e.g. *V. cholerae*) and various Gram-negative and -positive bacteria (Ng & Bassler, 2009). QS is characterized by a switch of the bacterial gene expression during passage from low to high cell densities (LCD->HCD) which is mediated by the synthesis and detection of small molecules called autoinducers that are secreted into the environment. In *V. cholerae*, at least two autoinducer molecules are produced: the intra-species cholera autoinducer 1 (CAI-1) and the universal autoinducer 2 (AI-2) (Miller *et al.*, 2002; Bassler *et al.*, 1997; Xavier & Bassler, 2003).

The regulatory pathway for QS is shown in Figure 10. The receptors for the autoinducers are two-component enzymes with both kinase and phosphatase activities. At a LCD, the amount of autoinducers is too low to be detected by their respective receptors and the receptors act as a kinase and autophosphorylate histidine residues in an ATP-dependent manner. This is the start of a phosphorylation cascade that ends with the phosphorylation and subsequent activation of the transcription factor LuxO. Phosphorylated LuxO (P-LuxO) activates the transcription of genes encoding four small regulatory RNAs (Qrr1-4) that target the mRNA of *hapR*, a gene coding for the master regulator of QS (Ng & Bassler, 2009). These sRNAs, together with the chaperone Hfq, destabilize the *hapR* mRNA by base pairing and repress HapR protein production (Lenz *et al.*, 2004). At a HCD, the concentration of the autoinducers in the extracellular space is above a certain threshold and binding of autoinducers to the respective receptors switch the activity of the receptors from kinase to phosphatase activity. Thus, the signalling cascade is reverted, LuxO is not activated and the sRNAs are not expressed. Consequently, the HapR protein is produced, which then positively or negatively regulates the expression of the target genes. Indeed, HapR, a homolog of LuxR from *V. harveyi*, is known to be involved in the regulation of several cellular mechanisms such as the repression of virulence and inhibition of biofilm formation (Ng & Bassler, 2009;

Jobling & Holmes, 1997; Zhu *et al*., 2002; Miller *et al*., 2002; Kovacikova & Skorupsky, 2002; Hammer & Bassler, 2003; Zhu & Mekalanos, 2003).

QS is also involved in the regulation of natural competence for transformation. This was discovered at the same time as natural competence because *V. cholerae* strains carrying a frameshift mutation in the *hapR* gene were not transformable but could be rescued by providing a functional copy of *hapR* (Meibom *et al*., 2005). Moreover the two autoinducers do not contribute equally to the regulation of natural competence; indeed the intra-species CAI-1 plays the main role (Suckow *et al*., 2011; Antonova *et al*., 2011).

The mechanism by which QS regulates natural competence for transformation was initially unknown but it appeared to be connected to the activity of Dns, an extracellular DNase. Natural transformation is negatively affected by the presence of Dns because the enzyme degrades extracellular DNA thereby preventing it from being taken up (Blokesch & Schoolnik, 2008; Seper *et al*. 2011).

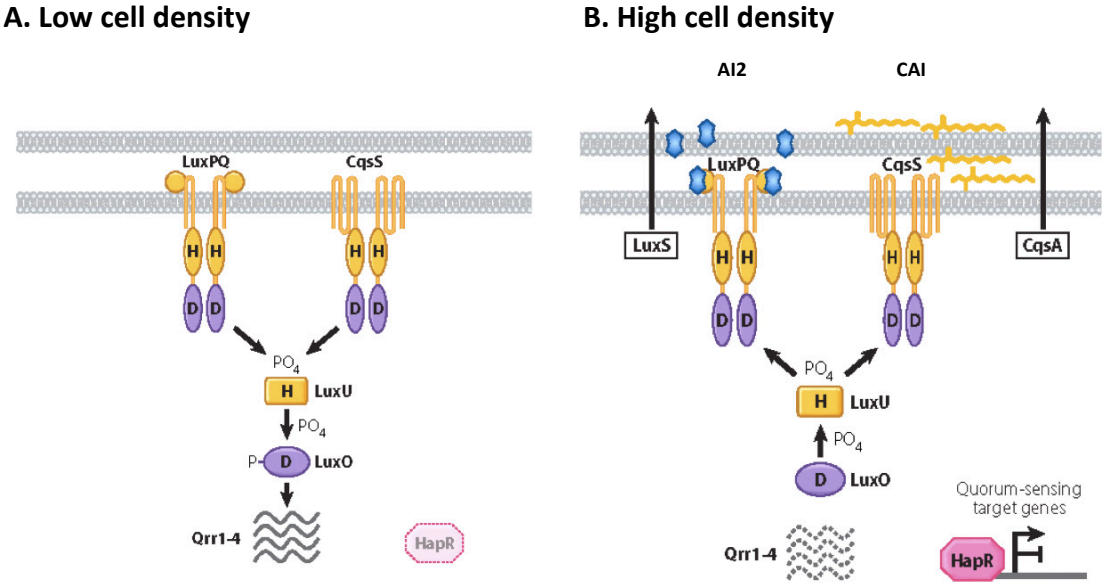

Figure 10. Quorum sensing pathway in *V. cholerae*. A. At low cell densities LuxPQ and CqsS, receptors of the universal autoinducer (AI-2) and of the intra-species cholera autoinducer 1 (CAI-1), respectively, act as a kinase and induce a phosphorylation signalling cascade. Phosphorylated LuxO (P-LuxO) activates the transcription of four small regulatory RNA (Qrr1-4) that base pair with the mRNA of *hapR* and cause its degradation. **B.** At high cell density the autoinducers, synthesized by the enzymes LuxS and CqsA, bind their respective receptors. Upon this binding the receptors act as phosphatase and revert the signalling cascade. Unphosphorylated LuxO does not initiate transcription of Qrr1-4 and HapR is produced (adapted from Ng & Bassler, 2009).

1.3.1.3 Carbon catabolite repression

Bacteria can adapt to environmental changes through complex regulatory mechanisms. In the presence of different sugars, bacteria develop the ability to use a particular preferred carbon source. This phenomenon, known as carbon catabolite repression (CCR), occurs through different mechanisms. In *E. coli* and in other species, the signalling cascade that leads to CCR is coupled to the phosphoenolpyruvate-carbohydrate phosphotransferase system (PTS), which is a multiprotein complex that allows the transport of several sugars into the cytoplasm concomitantly with their phosphorylation. The components of this system are EI, HPr and EII (subunits A-C) and the main players of the CCR signalling cascade are the PTS factor EIIA, the enzyme adenylate cyclase (AC or CyaA), the metabolite 3',5'- cyclic adenosine monophosphate (cAMP) and the cAMP receptor protein (CRP) (Deutscher *et al.*, 2006; Deutscher, 2008). The mechanism of CCR is shown in Figure 11.

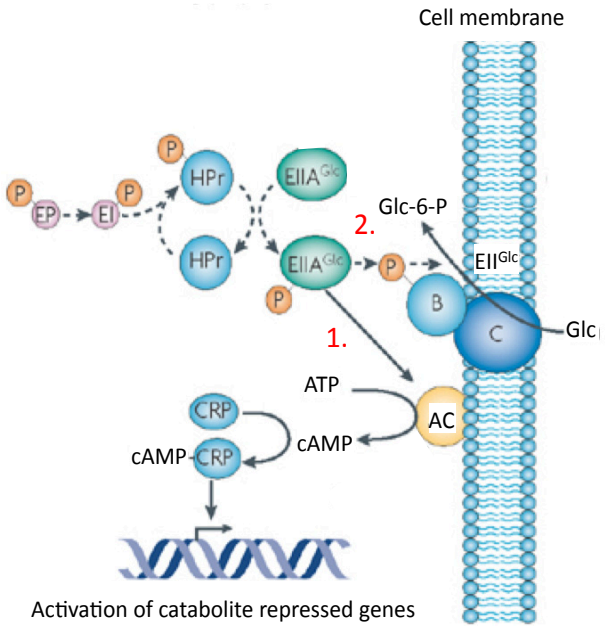

Figure 11. CCR in *E. coli*. The phosphorylation cascade begins with the auto-phosphorylation of the PTS component EI; the posphate group is provided by phosphoenol pyruvate (PEP). **1.** When the PTS system is not saturated by PTS sugars such as glucose, the last component of the phosphorylation cascade specific for glucose, $EIIA^{Glc}$, is kept in a phosphorylated state ($P\text{-}EIIA^{Glc}$) and activates the enzyme adenylate cyclase (AC), which synthesizes cAMP using ATP as the substrate. High levels of cAMP trigger the formation of an active complex between cAMP and the CRP protein (cAMP-CRP), which binds and activates the promoters of the catabolite repressed genes. **2.** When the PTS is saturated, $P\text{-}EIIA^{Glc}$ passes the phosphate group to the transported sugar (e.g. Glc). Unphosphorylated $EIIA^{Glc}$ cannot activate AC and cAMP is therefore not produced. Thus, CRP remains inactive and the catabolite repressed genes are not transcribed (modified from Görke & Stülke, 2008).

In the absence of a PTS sugar, the CRP protein forms an active complex with cAMP and activates the transcription of the catabolite repressed genes. Conversely, if glucose or another PTS sugar is transported, the AC enzyme is not activated, cAMP is not synthesized, the CRP protein remains inactive and the catabolism of the other carbon sources is repressed (Görke & Stülke, 2008).

The CRP protein, formerly known as catabolite activator protein (CAP), has been extensively studied in *E. coli*. The active CRP protein complex is a dimer of two identical subunits bound to the cAMP. This complex binds regulatory elements at CRP sites located within the promoter region of the target genes which enhances the ability of the RNA polymerase (RNAP) to bind and initiate the transcription at these CAP-dependent promoters (Harman, 2001).

The amino acid sequence of the CRP protein from *V. cholerae* (VcCRP) is very similar to that of *E. coli* (EcCRP) and VcCRP shows cAMP-dependency similar to EcCRP suggesting that the mechanism of promoter activation of CRP target genes is similar for the two bacterial species (Skorupski & Taylor, 1997; Chattopadhyay & Parrack, 2006). The genome of *V. cholerae* encodes for 25 PTS components and among these, nine are homologs of the EIIA component. In particular, $EIIA^{Glc}$ is specific for the transport of four different sugars: glucose, N-acetylglucosamine, sucrose and trehalose (Heidelberg *et al.*, 2000; Houot *et al.*, 2010).

CCR and natural competence

CCR is not limited to the regulation of catabolic genes. For example, the CRP-cAMP complex and the PTS components are known to be involved in the regulation of *V. cholerae* biofilm formation (Liang *et al.*, 2007; Fong & Yildiz, 2008; Houot *et al.*, 2010). The development of natural competence for transformation in *V. cholerae* is also subject to CCR. In the presence of a PTS sugar, the transformability of *V. cholerae* strains decreased significantly; furthermore *V. cholerae crp* or *cyaA* knock-out strains were non-transformable and had defects in the colonization of chitin surfaces (Meibom *et al.*, 2005; Blokesch, 2012a).

The importance of the CRP-cAMP complex in the development of natural transformation was initially demonstrated in *H. influenzae* (Chandler, 1992; Dorocicz

et al., 1993). The competence regulon of *H. influenzae* consists of 25 genes characterized by the presence of competence regulatory elements (CRE) in their promoter regions (Redfield *et al.*, 2005). The master regulator of transformation TfoX (also called Sxy) was initially supposed to lead to the expression of the competence genes by binding the CRE elements. However, CRE was recognized to be a CRP binding site, which was in agreement with the observation that TfoX did not possess any DNA-binding motifs (Macfadyen, 2000; Redfield *et al.*, 2005). The CRP binding sites identified upstream of the competence genes were later designated CRP-S (instead of CRE) due to their dependency on the Sxy protein (TfoX) and to clearly distinguish them from competence-unrelated and TfoX-independent canonical CRP binding sites designated as CRP-N (Redfield *et al.*, 2005; Cameron & Redfield, 2006). These authors proposed a model where the expression of the competence genes is due to the binding of the CRP protein (complexed to cAMP) at the CRP-S sites but together with Sxy (see Figure 12).

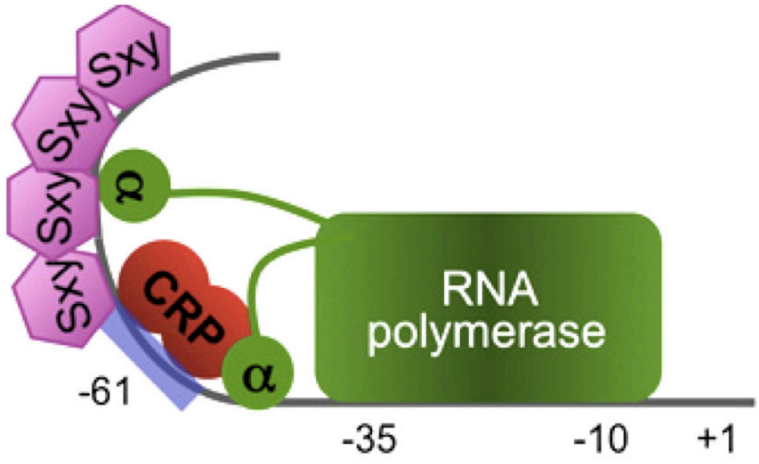

Figure 12. Model of activation at the promoters of the competence genes in *H. influenzae* (from Cameron & Redfield, 2008).

The competence genes of *H. influenzae* have orthologs in the *Enterobacteriaceae*, *Pasteurellaceae* and *Vibrionaceae* families. CRP-S and CRP-N consensus motifs were predicted for these γ-proteobacteria by cross-species sequence comparison (phylogenetic footprinting) and by specifically searching for shared motifs among the promoter regions (Cameron & Redfield, 2006). In

particular, the CRP-S consensus sequence of the *Vibrionaceae* family was *in silico* predicted by analysing the promoters of *V. cholerae* genes that were induced by both chitin and *tfoX* over-expression (Meibom *et al.*, 2004; Meibom *et al.*, 2005). The shared sequences were also found in the upstream region of the respective homologs in *V. parahaemolyticus* and *V. vulnificus*, which were pooled in order to construct the *Vibrionaceae* CRP-S consensus shown in Figure 13.

Figure 13. Consensus of the *in silico* predicted CRP-S site of the *Vibrionaceae* family (from Cameron & Redfield, 2006).

Despite the shared motifs used to construct the consensus of the CRP-S site was only found in five promoters (upstream regions of the *pilA*, *pilM*, *dprA*, *VC1612* and *VCA0140* genes), the upstream region of other competence genes showed similarity within the right part (Figure 13) of this consensus (Cameron & Redfield, 2006).

The significance of the predicted *in silico* competence-specific CRP-S sites of *V. cholerae* was unknown and investigated in this thesis.

Chapter 2.

Aims of the thesis

The appearance of new *V. cholerae* strains in the environment is suggested to be the result of HGT events. Among those HGT mechanisms, natural competence for transformation may have a prominent role in the emergence of new pathogenic strains (Blokesch & Schoolnik, 2007; Udden *et al*., 2008). Thus, it becomes clear that understanding the mechanism that controls natural competence is very important.

The global aim of this thesis was to investigate how natural competence is regulated in *V. cholerae*. Little information was available on the regulation of natural competence when we started this thesis project. We were aware that chitin is necessary to induce natural competence for transformation and that quorum sensing (QS) and likely also carbon catabolite repression (CCR) are involved in the development of this mechanism (Meibom *et al*., 2004; Meibom *et al*., 2005; Blokesch & Schoolnik, 2008). However, how these pathways are linked was unknown as was the mechanism that leads to the expression of these competence genes.

The first part of this thesis aimed to analyze for the first time the pattern of induction of the competence genes at single cell level. This experimental approach could provide important information not detectable when the gene expression is investigated at population level. In particular, based on the finding in *B. subtilis* that natural competence is activated only in a fraction of the bacterial population, we aimed to discover if a similar situation is present in *V. cholerae* (Nester & Stocker, 1963).

Competence genes are up-regulated by chitin due to the chitin-dependent induction of *tfoX,* a gene which encodes the master regulator of transformation

(TfoX). However, the signaling cascade downstream TfoX was unknown. Furthermore, the competence gene *comEA* was shown to be essential for natural transformation but was not induced by chitin in an initial study conducted in *V. cholerae* N16961; this strain is non-transformable because it carries a non-functional copy of the *hapR* gene, which encodes the master regulator of QS (Meibom *et al.*, 2004; Meibom *et al.*, 2005). Starting from this observation, we investigated whether the pathways involved in the regulation of natural competence are the same for all competence genes or not.

We were also interested in deciphering the link between QS and other regulatory pathways. Initial data concerning the activation of the competence regulon indicated that at a certain point, signals initiated by the availability of chitin converge with cell density signaling. Thus, by focusing on the QS pathway, we aimed to discover by which mechanisms HapR leads to the expression of the competence genes and to its positive contribution to natural transformation.

The final part of this project was dedicated to the elucidation of the CCR. The involvement of CCR in natural competence was initially suggested by Meibom and colleagues and recently demonstrated by Blokesch, who also showed that the master regulator of this pathway, the CRP protein, is necessary for the expression of the competence genes (Meibom *et al.*, 2005; Blokesch, 2012a). Nevertheless, the mechanism by which CRP activates the competence genes was still obscure. In the model described for the bacterium *H. influenzae*, CRP activates the expression of the competence genes by binding competence-specific regulatory elements called CRP-S sites; furthermore, it was also suggested that such binding occurs together with TfoX though definitive experimental proof is still missing (Cameron & Redfield, 2008; Chapter 1, Figure 12). Since CRP-S site were also *in silico* predicted for the *Vibrionaceae* family (Chapter 1, Figure 13, based on earlier microarray expression data), we inquired if we could experimentally show that the *H. influenzae*'s model applies to *V. cholerae*.

Summarizing, the aims of this thesis were to address the following questions:

1. Is natural competence for transformation induced in all the cells within a bacterial population?
2. Are all competence genes subject to the same regulatory pathways?
3. Does HapR directly or indirectly activate the transcription of the competence genes?
4. How is QS interconnected with the other pathways necessary for natural competence induction?
5. Do the *in silico* predicted CRP-S sites have a role in the regulation of the competence genes in *V. cholerae*?

A scheme of the regulatory pathways involved in natural competence is shown in Figure 1. Indeed, our data will significantly contribute to explain how *V. cholerae* cells integrate intercellular communication with environmental cues to then exploit the ability to acquire new genetic material leading to the evolution of increased fitness phenotypes.

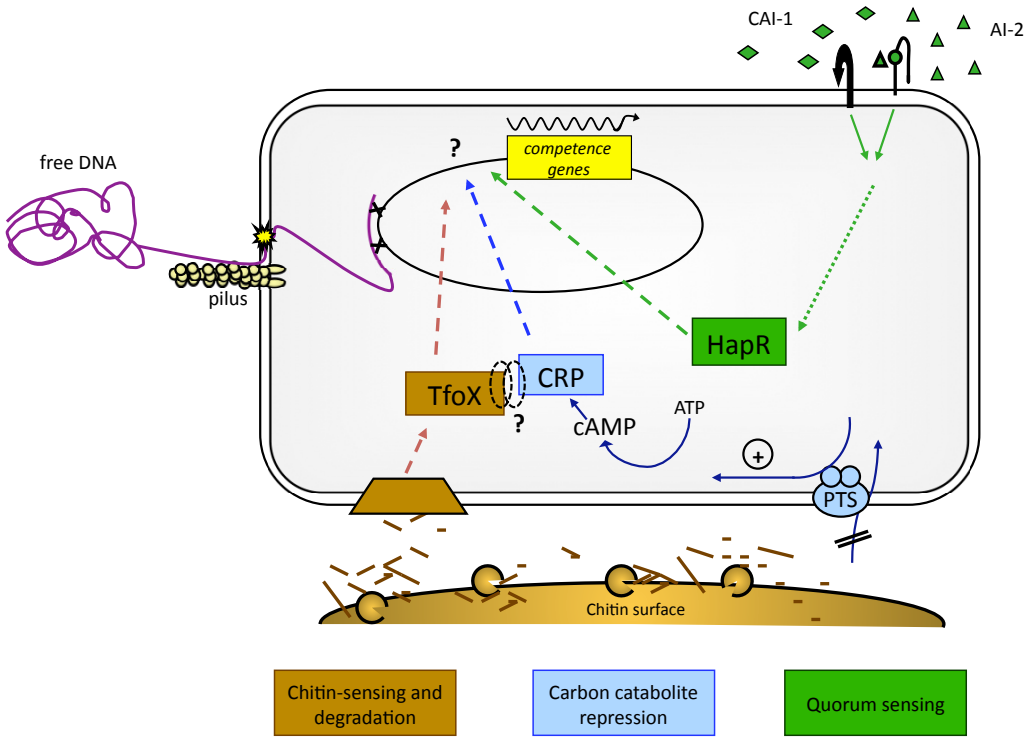

Figure 1. Model representation of pathways involved in the regulation of natural competence in *V. cholerae* (adapted from Blokesch, unpublished).

Chapter 3.

Results and discussion

3.1 Induction pattern of natural competence and its regulatory pathways

Overview: achievements and significance

In this section we provide new findings that contribute extensively to the understanding of the regulation of natural competence for transformation in *V. cholerae*. Our first aim was to investigate the induction pattern of natural competence. For this purpose we followed the expression of the competence genes using transcriptional reporter fusions. Initial attempts were performed by growing the bacteria on chitin beads to mimic the exoskeleton of the zooplankton. Under these conditions the competence genes were heterogeneously expressed within a given population. This observation prompted us to investigate the origin of this heterogeneity. Indeed, this heterogeneity could be the result of stochastic events, as observed for competence induction in *B. subtilis*, or the consequence of the heterogeneous availability of chitin-degradation products, PTS sugars, and QS signaling molecules. Notably, when we repeated the experiments using a soluble chitin source, to guarantee homogeneous growth conditions, the heterogeneous expression phenotype disappeared. This finding prompted us to conclude that stochastic events were not involved in the induction of the competence genes. Moreover, considering that the availability of chitin degradation products and autoinducers are most likely also not homogeneously distributed around the surface of zooplankton, we proposed that the heterogeneous induction pattern of natural competence genes reflects the situation in *V. cholerae*'s natural habitat.

We also optimized a method to artificially express *tfoX*, which encodes the master regulator of transformation, but without overproducing the protein. This method allowed us to induce natural competence and transformation in a chitin-independent manner and, compared to the very expensive soluble chitohexaose oligomer, represented a feasible method to examine phenotypes that are difficult to set up or analyze on chitin surfaces (e.g. microscopy and flow cytometry).

Apart from chitin, the CRP protein and the secondary messenger cAMP, two of the main players of CCR, are indispensable for natural competence (Blokesch, 2012a). However, it was unclear how CCR is linked to the chitin-sensing and -degradation pathway. Thus, we investigated the importance of cAMP in a chitin-independent manner. We demonstrated that cAMP is fundamental for the development of natural competence even if the induction of this mechanism is decoupled from chitin. Moreover, in the absence of the enzyme that degrades cAMP (cAMP-phosphodiesterase), a condition which should lead to rised cAMP levels, the bacterial transformability increased. Overall, these experiments emphasized the significant role of cAMP for the expression of the competence genes and thus for the occurrence of natural competence.

The next aim was to fully elucidate the contribution of QS to the network of natural competence for transformation. In a previous study Suckow *et al.* (2011) demonstrated that *V. cholerae* is unable to discriminate between self and foreign DNA at the level of the DNA uptake machinery; instead, the bacterium takes up any kind of DNA, even DNA which might never recombine due to a lack of homologous regions. However, the intra-species autoinducer CAI-1 is thought to facilitate the uptake of DNA belonging to the same and closely-related species thereby compensating for the lack of a proper discriminative DNA uptake apparatus. Despite the fact that the two autoinducers synthesized by *V. cholerae*, CAI-1 and the universal AI-2, converge in the same signaling cascade that leads to the production

of the master regulator of QS, HapR, only CAI-1 was necessary for the occurrence of natural competence (Suckow *et al.*, 2011).

We also confirmed these findings by inducing natural competence in a chitin-independent manner, and further investigated the role of CAI-1 in the expression of the competence genes. We showed that the expression of *comEA*, coding for a periplasmic DNA binding protein, which is important for DNA uptake into the periplasm (Seitz *et al.*, 2014), is QS-dependent; moreover, this expression mirrored the protein levels of HapR and proved to be dependent on the presence of CAI-1, but not to be influenced by the absence of AI-2. Notably, the expression of *pilA*, the gene coding for the major subunit of the DNA uptake machinery pilus (Seitz & Blokesch, 2013b), was not dependent on QS. These results strengthened the idea that QS, through CAI-1, is a checkpoint for the activation of natural transformation. Moreover, we showed for the first time that not all competence genes are subject to the same regulatory pathways but that at least two classes of competence genes exit in *V. cholerae*.

Another important finding resulted from our investigation of the production of the protein Dns in strains defective in the QS pathway. Dns is the extracellular nuclease that negatively influences natural transformation by degrading the extracellular DNA (Blokesch & Schoolnik, 2008; Suckow *et al.*, 2011; Seper *et al.*, 2011). In this study, we showed at the protein level that the proteins Dns and HapR are inversely present in the cell, which further indicates that HapR represses the gene encoding Dns.

Finally, we investigated which other competence genes were co-regulated by QS. Interestingly, we were able to show that the QS pathway regulates only a subset of those genes. In particular, HapR was necessary for the expression of *comEA* and *comEC*; the latter encodes an inner membrane transporter required for shutting the DNA into the cytosol of the competent bacteria. Our data therefore indicate that HapR has a double role in natural transformation: firstly, it represses the enzyme Dns thereby inhibiting the degradation of the extracellular DNA and secondly it drives the

expression of the two competence genes *comEA* and *comEC*. The mechanism how HapR regulates those genes was investigated by us and is presented later on (section 3.2).

The main achievements of this first section are summarized below:

1. The competence genes are homogeneously expressed within the population under homogeneous competence inducing conditions;
2. The induction pattern of natural competence in the aquatic environments most likely reflects the heterogeneity observed on chitin beads;
3. The metabolite cAMP is also fundamental to induce natural competence in the regulatory cascade downstream of *tfoX*;
4. Of the two autoinducers, CAI-1 is the main contributor to the QS signalling cascade that allows the production of HapR and as a consequence the expression of *comEA* and *comEC*;
5. HapR is necessary for the repression of Dns;
6. Only two competence genes, *comEA* and *comEC*, are co-regulated by QS.

This work provided novel insights into the regulation of natural competence for transformation and allowed us to propose a model of the regulatory network driving this mechanism of HGT. In this model, the environmental cues come from chitin, mediating induction of *tfoX,* which is the main input for natural competence induction. Furthermore, the CRP protein and its cofactor cAMP are also necessary for the expression of the competence genes. However, the repression of the nuclease and the onset of DNA uptake only occurs at high cell density, and we therefore conclude that the key signal for a functional uptake system is dependent on the intra-species autoinducer CAI-1.

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The Regulatory Network of Natural Competence and Transformation of *Vibrio cholerae*

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Abstract

The human pathogen *Vibrio cholerae* is an aquatic bacterium frequently encountered in rivers, lakes, estuaries, and coastal regions. Within these environmental reservoirs, the bacterium is often found associated with zooplankton and more specifically with their chitinous exoskeleton. Upon growth on such chitinous surfaces, *V. cholerae* initiates a developmental program termed "natural competence for genetic transformation." Natural competence for transformation is a mode of horizontal gene transfer in bacteria and contributes to the maintenance and evolution of bacterial genomes. In this study, we investigated competence gene expression within this organism at the single cell level. We provide evidence that under homogeneous inducing conditions the majority of the cells express competence genes. A more heterogeneous expression pattern was observable on chitin surfaces. We hypothesize that this was the case due to the heterogeneity around the chitin surface, which might vary extensively with respect to chitin degradation products and autoinducers; these molecules contribute to competence induction based on carbon catabolite repression and quorum-sensing pathways, respectively. Therefore, we investigated the contribution of these two signaling pathways to natural competence in detail using natural transformation assays, transcriptional reporter fusions, quantitative RT–PCR, and immunological detection of protein levels using Western blot analysis. The results illustrate that all tested competence genes are dependent on the transformation regulator TfoX. Furthermore, intracellular cAMP levels play a major role in natural transformation. Finally, we demonstrate that only a minority of genes involved in natural transformation are regulated in a quorum-sensing-dependent manner and that these genes determine the fate of the surrounding DNA. We conclude with a model of the regulatory circuit of chitin-induced natural competence in *V. cholerae*.

Citation: Lo Scrudato M, Blokesch M (2012) The Regulatory Network of Natural Competence and Transformation of *Vibrio cholerae*. PLoS Genet 8(6): e1002778. doi:10.1371/journal.pgen.1002778

Editor: Josep Casadesús, Universidad de Sevilla, Spain

Received January 13, 2012; **Accepted** May 3, 2012; **Published** June 21, 2012

Funding: This work was supported by Swiss National Science Foundation (SNSF) grant 31003A_127029. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

The bacterium *Vibrio cholerae* is a facultative pathogen and the causative agent of the disease cholera. Cholera is far from being extinct and is, in fact, considered a re-emerging disease [1]. The destructive capacity of cholera is demonstrated by its current outbreak in Haiti. According to a recent health bulletin issued by the Ministère de la Santé Publique et de la Population (MSPP) of Haiti and the PAHO, 515'699 cholera cases have been reported from Haiti up to November 30th 2011 with more than 6'942 deaths. This epidemic highlights the fact that new modeling approaches are required to allow for the prediction of cholera outbreaks in time and space [2–5]. However, it also initiated discussions on the origin of the *V. cholerae* strain, which appears more closely related to south Asian strains than to Latin American and U.S. Gulf Coast isolates [6]. This study by Chin *et al.* [6] once more reminded us of the differentiation power that whole genome sequencing provides. Indeed an earlier study by Rita Colwell and collaborators compared 23 *V. cholerae* strains isolated over the past 98 years using whole genome sequencing [7]. These authors concluded that "*V. cholerae* undergoes extensive genetic recombination via lateral gene transfer". It is therefore of major importance to understand the mechanisms underlying horizontal gene transfer (HGT).

Natural competence for transformation, as one of the three modes of HGT in bacteria, describes the physiological state that allows a bacterium to take up free DNA from the environment. If the internalized DNA is recombined into the chromosome, the bacterium is considered naturally transformed. *V. cholerae* commonly occurs in aquatic ecosystems, its true habitat, where it intimately associates with zooplankton and their chitinous exoskeleton [8–10]. In this context, it has been shown that chitin, the polymer used as the building block of planktonic exoskeletons, induces natural competence for transformation of *V. cholerae* [11]. Thus, HGT is tightly linked to the environmental niche of *V. cholerae* and potentially also to the niche of many other *Vibrio* species. In fact, three other species of the genus *Vibrio*, *V. fischeri*, *V. vulnificus* and *V. parahaemolyticus*, are naturally transformable in a chitin-dependent manner [12–14].

Transforming DNA can be used to repair damaged genes and, therefore, contributes to genome maintenance or to the acquisition of new alleles/genes, which lead to genetic diversity and evolution. Indeed, experimental laboratory microcosm experiments that simulate aquatic environments have succeeded in recapitulating a *V. cholerae* O1-to-O139 serogroup conversion by means of natural transformation [15]. This result provides a potential explanation for the devastating occurrence of the O139 serogroup variant of *V. cholerae*. Today this strain is almost undetectable in endemic

Author Summary

The human pathogen *Vibrio cholerae* is an aquatic bacterium often encountered in rivers, estuaries, and coastal regions. Within this environmental niche, the bacterium often associates with the chitinous exoskeleton of zooplankton. Upon colonization of these chitinous surfaces, *V. cholerae* switches on a developmental program known as natural competence for genetic transformation. Natural competence for transformation is a mode of horizontal gene transfer that allows bacteria to acquire new genes derived from free DNA, which is released by other members within the same habitat. The evolutionary consequences could be that the bacterial recipient becomes better adapted to its environmental niche or, in a worst-case scenario, more pathogenic for man. The results of this study show that, under optimal conditions, the majority of cells within a *V. cholerae* population express competence genes. However, in an aquatic environment, a combination of different ecological factors might lead to heterogeneity in the competence phenotype. Therefore, we investigated the role of extracellular and intracellular signaling molecules with respect to competence induction. This report illustrates that at least three interconnected signaling cascades are required for competence induction, which are based on bacterial metabolism and group behavior.

regions even though researchers have feared its occurrence as the onset of a new and, therefore 8th, cholera pandemic [16]. However, an important lesson can be learned from the emergence of this new strain: by means of horizontal gene transfer (HGT), *Vibrio* species may exchange genetic material and become more virulent to mankind.

Chitin-induced natural competence and transformation is poorly understood in spite of its importance. Based on a few suggestive experiments on how natural competence could be regulated in *V. cholerae*, the authors of a previous study proposed a model that involved at least three regulatory pathways [11]: 1) induction by chitin, 2) catabolite repression, and 3) quorum-sensing (QS). We and others followed up on this study and provided further evidence for an involvement of these three pathways [17–23]. However, all of these studies have only looked at a population-wide level. This could lead to a lack of information on how competence is regulated within a single cell. This is exemplified in one of the best-studied naturally competent bacterial species, *Bacillus subtilis*, for which it is known that "a majority of the bacteria being insusceptible and a minority being highly susceptible to transformation" [24]. David Dubnau and collaborators explained why only 10–20% of cells within a *B. subtilis* population enter the competence state, and demonstrated that such bistability is caused by intrinsic noise in competence gene expression [25,26]. Here we show, for the first time, that under homogeneous competence–inducing conditions *V. cholerae* displays a homogeneous expression pattern as the vast majority of cell within a population scored positive for expression of competence genes.

Taking this important finding into consideration, we then moved on to establish an inducible competence system for *V. cholerae*, which is based on low levels of TfoX production and not on *tfoX* overexpression as previously done. To date all studies on natural competence and transformation in *V. cholerae* have only looked at single genes involved in the competence program, at single pathways, and at varying inter-experimental conditions (e.g., chitin surface transformation phenotypes compared to artificial competence induction with plasmids in rich medium etc.) [17,20–23]. This inducible and chromosomally encoded competence system allowed us to look at different aspects of the regulatory network of natural competence under standardized conditions. Based on these new data, we propose a model of how the regulatory network of natural competence functions in *V. cholerae*.

The goals of our study were to 1) investigate whether natural competence is induced in a whole population under natural or optimized conditions, 2) establish a homogeneous competence-inducing system to investigate the contribution of separate and interconnected regulatory pathways to competence induction, and 3) test whether different competence genes are subject to the same regulatory circuits. We achieved these aims by using transcriptional reporter fusion constructs of representative competence genes. We combined these fluorescent reporter fusions with the following detection methods: epifluorescence microscopy and flow cytometry, which allowed us to visualize the expression of fluorescent reporter genes at the level of single cells and to quantify gene expression accordingly; and fluorescent plate reading, which we used to investigate a plethora of regulatory mutants and regulated genes based on population average fluorescent value measurements.

Results/Discussion

Visualization of competence gene expression upon chitin surface colonization

To better understand whether natural competence of *V. cholerae* is a developmental program followed by (almost) all members of a population or rather a state, which only a subpopulation acquires, we investigated gene expression at the single cell level. Therefore, we transcriptionally fused the promoter regions of competence genes to those genes encoding fluorescent proteins (FPs). Our choice of FPs was GFP-mut3* [27] and DsRed.T3[DNT] [28,29] as both of them have been optimized for fluorescence intensity and can be visualized within the same cell (i.e., the excitation/emission spectra are adequately separated). We initially focused on two promoter regions: the upstream region of the *pilA-D* operon [30], hereafter referred to as the *pilA* promoter, and the region upstream of *comEA*. Both of these genes, *pilA* and *comEA*, are upregulated on chitin ([11,31]; Blokesch and Schoolnik, unpublished) and essential for natural transformation to occur [11]. *PilA* encodes a major pilin, which, as a part of a hypothetical type IV-like pilus [30], is most likely involved in the DNA uptake process. The same involvement in DNA uptake holds true for ComEA, which shows homology to ComEA of *Bacillus subtilis* [32] as it also contains a helix-hairpin-helix (HhH) motif (pfam12836). HhH motifs have been previously described as short DNA-binding domains that bind DNA in a non-sequence-specific manner [33]. How exactly the DNA uptake, including the involvement of the type IV-like pilus and ComEA, functions is so far unknown for *V. cholerae* and for other naturally competent bacteria.

With these reporter fusions in hand, we moved on to visualize competence gene expression. We first tested the expression in *V. cholerae* strains after allowing them to colonize chitin beads. Chitin beads mimic the natural environment of *V. cholerae* in which the bacteria are often found associated with the chitinous exoskeletons of zooplankton [10]. In contrast to other competence-inducing chitin surfaces, such as crab shell fragments or chitin flakes [11,19], chitin beads are amenable to light microscopy. As shown in Figure 1, no significant green or red fluorescence was detectable by epifluorescence microscopy for *V. cholerae* cells grown on chitin beads if the bacteria carried the promoter-less FP reporter plasmid (vector control; panel I). In contrast, bright fluorescence signals

were visible when the FP genes were driven by either of the two promoters belonging to *pilA* or *comEA* (panel II and reciprocal with respect to the FP-fusions in panel III). From the obtained images, it was apparent that not all of the bacteria within the population were fluorescent and, therefore, expressing the competence genes at detectable levels (Figure 1). Based on the finding that not all bacteria appeared as fluorescent we constructed another reporter as positive control, which consisted of the promoter preceeding the housekeeping gene *gyrA* (encoding gyrase) transcriptionally fused to *gfp*. This fusion was cloned onto the same plasmid as the P_{comEA}-*dsRed* fusion (see Material and Methods). We tested this reporter strain in our chitin bead colonization assay (Figure 1, panel IV). In contrast to *dsRed* being driven by the *comEA* promoter the *gyrA* promoter led to detectable *gfp* expression in a significantly larger fraction of cells. The same expression pattern as for *gyrA* was observable for three additional transcriptional reporter fusions containing the promoter region of *recA*, *clpX*, and *ftsH*, respectively (Figure S1). We included these reporter strains in our study as the expression of the housekeeping gene *gyrA* is most likely controlled by DNA supercoiling as it was demonstrated for *E. coli* [34] and/or might be cell cycle-dependent as shown for *Caulobacter crescentus* [35]. The rational for choosing *recA*, *clpX*, and *ftsH* as additional positive controls was based on the fact that other researchers have already used these genes to normalize quantitative RT-PCR expression data. Furthermore, expression of none of these genes was significantly changed in microarray expression studies using different *V. cholerae* mutant strains ([11,36] and Blokesch and Schoolnik, unpublished) or using different growth conditions (e.g. comparing rabbit ileal loop grown cells versus exponential *in vitro* cultures of *V. cholerae* [37]).

We considered three different reasons for the finding that competence genes are only expressed at detectable levels in a fraction of the population: 1) competence gene expression in *V. cholerae* is a bistable phenomenon, which is similar to *B. subtilis* [25]; 2) the environment around the chitinous surface is heterogeneous and, thus, does not lead to competence induction in all cells; and 3) the fluorescence signal in cells that appear as non-induced for *pilA* and *comEA* expression is too weak to be detected with our epifluorescence microscopy settings.

To follow up on these three possibilities, we aimed at differentiating between the existence of an intrinsic bistable switch versus the idea of a heterogeneous expression pattern due to heterogeneous conditions and to concomitantly judge whether the seemingly uninduced cells observed on the chitinous surfaces (Figure 1) were the result of experimental limitations in our system.

Population-wide expression of competence genes under homogeneous conditions

First, we wanted to test if we would observe a bistable competence gene expression pattern under homogeneous growth conditions. Therefore, we changed the chitin substrate from chitin beads (an insoluble GlcNAc polymer) to soluble hexa-N-acetylchitohexaose (from here on referred to as $GlcNAc_6$). This chitin oligomer has been used before to induce natural competence in *V. cholerae* [11,23]. As a control, we grew the same *V. cholerae* reporter strains under identical minimal medium conditions (defined artificial seawater medium, DASW; [11]) but changed the main carbon source from $GlcNAc_6$ to the non-competence inducing chitin monomer N-acetylglucosamine (GlcNAc). All strains were grown to the same optical density before being either visualized by epifluorescence microscopy (Figure S2) or quantified with respect to their fluorescence intensity using flow cytometry (Figure 2). As shown in Figure S2A and S2B, we did not detect significant fluorescent signals for the promoterless reporter control using microscopy, which is in accordance with the low levels of fluorescent signals measured by flow cytometry (Figure 2A). Thus, the fluorescence intensity of these bacteria (panel A, flow cytometry graphs) was considered background. For the strains grown with GlcNAc, FP expression driven by the *comEA* promoter was not detectable using microscopy (Figure S2C, S2E, S2G including the respective image analysis) and quantified as extremely low fluorescent signals using flow cytometry (Figure 2B–2D upper row). However, weak *pilA* promoter-driven *gfp* expression in the presence of GlcNAc was observed after an extended exposure time (Figure S2C). We confirmed this basal *pilA* promoter-driven *gfp* expression in the presence of GlcNAc by flow cytometry (mean FU = 7.2×10^2; Figure 2B, upper row). Swapping the FP reporter gene behind the *pilA* promoter from *gfp* to *dsRed* resulted in undetectable red fluorescence using our epifluorescence microscopy settings (Figure S2E); however, an increased *pilA* promoter-driven expression of *dsRed* compared to the promoterless reporter plasmid control (Figure 2A) was detectable by flow cytometry (Figure 2C, upper row), confirming the low level of *pilA* expression under non-competence inducing conditions.

In cells grown under competence-inducing conditions (e.g. in the presence of $GlcNAc_6$), the reporter strains displayed strong *pilA* and *comEA* promoter-driven fluorescent signals (Figure S2D, S2F, S2H). This was the case for the majority of the cells and only a minority appeared as non-fluorescent under these conditions. There was a distribution of fluorescence intensities as depicted in the flow cytometry graphs (Figure 2B–2D, lower row) but the distribution was not bimodal. To further investigate whether the non-fluorescent-appearing cells in the microscopy images (Figure S2) were meaningful with respect to competence expression, we again investigated the behavior of the housekeeping gene reporter strains under these homogenous competence-inducing conditions (for *gyrA* see Figure 2D and Figure S2G and S2H; or for *recA*, *clpX*, and *ftsH* see Figure S3). The same expression pattern as for the competence genes was observed for these strains with a minority of cells not displaying any detectable fluorescence using our epifluorescence microscopy and image display settings. Thus, and also based on the image analysis (Figure S2) and on the flow cytometry measurements (Figure 2), this minority of non-fluorescent-appearing bacteria probably corresponds to cells, which fluoresce at lower levels (mostly with a good correlation between both FPs; Figure S2).

An issue that we had to consider was the fact that our FP reporter constructs were plasmid-encoded. Indeed, plasmid copy numbers can change in *V. cholerae* according to growth rate [38]. However, in our experiments we mainly looked at different strains but under similar growth conditions (and the biological replicates were highly reproducible; Figure S4). Furthermore, a recent study by Silander *et al.* provided evidence that plasmid-based systems are useful to study gene expression in bacteria; they observed that both the mean and the variation of expression correlated well between both settings [39].

A chromosomally encoded inducible competence system allows quantification of gene expression

Based on the data described above, we concluded that bistability of competence gene expression is unlikely in a population of *V. cholerae* cells under homogeneous competence-inducing conditions. Therefore, it seemed feasible to further investigate the regulatory circuit at a population-wide level given that homogeneous, competence-inducing growth conditions were provided. Unfortunately, $GlcNAc_6$, a competence-inducer, has recently been discontinued by the Seikagaku Corporation, and multiple and large-scale experiments using this commercially available com-

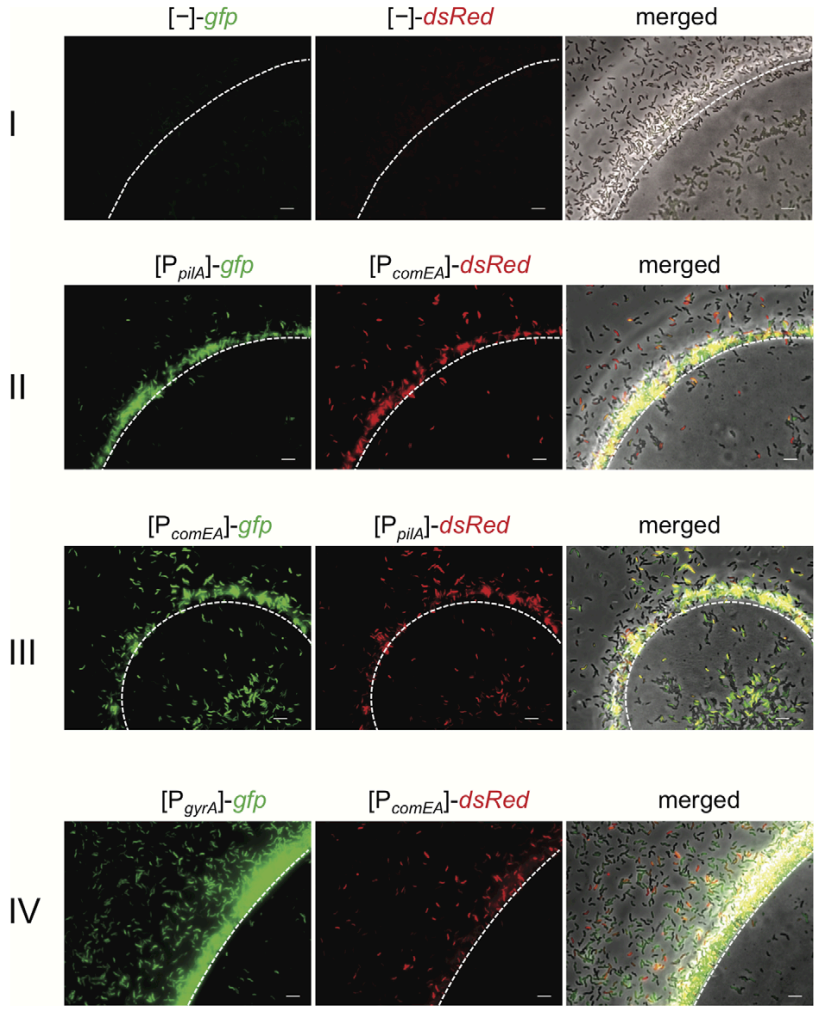

Figure 1. Visualization of competence gene expression on chitin surfaces. *V. cholerae* cells were grown on chitin beads as previously described [23]. The white dashed line notes the edge of the chitin surface. The bacteria carried diverse transcriptional FP reporter fusions. I: vector control containing promoter-less *gfp* and *dsRed*; II: plasmid containing *gfp* driven by the *pilA* promoter and *dsRed* downstream of the *comEA* promoter region; III: swapped reporter genes in comparison to II; IV: plasmid containing *gfp* driven by the *gyrA* promoter and *dsRed* driven by the *comEA* promoter. Bacteria were grown statically for 48 h before pictures were taken. The order of the images here and in the following figures is (from left to right): green channel (GFP), red channel (dsRed), and merged image composed of a phase contrast image overlaid by both fluorescence channel images. Scale bar = 5 μm.
doi:10.1371/journal.pgen.1002778.g001

pound are extremely costly. Furthermore, shorter GlcNAc oligomers such as $GlcNAc_2$ often result in large variations with respect to transformation frequencies (M. Blokesch, unpublished), which is most likely due to GlcNAc monomer impurities within the preparation that exert catabolite repression on natural transformation [23]. Therefore, we thought of establishing a chitin-independent, competence-inducing system. One possibility was to artificially overexpress the major regulator of transformation, TfoX, from a plasmid as previously performed [11,17,22,23]. However, we observed major disadvantages using this method. First, the morphology of some of the cells changed towards a filamentous form after competence induction due to the plasmid-maintaining antibiotic ampicillin (Blokesch, unpublished). Second, TfoX overexpression from a multi-copy plasmid resulted in the induction of heat shock proteins and chaperones [11], which might be indicative of stress conditions. Indeed, the toxicity of TfoX overexpression has been previously described for *Escherichia coli* and *Haemophilus influenzae* [40,41]. Third, we wanted to avoid working with *V. cholerae* cells containing two different plasmids within the same cell (*tfoX* and FP reporter fusions-carrying). Therefore, we constructed a chromosomally encoded competence induction system, which is based on inducible low-level TfoX production. The system was composed of *tfoX* under the control of an arabinose-inducible promoter (P_{BAD}) and the gene encoding AraC, which act as a repressor or initiator of gene expression in the absence or presence of L-arabinose, respectively [42]. Both of these elements were cloned into a mini-Tn7 transposon [43], which integrates into the large chromosome of *V. cholerae* (later

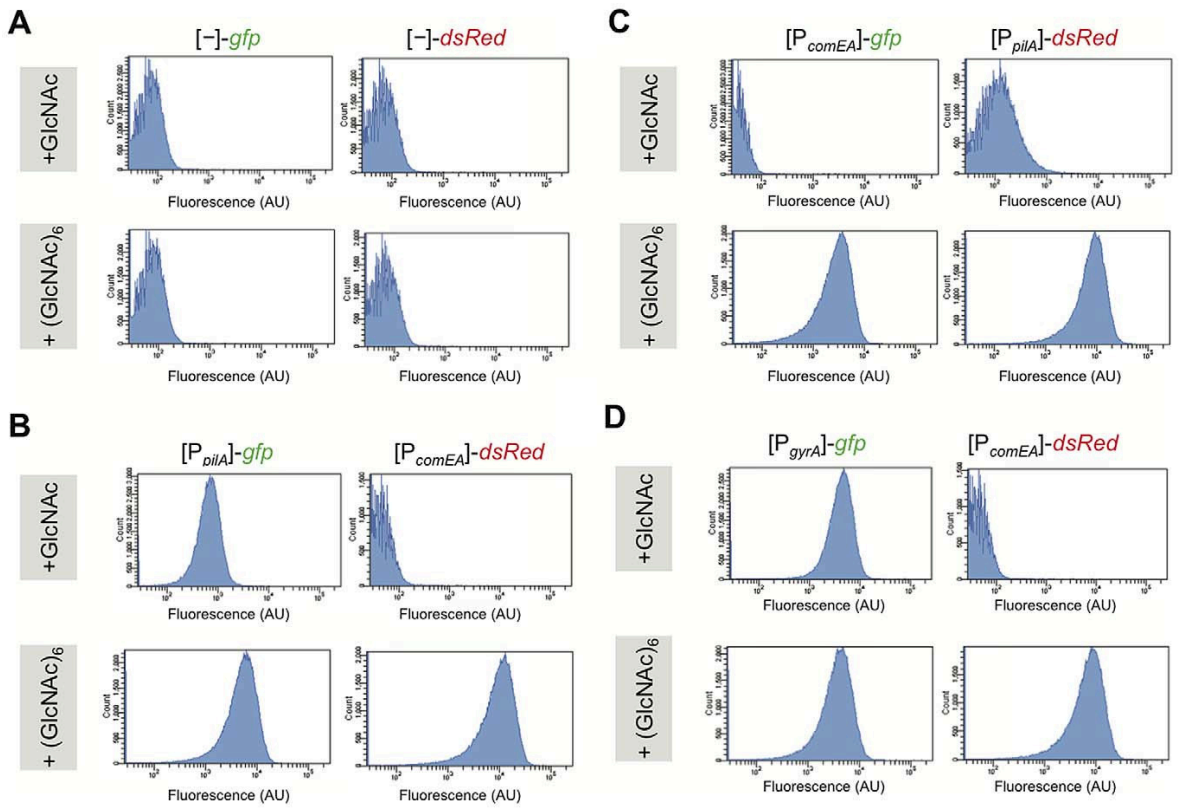

Figure 2. Competence genes are expressed by the majority of cells under homogeneous competence-inducing conditions. *V. cholerae* strains were grown aerobically in defined artificial seawater medium with the addition of either N-acetylglucosamine (GlcNAc) or hexa-N-acetylchitohexaose $(GlcNAc)_6$ as sole carbon source. The latter is known as a potent inducer of natural competence/transformation [11]. Competence gene expression was quantified for fluorescence intensities using flow cytometry. Reporter fusions are indicated above each panel. Panel A: promoter-less *gfp* and *dsRed* reporter; panel B: [P_{pilA}]-*gfp*/[P_{comEA}]-*dsRed*; and panel C: [P_{comEA}]-*gfp* and [P_{pilA}]-*dsRed*. Panel D: [P_{gyrA}]-*gfp*/[P_{comEA}]-*dsRed*. The flow cytometry graphs indicate the number of cell counts on the y-axis and the fluorescence signal intensity (as arbitrary units, AU) on the x-axis.
doi:10.1371/journal.pgen.1002778.g002

referred to as Tn*tfoX*). We transferred this transposon by triparental mating into the *V. cholerae* wild type strain A1552 and tested the respective strain for natural transformability in LB medium (Figure 3). By adding a low amount of L-arabinose (0.02%), we obtained transformation frequencies that were two orders of magnitude higher than what has been described for overproduced *tfoX* in *V. cholerae* [11] (Figure 3A). Furthermore, the transformation frequency (2.1×10^{-4}) was in the same range as the frequencies we usually obtain using optimized chitin-inducing conditions (3.1×10^{-4}; [19]). We were unable to detect any naturally transformed colony-forming units in the inducer-free control culture (shown for the wild type in Figure 3A but likewise tested in all other strains). We also examined the abundance of TfoX at the protein level. To do so we grew the *V. cholerae* strain A1552-Tn*tfoX* in LB medium in the absence or presence of different L-arabinose concentrations followed by western blot analysis of cellular proteins using antibodies against TfoX (Figure S5). In parallel we grew a strain containing inducible *tfoX* on a plasmid similar to the *tfoX*-overexpression system described earlier [11]. As shown in Figure S5 we observed a major difference in TfoX protein levels comparing the previous [11] and current experimental setup as indicated by the two arrows. This reassured us that this system was not heavily overproducing TfoX and was therefore adequate for further analysis to establish the genetic interactions downstream of TfoX.

We first wanted to visualize competence gene expression in this chitin-independent system. We transferred the respective FP reporter fusion constructs into a wild type *V. cholerae* strain carrying the chromosomally encoded *tfoX* construct (A1552-Tn*tfoX*) and visualized FP gene expression by epifluorescence microscopy (Figure 3B). As can be appreciated from the images in Figure 3B (middle and lower part), the *pilA*- and *comEA* promoter-driven expression pattern of the FP reporters under such chitin-independent, competence-inducing conditions mirrored what we observed under the $GlcNAc_6$-mediated induction of competence (Figure S2). As described above for the chitin-dependent experiment, only a minority of cells did not display any detectable fluorescence using this microscopy technique, which was also the case for *gyrA* promoter-driven FP reporter expression (data not shown). The fluorescent signal was below the detection limit in cells grown in the absence of inducer (Figure 3B, -ara) or in cells harboring the promoter-less plasmid as a control (Figure 3B, upper two rows).

The fluorescent signal was quantified using a 96-well plate reader (Figure 3C). A statistically significant increase in fluorescence intensity was observed upon induction of competence for all promoter-driven FP reporter fusion constructs (Figure 3C, middle and right columns). No significant difference in fluorescence signals between competence-uninduced and competence-induced

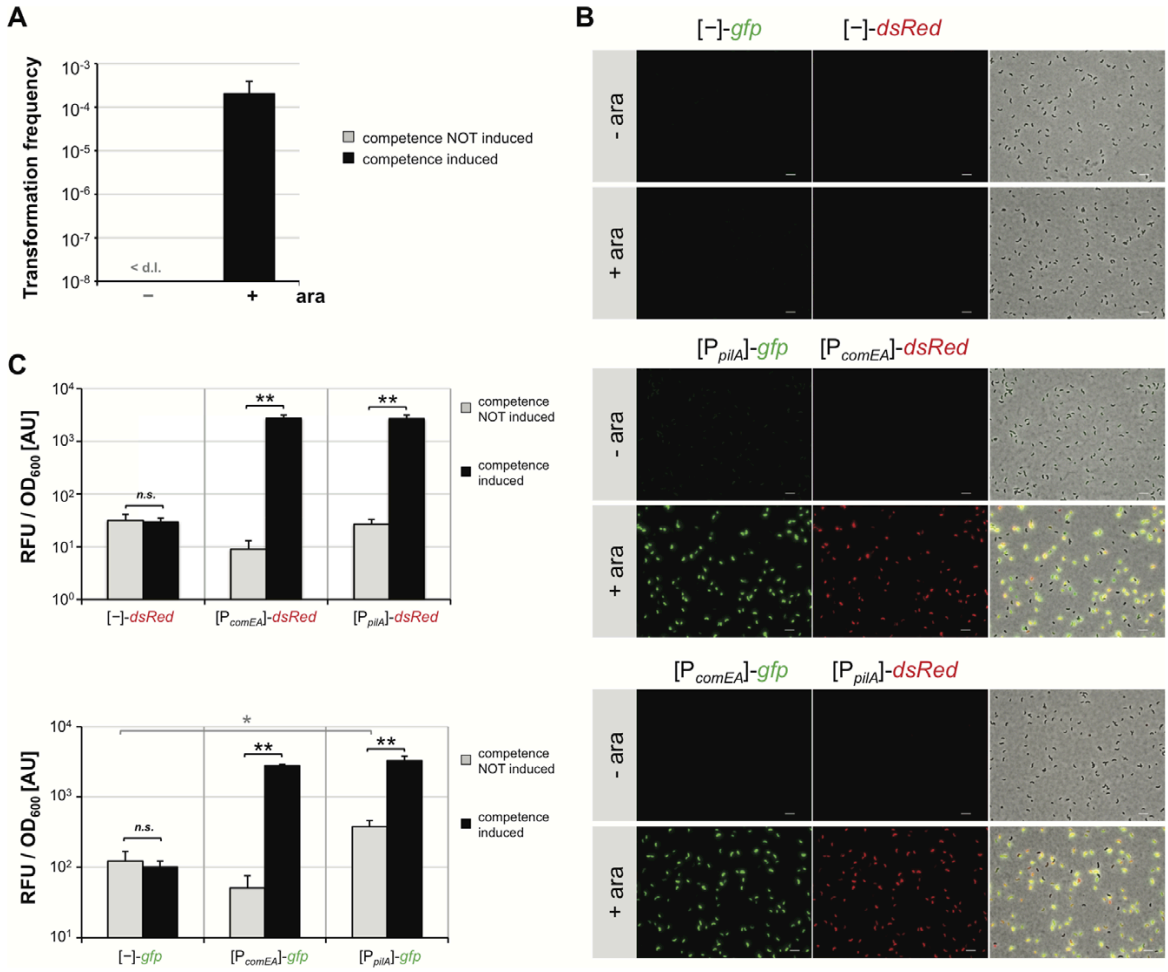

Figure 3. Artificial induction of natural transformation by expression of the competence regulatory gene *tfoX in cis*. *V. cholerae* cells were grown in rich medium in the absence (−) or presence (+) of the artificial inducer arabinose (0.02%). Cells were either tested for natural transformability (panel A) or competence gene promoter activity based on FP reporters (panels B and C). Panel A: Transformation frequencies are given on the y-axis for competence-uninduced (−) and competence-induced (+) bacteria. <d.l. = below detection limit. Panel B and C: *V. cholerae* cells harboring the different transcriptional reporter fusions were grown without or with competence induction. Bacteria were either visualized by epifluorescence microscopy (panel B; image arrangements as in Figure 1; Scale bar = 5 µm) or measured with respect to relative fluorescence units (RFU) and optical density at 600 nm (panel C). Panel C: RFU per OD_{600} values are given on the y-axis. All experiments in Figure 3 were repeated at least three independent times. Error bars reflect standard deviations. Statistics were applied using the Student's *t* test. * $P<0.05$, ** $P<0.01$, *n.s.* = not significant.
doi:10.1371/journal.pgen.1002778.g003

conditions was observed for the promoter-less FP reporter control (Figure 3C). In agreement with the chitin data described above, we detected a statistically significant increase in *pilA*-driven *gfp* expression compared to the promoter-less construct even in the absence of inducer. Therefore, we conclude that this basal expression of *pilA* is TfoX-independent.

Induction of natural competence is dependent on the cAMP level within cells

We then moved on to investigate the regulatory network of natural competence in *V. cholerae* in further detail. The first assay aimed at testing whether carbon catabolite repression (CCR) plays a role in this chitin-independent setup. Carbon catabolite repression occurs if preferred phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS)-transported sugars are abundant. However, in their absence the PTS systems are unsaturated and indirectly lead to the activation of the enzyme adenylate cyclase, which subsequently synthesizes cAMP within the cell (for review on CCR see [44]). We were mostly interested in *V. cholerae* strains that are impaired in this synthesis or in the degradation of cAMP. The concentration of cAMP within cells is accomplished by interplay between adenylate cyclase (CyaA) and cAMP-degrading phosphodiesterases (CpdA). A recent study by Kim *et al.* demonstrated the importance of CpdA in balancing the intracellular cAMP level in *Vibrio vulnificus* [45]. As the *cpdA* gene of *V. cholerae* is located at the same chromosomal locus (as analyzed using *SynTView*, a synteny viewer developed by the Genomic and Genetic Department of Institute Pasteur, Paris) and *cpdA*/CpdA display 64%/68% identity (76%/82% similarity) at the DNA and

protein levels, respectively, the functionality of the protein is most likely identical in both organisms. To disrupt the equilibrium between cAMP production and degradation, the *cpdA* gene in *V. cholerae* was deleted (Table 1). Although the production of cAMP does not change in this mutant, cAMP degradation should be impaired, resulting in the accumulation of cAMP within the cell. We tested this strain in a chitin surface colonization assay [23] and observed a hyper-colonization phenotype consistent with increased intracellular cAMP levels (data not shown). More importantly, we transferred the Tn*tfoX* transposon into this *V. cholerae* strain as well as a strain lacking adenylate cyclase (Δ*cyaA*) and tested both strains with respect to natural transformability (Table 2) and *pilA/comEA* promoter-driven FP gene expression (Figure S6). The results confirmed part of what we had previously demonstrated on chitin surfaces [23], namely that adenylate cyclase is essential for natural transformation even under rich culture medium conditions. However, in this study we extended this knowledge by showing that a statistically significant increase in natural transformability occurred in the newly constructed *cpdA* mutant compared to the wild type parental strain (Table 2). With respect to competence gene induction, *pilA* and *comEA* promoter-driven FP gene expression was abolished in the absence of cAMP (Figure S6). This was in contrast to the fluorescent signal measured for the *cpdA* mutant, that is, both the *pilA* and the *comEA* promoter efficiently drove FP gene expression in this genetic background upon competence induction (Figure S6). These data highlight the necessity of cAMP for competence gene expression even when competence induction is uncoupled from chitin surface colonization and chitin degradation (e.g., from metabolism of carbon sources).

Quorum-sensing only regulates a subset of competence genes

The next question we wanted to address was with respect to QS and the involved autoinducer molecules. We recently showed that the species-specific cholera autoinducer 1 (CAI-1; [46,47]) plays a major role in natural competence for transformation and suggested that CAI-1 could be considered a competence pheromone [22]. We showed that the absence of the non-species-specific autoinducer 2 (AI-2; [48]) had no statistically significant effect on natural transformation on chitin surfaces, whereas strains devoid of CAI-1 synthesis were rarely transformable, and, even then, only at very low transformation frequencies [22]. However, as described above, chitin surfaces appear to be a rather heterogeneous environment, and cells might not all encounter the same autoinducer concentration in time and space, making chitin surface experiments difficult to conclusively judge the involvement of QS in the regulatory circuit of *V. cholerae*. Therefore, we investigated the role of QS in natural competence and transformation using our homogeneous competence-inducing system (Figure 4). We constructed Tn*tfoX*-containing *V. cholerae* deletion strains, which were devoid of either or both of the autoinducer-synthesizing enzymes CqsA and LuxS, or which lacked the gene encoding the major regulator of QS, HapR. These strains were grown in LB medium in the presence or absence of the competence-inducer arabinose and scored for natural transformability or competence gene promoter-driven FP expression (Figure 4). In the absence of inducer, the transformation frequency was consistently below the level of detection in all strains (Figure 4A). In the absence of the AI-2-producing enzyme LuxS, only a minor and statistically not significant decrease in transformation frequency upon competence induction was detectable when compared to the wild type parental strain (Figure 4A). More importantly, the dependency on CAI-1 was even enhanced when compared to our previous study on chitin surfaces, in that a deletion in the gene *cqsA* completely abolished natural transformation. This was also the case in strains devoid of both autoinducer synthases (CqsA and LuxS) and the major regulator of QS, HapR (Figure 4A). We argue that few occasionally detectable transformants in a CAI-1 negative mutant in our previous study on chitin flake surfaces [22] were the result of the heterogeneity of the chitin surface environment in which at least three components are not evenly distributed: autoinducers, transforming DNA and nuclease. However, under homogeneous conditions as tested here, a full dependency on CAI-1 is apparent.

We then visualized and quantified competence gene expression in these strains using the above-described FP report fusions (Figure 4B, 4C). The data for *comEA* promoter-driven FP expression (Figure 4B) mirrored the data of the transformation assay (Figure 4A). Under non-competence-inducing conditions, only the background fluorescence was measurable (in the range of the vector control shown in Figure 3). There was no statistically significant difference between the fluorescence signal detected in the wild type strain and the signal derived from the *luxS*-deficient strain upon competence-inducing conditions. A highly significant reduction of *comEA* promoter-driven FP gene expression was detected in the *cqsA*, *cqsA/luxS* and *hapR* negative strains. We also measured for the first time *pilA* promoter-driven FP expression in the different QS mutants and thus in the presence or absence of the two autoinducers. We observed that the expression pattern looked completely different from the *comEA* data; though the fluorescent signal increased upon competence induction, the fluorescence units were in the same range for all strains tested (Figure 4C). Therefore, we conclude that, in contrast to *comEA*, *pilA* is not subject to QS-dependent regulation.

Taken together, we provide evidence that CAI-1 is essential for *comEA* expression and natural transformation under homogeneous competence-inducing conditions. These data are in slight contrast to another study where a gradual decrease in *comEA* expression and natural transformation from a wild type *V. cholerae* strain towards an AI-2- and CAI-1-deficient strain, respectively, was displayed [21]. The authors of this study concluded that not only CAI-1 but also AI-2 contributes to natural transformation. We believe that the discrepancy between studies (the study described here, [21] and [22]) could reflect the different *V. cholerae* O1 El Tor strains used in both studies (A1552 here and in [22], versus C6707str in [21]). This hypothesis is in excellent agreement with a recent finding by Fong and Yildiz [36], who showed that the cAMP-CRP-mediated negative regulation of the biofilm regulatory gene *vpsR* only occurred in three out of four tested *V. cholerae* strains, namely strains A1552, N16961 and MO10. In contrast to this result, *V. cholerae* strain C6706 displayed no such regulation [36], highlighting the fact that different regulatory circuits exist in these *V. cholerae* strains.

The amount of HapR within cells dictates *comEA* expression and nuclease repression

The involvement of QS in natural transformation has previously been demonstrated by elucidating a role for HapR in the repression of a gene encoding the extracellular nuclease Dns [17]. This conclusion was mainly based on comparing a wild type *V. cholerae* strain to a ΔhapR mutant with respect to *dns* gene expression or nuclease activity. However, a direct correlation between HapR protein levels within the cell, *dns* repression, and *comEA* induction has never been demonstrated. We addressed this missing information by performing western blot analysis of non-competence-induced and competence-induced cells to detect the HapR protein within different QS mutant strains (Figure 5A). Whereas the amount of HapR did not differ between *tfoX*-induced

Table 1. Bacterial strains and plasmids.

Strains or plasmids	Genotype*	References
Strains (*V. cholerae*)		
A1552	Wild type, O1 El Tor Inaba, Rif^R	[68]
A1552-LacZ-Kan	A1552 strain with *aph* cassette in *lacZ* gene; Rif^R, Kan^R	[19,69]
ΔcyaA	A1552ΔVC0122, Rif^R	[23]
ΔcpdA	A1552ΔVC2433, Rif^R	this study
ΔcqsA	A1552ΔVC0523, Rif^R	[22]
ΔluxS	A1552ΔVC0557, Rif^R	[70]
ΔcqsAΔluxS	A1552ΔVC0523ΔVC0557, Rif^R	[22]
ΔhapR	A1552ΔVC0583, Rif^R	[11]
A1552-Tn*tfoX*	A1552 containing mini-Tn7-*araC*-P_{BAD}-*tfoX*; Rif^R, $Gent^R$	this study
ΔcyaA-Tn*tfoX*	A1552ΔcyaA containing mini-Tn7-*araC*-P_{BAD}-*tfoX*; Rif^R, $Gent^R$	this study
ΔcpdA-Tn*tfoX*	A1552ΔcpdA containing mini-Tn7-*araC*-P_{BAD}-*tfoX*; Rif^R, $Gent^R$	this study
ΔcsqA-Tn*tfoX*	A1552ΔcsqA containing mini-Tn7-*araC*-P_{BAD}-*tfoX*; Rif^R, $Gent^R$	this study
ΔluxS-Tn*tfoX*	A1552ΔluxS containing mini-Tn7-*araC*-P_{BAD}-*tfoX*; Rif^R, $Gent^R$	this study
ΔcqsAΔluxS-Tn*tfoX*	A1552ΔcqsAΔluxS containing mini-Tn7-*araC*-P_{BAD}-*tfoX*; Rif^R, $Gent^R$	this study
ΔhapR-Tn*tfoX*	A1552ΔhapR containing mini-Tn7-*araC*-P_{BAD}-*tfoX*; Rif^R, $Gent^R$	this study
Plasmids		
pBR322	Amp^R, Tc^R	[61]
pGP704-Sac28	Suicide vector, ori R6K *sacB*, Amp^R	[31]
pGP704-28-SacB-ΔcpdA	pGP704-Sac28 with a gene fragment resulting in a	
351-bp deletion of VC2433 (*cpdA*)	this study	
pVSV209	Kan^R, *rfp* (DsRed.T3[DNT]), transcriptional terminators-(*Avr*II, *Sal*I, *Stu*I)-promoterless Cm^R and *gfp;* $oriV_{R6K}$;	[29]
pBKdsGFP	Derivative of pBR322; promoterless *gfp* preceded by MCS; Kan^R, *rfp* (DsRed.T3[DNT]), transcriptional terminators-(*Avr*II, *Sal*I, *Stu*I) derived from pVSV209	this study
pBR-Tet_MCSI	pBR322 derivative deleted for Tet promoter and part of tet^R gene; Amp^R	this study
pBR-Tet_MCSI-GFP	Promoterless *gfp* from pBKdsGFP cloned into *Aat*II/*Eco*RI site of pBR-Tet_MCSI; Amp^R	this study
pBR-Tet_MCSI-GFP_dsRed	Promoterless *rfp* (DsRed.T3[DNT]) from pBKdsGFP cloned into *Eco*RV/*Bam*HI site of pBR-Tet-MCSI-GFP; Amp^R	this study
pBR-GFP_dsRed_Kan	*aph* gene (from pBKdsGFP) cloned into *BamH*I site of pBR-Tet-MCSI-GFP_dsRed; Amp^R, Kan^R	this study
pBR-GFP-[P_{comEA}]dsRed-Kan	Upstream region of *comEA* (~200 bp) cloned into *Stu*I site of pBR-GFP_dsRed_Kan; Amp^R, Kan^R	this study
pBR-[P_{pilA}]GFP-[P_{comEA}]dsRed-Kan	Upstream + part of coding region of *pilA* gene (~600 bp) cloned into *Sma*I site of pBR-GFP-[P_{comEA}]dsRed-Kan; Amp^R, Kan^R	this study
pBR-[P_{VC0047}]GFP-[P_{comEA}]dsRed-Kan	Upstream region of *VC0047* gene (~500 bp) cloned into *Eco*RI/*Eco*RV site of pBR-GFP-[P_{comEA}]dsRed-Kan; Amp^R, Kan^R	this study
pBR-[P_{hapA}]GFP-[P_{comEA}]dsRed-Kan	Upstream region of *hapA* gene (~200 bp) cloned into *Eco*RI/*Xma*I site of pBR-GFP-[P_{comEA}]dsRed-Kan; Amp^R, Kan^R	this study
pBR-[P_{gyrA}]GFP-[P_{comEA}]dsRed-Kan	Upstream region of *gyrA* gene (~200 bp) cloned into *Eco*RI/*Xma*I site of pBR-GFP-[P_{comEA}]dsRed-Kan; Amp^R, Kan^R	this study
pBR-[P_{recA}]GFP-[P_{comEA}]dsRed-Kan	Upstream region of *recA* gene (~220 bp) cloned into *Eco*RI/*Xma*I site of pBR-GFP-[P_{comEA}]dsRed-Kan; Amp^R, Kan^R	this study
pBR-[P_{clpX}]GFP-[P_{comEA}]dsRed-Kan	Upstream region of *clpX* gene (~200 bp) cloned into *Eco*RI/*Xma*I site of pBR-GFP-[P_{comEA}]dsRed-Kan; Amp^R, Kan^R	this study
pBR-[P_{ftsH}]GFP-[P_{comEA}]dsRed-Kan	Upstream region of *ftsH* gene (~180 bp) cloned into *Eco*RI/*Xma*I site of pBR-GFP-[P_{comEA}]dsRed-Kan; Amp^R, Kan^R	this study
pBR-[P_{comEC}]GFP-[P_{comEA}]dsRed-Kan	Upstream region of *comEC* gene (~200 bp) cloned into *Eco*RI/*Xma*I site of pBR-GFP-[P_{comEA}]dsRed-Kan; Amp^R, Kan^R	this study
pBR-[P_{comEA}]GFP-[P_{pilA}]dsRed-Kan	PCR product [P_{comEA}]-[P_{pilA}], using pBR-[P_{pilA}]GFP-[P_{comEA}]dsRED-Kan as template, cloned into *Xma*I/*Stu*I site of pBR-GFP_dsRed_Kan; Amp^R, Kan^R	this study
pBAD-*tfoX*-stop	*VC1153* (*tfoX*) in pBAD/Myc-HisA without tag; arabinose-inducible; Amp^R	[22]
pUX-BF13	*ori*R6K, helper plasmid with Tn*7* transposition function; Amp^R	[60]

Table 1. Cont.

Strains or plasmids	Genotype*	References
pGP704::Tn7	pGP704 with mini-Tn7	Schoolnik lab collection; [37]
pGP704-mTn7-*araC-tfoX*	pGP704 with mini-Tn7 carrying *araC* and P_{BAD}-driven *tfoX;* Amp^R	this study

*VC numbers according to [55].
doi:10.1371/journal.pgen.1002778.t001

and *tfoX*-uninduced cells significant differences between the tested strains were observable (Figure 5A). That is, whereas the HapR level was only slightly reduced in an AI-2-deficient strain (ΔluxS), HapR was almost undetectable in a strain lacking CAI-1 (ΔcqsA) (Figure 5A). Better detection was only possible upon overexposure of the film and such an overexposure did not reveal any HapR protein in the dual autoinducer mutant strain (ΔcqsAΔluxS) or the *hapR* negative control (Figure S7). This HapR protein pattern directly reflects the *comEA*-promoter driven FP expression data quantified in Figure 4B and also indicates that CAI-1 is the stronger autoinducer compared to AI-2 in *V. cholerae* strain A1552 (consistent with what was shown for strain C6707 using a heterologous read-out [49]). We also tested the expression of another QS-dependent but competence-independent gene, *hapA*, by transcriptionally fusing the *hapA* promoter to *gfp*. The *hapA* gene encodes a hemagglutinin protease (HA protease) and is positively regulated by HapR [50]. When we compared the *comEA* expression data (Figure 4B) to those of *hapA* (Figure 5B) a very similar expression pattern was observable under competence inducing conditions. We hypothesize that the low amount of HapR present in the *cqsA* mutant (Figure 5A and Figure S7) is not sufficient to activate expression of either *comEA* or *hapA*. A potential reason for this might be that HapR displays only a weak affinity for these promoters, which is consistent with a LuxR promoter affinity model described for *Vibrio harveyi* [51].

We also tested the impact of the HapR level on the protein amount of the nuclease Dns (Figure 5C) and observed an inverse correlation: Dns repression only occurred in those strains in which we detected high levels of HapR protein (e.g. WT and ΔluxS in Figure 5A). This is in good agreement with the absence of any transformants in a *cqsA* mutant (Figure 3A), as the abundance of the nuclease in this strain would avoid uptake of intact DNA. This is the first time that a direct correlation between HapR protein levels, nuclease levels and *comEA* expression has been shown, which is the critical link between QS and natural competence/transformation.

Investigation of other *tfoX* and QS–regulated competence genes

Finally, we were curious to determine whether this chitin-independent, competence-inducing system would allow us to investigate other genes that are potentially involved in natural transformation. We initially focused on two potential promoter regions belonging to genes *VC0047* and *comEC*. The first promoter precedes *VC0047*, which is part of a four-gene operon (*VC0047-50*). The gene *VC0048* in this cluster encodes DprA, which is essential for natural transformation of *V. cholerae* [22]. The function of DprA in *Streptococcus pneumoniae* and probably also in *V. cholerae* is to protect the incoming single-stranded DNA from degradation and to convey the DNA to RecA-mediated recombination [52]. As shown in Figure 6A, a *tfoX*-dependent expression pattern was detectable in our *VC0047* promoter-driven *gfp* reporter strain.

Another gene that we have previously shown to be essential for transformation of *V. cholerae* is *comEC* ([22]; annotated as inner membrane transporter). As so far nothing was known about its regulation we sought to investigate whether *comEC* is regulated in a TfoX-dependent manner. Using a *comEC* promoter-driven *gfp* reporter strain we were able to measure a slight but statistically significant increase in *comEC* upon competence induction (Figure 6A). This is the first time that *comEC* has been shown to belong to the chitin-/TfoX-regulon in *V. cholerae* and as such being co-regulated with other competence genes.

We were also interested in the regulation of the *pilM-Q* operon as *pilQ* is also required for natural transformation [11,22]. Thus, we fused the *pilM* promoter region to *gfp* and determined FP expression under competence inducing conditions. Unfortunately, the signal intensity was too low to allow us to unambiguously judge *pilM* promoter-driven expression. To overcome this obstacle we

Table 2. Natural transformation is dependent on cAMP, which is produced by adenylate cyclase (CyaA) and degraded by cAMP-degrading phosphodiesterase (CpdA).

V. cholerae strain	Competence induced[#]	Transformation frequency (±SD)
A1552-Tn*tfoX*	No	<d.l.
A1552-Tn*tfoX*	Yes	2.1×10^{-4} ($\pm1.9\times10^{-4}$)
ΔcyaA-Tn*tfoX*	No	<d.l.
ΔcyaA-Tn*tfoX*	Yes	<d.l.
ΔcpdA-Tn*tfoX*	No	<d.l.
ΔcpdA-Tn*tfoX*	Yes	7.1×10^{-4} ($\pm4.7\times10^{-4}$)*

[#]Competence was induced by the addition of 0.02% arabinose wherever indicated.
<d.l.: below detection limit;
*Statistically significant difference in comparison to the wild type strain A1552-Tn*tfoX*; $P<0.05$; Average of at least three independent experiments.
doi:10.1371/journal.pgen.1002778.t002

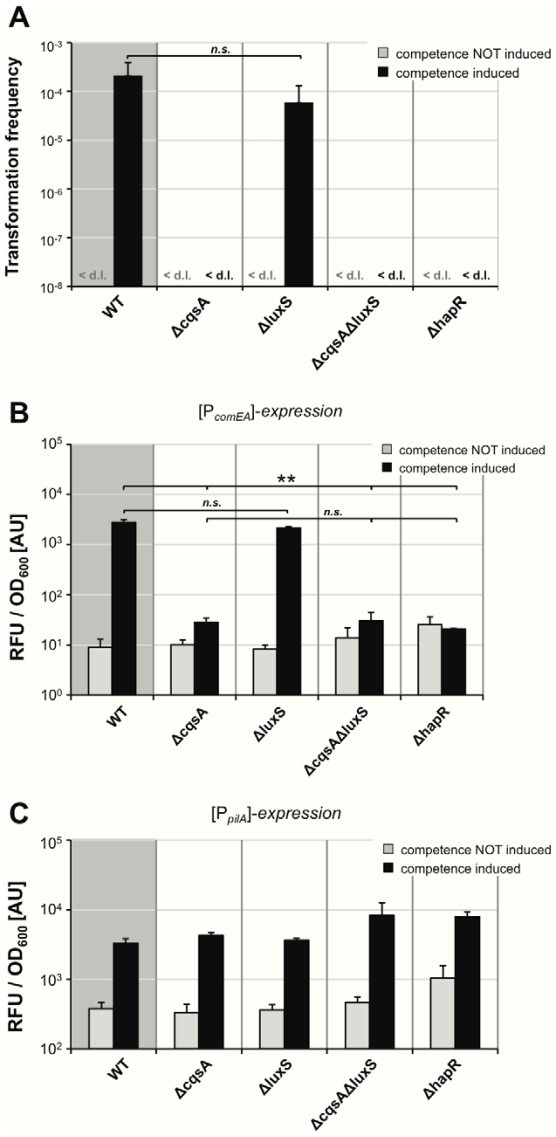

Figure 4. Quorum sensing only regulates a subset of competence genes. The *tfoX*-expression construct was transferred onto the chromosome of mutant *V. cholerae* strains, which were defective in the quorum-sensing circuit. Strains were grown in LB medium with or without 0.02% arabinose and tested for natural transformability (panel A), *comEA* (panel B), and *pilA* promoter-driven FP expression (panel C) as described for Figure 3. Experiments were repeated at least three times. Statistically significant differences were calculated using the Student's *t* test. * $P<0.05$, ** $P<0.01$, *n.s.* = not significant. <d.l. = below detection limit.
doi:10.1371/journal.pgen.1002778.g004

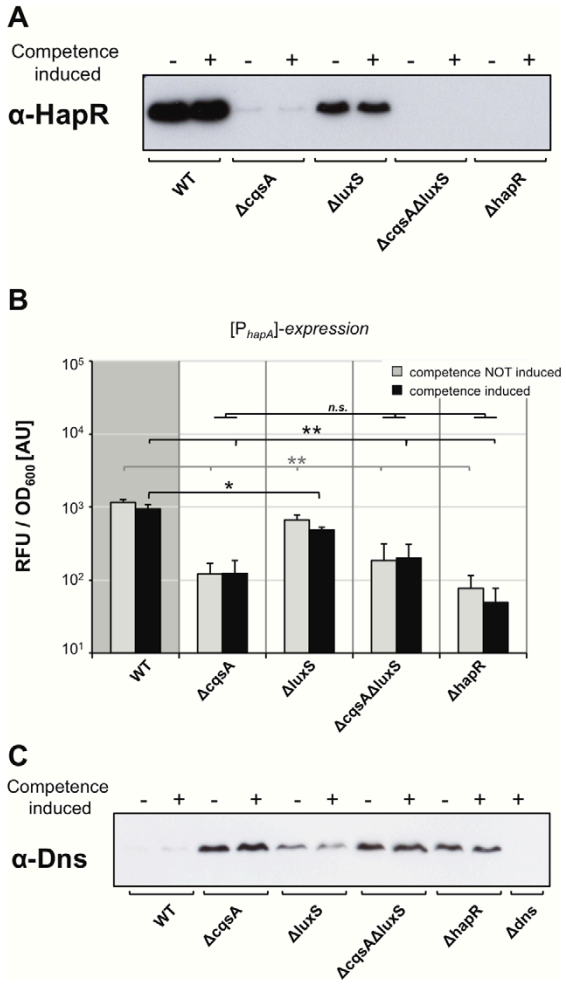

Figure 5. Correlation among HapR protein levels, *hapA* gene expression, and the nuclease Dns. Panel A and C: Proteins of the indicated strains, each containing artificially inducible *tfoX* on the chromosome, were separated by SDS-PAGE. After blotting, the relative abundance of proteins HapR (panel A) or Dns (panel C) were determined by detection with protein-specific antibodies. For each sample 6 μg (panel A) and 12 μg total protein (panel C), respectively, were applied per lane. Strains were tested under non-competence-inducing and competence-inducing conditions as indicated above each image. Panel B: HapR-dependent expression of *hapA* promoter-driven gene expression was quantified as described in Figure 3 for *comEA*/*pilA*. The growth conditions were as described for Figure 4. Averages of three independent experiments are indicated. Error bars indicate standard deviations. Statistically significant differences were calculated using the Student's *t* test. * $P<0.05$, ** $P<0.01$, *n.s.* = not significant.
doi:10.1371/journal.pgen.1002778.g005

established quantitative RT-PCR in our laboratory to further monitor competence gene expression using our chitin-independent system. We first compared competence-uninduced to competence-induced cells with respect to expression of *comEA*, *pilA*, *pilM*, *VC0047*, *dprA and comEC*. As indicated in Figure 6B all of these genes were significantly induced upon competence-induction. This again confirmed the TfoX-dependent regulation of *comEC* shown in Figure 6A, which was missed in previous chitin/TfoX-dependent expression studies [11,31]. We suggest that this was the case, as the change in expression did not pass the significance filter in these microarray expression studies. Indeed, the fold-difference for *comEC* expression upon competence induction was only 2.7 (Figure 6B). Interestingly, this 2.7-fold increase in *comEC* expression is in the same range as what we observed using the *comEC* FP reporter construct (1.8-fold change; Figure 6A), though the latter system was plasmid-based.

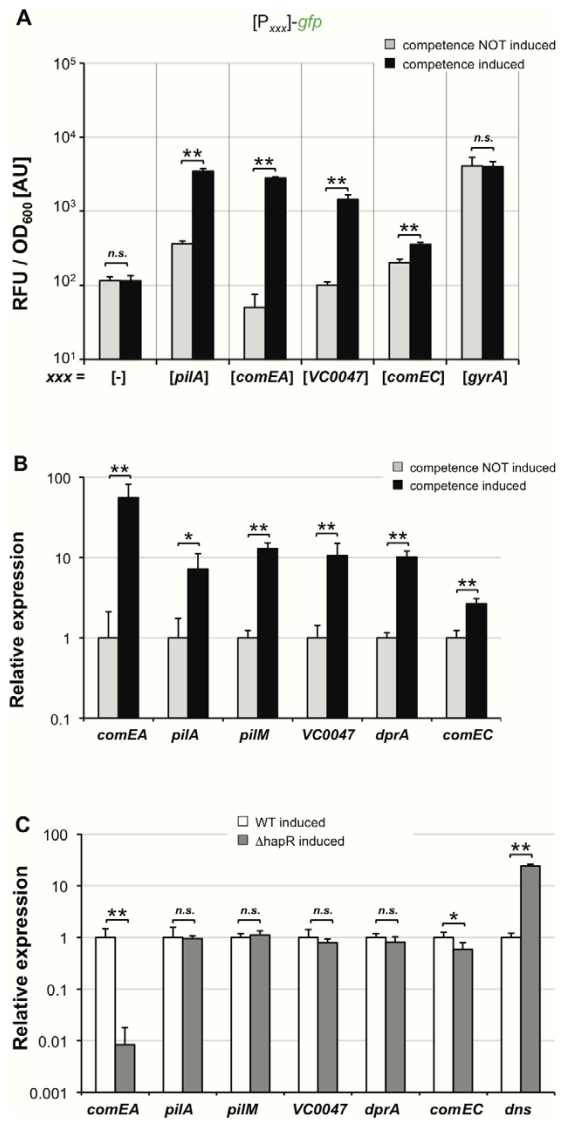

Figure 6. TfoX drives expression of QS–dependent and QS–independent competence genes. *V. cholerae* wild type strain containing artificially inducible *tfoX in cis* was tested for the expression of different competence genes. Panel A: Different transcriptional FP reporter fusions were tested for TfoX-dependent induction. These fusions were composed of the potential promoter region of the respective competence gene(s) (x-axis) fused to *gfp*. The housekeeping gene *gyrA* was tested as control. Relative fluorescence per OD_{600} unit is given on the y-axis. Panel B and C: qRT-PCR data comparing the relative expression of the indicated genes in a wild type strain under competence non-inducing and competence-inducing conditions (panel B). In panel C both the wild type strain and the hapR mutant were tested for competence gene expression under *tfoX*-expressing conditions. All panels depict averages of at least three independent experiments and error bars indicate standard deviations. Statistically significant differences were determined using Student's *t* tests. * $P<0.05$, ** $P<0.01$, *n.s.* = not significant.
doi:10.1371/journal.pgen.1002778.g006

Finally, we wanted to test whether any of these other competence genes is also regulated in a QS-dependent manner. We therefore tested and compared the expression of these genes under competence-inducing conditions in a wild type and *hapR* negative strain, respectively (Figure 6C). Apart from *comEA* and *dns*, only *comEC* turned out as also HapR-dependent (Figure 6C, $P = 0.0157$). This is in nice agreement with our regulatory model in which the fate of the surrounding DNA is determined by QS (Figure 7 and conclusion below).

Concluding remarks and proposed model of the regulatory network

In this study, we analyzed the regulatory network of chitin-induced natural competence and transformation in *V. cholerae* in its full complexity (Figure 7). Our results suggest that under homogeneous conditions bistability is unlikely for *V. cholerae*. However, the conditions might be less homogeneous in time and space around biotic surfaces (e.g., different concentrations of autoinducers and PTS sugars, which interfere with natural competence via QS and CCR, respectively). Such "environmental heterogeneity" might foster a non-synchronized response by the chitin-associated bacteria. We hypothesize that due to such a heterogeneity competence gene expression appeared absent in a subpopulation of bacteria grown on chitin beads, whereas housekeeping genes were almost uniformly expressed throughout the population (Figure 1 and Figure S1).

Based on the results obtained in this study and combined with the knowledge from earlier studies by us and others [11,17,22,23], we developed a model for the regulatory network of natural competence and transformation (Figure 7). The model predicts the interplay between three pathways for the initiation of competence: chitin sensing followed by TfoX activation, CCR, and QS. The first pathway, the dependency on a chitin surface or on chitin oligomers (e.g., $GlcNAc_{2-6}$) for competence induction, was discovered as these compounds lead to an upregulation of potential competence genes. Within these competence genes were the so-called *pil* genes, which encode for a type IV pilus that is potentially involved in the DNA uptake process [22,31] (Figure 7). Chitin also led to an induction of the gene encoding the main regulator of natural transformation, TfoX [11,31]. This finding is supported by recent studies that show that chitin oligomers ($GlcNAc_{>2}$) lead to an increase of *tfoX* transcription but also to its enhanced translation [18]. The latter effect could be explained after the discovery of a chitin-induced small RNA TfoR, which activates translation of TfoX mRNA and, therefore, contributes to the induction of natural competence [20]. Attempts from our group to look at transcriptional reporter fusions between *tfoX* and *gfp* were unsuccessful, which was most likely due to the low activity of the *tfoX* promoter (M. Lo Scrudato, M. Grasser, M. Blokesch, unpublished). The flow of information in this part of the regulatory circuit (e.g., chitin sensing) is therefore as follows (Figure 7): the presence of chitin is sensed by the chitin sensor ChiS, due to chitinase-released GlcNAc di-/oligomers [31,53], and the signal is then transferred via TfoR towards the production of TfoX.

As this chitin-dependent pathway is well established, we excluded it in the second part of this study and designed a chitin-independent, competence-inducing system, which is based on artifical *tfoX* expression (though not overproduction). With this chitin-independent system, we obtained transformants at comparable frequencies to our optimized chitin-induced transformation protocol [19] and at 10- to 10,000-fold higher frequencies than recent studies by other groups [18,20,21]. As the induction occurs under homogeneous conditions in this system, it allowed us to us to better investigate the two other pathways involved in natural competence regulation, CCR and QS.

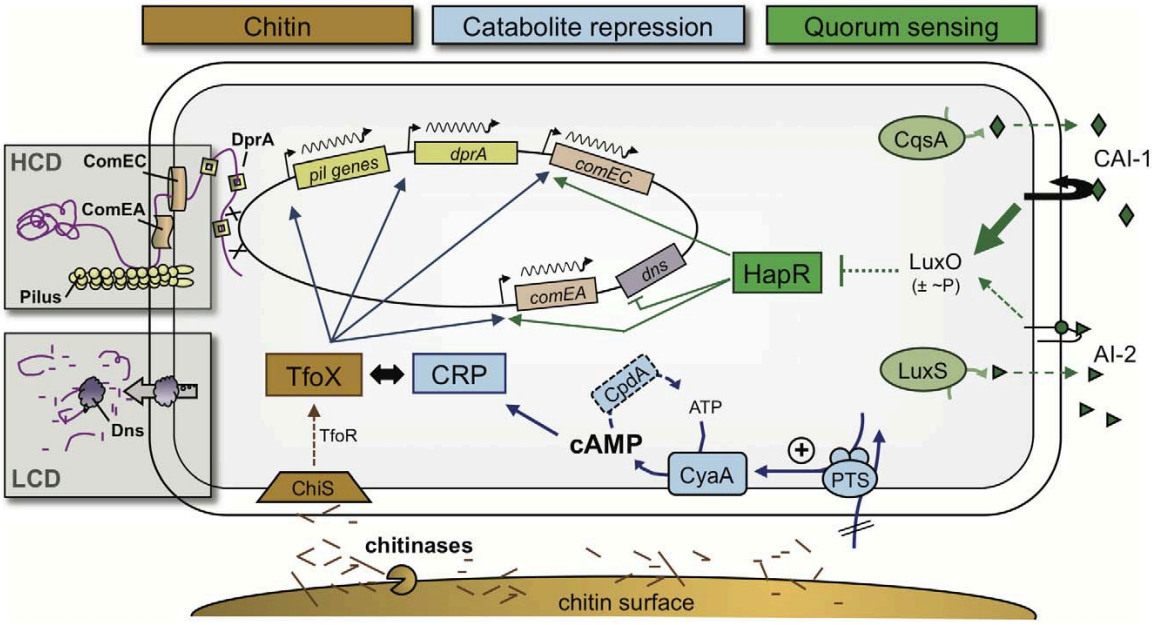

Figure 7. Model of the regulatory network of natural competence and transformation of *V. cholerae*. At least three extracellular and intracellular signaling molecules must be present to allow natural transformation to occur in *V. cholerae*. 1) Chitin degradation products such as chitin oligomers, which lead to the induction of the sRNA TfoR and the main regulator of transformation TfoX (chitin pathway shown in brown). 2) The secondary messenger cAMP, which has to accumulate within cells (CCR pathway shown in blue). 3) Extracellular autoinducers, with an emphasis on the stronger autoinducer CAI-1, which feed into the quorum-sensing circuit (shown in green). Whereas chitin- and TfoX-dependent induction and the requirement for cAMP and CRP are universal for all, so far investigated, competence genes, the QS-dependent circuit regulates only a subset of those, such as *comEA* and *comEC*. Therefore, QS acts as a switch in gene expression and is responsible for the final fate of the surrounding DNA (boxed areas). At a low cell density (LCD), the DNA (shown in purple) is degraded by the nuclease Dns. As a consequence, the cells are non-transformable. At a high cell density (HCD) and, therefore, high abundance of the autoinducer CAI-1, the nuclease gene *dns* is transcriptionally repressed, whereas *comEA* and *comEC* are activated. ComEA as well as ComEC then contribute to the DNA uptake process, probably due to their ability to shuffle the DNA through the periplasmic space and the inner membrane, respectively.
doi:10.1371/journal.pgen.1002778.g007

Based on experimental data showing that glucose interferes with chitin-induced transformation, Meibom *et al.* suggested that catabolite repression might be involved in the competence phenotype [11]. This hypothesis was recently extended as we showed that competing PTS-dependent carbon sources indeed repress natural transformation [23]. Such sugars are known to play a role in the intracellular accumulation of the secondary messenger cAMP, which, together with the cAMP receptor protein CRP, contributes to chitin surface colonization, chitin degradation and natural competence [23]. In this current study, we circumvented the problem that CCR mutants are often impaired for colonization and growth on chitin as a sole carbon source [23] by uncoupling natural competence-induction from chitin. This allowed us to better understand the dependency on cAMP for competence gene expression. Indeed we observed a change in natural transformability upon creating an imbalance in the intracellular cAMP pool, either by inhibiting cAMP production or, alternatively, by avoiding cAMP degradation. The latter effect was accomplished by deleting the gene encoding the cAMP phosphodiesterase, an enzyme that has never been studied in *V. cholerae* before. We also confirmed that the TfoX-induced expression of *pilA* and *comEA* requires cAMP, which is consistent with the idea proposed, but not yet unequivocally demonstrated, for *Haemophilus influenzae* that TfoX and cAMP-CRP act in concert to induce competence genes [40,41,54] (Figure 7).

The third pathway that participates in natural competence induction is quorum-sensing (QS). The involvement of QS in competence initiation was initially speculated based on different facts. First, the first sequenced strain of *V. cholerae* N16961 [55] was non-transformable [11]. In this strain, the indigenous *hapR* gene contains a frameshift mutation that renders it non-functional [56]. This deficiency could be overcome by providing a functional copy of *hapR* back in *cis* [11]. The second line of evidence for the involvement of QS in natural competence and transformation came from the fact that cells were more efficiently transformable after longer growth on chitin surfaces, which is equivalent to higher cell densities [11]. However, such a finding could also be explained by elevated intracellular cAMP levels after extended growth on chitin. The involvement of QS in natural transformation was more directly demonstrated by elucidating a role for HapR in the repression of a gene encoding the extracellular nuclease Dns [17]. The finding that HapR "acts as a negative regulator for *dns* transcription" was recently also confirmed by others [57]. The study by Blokesch and Schoolnik unambiguously demonstrated that the HapR-induced repression of the nuclease is the main, but not the only, contribution of QS to natural transformability, and the authors proposed that HapR also acts as a positive regulator of *comEA* [17]. This speculation was based on earlier microarray expression data, which showed that *comEA* expression upon growth on chitin was significantly reduced in the absence of HapR [11]. A QS-dependent regulation of *comEA* has

recently been demonstrated [21] but differed from the data presented here as discussed above. In the current study, we extended this analysis by studying natural transformation and competence gene expression using the same competence-inducing conditions. Furthermore, we simultaneously monitored the expression of two competence genes, namely *comEA* and *pilA*, and compared their expression levels in different QS mutants. Based on the data provided, we conclude that *comEA* but not *pilA* is regulated by QS (Figure 7). We also showed for the first time a direct link between intracellular HapR protein concentrations, nuclease repression and *comEA* induction (Figure 4 and Figure 5).

Finally, we tested other competence genes, namely *pilM*, *VC0047*, *dprA*, and *comEC*, with respect to their expression levels; for these genes less or no information concerning their regulation was known before this study. We conclusively showed that all tested competence genes were dependent on induction by TfoX but only a part of them was also co-regulated in a QS-dependent manner (Figure 6). In fact only three genes showed a significantly different expression under competence inducing conditions in a wild type strain compared to a *hapR* mutant, namely *dns*, *comEA* and *comEC* (Figure 7). The encoded proteins are all directly involved in the fate of the surrounding DNA: whereas Dns degrades free DNA at low cell density, ComEA and ComEC are required for the DNA uptake process once high cell density is reached on the chitin surface (Figure 7). Further studies will follow to provide a better insight into the DNA uptake process itself.

Materials and Methods

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains DH5α [58] and One Shot PIR1 or PIR2 (Invitrogen) were used as hosts for cloning purposes. *E. coli* strain S17-1λpir [59] served as mating donor for plasmid transfers between *E. coli* and *V. cholerae*.

Media and growth conditions

Overnight cultures were grown in LB medium under aerobic conditions. Defined artificial seawater medium (DASW) [11] supplemented with vitamins (MEM, Gibco) and 0.1% casamino acids (Becton, Dickson and Company) was used for static growth of *V. cholerae* on chitin beads (New England Biolabs) or for growth under shaking conditions with N-acetylglucosamine (GlcNAc) or hexa-N-acetylchitohexaose $(GlcNAc)_6$ (obtained from Seikagaku Corporation via Northstar BioProducts and LuBioScience, Lucerne, Switzerland) as sole carbon source. Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar plates were prepared following the manufacturer's instructions (Fluka) and were used to counter-select *E. coli* strains after triparental mating with *V. cholerae*. Experiments for artificial *tfoX* expression were performed in LB medium with or without the addition of 0.02% arabinose. LB medium and LB agar plates were supplemented with antibiotics wherever required. The final concentrations of antibiotics were 75 μg/ml for kanamycin, 50 or 100 μg/ml for ampicillin and 50 μg/ml for gentamicin. *V. cholerae* cells were always grown at 30°C.

Construction of *V. cholerae* strains

The *V. cholerae cpdA* deletion strain was constructed using plasmid pGP704-28-SacB-ΔcpdA (Table 1) and the gene disruption method described previously [31]. The oligonucleotides used for the construction of the deletion plasmid are indicated in Table S1.

V. cholerae strains carrying artificially inducible *tfoX* on the chromosome (e.g., in *cis*) were created by triparental mating between the respective *V. cholerae* strain (Table 1), *E. coli* strain S17λpir/pUX-BF13 (providing the transposase function; [60]), or *E. coli* strain S17λpir/pGP704-mTn7-*araC-tfoX*. The latter plasmid consists of the suicide vector pGP704 as the backbone and the mini-Tn7 transposon [43] containing the gene cluster *araC*-P_{BAD}-*tfoX* as cargo. This gene cluster was amplified by PCR from the plasmid pBAD-*tfoX*-stop [22] (primers indicated in Table S1).

Construction of transcriptional reporter fusions

The FP reporter constructs are all based on plasmid pBR322 [61]. Initially, this plasmid was modified by (partial) deletion of the tetracycline resistance cassette as well as the constitutive promoter P_{Tet}, resulting in plasmid pBR-Tet_MCSI (Table 1). A kanamycin resistance cassette (*aph*) as well as promoter-less versions of the genes *gfp* and *dsRed* (DsRed.T3[DNT]), both pointing in the opposite direction, were inserted into pBR-Tet_MCSI to yield plasmid pBR-GFP_dsRed_Kan. Details of all plasmids are included in Table 1. The primers used for construction of the plasmids are listed in Table S1 (synthesized by Microsynth, Switzerland). PCR mixtures, PCR programs, restriction enzyme digestions, primer phosphorylation, and ligations followed standard protocols recommended by the manufacturers of the enzymes (Roche, Switzerland and New England Biolabs via Bioconcept, Switzerland).

Growth of *V. cholerae* on chitin beads

V. cholerae strains were grown aerobically in LB medium until an OD_{600} of ~0.3. Cells were harvested, washed in DASW medium and mixed with an equal volume of prewashed chitin beads (New England Biolabs) and three volumes of DASW (final volume 1 ml). The mixture was supplemented with vitamins, 0.1% casamino acids, and kanamycin (for plasmid maintenance). The bacteria were grown as standing cultures in 12-well plates. Bacteria were visualized by epifluorescence microscopy after 24 and 48 hours of growth, respectively.

Epifluorescence microscopy settings

Specifications of the epifluorescence microscope were as follows: Zeiss Axio Imager M2 microscope; Zeiss High Resolution Microscopy Camera AxioCam MRm; Illuminator HXP 120 as fluorescence light source (metal halide); objective used in this study: Plan-Apochromat 100×/1.40 Oil Ph3 M27 (WD = 0.17 mm). Filters relevant to this study were Zeiss Filter set 63 HE mRFP shift free; EX BP 572/25, BS FT 590, EM BP 629/62 and Zeiss filter set 38 Endow GFP shift free; EX BP 470/40, BS FT 495, EM BP 525/50. Image acquisition was done using the Zeiss AxioVision software. Images were rotated, cropped and uniformly enhanced with respect to contrast and brightness using Zeiss AxioVision and Adobe Photoshop CS3.

Microscopy image analysis was performed using the Matlab-based MicrobeTracker Suite [62] and according to the instructions given by the inventors (http://microbetracker.org/). Fluorescence intensities were normalized with respect to the area of the cell and the exposure time.

Induction of natural competence by hexa-N-acetylchitohexaose and flow cytometry

Chitin-dependent but surface-independent growth of *V. cholerae* was performed as previously described [23] using 2 mM of hexa-N-acetylchitohexaose $GlcNAc_6$ (obtained from Seikagaku Corporation via Northstar BioProducts and LuBioScience, Lucerne, Switzerland) as sole carbon source. The same strains were grown in parallel with N-acetylglucosamine (GlcNAc; control). Bacteria

were harvested at an OD_{600} of 0.8. Bacteria were either immediately visualized by epifluorescence microscopy or fixed in 2% paraformaldehyde for 30 min. Fixed samples were washed, diluted in PBS (1:5), and analyzed by flow cytometry using a BD LSR II Flow cytometer. BD FACSDiva software was used for data acquisition. GFP signals were excited with a blue laser (488 nm) and detected with a 525/50 filter. DsRed.T3[DNT] was excited with a green laser (561 nm) and detected with a 585/15 filter. For each sample, 100,000 events were counted in total. Biologically independent experimental replicates (three for Figure 1 and Figure 2; two for Figure S1 and Figure S3) were performed within two weeks. One representative experiment is depicted in Figure 2 and the averages of the mean fluorescence intensities from three different biological replicates are shown in Figure S4.

Chitin-independent induction of competence

Strains used for chitin-independent competence-induction all carried inducible *tfoX* (*araC*-P_{BAD}-*tfoX*) on a mini-Tn7 transposon [43] within the chromosome. Induction was accomplished by growth in LB supplemented with 0.02% arabinose.

For transformation assays, cells were grown until an OD_{600}~1.0. At that point, aliquots of 0.5 ml cultures were transferred to 1.5-ml tubes and supplemented with 2 μg/ml transforming DNA (gDNA of strain A1552-LacZ-Kan; [19]). Tubes were shaken horizontally for 5 h. Transformed cells and total colony forming units (CFU) were enumerated by a variation of a previously described method [63]. Briefly, the cultures underwent a serial dilution, and 5 μl of each dilution step was spotted in duplicate or triplicate on plain LB or LB containing 75 μg/ml kanamycin plates, respectively. Transformation frequencies were calculated as number of transformants divided by total number of CFUs. Each experiment was repeated at least three independent times, and the averages of all experiments are given in the figures (± standard deviations). Statistical analyses of transformation frequencies were performed on log-transformed data [64] using a two-tailed Student's *t* test.

Strains were grown in LB medium with or without 0.02% arabinose to investigate transcriptional FP reporter fusions. After 24 h, the cells were either visualized by epifluorescence microscopy or measured for relative fluorescence using a Tecan Infinite M200 plate reader. Parameters for detection of GFP were: excitation (Ex) at 485 nm (9 nm bandwidth) and emission (Em) at 515 nm (20 nm bandwidth). DsRed.T3[DNT] was detected using Ex 560 (9)/Em 587 (20) nm, as previously described for DsRed.T3 [28]. The samples were also measured with respect to their OD_{600}, and results are given as relative fluorescence units (RFU) divided by OD_{600} values. The averages of three biological replicates are shown. Error bars indicate standard deviations. Statistically significant differences were analyzed using two-tailed Student's *t* tests.

Quantitative reverse transcription PCR (qRT-PCR)

V. cholerae strains were grown in 10 ml LB in the absence or presence of 0.02% arabinose until they reached an optical density of ~1.7. At that time 5 ml of each culture was harvested and lysed in 1 ml Tri Reagent (Sigma). The samples were stored at −80°C. RNA isolation, DNase treatment, and reverse transcription using 1 μg of total RNA as template was done as previously described [23]. The obtained cDNA was diluted 40-fold and served as template in the qPCR. The primers used for the qPCR are indicated in Table S1. The qPCR mix was based on the *Fast Start Essential DNA Green Master Mix* (Roche, Switzerland), a ready-to use hot start reaction mix optimized for qPCR using the Light Cycler Nano system from Roche. The qPCR mix further contained 0.5 μM of each primer. The qPCR run using the Light Cycler Nano was performed according to these parameters: a denaturation step at 95°C for 10 min followed by 40 cycles of 95°C for 10 s, 60°C for 20 s, 72°C for 20 s. Each run was finished with a melting-curve ranging from 50°C to 95°C to validate specific amplification, which was also initially confirmed for each primer pair by standard PCR and visualization of PCR fragments in agarose gels. For each sample a reverse transcriptase-negative control was also performed while doing the reverse transcription and the respective samples were analyzed with at least three independent primer pairs to exclude residual DNA contaminations. A standard curve was prepared for each primer pair using purified genomic DNA of *V. cholerae* A1552 diluted in PCR grade water (from 1000 to 0.1 pg gDNA template). A negative control lacking any template was also tested for each primer pair. The expression values were normalized against expression of the housekeeping gene *gyrA* as previously described [65]. However, as discussed above the expression of *gyrA* might be dependent on DNA supercoiling and the cell cycle as shown for other bacteria [34,35]. We therefore compared the relative expression of the four housekeeping genes *gyrA*, *recA*, *clpX*, *ftsH* as well as *comEA* in two different *V. cholerae* strains (WT and ΔhapR). The expression patterns were extremely similar no matter whether we normalized the expression data against *gyrA* expression (Figure S8A) or against *recA* expression (Figure S8B) as internal controls. The results were analyzed using the Light Cycler Nano software.

Preparation of cell lysates and determination of total protein concentration

For the preparation of cell lysates, bacteria were grown aerobically in LB medium in the absence or presence of 0.02% arabinose. Cells were harvested after reaching an OD_{600} of ~1.5, resuspended in SDS-loading buffer (2×Laemmli buffer without β-mercaptoethanol and bromophenol blue), and boiled at 98°C for 15 min. Total protein concentration was quantified using the Pierce BCA Protein Assay kit (Thermo Scientific) before the addition of β-mercaptoethanol and bromophenol blue (7.5% and 0.01% final concentrations, respectively).

Generation of antibodies against HapR and Dns

Antibodies raised against the peptides derived from the proteins HapR, Dns, and TfoX were produced by Biomatik (Canada). The polyclonal antibody production service included suggestions for the design of two peptides per protein, peptide synthesis, conjugation of the peptides to the carrier proteins (keyhole limpet hemocyanin), and immunization of two rabbits per peptide mix. Polyclonal antibodies were affinity purified against the antigen and checked by ELISA. Each antibody was first validated for potential cross-reactions at the same size as the target protein using western blot analysis of the respective know-out strains.

Electrophoretic separations and Western blotting

Separation of proteins under denaturing conditions was conducted by SDS-PAGE using 15% acrylamide gels [66,67]. The amount of total protein loaded per lane was 6 μg, 12 μg, and 50 μg for HapR, Dns, and TfoX detection, respectively. For western blot analysis, the proteins were transferred onto PVDF western blotting membranes (Roche), stained with amido black to verify transfer efficiency, incubated in blocking buffer, and reacted with primary antibodies directed against HapR (1:5000), Dns (1:1000), or TfoX (1:2000). Detection of the primary antibody was performed using a secondary goat anti-rabbit IgG antibody conjugated to peroxidase (Sigma A9169; used at a 1:20,000

dilution). Signals were revealed using Lumi-Light^{PLUS} Western Blotting substrate (Roche, Switzerland) and were recorded by exposure to chemiluminescence-detecting films (Amersham Hyperfilm ECL, GE Healthcare).

Supporting Information

Figure S1 Visualization of housekeeping compared to competence gene expression on chitin surfaces. *V. cholerae* cells were grown on chitin beads and were visualized as described for Figure 1. The diverse transcriptional FP reporter tested were: I: the vector control containing promoter-less *gfp* and *dsRed*; II to V: a reporter constructs containing *gfp* driven by the *gyrA* promoter, the *recA* promoter, the *clpX* promoter and the *ftsH* promoter, respectively, oppositely oriented to the *comEA* promoter-driven *dsRed* gene. Bacteria were grown statically for 24 h before pictures were taken. The order of the images is the same as for Figure 1. Scale bar = 5 μm.
(TIF)

Figure S2 Homogeneous expression of competence genes under homogeneous competence-inducing conditions. *V. cholerae* strains were grown in the presence of either GlcNAc or $(GlcNAc)_6$ as described for Figure 2. Competence gene expression was visualized using epifluorescence microscopy. Images in the upper row show the pictures taken in the green (left) and red (right) channel. The image on the lower left shows a merged image of the phase contrast picture and the images of both fluorescence channels. The signals in the original images were also quantified using MicrobeTracker [62]. Thus, graphs below microscopy images show histograms of the fluorescent intensities (log-transformed; including a two-period moving average trendline); the plot in the lower right shows the correlation of the bacteria with respect to both FPs (given as log scale). Reporter fusions are indicated above each panel. Panel A&B: promoter-less *gfp* and *dsRed* reporter; panel C&D: [P_{pilA}]-*gfp*/[P_{comEA}]-*dsRed*; and panel E&F: [P_{comEA}]-*gfp* and [P_{pilA}]-*dsRed*. Panel G&H: [P_{gyrA}]-*gfp*/[P_{comEA}]-*dsRed*. Scale bar in all images = 5 μm.
(PDF)

Figure S3 Housekeeping genes are expressed in the majority of cells under homogeneous conditions. The indicated bacterial reporter strains were grown aerobically in DASW medium with $GlcNAc_6$ as inducer of competence. Promoter-driven FP gene expression was either visualized by epifluorescence microscopy (images) or quantified for fluorescence intensities using flow cytometry (graphs below the fluorescence images). Scale bar in all images = 5 μm.
(PDF)

Figure S4 Reproducibility of mean fluorescence intensities as quantified by flow cytometry. The mean fluorescence intensity for promoter-driven *gfp* expression (panel A) and the corresponding *dsRed* expression (panel B) was measured in the three biological replicates of the experiment corresponding to Figure 2. Average values are indicated for competence non-inducing and competence-inducing conditions and errors bars represent the standard deviations.
(TIF)

Figure S5 Artifical *tfoX* induction in *cis* does not lead to TfoX overproduction. 50 μg of total protein derived from the two indicated strains, which were grown in the presence of the indicated concentration of L-arabinose, were separated by SDS-PAGE. After blotting, the abundance of the TfoX protein was determined with protein-specific antibodies. The position of TfoX is indicated on the right. The upper image was obtained after 10 min of film exposure. For the lower image the film was exposed for 60 min. The white arrow at the bottom indicates the conditions used in earlier studies, whereas the gray arrows reflects the chromosomally encoded inducible but not overproducing *tfoX* system described in this study.
(TIF)

Figure S6 TfoX-dependent competence induction requires cAMP within the cells. Wild type *V. cholerae* cells or derivatives of the parental strain lacking adenylate cyclase (CyaA) or cAMP phosphodiesterase (CpdA) harboring *cis*-encoded *tfoX* were grown in rich medium in the absence (gray bars) or presence of the inducer arabinose (black bars). Bacteria were scored for *comEA*- (panel A) and *pilA*- (panel B) driven expression using a 96-well plate reader. The relative fluorescence units were normalized to the OD_{600} values. Average are from three independent replicates. <d.l. = below detection level.
(TIF)

Figure S7 Detection of HapR within different QS mutant strains. Proteins of the indicated strains, each containing artificially inducible *tfoX* on the chromosome, were separated by SDS-PAGE. After blotting, the relative abundance of the HapR protein was determined by detection with protein-specific antibodies. For each sample, 6 μg of total protein was applied per lane. Strains were tested under non-competence-inducing and competence-inducing conditions as indicated above the figure. The image represents an overexposed film (in comparison to Figure 5A) to detect weaker signals.
(TIF)

Figure S8 Normalization of qRT-PCR data using either *gyrA* or *recA* as internal controls results in comparable expression patterns. qRT-PCR data comparing the relative expression of the genes *gyrA*/*recA* (lane 2 in panel A and B, respectively), *clpX* (lane 3), *ftsH* (lane 4), and the competence gene *comEA* (lane 5) in a ΔhapR-Tn*tfoX* strain compared to the normalized expression in the wild type strain A1552-Tn*tfoX* (lane 1). Both strains were grown under competence inducing conditions. Both panels show averages of three independent biological replicates and error bars indicate standard deviations.
(TIF)

Table S1 Primers used in this study.
(DOCX)

Acknowledgments

We are grateful for technical assistance by Olga de Souza Silva and Sandrine Borgeaud and for preliminary data from Melinda Grosser (as part of EPFL's International Undergraduate Summer Research Program 2010). The authors like to thank Eric Stabb for plasmid pVSV209. A special "thank you" goes to the staff members of the flow cytometry facility (FCCF; in particular, to Sintia Winkler and Miguel Garcia) and to the Bioimaging facility (PT-BIOP; Arne Seitz) at the School of Life Sciences of EPFL.

Author Contributions

Conceived and designed the experiments: MLS MB. Performed the experiments: MLS MB. Analyzed the data: MB. Contributed reagents/materials/analysis tools: MLS MB. Wrote the paper: MB.

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3.2 A link between environmental signals and bacterial communication

Overview: achievements and significance

In this study we thoroughly investigated the regulatory pathway of QS and in particular the signalling cascade downstream the QS master regulator HapR. Previously, we demonstrated that only a subset of the competence genes is QS-dependent, *comEA* and *comEC*. Starting from this finding, we aimed at deciphering the mechanisms by which HapR positively regulates these competence genes.

Due to the higher expression levels in wild type competence-induced *V. cholerae* cells, we focused mostly on *comEA* and showed that the negative transformation phenotype of the *hapR* minus strain could be partially rescued by *in trans* over-expression of *comEA*. This finding was in agreement with the HapR-dependency of *comEA* expression and prompted us to suggest that HapR directly regulates *comEA*. Following this hypothesis we identified a putative HapR binding site upstream the start codon of *comEA*. When we mutagenized this site, the transformability of the strain did not change. However, this information was not entirely conclusive since we could not exclude that HapR binds a different sequence upstream of *comEA*.

Next, we decided to assay the *in vitro* ability of HapR to bind the upstream region of *comEA* using an electrophoretic mobility shift assay (EMSA). For this purpose we employed a purified tagged version of the protein HapR ascertained for its *in vivo* functionality. No shift of the *comEA* promoter DNA probe was provoked by HapR suggesting that the protein does not bind this region. The same result was obtained for the upstream region of *comEC*. However, we were able to show that HapR binds the upstream region of the extracellular nuclease-encoding gene *dns in vitro*. Moreover we localized this binding to two regions upstream of the *dns* gene, which were bound by HapR independently from each other. Within these regions we identified two motifs resembling the *in silico* predicted consensus of the HapR

binding sites (Tsou *et al.*, 2009). We concluded that at least two HapR binding sites exist within the promoter of *dns*. These experiments were essential to infer that HapR directly represses *dns* and most likely indirectly regulates the expression of the competence genes *comEA* and *comEC*.

Since HapR did not bind the upstream region of *comEA* we took another possibility into consideration: an intermediate regulator might exist between HapR and the QS-dependent competence genes. As an educated guess we considered the gene *VC0396* as gene encoding such a potential intermediate regulator because *VC0396* was among the genes up-regulated by TfoX overexpression (Meibom *et al.*, 2005) and encodes a putative transcriptional regulator of the LuxR family. To test this hypothesis, we first demonstrated that *VC0396*'s expression was dependent on both, QS and TfoX; we therefore named the gene *qstR* for QS and TfoX-dependent *r*egulator. In particular, we showed that the expression of *qstR* was up-regulated by chitin-induced TfoX but only in the presence of HapR. These results suggested that QstR could represent the connection between the two pathways of QS and chitin-sensing and -degradation.

The importance of *qstR* in natural competence for transformation became clear when we tested the transformability of the *qstR* minus strain; in fact this strain was severely impaired for natural transformation. Using epistasis experiments we then provided evidence that QstR acts downstream of HapR.

The next question we addressed was whether HapR directly or indirectly regulates *qstR*. Thus, we tested the ability of HapR to bind the upstream region of *qstR in vitro*. The EMSA showed a DNA shift in the presence of HapR thereby indicating that it directly regulates *qstR.* Moreover, we were able to map the HapR binding site within a stretch of 50 bp upstream *qstR*. Interestingly, we identified a motif resembling an *in silico* predicted HapR binding site (Tsou *et al.*, 2009) within this DNA region. And indeed, site-directed mutagenesis of this motif abolished HapR's ability for binding indicating that the motif represents a real HapR binding site.

Similarly to the *hapR* minus strain, the transformability of a *qstR* minus strain could also be partially rescued by *in trans* over-expression of *comEA*. This indicated that QstR acts upstream of *comEA.* Indeed, by measuring the relative expression of several competence genes in a *qstR* mutant we demonstrated that QstR (directly or indirectly) regulates the transcription of *comEA* and *comEC* but not of other tested competence genes.

The main findings of this section can be summarized as follow:

1. HapR directly regulates *dns* and indirectly activates the competence genes *comEA* and *comEC*;
2. The newly identified transcription factor QstR is dependent on the simultaneous activity of TfoX and HapR;
3. QstR is necessary for the occurrence of natural competence;
4. HapR directly activates *qstR* by binding to its upstream region*;*
5. QstR is necessary for the expression of the QS-dependent competence genes *comEA* and *comEC*.

Overall this study proved that the QS and the chitin-sensing and -degradation pathways converge on the expression of *qstR* to determine the fate of the extracellular DNA. Indeed, the production of the ComEA and ComEC proteins, both of which are necessary for the translocation of the incoming DNA across the outer and inner membranes, respectively, does not occur until QstR is expressed. Thus, both pathways are required to enable the cells to take up the extracellular DNA. This represents an important example of how bacteria integrate signals of different origins to scrupulously and minutely regulate a specific cellular mechanism.

3644–3658 *Nucleic Acids Research, 2013, Vol. 41, No. 6* *Published online 4 February 2013*
doi:10.1093/nar/gkt041

A transcriptional regulator linking quorum sensing and chitin induction to render *Vibrio cholerae* naturally transformable

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Received December 15, 2012; Revised January 6, 2013; Accepted January 8, 2013

ABSTRACT

The human pathogen *Vibrio cholerae* is an aquatic bacterium associated with zooplankton and their chitinous exoskeletons. On chitinous surfaces, *V. cholerae* initiates a developmental programme, known as natural competence, to mediate transformation, which is a mode of horizontal gene transfer. Competence facilitates the uptake of free DNA and recombination into the bacterial genome. Recent studies have indicated that chitin surfaces are required, but not sufficient to induce competence. Two additional regulatory pathways, i.e. catabolite repression and quorum sensing (QS), are components of the regulatory network that controls natural competence in *V. cholerae*. In this study, we investigated the link between chitin induction and QS. We show that the major regulators of these two pathways, TfoX and HapR, are both involved in the activation of a gene encoding a transcriptional regulator of the LuxR-type family, which we named QS and TfoX-dependent regulator (QstR). We demonstrate that HapR binds the promoter of *qstR* in a site-specific manner, indicating a role for HapR as an activator of *qstR*. In addition, epistasis experiments indicate that QstR compensates for the absence of HapR. We also provide evidence that QstR is required for the proper expression of a small but essential subset of competence genes and propose a new regulatory model in which QstR links chitin-induced TfoX activity with QS.

INTRODUCTION

The bacterium *Vibrio cholerae* is a facultative pathogen and the causative agent of cholera. Cholera is far from extinction and is even considered a re-emerging disease (1). *V. cholerae* commonly occurs in aquatic ecosystems, its true habitat, where it intimately associates with zooplankton and their chitinous exoskeletons. Chitin induces natural competence for transformation in *V. cholerae* (2), a mode of horizontal gene transfer. In this state, the bacterium can import and recombine DNA from the environment, thereby becoming naturally transformed. Chitin-induced natural competence is not only specific for *V. cholerae* but is also conserved in other species of the genus *Vibrio* and has been experimentally demonstrated for *Vibrio fischeri*, *Vibrio vulnificus* and *Vibrio parahaemolyticus* (3–5).

Recent studies have demonstrated that there is a strong link between natural competence/transformation and the environmental niche of the bacterium (2,6–9). More specifically, it was shown that chitin sensing and degradation, quorum sensing (QS) and carbon catabolite repression contribute to the onset of competence [for a recent review, see (10)]. Nevertheless, how these pathways are interconnected with respect to competence induction and natural transformation remains still poorly understood. Here, we describe a regulatory protein, which we named QS and TfoX-dependent regulator (QstR), as an intermediate regulator for natural competence induction and transformation, thereby linking chitin induction and QS (Figure 1).

The induction of *tfoX*, which encodes the major regulator of transformation in *V. cholerae*, in the presence of chitin was first demonstrated using microarray expression profiling (14). Indeed, *tfoX* was significantly upregulated upon the growth of *V. cholerae* on crab shell surfaces or, alternatively, in liquid cultures supplemented with N-acetylglucosamine oligomers ($n > 2$), but not on supplementation with the N-acetylglucosamine monomer (14). In 2005, experiments demonstrated for the first time that chitin renders *V. cholerae* naturally transformable and that this phenotype is fully dependent on TfoX (2). The authors of that previous study also showed that *tfoX* overexpression is sufficient to render *V. cholerae* naturally transformable, even in the absence of chitin as an inducer (2) (Figure 1, chitin independent). Subsequent studies have

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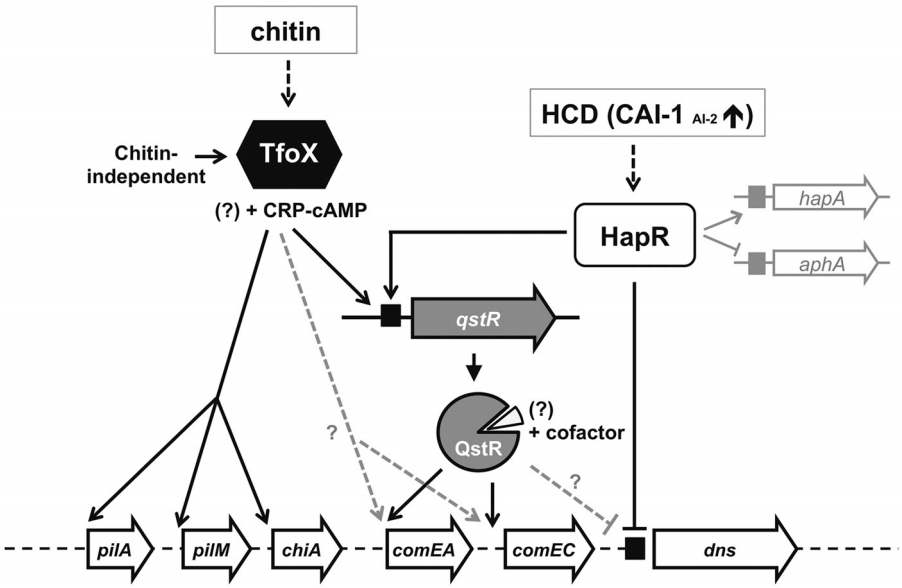

Figure 1. Schematic representation of the regulatory circuitry of natural competence and transformation of *V. cholerae*. Upon growth on chitin surfaces (or chitin-independent artifical induction), the expression of *tfoX*, encoding for the main regulator of transformation TfoX, occurs. Concomitantly with cAMP binding to CRP, TfoX most likely induces the expression of the competence genes, which include the genes encoding the assembly machinery and structural components of a type IV pilus (*pil* genes) in *V. cholerae*. TfoX also positively regulates chitin metabolism genes, such as those encoding chitinases (*chiA-1* and *chiA-2* depicted as *chiA* in the scheme). In this study, we provided evidence for the existence of an intermediate transcription factor downstream of TfoX, QstR, which is required for the expression of a small subset of competence genes (*comEA* and *comEC*). We showed that the expression of these genes, which are also dependent on the QS circuitry, is mediated through QstR, which itself is dependent on the main regulator of QS, HapR. QstR thus links the TfoX- and QS-dependent signalling in *V. cholerae*. At this point, an additional regulation of *comEA*/*comEC* by TfoX/CRP-cAMP cannot be excluded and is indicated by the grey dashed arrow. HapR is primarily produced in the presence of high levels of the CAI-1, (whereas AI-2 only plays a minor role in the production of HapR) (8), reflecting the high cell density (HCD) of the population (11). Earlier studies have demonstrated that HapR binds to the promoter sequences of the two competence-unrelated genes (*aphA* and *hapA*) (12,13), which we used as controls in this study. Here, we identified putative HapR binding sites upstream of *qstR* and *dns* (black boxes) based on the *in vitro* binding of HapR to these promoter regions and previous *in silico* predictions (grey boxes) (13).

confirmed the requirement of chitin oligomers for *tfoX* induction (15). Furthermore, Yamamoto *et al.* (16) provided evidence for the involvement of a small regulatory RNA, TfoR, which acts as an activator of *tfoX* translation upon chitin induction [reviewed in (10)]. How the regulatory protein TfoX acts on downstream genes remains unknown. However, as the secondary messenger Adenosine 3′,5′-cyclic monophosphate (cyclic AMP or cAMP) and its receptor protein CRP are also crucial for natural competence and transformation of *V. cholerae* (9), the current idea with respect to TfoX-mediated competence induction is based on a model proposed by Redfield for another naturally competent bacterium, *Haemophilus influenzae* (17–19) (Figure 1). In this organism, the TfoX-homolog Sxy is required for a CRP-cAMP-dependent induction of the 'Sxy-dependent cyclic AMP receptor [CRP-S] regulon' (20).

The third pathway that is crucial for natural competence and transformation of *V. cholerae* is QS (2,6–8,21–23). Bassler and collaborators have extensively studied QS in *V. cholerae* for many years [for a recent review, see (11)]. These studies have indicated that the regulatory circuitry of QS is incredibly complex, as it includes at least two different autoinducer molecules, i.e. cholera autoinducer 1 (CAI-1) and autoinducer 2 (AI-2) (24–26), receptor proteins acting as kinases/phosphatases, small regulatory RNAs (27) and many other regulatory elements (11,28). Blokesch and Schoolnik demonstrated one mechanism underlying the natural transformation-negative phenotype of QS defective *V. cholerae* strains and the interconnection between QS and natural competence and transformation (7). These authors showed that on increased cell density, the major regulator of QS, HapR, represses (directly or indirectly) the gene *dns*, which encodes an extracellular nuclease (7) (Figure 1). As this nuclease degrades surrounding DNA [(7), and recently confirmed in (29)] that could potentially act as transforming material, its repression is crucial for natural transformation. Furthermore, based on previous microarray expression data (2), it was also speculated that HapR acts as an activator for the expression of the essential competence gene *comEA* (7,21). ComEA is predicted to encode a periplasmic DNA-binding protein, which is involved in DNA uptake (2,7,21). The reduced expression of *comEA* in QS mutants was later confirmed experimentally (8,22).

Lo Scrudato and Blokesch largely extended our view on the connection between QS and natural competence and transformation, providing evidence that HapR and Dns are inversely correlated at the protein level and demonstrating that QS and TfoX activity co-regulate only a minority of competence genes (8). Specifically, this study indicated that apart from *comEA*, only one other tested competence gene, *comEC*, also requires HapR for TfoX-dependent expression (8). ComEA, which is most

likely a periplasmic DNA-binding protein (2), and ComEC, which encodes an inner membrane transporter (21), play a major role in the DNA uptake process, as both of them are supposed to directly interact with the incoming DNA (23). In contrast, the expression of the competence genes with a potential role in scaffolding or type IV-like pilus assembly [e.g., *pilA* and *pilM*; (2,21,30)] was QS independent (8). Interestingly, Suckow *et al.* (21) and Lo Scrudato and Blokesch (8) unambiguously showed that natural transformation was almost exclusively dependent on the major autoinducer of *V. cholerae*, CAI-1. These data were consistent with the previous results of Zhu and Mekalanos (31) who investigated QS-dependent biofilm formation in *V. cholerae*. These authors wrote, 'AI-2 signals are largely dispensable, while CAI-1 signalling is important for regulating biofilm formation' (31). The extremely minor contribution of AI-2 to natural transformation was also reflected in another recent study (22). The data of this study showed that natural transformation was also highly reduced in a CAI-1 negative strain, whereas the transformation frequencies did not drop significantly below that level in a strain lacking both autoinducers (e.g. CAI-1 and AI-2 negative) (22). Furthermore, natural transformation occurred even in the absence of both autoinducers, which was not observed in our previous studies (8,21). Based on the strong connection between CAI-1 and natural competence/transformation, we proposed that CAI-1 acts as a competence pheromone (8,21,23). Notably, another gram-negative and naturally competent bacterium, *Legionella pneumophila* (32), also produces a α-hydroxyketone (AHK) autoinducer called *Legionella* autoinducer 1 [LAI-1, a 3-hydroxypentadecane-4-one; (33)], which is similar to CAI-1 [a 3-hydroxytridecane-4-one; (26)] [for a recent review on AHK, see (34)]. Based on this and the hypothesis that CAI-1 is a competence pheromone (8,21), Seitz and Blokesch (10) recently wrote that 'it is tempting to speculate that α-hydroxyketone signalling molecules are commonly involved in the regulation of natural competence'. Indeed, Kessler *et al.* (35) recently showed that LAI-1 and its respective sensor kinases play a major role in the natural competence of *L. pneumophila*, although in a reciprocal manner from that of *V. cholerae*. But even though a lot of information is available concerning the upstream regulatory circuitry of the major regulator of QS, HapR, and its contribution to natural competence and transformation, the downstream signalling pathway has not been experimentally challenged. This report is the first to demonstrate that HapR directly binds the promoter region of *dns*, but not that of *comEA*. In contrast, HapR binds the promoter region and activates the expression of a gene encoding an intermediate regulatory protein, QstR. The expression of *qstR* also requires TfoX, apart from HapR, thus linking QS and (chitin-induced) TfoX induction in *V. cholerae*. Moreover, we demonstrate that the artificial expression of *qstR* restores natural competence and transformation in *hapR*-deficient strains, further confirming a role for QstR as an intermediate regulator in the natural competence and transformation of *V. cholerae*.

MATERIALS AND METHODS

Media and growth conditions

Vibrio cholerae and *Escherichia coli* strains were grown in Luria both (LB) medium at 30°C with shaking, unless otherwise stated. Antibiotics were added for plasmid maintenance or transformants/transconjugants selection at concentrations of 50 or 100 μg/ml for ampicillin, 75 μg/ml for kanamycin and at 50 μg/ml for gentamicin. Thiosulfate-citrate-bile salts-sucrose agar plates were used to counter-select *E. coli* after bi-/triparental mating with *V. cholerae*; the thiosulfate-citrate-bile salts-sucrose agar plates were prepared according to the manufacturer's instructions (Sigma-Aldrich/Fluka, Buchs, Switzerland). NaCl-free LB medium containing 6% sucrose was prepared for sucrose-based *sacB*-counter-selection.

Bacterial strains and plasmids

The *V. cholerae* strains and plasmids used in this study are indicated in Table 1. The *E. coli* strains DH5α (43) and S17-1λpir (44) were used for cloning and as a donor strain in bacterial mating experiments, respectively. *E. coli* Origami 2^{TM} (DE3) pLysS (Novagen) was used as a host for HapR protein expression.

Construction of *V. cholerae* mutant strains

The gene *qstR* (VC0396) was deleted from the parental strain A1552 using the gene disruption method based on the counter-selectable plasmid pGP704-Sac28, as previously described (14). The oligonucleotides used to construct the respective plasmid are indicated in Supplementary Table S1.

Construction of plasmids used to investigate the putative HapR binding site upstream *comEA*

Most of the plasmids used in this study were derived from the plasmid pBR322 (39) (Table 1). Initially, pBR322 was modified through the partial deletion of the tetracycline resistance cassette and the constitutive promoter P_{Tet}, resulting in the plasmid pBR-Tet_MCSII (Table 1). The primers P[VC1917]-GFP#1-*NotI* and VC1917-down-*NotI* (Supplementary Table S1) were used in polymerase chain reaction (PCR) to amplify *comEA* preceded by 900 bp of its upstream region. The genomic DNA (gDNA) from the *V. cholerae* strain A1552 was used as a template. The PCR fragment was digested with *Not*I and cloned into the equally digested vector pBR-Tet_MCSII, resulting in the plasmid pBR-[own]*comEA*.

The plasmid pBR-[own]*comEA* was digested with *Aat*II and *Bgl*II to shorten the upstream region of *comEA*. Self-ligation resulted in the plasmid pBR-[P_{wt}]*comEA*, carrying *comEA* and 200 bp of its upstream region (Table 1). Site-directed mutagenesis using inverse PCR on plasmid pBR-[P_{wt}]*comEA* generated the plasmids pBR-[$P_{mut\ L}$]*comEA*, pBR-[$P_{mut\ R}$]*comEA* and pBR-[$P_{mut\ L/R}$]*comEA*, carrying mutations in the putative HapR binding site upstream *comEA* (see oligonucleotides in Supplementary Table S1).

Table 1. Bacterial strains and plasmids

Strains or plasmids	Genotype[a]	Reference
Strains (*V. cholerae*)		
A1552	Wild-type (WT), O1 El Tor Inaba, Rif^R	(36)
A1552-LacZ-Kan	A1552 strain with *aph* cassette in *lacZ* gene; Rif^R, Kan^R	(37,38)
A1552-Tn*tfoX*	A1552 containing mini-Tn7-*araC*-P_{BAD}-*tfoX*; Rif^R, $Gent^R$	(8)
ΔhapR	A1552ΔVC0583, Rif^R	(2)
ΔhapR-Tn*tfoX*	A1552ΔhapR containing mini-Tn7-*araC*-P_{BAD}-*tfoX*; Rif^R, $Gent^R$	(8)
ΔcomEA	A1552ΔVC1917 [=A1552*VC1917* in (2)], Rif^R	(2)
ΔcomEA-Tn*tfoX*	A1552ΔcomEA containing mini-Tn7-*araC*-P_{BAD}-*tfoX*; Rif^R, $Gent^R$	This study
ΔqstR	A1552ΔVC0396, Rif^R	This study
ΔqstR-Tn*tfoX*	A1552ΔqstR containing mini-Tn7-*araC*-P_{BAD}-*tfoX*; Rif^R, $Gent^R$	This study
Plasmids		
pBR322	Amp^R, Tc^R	(39)
pGP704-Sac28	Suicide vector, ori R6K *sacB*, Amp^R	(14)
pGP704-28-SacB-ΔqstR	pGP704-Sac28 with a gene fragment resulting in a 402-bp deletion (incl. stop codon) within VC0396 (*qstR*)	This study
pBAD/Myc-HisA	pBR322-derived expression vector; *araBAD* promoter (P_{BAD}); Amp^R	Invitrogen
pBAD-*comEA*	*comEA* gene cloned into pBAD/Myc-HisA, arabinose inducible; Amp^R	This study
pBAD-*hapR*	*hapR* gene cloned into pBAD/Myc-HisA, arabinose inducible; Amp^R	This study
pBAD-*hapR*-N-Strep	*hapR* gene preceded by sequence encoding *Strep*-tagII® cloned into pBAD/Myc-HisA, arabinose inducible; Amp^R	This study
pBAD-*qstR*	*qstR* gene cloned into pBAD/Myc-HisA, arabinose inducible; Amp^R	This study
pUX-BF13	*ori*R6K, helper plasmid with Tn*7* transposition function; Amp^R	(40)
pGP704::Tn7	pGP704 with mini-Tn7	Schoolnik laboratory collection; (41)
pGP704-mTn7-*araC-tfoX*	pGP704 with mini-Tn7 carrying *araC* and P_{BAD}-driven *tfoX;* Amp^R	(8)
pBR-Tet_MCSI	pBR322 derivative deleted for Tet promoter and part of tet^R gene; Amp^R	(8)
pBR-Tet_MCSII	pBR322 derivative deleted for Tet promoter and part of tet^R gene; new MCS included; Amp^R	This study
pBR-[own]*comEA*	*comEA* gene preceded by 900 bp of upstream region cloned into pBR-Tet_MCSII; Amp^R	This study
pBR-[P_{WT}]*comEA*	*comEA* gene preceded by 200 bp of upstream region; Amp^R	This study
pBR-[$P_{mut\ L}$]*comEA*	plasmid generated by inverse PCR of pBR-[P_{WT}]*comEA*; mutated within the left part of the putative HapR binding site upstream *comEA*; Amp^R	This study
pBR-[$P_{mut\ R}$]*comEA*	plasmid generated by inverse PCR of pBR-[P_{WT}]*comEA*; mutated within the right part of the putative HapR binding site upstream *comEA*; Amp^R	This study
pBR-[$P_{mut\ L/R}$]*comEA*	plasmid generated by inverse PCR of pBR-[P_{WT}]*comEA*; mutated within the left and right part of the putative HapR binding site upstream *comEA*; Amp^R	This study
pBR-[P_{dns}-100-50]	DNA sequence corresponding to the −100 to −50 bp region upstream of *dns* was inserted into pBR-Tet_MCSI by inverse PCR; Amp^R	This study
pBR-[P_{dns}-50-1]	DNA sequence corresponding to the −50 to −1 bp region upstream of *dns* was inserted into pBR-Tet_MCSI by inverse PCR; Amp^R	This study
pBR-[P_{qstR}-150-120]	DNA sequence corresponding to the −150 to −120 bp region upstream of *qstR* was inserted into pBR-Tet_MCSI by inverse PCR; Amp^R	This study
pBR-[P_{qstR}-150-102]	DNA sequence corresponding to the −150 to −102 bp region upstream of *qstR* was inserted into pBR-Tet_MCSI by inverse PCR; Amp^R	This study
pBR-[P_{qstR}-150-102]_mut_*Age*I	plasmid generated by inverse PCR of pBR- [P_{qstR}-150–102]; site-directly mutated in two bases as indicated in Figure 6D thereby creating an *Age*I restriction enzyme recognition site; Amp^R	This study

[a]VC numbers according to (42).

Construction of complementing plasmids and plasmid pBAD-*hapR*-N-Strep

The genes *hapR*, *comEA* and *qstR* were amplified with the respective primers indicated in Supplementary Table S1 using gDNA from *V. cholerae* strain A1552 as a template. The *Nco*I and *Eco*RI-digested PCR products were cloned into the equally digested plasmid pBAD/Myc-HisA to generate plasmids pBAD-*hapR*, pBAD-*comEA* and pBAD-*qstR* (Table 1). The *Strep*-tagII® encoding sequence was added to *hapR* through inverse PCR using the oligonucleotides hapR-N-Strep-fw and hapR-N-Strep-bw (Supplementary Table S1) and pBAD-*hapR* as a template, yielding plasmid pBAD-*hapR*-N-Strep.

Construction of plasmids containing the putative HapR binding sites upstream of *dns* and *qstR*

The short DNA segments upstream of *dns* and *qstR*, respectively (30–50 bp), were introduced as overhangs in the oligonucleotides indicated in Supplementary Table S1. The primers were used to amplify pBR-Tet_MCSI through inverse PCR to generate the plasmids pBR-[P_{dns}-100-50], pBR-[P_{dns}-50-1], pBR-[P_{qstR}-150-120] and pBR-[P_{qstR}-150-102] (Table 1). For the electrophoretic mobility shift assay (EMSA) experiments, the respective inserted DNA regions, flanked by DNA sequences derived from the plasmid (~200-bp fragments), were PCR amplified using the primers pBR-TET_MCS-before and

pBR-TET_MCS-after (Supplementary Table S1). The plasmid pBR-[P_{qstR}-150-102]_mut_*Age*I, carrying mutations in the promoter region −150 to −102 upstream of the start codon of *qstR* (P_{qstR}; see Figure 6D), was constructed through inverse PCR using the oligonucleotides PVC0396_AgeI_fw and PVC0396_AgeI_bw and the plasmid pBR-[P_{qstR}-150-102] as a template.

Natural transformation assay (chitin-dependent and chitin-independent)

Chitin-dependent natural transformation assays were done on chitin flakes as previously described by Marvig and Blokesch (37,45). Chitin-independent induction of natural competence and scoring of transformants was performed using *V. cholerae* strains carrying an inducible chromosomal copy of TfoX as described by Lo Scrudato and Blokesch (8). Statistical analyses of transformation data were done on log-transformed data (46) using a two-tailed Student's *t*-test.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blotting

The proteins were separated through sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then either stained for protein using Coomassie blue or subjected to western blotting as previously described (8). Primary antibodies against HapR (8) and against the *Strep*-tagII sequence (α-Strep-MAB classic, IBA GmbH, Göttingen, Germany) were diluted at 1:10 000 and 1:1000, respectively. Goat anti-rabbit horseradish peroxidase (HRP) (diluted 1:20 000; Sigma-Aldrich, Switzerland) and goat anti-mouse HRP (diluted 1:5000; Sigma-Aldrich, Switzerland) were used as secondary antibodies. Lumi-Light^{PLUS} western blotting substrate (Roche, Rotkreuz, Switzerland) or Western Lightning®-ECL (PerkinElmer, Schwerzenbach, Switzerland) were used as HRP substrates, and the luminescence signals were detected using chemiluminescence-detecting films (Amersham Hyperfilm ECL, GE Healthcare via VWR, Dietikon, Switzerland).

Purification of HapR-N-Strep

HapR-N-Strep protein was expressed in the *E. coli* strain Origami^{TM} 2 (DE3) pLysS (Novagen) carrying the plasmid pBAD-*hapR*-N-Strep. The cells were grown in LB medium at 30°C until the culture reached an optical density of ~0.8 at 600 nm. At that time, the expression was induced on the addition of 0.2% arabinose to the culture medium. After induction, the bacteria were further cultivated at 16°C overnight. The cells were collected through centrifugation and resuspended in lysis buffer (50 mM sodium phosphate pH 8, 125 mM NaCl and 1% Triton) containing a protease inhibitor cocktail (Roche, Rotkreuz, Switzerland). The cells were disrupted using several passages through a French press cell. The crude extract was clarified through centrifugation (17 000 rpm for 30 min at 4°C), and the supernatant was loaded onto two individual *Strep*-Tactin® Sepharose columns (1 ml of column volume; IBA GmbH, Göttingen, Germany). Each column was washed with 5 volumes of washing buffer (100 mM Tris–HCl, pH 8.0, 150 mM NaCl and 1 mM ethylenediaminetetraacetic acid) to remove the unbound proteins. The protein was eluted in six fractions (each at 0.5× column volume) of the same buffer containing 2.5 mM D-desthiobiotin. The respective fractions obtained from both columns were combined and loaded onto a 15% sodium dodecyl sulfate-polyacrylamide gel for electrophoretic separation. The proteins within the gel were stained with Coomassie blue (BIO-RAD). The fraction with the highest amount of protein was selected and concentrated using centrifugal filter columns (Roti®-Spin MINI-10, Carl Roth, Germany). The protein concentration was determined using the Pierce BCA Protein Assay kit (Thermo Scientific). The HapR-N-Strep protein was stored at −20°C in elution buffer containing 10% glycerol and 0.3 mM Dithiothreitol (DTT).

EMSA

The protocol for EMSA was established based on similar previously described protocols (12,13). Briefly, DNA fragments were obtained through the PCR amplification of gDNA from *V. cholerae* or plasmid DNA sequences using the primers listed in Supplementary Table S1. The amplicons were visualized on a 1.2% agarose gel. The DNA fragments were (gel-) purified using either GenElute^{TM} PCR Clean-Up or Gel Extraction kits (Sigma-Aldrich, Switzerland). The *in vitro* binding of HapR to the DNA probes was facilitated through incubation of the purified protein with the DNA fragments in reaction buffer (10 mM Tris–HCl pH 8.0, 2.5% glycerol, 0.5 mM ethylenediaminetetraacetic acid, 100 mM KCl, 1 mM $MgCl_2$ and 2 mM DTT) for 20 min at 30°C. After the incubation, bromophenol blue and 2.5% glycerol were added to the reactions. The samples were loaded onto a pre-run (in 0.5× TBE) 8% polyacrylamide gel. The electrophoretic separation occurred for 1 h 40 min at 100 V. The proteins were transferred from the gel to a polyvinylidene difluoride membrane for western blotting where indicated. All other gels were soaked for 30 min in a solution containing 0.2 μg/ml of ethidium bromide to stain DNA. The pictures were captured with an UV transilluminator (G:BOX, Syngene). The images were rotated, cropped and uniformly adjusted for brightness and contrast using Adobe Photoshop.

Quantitative reverse transcription PCR (qRT-PCR)

The *V. cholerae* strains were grown for several hours in LB medium, as previously described (8). Where indicated, 0.02% arabinose was added to the medium to artificially induce natural competence (as TfoX is driven by the P_{BAD} promoter in this system) (8). The cell harvest, RNA preparation, and reverse transcription followed by quantitative PCR (qRT-PCR) (LightCycler® Nano, Roche) were performed as described earlier (8), except the cDNA was prepared using the Transcriptor Universal cDNA Master mix (Roche, Rotkreuz, Switzerland). The expression values are given relative to the expression of the reference gene *gyrA* (8). The gene-specific primers used for qRT-PCR are indicated in reference (8) and in Supplementary Table S1.

RESULTS

The artificial expression of *comEA* increases natural transformation in a HapR-negative strain

Natural competence and transformation of *V. cholerae* occurs at a high cell density, as described earlier in the text. We previously showed that the expression of *comEA* is dependent on HapR, whereas many of the other competence genes, such as *pilA*, are expressed independently of this regulatory protein (8) (Figure 1). Based on this finding, we wanted to determine whether the transformability of a *V. cholerae HapR* mutant strain could be rescued solely through mimicking the HapR-mediated activation of *comEA*. Thus, strain ΔhapR-Tn*tfoX* carrying the inducible plasmid pBAD-*comEA* was subjected to a chitin-independent natural transformation assay, as previously described (8). As shown in Figure 2, the artificial expression of *comEA* partly rescued the transformation phenotype in the *hapR* minus strain (lane 4). This result is consistent with that of a previous study showing that the natural transformation in *HapR* mutant strains could be partially rescued through deletion of *dns*, the gene repressed by HapR (7). However, it is unknown whether HapR or intermediate regulators directly influence *comEA* and *dns* expression.

A putative HapR binding site upstream of *comEA* is not essential for natural transformation

The direct binding of HapR to the promoter regions of the virulence regulator gene *aphA* and the gene encoding the hemagglutinin protease (HA protease) *hapA* has been demonstrated *in vitro* (12,13) (Figure 1). Furthermore, Tsou *et al.* (13) identified two distinct HapR-binding motifs, motif 1 and motif 2, using bioinformatics tools and experimental validation. We wanted to determine whether a similar motif would also be present in the upstream region of *comEA*, indicating the direct regulation of *comEA* through HapR. Indeed, we identified a DNA region located between 122 and 103 bp upstream of the *comEA* start codon, which highly resembled motif 1 (13) (Supplementary Figure S1A). To characterize the significance of this motif, we generated the plasmid pBR-[P_{WT}]*comEA*, carrying *comEA* preceded by 200 bp of its indigenous upstream region. This plasmid was used as a template for the site-directed mutagenesis of the left, right or both segments of the putative HapR binding site. The resulting plasmids, pBR-[$P_{mut\ L}$]*comEA*, pBR-[$P_{mut\ R}$]*comEA* and pBR-[$P_{mut\ L/R}$]*comEA*, were assessed for their ability to complement *V. cholerae* strains lacking the *comEA* gene on the chromosome in a chitin-independent transformation assay. As shown in Supplementary Figure S1, the transformation frequencies of all strains carrying mutations in the putative HapR binding site (panel B, lanes 4–6) were comparable with the frequency of the strain complemented with *comEA* preceded by its indigenous upstream region (lane 3). Similar results were obtained when natural competence was induced on a chitin surface using a previously described method (37). Indeed, in this chitin-dependent transformation assay, the complementation worked even better (Supplementary Figure S1C). The most likely explanation for this difference in complementation efficiency is that the plasmids used in this study were derivatives of pBR322, which changes copy number under different growth conditions/growth rates (47). These results demonstrate that the putative HapR binding site is negligible for the regulation of *comEA*. However, it remains unknown whether HapR binds to a different sequence within the upstream region of *comEA* or whether HapR indirectly regulates this competence gene.

Direct and indirect regulation of competence genes by HapR

To determine whether HapR directly regulates certain genes that play a role in competence and transformation, we assessed the binding of HapR to the respective promoter regions of these genes *in vitro*. First, we purified the HapR protein using a tagged version of HapR through the cloning of plasmid pBAD-*hapR*-N-Strep (fusion between the sequence encoding for the *Strep*-tagII peptide and *hapR*). We then determined whether *hapR*-N-Strep could complement the transformation-negative phenotype of a *V. cholerae hapR* minus strain. As depicted in Supplementary Figure S2, the transformation frequencies of the strain ΔhapR-Tn*tfoX* carrying *hapR* (lane 3) or *hapR*-N-Strep (lane 4) were almost identical, highlighting the *in vivo* functionality of this translational fusion.

Assured that HapR-N-Strep was fully functional *in vivo*, we heterologously expressed this gene in *E. coli* strain Origami 2^{TM} (DE3) pLysS. The successful but low-level expression of HapR was verified through PAGE and western blot analyses (Supplementary Figure S2B). The N-terminally tagged protein was purified using a *Strep*-Tactin® Sepharose column (see 'Material and Methods' section for details; Supplementary Figure S2C) and stored at −20°C.

Next, we characterized the *in vitro* binding of the purified HapR protein to specific DNA fragments using an EMSA. We first tested the *in vitro* binding ability of HapR to the *comEA* promoter region (the features of this and other tested DNA probes are indicated in Supplementary Tables S2–S4). As indicated in Figure 3A (lanes 5–8), HapR did not shift the *comEA* promoter (P_{comEA})-specific DNA fragment, even at the highest tested protein concentration (lane 8). The same result was obtained for another *V. cholerae* competence gene, *comEC* (21), whose expression is also influenced through HapR *in vivo* (8) (Figure 3A, lanes 9–12). In contrast, the promoter region of *aphA*, to which HapR binds *in vitro* (12), was successfully used as positive control in this assay (Figure 3A, lanes 1–4). We also examined the promoter of the competence gene *pilA*, which is not a component of the QS regulon in *V. cholerae* (8) (Figure 1). In this experiment, we used a longer DNA fragment of ~600 bp, as this fragment was previously used to report *pilA* transcription using transcriptional reporter fusions (8). Consistent with the HapR-independent expression *in vivo*, HapR did not bind the *pilA* promoter *in vitro* (Figure 3B, lanes 5–8). A similar-sized fragment spanning the *aphA* promoter region

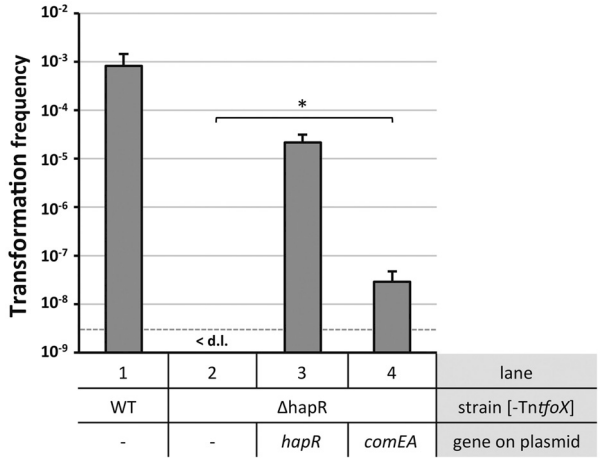

Figure 2. Artificial expression of *comEA* increases natural transformation in *hapR* negative strains. *V. cholerae* strains were tested for natural transformability through the artificial expressing of the transformation regulatory gene, *tfoX*, using 0.02% arabinose as inducer. Plasmid-encoded and P_{BAD}-driven genes were simultaneously induced. The tested strains were either A1552-Tn*tfoX* (WT-Tn*tfoX*, lane 1) or a *hapR* minus variant (ΔhapR-Tn*tfoX*, lanes 2–4) all harbouring various plasmids. These plasmids were either the empty vector as control (lanes 1 and 2), plasmid pBAD-*hapR* (lane 3) or plasmid pBAD-*comEA* (lane 4). The natural transformation frequencies are indicated on the *y*-axis. The experiments were independently repeated three times, and the error bars reflect standard deviations. <d.l.: below detection limit (average d.l. of strain ΔhapR-Tn*tfoX* was 2.9×10^{-9}, as indicated with a dashed grey line). Statistically significant differences were determined using Student's *t*-test. $^{*}P < 0.05$; for strain ΔhapR-Tn*tfoX*, the value of the detection limit was used for statistical analysis.

(*aphA**) served as a positive control (lanes 1–4) to facilitate the proper comparison with the P_{pilA} probe. From these data, we concluded that HapR neither binds to the *comEA* and *comEC* promoter regions nor to the *pilA* promoter region.

The other QS-regulated gene that plays a major role in natural transformation is *dns* (7) (Figure 1). To determine whether HapR directly or indirectly mediates the repression of *dns*, we repeated the mobility shift assays using the *dns* promoter region as a DNA template (Figure 4). An ~250 bp fragment covering the region −203 bp to +48 bp from the start codon of *dns* was first tested (Figure 4A). Interestingly, HapR was able to shift the DNA fragment, although only at higher HapR protein concentrations than those sufficient to shift the positive control fragment (Figure 3A). We also included a non-related negative control in this assay [the *recA* promoter region; (8)], which did not show any mobility shift (data not shown). A western blot analysis of the *dns*-specific EMSA gel followed by the detection of HapR with α-HapR-specific antibodies confirmed that HapR protein co-localizes with the shifted DNA fragment (data not shown).

To identify the putative HapR binding site within the *dns* promoter region, we PCR amplified several fragments around the *dns* start codon as indicated in Figure 4B (fragments A–H; details about the fragments can be found in Supplementary Table S3). All fragments were tested for HapR-dependent mobility shifts, and the results are indicated in the table on the left of Figure 4B. Apart from the outermost fragments (fragments A, G and H), all other probes remained bound to the HapR protein (Figure 4B). These fragments contained either the DNA sequence from −100 to −50 bp and/or the region from −50 to −1 bp, with respect to the start codon. Thus, we cloned these two sub-regions into vector pBR-Tet_MCSI (Table 1). The resulting plasmids were then used as PCR templates to obtain DNA probes of ~200 bp in length (Figure 4B, fragments I and J). To ensure that HapR did not bind to the vector sequence, we also used a DNA fragment solely derived from vector pBR-Tet_MCSI as a negative control (Figure 4B, fragment K). The resulting EMSA patterns, after *in vitro* incubation with HapR, are depicted in Figure 4C. These data indicated that HapR binds to at least two separate HapR binding sites within the *dns* promoter *in vitro*. Although we did not find any obvious HapR binding site for the *dns* sub-region of DNA fragment J, we identified a putative HapR binding site resembling motif 2 (13) within DNA fragment I (−71 bp to −56 bp from the *dns* start codon but on the complementary strand; Supplementary Figure S3).

Despite both *comEA* and *dns* being HapR-dependent *in vivo* (8), the *in vitro* binding of HapR to the respective promoter region could only be demonstrated for *dns*. Thus, we concluded that HapR regulates *comEA* (and most likely also *comEC*) in an indirect manner.

A QS- and *tfoX*-dependent regulatory protein plays a major role in the natural competence and transformation of *V. cholerae*

Based on the results described earlier in the text, we hypothesized that an intermediary regulatory protein might exist between HapR and the QS-dependent expression of *comEA*. Based on previous microarray expression-profiling data (2), we identified VC0396, which potentially encodes an intermediate protein in the competence regulatory network. VC0396 was initially annotated as a 'transcriptional regulator of the LuxR family' (42). Hereafter, we refer to this gene as QS and TfoX-dependent regulator (*qstR*). We validated the expression of *qstR* in our chitin-independent experimental model using qRT-PCR. Comparable with other competence genes, *qstR* was induced upon artificial low-level *tfoX* expression (Figure 5, highlighted results). This TfoX-dependent induction was not observed in a *hapR*-deficient background strain (Figure 5), which is consistent with the HapR-mediated regulation of *qstR*. Furthermore, a comparison of the expression pattern of *qstR* revealed high similarities with the expression pattern observed for *comEA* (Figure 5). Thus, these results confirmed our hypothesis that QstR could be an intermediary regulatory protein that signals between HapR and *comEA*.

The qRT-PCR data suggested the involvement of QstR in natural competence and transformation; therefore, we deleted the *qstR* gene in the *V. cholerae* wild-type strain A1552 and in the strain A1552-Tn*tfoX*. The deletion of *qstR* did not result in any obvious

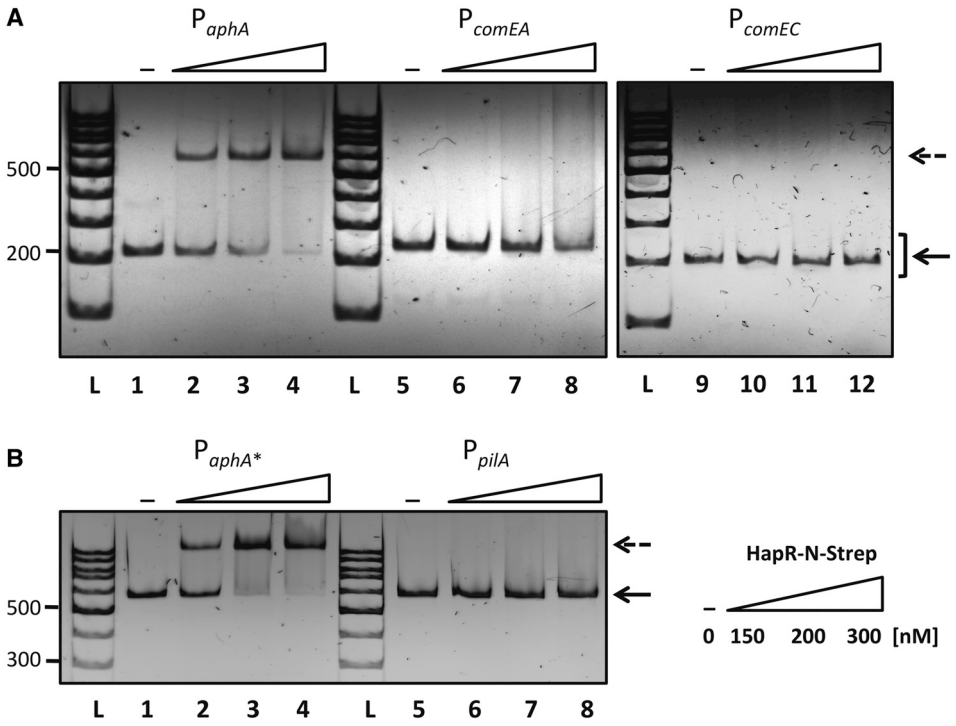

Figure 3. HapR does not bind to the *comEA*, *comEC* and *pilA* promoters *in vitro*. EMSA using the *comEA* (P_{comEA}), *comEC* (P_{comEC}) (panel **A**) and the *pilA* (P_{pilA}) (panel **B**) upstream regions as a probe did not show any bandshift. The *aphA* promoter was used as a positive control (P_{aphA}; *indicates the longer fragment used in panel B as described in the text). A total of 40 ng (panel A)/80 ng (panel B) of DNA fragments were incubated without (−) or with increasing amounts of HapR-N-Strep protein, as schematized in the figure. L: DNA ladder (representative bp are indicated on the left). Solid arrow: unbound DNA probe. Dashed arrow: bound/shifted DNA.

competence-independent phenotype [e.g. growth in rich medium, colony morphology, chitin colonization (9), etc.]. We therefore examined whether the lack of *qstR* influences chitin-induced or chitin-independent natural transformation (Table 2). Indeed, for the *qstR* minus strain, transformation was below (chitin-dependent) or at the limit of detection (chitin-independent assay) and therefore reduced by at least four orders of magnitude compared with the respective parental strain (Table 2), highlighting the importance of QstR for natural competence and transformation. The extremely rare transformants observed under chitin-independent competence-inducing conditions (Table 2) were most likely a reflection of low levels of *comEA* transcription even in the absence of QstR, whereas a *comEA* knockout strain was never transformable in our assay.

Next, we complemented the deletion strain by providing the *qstR* gene *in trans*, which restored natural transformation (Table 2). Consistent with our hypothesis that QstR acts as an upstream regulator of *comEA* (Figure 1), the exogenous expression of *comEA* also significantly increased natural transformability in the ΔqstR genetic background (Table 2). The transformation frequency in this setting (e.g. ΔqstR/pBAD-*comEA*) was ∼10-fold higher than in strain ΔhapR/pBAD-*comEA* (Figure 2), which is consistent with HapR-mediated repression of *dns* in the *qstR* mutant. To demonstrate that QstR acts downstream of HapR, we also artificially expressed *qstR* from a plasmid in the *hapR* minus strain. Indeed, the exogenous expression of *qstR in trans* in the ΔhapR-Tn*tfoX* strain rescued natural transformation to the same extent as the *hapR* gene itself (Table 2). We therefore propose that HapR directly or indirectly influences *qstR* expression and that QstR regulates QS-dependent competence and transformation genes (Figure 1). Furthermore, as the overexpression of *qstR* in a *hapR* background apparently also alleviated the transformation barrier exerted by the Dns nuclease, we suggest that QstR might also be involved in the repression of *dns* (Figure 1).

HapR binds to the *qstR* promoter region

Next, we examined whether HapR regulates *qstR* in a direct or indirect manner. We repeated the *in vitro* HapR binding assay as described earlier in the text, using the upstream region of *qstR* as a probe. As indicated in Figure 6, HapR bound to the DNA fragment causing a shift in mobility.

To identify the location of the putative HapR binding site(s), we followed the strategy described for Figure 4, examining a plethora of DNA fragments derived from −359 bp upstream to +105 bp downstream of the start codon of *qstR* (Figure 6B). The probes (fragments A–G; details in Supplementary Table S4) were tested for *in vitro* binding to HapR (EMSA), as indicated in the figure. All shifted fragments shared the sequence spanning the region from −150 to −102 bp upstream *qstR*

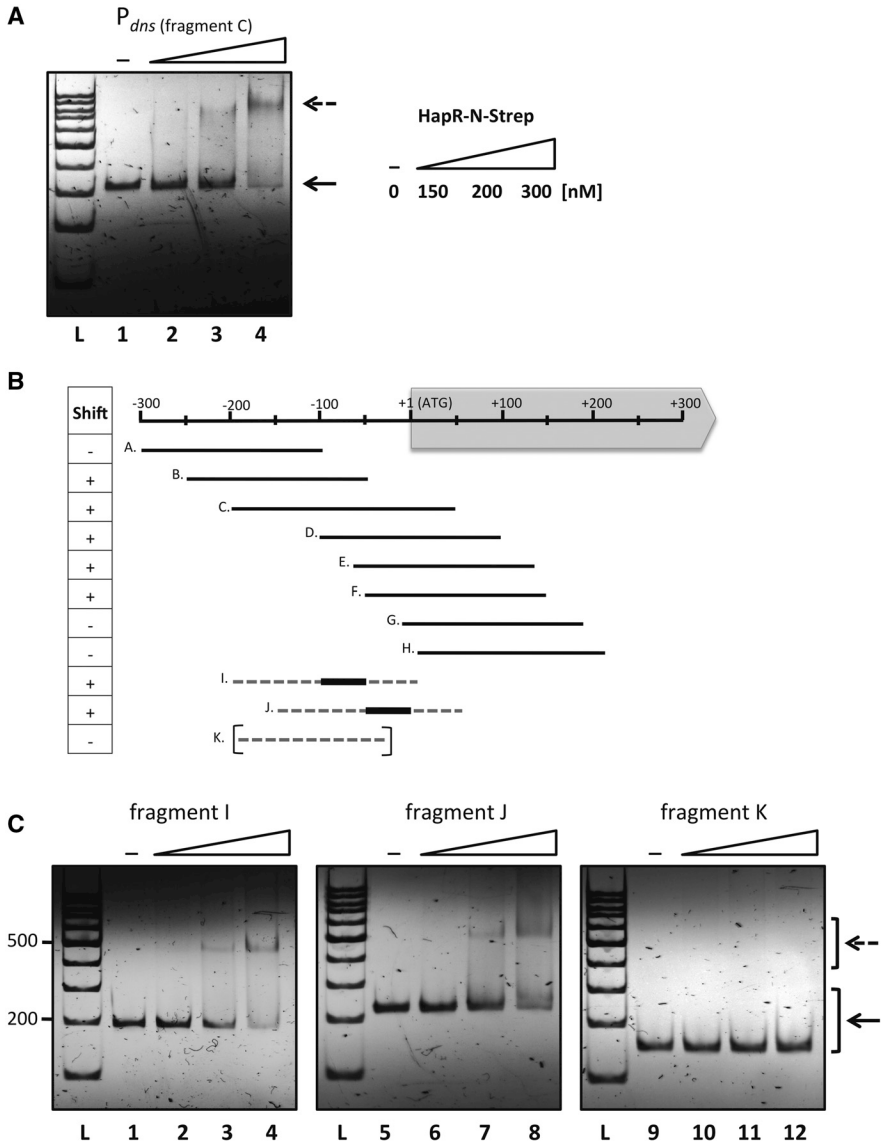

Figure 4. The HapR protein binds to the *dns* upstream region. (Panel **A**) Binding of HapR to the upstream region of *dns* results in a shifted DNA fragment. Lanes 1–4: EMSA of the *dns* promoter fragment covering the region −203 to +48 bp with respect to the "ATG" start codon (corresponding to fragment C shown in panel B). The increasing amounts of HapR-N-Strep protein are depicted on the right of the image. (Panel **B**) Schematic representation of the DNA region surrounding the *dns* start codon. The tested DNA fragments (A. to K.) spanning the respective region are depicted below the scheme. The dashed line in fragments I. to K. represents unrelated and plasmid-derived DNA. All fragments were tested for HapR-N-Strep-mediated *in vitro* binding using EMSA, and the EMSA results are indicated in the left column. The large grey arrow depicts the *dns* gene (not to scale). (Panel **C**) HapR binding site(s) were associated with two 50 bp regions located within the *dns* promoter. DNA fragments (40 ng) of ∼200 bp length containing short parts of *dns* upstream region (−100 to −50 bp for fragment I; −50 to −1 bp for fragment J) surrounded by unrelated and plasmid-derived DNA were subjected to EMSA using increasing amounts of HapR-N-Strep, as indicated in panel A. The negative control (fragment K) did not contain any P_{dns}-derived DNA sequence. L: DNA ladder. Solid arrow: unbound DNA probe. Dashed arrow: bound/shifted DNA.

(Figure 6B and Supplementary Table S4). Thus, we subcloned two fragments, i.e. the regions spanning either −150 to −120 bp or −150 to −102 bp upstream of the ATG start codon into vector pBR-Tet_MCSI (Table 1). The resulting plasmids were used as PCR templates to obtain linear DNA fragments of ∼200 bp in length (fragments H and I in Figure 6B). Fragment H, containing the shorter stretch of the P_{qstR} region, did not exhibit *in vitro* binding to HapR (Figure 6C). In contrast to fragment H, the ∼50 bp of the *qstR* upstream region within fragment I, were sufficient to facilitate HapR binding. On further examination of this particular DNA region, we identified a sequence resembling *in silico* predicted motif 2 (13) [Figure 6D; note that the simplified consensus was slightly modified

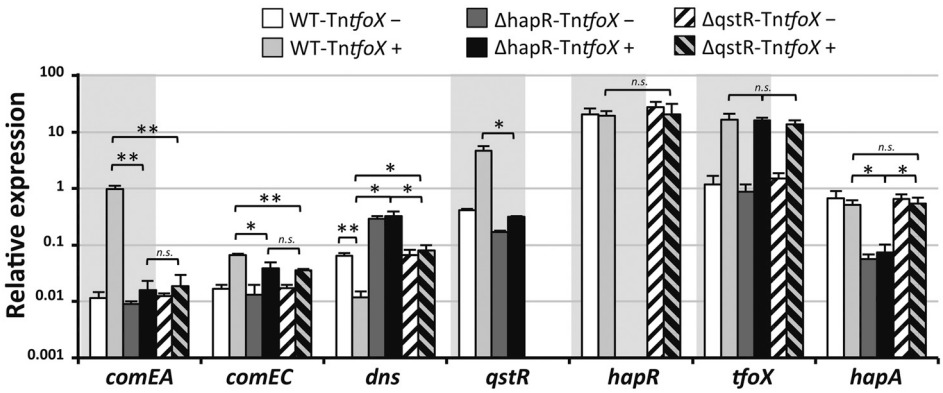

Figure 5. QstR is required for the induction of *comEA* and *comEC*. qRT-PCR data showing the expression of the indicated genes relative to *gyrA* in the wild-type background strain A1552-Tn*tfoX* and its *hapR* or *qstR* knockout derivatives (ΔhapR-Tn*tfoX* and ΔqstR-Tn*tfoX*). All three strains were grown under competence non-inducing (Tn*tfoX* −) and competence-inducing (Tn*tfoX*+) conditions. The highlighted results (shaded boxes) are first discussed in the text and indicate that the expression of *qstR* is TfoX- and HapR-dependent. The data represent the averages of three biological replicates. The error bars indicate standard deviations. Statistically significant differences were determined using Student's *t*-tests. $*P < 0.05$, $**P < 0.01$, *n.s.* = not significant.

Table 2. QstR plays a major role in the natural transformation of *V. cholerae*

V. cholerae strain	± plasmid (gene on plasmid)	Assay	Transformation frequency[x] (± SD)[y]
A1552	–	Chitin-induced transformation [assayed on chitin flakes; (37)]	1.3×10^{-4} ($\pm 5.7 \times 10^{-5}$)
ΔqstR	–		<d.l. (d.l. = 1.3×10^{-8})
A1552-Tn*tfoX*	–	Artificial and chitin-independent expression of *tfoX* (8)	2×10^{-4} ($\pm 7.5 \times 10^{-5}$)$^{a = **}$
ΔqstR-Tn*tfoX*	–		1.5×10^{-8} ($\pm 3.6 \times 10^{-9}$)$^{a = **}$
A1552-Tn*tfoX*	+ (no gene; vector control)		8.1×10^{-4} ($\pm 6.2 \times 10^{-4}$)$^{b = **}$
ΔqstR-Tn*tfoX*	+ (no gene; vector control)		1.2×10^{-8} ($\pm 9.1 \times 10^{-9}$)$^{b = **/c = **/d = **}$
ΔqstR-Tn*tfoX*	+ (*qstR*)		4.4×10^{-5} ($\pm 2.0 \times 10^{-5}$)$^{c = **/e = **}$
ΔqstR-Tn*tfoX*	+ (*comEA*)		1.8×10^{-7} ($\pm 8.4 \times 10^{-8}$)$^{d = **/e = **}$
ΔhapR-Tn*tfoX*	+ (no gene; vector control)		<d.l. (d.l. = 3.1×10^{-9})
ΔhapR-Tn*tfoX*	+ (*hapR*)		3.9×10^{-5} ($\pm 2.8 \times 10^{-5}$)$^{f = n.s.}$
ΔhapR-Tn*tfoX*	+ (*qstR*)		1.5×10^{-5} ($\pm 9.2 \times 10^{-6}$)$^{f = n.s.}$

[x]Average of at least three independent experiments.
[y]Statistically significant differences between transformation data indicated with the same characters were determined using Student's *t*-tests.
$**P < 0.01$.
n.s. = not significantly different.
<d.l.: below detection limit.

from (13) to facilitate alignment with the putative motif upstream of *qstR*]. Indeed, after site-directed mutagenesis of two conserved bases within this motif (Figure 6D), the capability of HapR to shift the DNA fragment in the assay was lost (Figure 6C). Taken together, these results demonstrate that HapR directly binds to the *qstR* upstream region, and that this binding occurs in a sequence-specific manner, despite the fact that the motif does not perfectly match the *in silico* predicted consensus (13).

QstR is essential for the TfoX-dependent induction of QS-dependent competence genes

The data provided earlier in the text indicated that HapR binds to the *qstR* promoter region. However, which competence genes does QstR subsequently regulate? To answer this question, we compared the expression levels of a plethora of competence and chitin metabolism genes, both under competence non-inducing and competence-

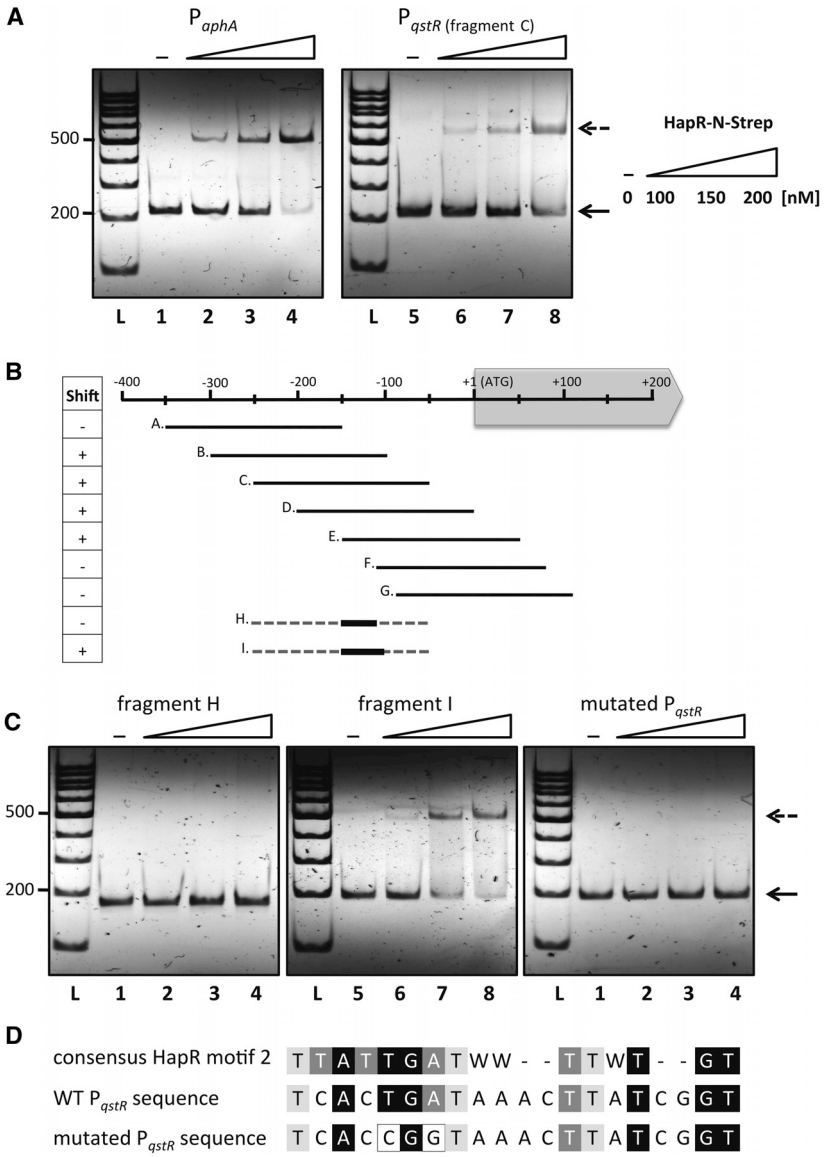

Figure 6. The HapR protein binds to the promoter region of *qstR*. (Panel **A**) HapR's ability to bind to the upstream region of *qstR* was tested by EMSA. A DNA fragment covering the region −248 to −47 bp upstream the start codon of *qstR* (corresponding to fragment C shown in panel B) was used as a probe for the *in vitro* binding of HapR. The *aphA* promoter was used as a positive control. The concentration of HapR protein used in each lane is indicated above the images and schematized on the right of the figure. (Panel **B**) Scheme representing the DNA region, which surrounds the *qstR* start codon. DNA fragments (A. to I.) spanning regions upstream and within *qstR* are depicted below the scheme. A dashed line indicates the unrelated and plasmid-derived DNA of fragments H. and I. All fragments (A. to I.) were tested using EMSA, and the results are indicated in the left column. The large grey arrow depicts the *qstR* gene (not to scale). (Panel **C**) A HapR binding site was located within a 50 bp stretch upstream the *qstR* gene and site-directed mutagenesis abolished HapR's ability to bind the *qstR* promoter. A total of 40 ng of DNA fragments (~200 bp in length) containing short parts of the *qstR* upstream region (−150 to −120 bp for fragment H; −150 to −102 bp for fragment I; and a mutated version thereof as indicated in panel D) surrounded by plasmid-derived and therefore *qstR*-unrelated DNA were subjected to EMSA. Only fragment I, containing the longer *qstR* upstream region, bound to HapR *in vitro,* resulting in a bandshift. The amounts of HapR-N-Strep used were as indicated in panel A. L: DNA ladder. Solid arrow: unbound DNA probe. Dashed arrow: bound/shifted DNA. Panel **D**: A HapR binding motif exists within the *qstR* promoter region. Simplified scheme of the *in silico* predicted HapR binding motif 2 [with slight modification from (13); e.g. four gaps (−) were introduced in the consensus sequence to allow proper alignment with the *qstR* promoter sequence]. A similar sequence located upstream of *qstR* (−130 to −111 bp from the start codon) is illustrated in the middle row. This DNA sequence has been modified through site-directed mutagenesis (boxed residues), resulting in a mutated P_{qstR} sequence as depicted in the lower row. The shadings indicate highly conserved (black background), medium conserved (dark grey background) and low conserved (light grey background) bp. Non-conserved bp are not shaded. 'W' stands for A/T.

inducing conditions in three different strains. Apart from the *tfoX*-carrying transposon Tn*tfoX* (8) the genetic backgrounds of these strains were either wild-type (A1552-Tn*tfoX*), *hapR*-negative (ΔhapR-Tn*tfoX*) or *qstR*-negative (ΔqstR-Tn*tfoX*) (Figure 5). The results showed that the expression of *comEA* and *comEC* is significantly lower under competence-inducing conditions in the two knockout strains compared with that observed in the corresponding wild-type strain, although the expression levels were comparable between the two strains. In contrast, the competence-independent background expression of *comEA* and *comEC* did not differ among any of the three strains tested (Figure 5). The results were completely different for *dns*. The expression of *dns* was significantly reduced in the wild-type background strain upon *tfoX* induction, although the *hapR* transcript levels did not change (Figure 5). Furthermore, the expression level of *hapA*, as a competence-unrelated but HapR-dependent gene and therefore a direct reflection of HapR protein activity (Figure 1), did not change on competence induction. Consistent with HapR acting as repressor of *dns*, the *dns* expression levels were significantly increased in the *hapR* minus background strain, independently of the status of *tfoX* (induced or not) (Figure 5). For the newly identified regulator QstR, we observed a phenotype with respect to *dns* expression, which neither reflected the observations of the wild-type strain (e.g. TfoX-dependent repression of *dns*) nor those of the *hapR* minus strain (constitutively high expression of *dns*). In contrast, under competence-non-inducing conditions, *dns* was repressed through HapR in the *qstR* negative strain, but further repression on TfoX induction did not occur (Figure 5). We therefore speculate that QstR might exert additional repression on *dns* (as depicted with a dashed repression arrow in Figure 1). In contrast to the expression signatures of the QS-dependent genes *comEA*, *comEC* and *dns* (2,7,8), none of the other tested genes involved in competence [*pilA*, *pilM*, *VC0047*, *dprA*; (2,21)] or in the metabolism of chitin in *V. cholerae* [*chiA-1*, *chiA-2*, *VCA0700*; (9,14)] showed any significant difference between the three tested strains under competence-inducing conditions (Supplementary Figure S4).

DISCUSSION

We recently established a chitin-independent experimental model to study the regulatory pathway of natural competence and transformation of *V. cholerae* (8). This assay is based on the artificial expression of low levels of the major regulator of transformation, TfoX, from a single chromosomal copy (8). The advantages over competence induction on chitin surfaces were as follows: (i) chitin sensing, chitin surface colonization and chitin degradation can be uncoupled from competence induction and (ii) the experiments were highly reproducible (see the 'Results' section) for the investigation of the events downstream of TfoX owing to a more homogeneous response within the bacterial population (8). In this study, we used the system to identify a new regulatory protein involved in the natural competence and transformation of *V. cholerae*, i.e. QstR, and determine some of its downstream target genes. We demonstrated that *qstR* expression is mediated through the two master regulators of QS and transformation, HapR and TfoX, respectively, thereby connecting these two pathways (Figure 1). Furthermore, using bacterial genetics and biochemical approaches, we provided evidence that QstR acts downstream of HapR and that HapR binds to a specific DNA sequence motif in the *qstR* promoter region. This motif resembles one of two *in silico* predicted HapR consensus binding sites (13), and site-directed mutagenesis abolished the *in vitro* binding of HapR to this promoter region. Finally and in accordance with its role downstream of HapR, we provided data demonstrating that QstR is only required for the regulation of a subset of competence genes. Indeed, consistent with previous data (8), we demonstrated here that the competence and chitin metabolism genes, apart from *comEA* and *comEC*, were fully inducible through TfoX even in the absence of HapR and QstR (Figure 5), including the genes *chiA-1* and *pilA*, both of which were recently suggested by Antonova *et al.* (48) as positively regulated through HapR. Apart from the HapR-independent expression of *pilA* and *chiA-1*, we did not observe any *in vitro* binding of HapR to the *pilA* promoter, again suggesting that *pilA* expression occurs independently of HapR. We previously suggested that these discrepancies between studies might reflect the differences in the strains of *V. cholerae* O1 El Tor employed in different research laboratories (8). However, the data provided here are strongly supported by the results derived from yet another O1 El Tor strain, i.e. the first sequenced strain of *V. cholerae* N16961 (42). Microarray expression data derived after chitin-induction of strain N16961 showed the highly significant induction of *pilA*, *chiA-1* and other competence- and chitin metabolism-related genes (14). Notably, the *V. cholerae* strain N16961 has a frameshift mutation within *hapR*, which abolishes QS (49,50) and consequently also natural transformation (2,23). Therefore, the expression data derived for this QS-defective strain N16961 are consistent with the HapR- (8) and QstR- (this study) independent expression of *pilA*, *chiA* and other competence and chitin metabolism genes (apart from *comEA* and *comEC*) within *V. cholerae* strain A1552 (23,36) used in this study.

The mechanism of how QstR regulates *comEA*, *comEC* and potentially *dns* remains unknown. Our preliminary data demonstrated that a tagged version of the QstR protein, which showed full functionality *in vivo*, did not bind to the *comEA* or *comEC* promoter region *in vitro* (data not shown). One possible explanation for this might be that instead of directly binding the *comEA* promoter, QstR somehow enhances the activity of TfoX (+/− CRP-cAMP) *in vivo*. However, the overexpression of *qstR* from a plasmid under non-competence-inducing conditions triggered *comEA* expression, even though not to the same level as observed under competence-inducing conditions (data not shown). Thus, we hypothesize that a (competence-specific) cofactor might be required for the full functionality of QstR *in vivo* and *in vitro* (Figure 1). QstR, together with its cofactor, might further repress

dns, as the qRT-PCR data suggest (Figure 5). The requirement of a cofactor for the full activity of QstR is consistent with QstR being a LuxR-type regulatory protein. This annotation is based on the C-terminally located DNA-binding domain (LuxR_C_like [cd06170]; NCBI Conserved Domain database). According to the Pfam protein family database (51), 'the LuxR-type DNA-binding helix-turn-helix (HTH) domain is a DNA-binding, HTH domain of about 65 amino acids. It is present in transcription regulators of the LuxR/FixJ family of response regulators'. This database further states that the HTH domain is typically located at the C-terminal region of the protein, whereas the N-terminal region of the protein often contains autoinducer binding, ligand binding or response regulatory domains. As discussed earlier in the text, the autoinducer CAI-1 plays a pivotal role in natural competence and transformation of *V. cholerae*; therefore, it is tempting to speculate that QstR directly senses CAI-1. However, our preliminary data do not support this notion. We will further explore this hypothesis in the future.

VpsT is another prominent transcriptional regulator of the LuxR family in *V. cholerae*. Indeed, QstR and VpsT share 35% sequence identity but only within the C-terminal HTH domain. VpsT has been identified in the Yildiz group and is required for the expression of the *Vibrio* polysaccharide (*vps*) gene cluster (52,53). More precisely, VPS production in *V. cholerae* is tightly linked to the signalling pathway of the secondary messenger molecule cyclic di-GMP (c-di-GMP) (54,55). Indeed, it was shown that the regulatory protein VpsT inversely regulates motility and the VPS matrix production required for biofilm formation in *V. cholerae* owing to its ability to directly sense c-di-GMP (56). On the crystallization of VpsT, the authors identified a four-residue-long c-di-GMP binding motif (W[F/L/M][T/S]R) and provided evidence that the binding of c-di-GMP stabilizes the VpsT dimer. Krasteva *et al.* (56) also reported that 'a subclass of VpsT and/or CsgD homologs exists with a proline substitution in position 3 (W[F/L/M]PR)', which is indeed the case for QstR. Thus, it is unlikely that QstR binds c-di-GMP as a cofactor in a similar manner as VpsT.

In a recent study, Ferreira *et al.* (57) examined homologs of the LuxR/VpsT/CsgD family of transcriptional regulator and c-di-GMP signalling in another *Vibrio* species, *V. parahaemolyticus*. These authors compared the microarray gene expression profiles of a wild-type and a mutant strain, with a deletion in the Scr system that affects the cellular levels of c-di-GMP (57). One of the few differentially regulated genes encoding a transcription factor was VP2710, a QstR homolog in *V. parahaemolyticus*. Indeed, based on a Basic Local Alignment Search Tool comparison, QstR and VP2710 share 52% identity (72% similarity), and GenoList (58) indicates VP2710 as the closest match of QstR in *V. parahaemolyticus*. Furthermore, *qstR* and *VP2710* are both located in close proximity to the *msh* gene cluster (see later in the text), which is indicative of VP2710 playing a similar role in the regulation of natural competence and transformation in this organism. The microarray data by Ferreira *et al.* (57) suggest that the expression of the essential competence genes [e.g. homologs to the *V. cholerae* counterparts, which we identified through comparative genomics using GenoList (58)] was not altered under the tested experimental conditions, potentially reflecting that the main regulator of competence, TfoX, was not concomitantly expressed. Notably, natural transformation was only demonstrated in *V. parahaemolyticus* under chitin-inducing conditions (4) based on a previously published protocol established for *V. cholerae* (2). QstR homologues are also present in many other *Vibrios* such as *Vibrio mimicus* (ZP_06040190.1; 96% identity), *Vibrio furnissii* (VFA_000366/ZP_05876252.1; 71% identity), *Vibrio anguillarum* (VAA_01740/YP_004567366.1; 67% identity), *Vibrio harveyi* (VIBHAR_03706/YP_001446847.1; 52% identity) and *Vibrio vulnificus* (VV1_1429/AAO09869.1; 47% identity).

Basic Local Alignment Search Tool searches using the QstR amino acid sequence showed the partial annotation of these proteins as homologues of the CsgD family of LuxR-type transcriptional regulators. CsgD is a key regulator in *Salmonella enterica* and *E. coli*, required as a positive regulator for the expression of genes encoding curli fimbriae and the synthesis of the exopolysaccharide cellulose; both curli and cellulose are implicated in biofilm formation [for a review, see (59)]. Interestingly, in *V. cholerae qstR* (VC0396) is located in close proximity (though in opposite orientation) to the mannose-sensitive haemagglutinin pilus-encoding gene cluster (VC0398 to VC0414) (60,61). This type IV pilus participates in the initiation of biofilm formation on abiotic and biotic surfaces within the aquatic environment of *V. cholerae* [(62–64); for a review, see (65)]. Based on the genetic linkage between *qstR* and the *msh* cluster and the correlation between *msh*-dependent chitin colonization (14,64) and chitin-induced competence (2), we examined the expression of *mshA*, a gene encoding the major pilin subunit of the mannose-sensitive haemagglutinin pilus (66), under competence non-inducing and competence-inducing conditions (according to the experimental approach described for Figure 5). The relative expression of *mshA* was 57.4 ($\pm$1.2) and 63.7 ($\pm$13.7) in the wild-type background strain (A1552-TntfoX) and 63.0 ($\pm$17.6) and 63.6 ($\pm$20.8) in its *qstR* negative counterpart (ΔqstR-Tn*tfoX*) under non-inducing and inducing conditions, respectively. No statistically significant differences were observed for these values between the strains and without or with *tfoX* expression (average of three biological replicates). Furthermore, we observed a strong chitin surface colonization phenotype (9) for an *mshA* mutant, i.e. absence of surface colonization accompanied by increased motility; this phenotype was not observed for the *qstR* mutant, which exhibited behaviours similar to the WT in this assay (data not shown). However, the *hapR* negative strain (ΔhapR-Tn*tfoX*) showed a statistically significant increase of ~2-fold in *mshA* expression compared with that of WT under both *tfoX*-non-inducing (relative expression = 113.3 $\pm$ 7.5) and *tfoX*-inducing (100.5 $\pm$ 6.7) conditions. We conclude that HapR is involved in the regulation of the *msh* gene cluster and that this regulation is independent of QstR. Notably, Marsh and Taylor (61) speculated that the *msh* gene cluster has been horizontally

acquired as the flanking genes are adjacent to each other in *E. coli* and as the region is flanked by a 7-bp direct repeat. This could explain why *qstR* is located proximal to the *msh* cluster but is not involved in its regulation.

In summary, our study demonstrates that QstR is a new transcription factor involved in the regulatory circuitry of natural competence and transformation. We provide evidence that QstR is dependent on the QS regulator HapR, and that it acts downstream of HapR. The expression of *qstR* is also dependent on *tfoX* expression. QstR is required for the downstream expression of competence genes, which we have previously shown to be QS regulated (e.g. *comEA* and *comEC*) (8). We also propose that QstR functions most efficiently in the presence of a cofactor, and future studies will elucidate the nature of such a potential factor.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1–4 and Supplementary Figures 1–4.

ACKNOWLEDGEMENTS

The authors would like to thank Sandrine Borgeaud and Clémentine Thévoz for technical assistance. They also acknowledge Patrick Seitz for provision of *V. cholerae* strains and Juliane Kühn for contributing primers to this work. They thank three anonymous reviewers for excellent suggestions on the manuscript.

FUNDING

Swiss National Science Foundation [31003A_127029 and 31003A_143356]. Funding for open access charge: Ecole Polytechnique Fédérale de Lausanne (EPFL).

Conflict of interest statement. None declared.

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3.3 Regulatory elements of the promoter of the competence genes

Overview: achievements and significance

This final part of the thesis project attempted to decipher which regulatory elements are important for the transcription of the competence genes. The proteins TfoX and CRP, global regulators of chitin-transformation and CCR, are necessary for the expression of the competence genes. Moreover, in the previous section we demonstrated that, in addition to those proteins, the QS pathway through the QstR protein is required for the expression of a subset of the competence genes (e.g. *comEA* and *comEC*). In *H. influenzae* the CRP protein binds to competence-specific CRP binding motifs called CRP-S sites (Redfield *et al.*, 2005). Moreover, Cameron and Redfield suggested that the binding of CRP to this site occurs together with TfoX (Sxy) protein (Cameron & Redfield, 2008; Chapter 1, Figure 12). CRP-S sites were also predicted for the *Vibrionaceae* family (Cameron & Redfield, 2006; Chapter 1, Figure 13) but their *in vivo* function has not been demonstrated.

Our aim was to investigate the role of the *in silico* predicted CRP-S sites in the expression of the competence genes of *V. cholerae*. To do so we again focused on the competence gene *comEA*.

First, we mapped the potential promoter region of *comEA* by demonstrating that the minimal region required for natural transformability is a stretch of 134bp upstream of *comEA* open reading frame. Within this region we identified a sequence similar to the *in silico* predicted consensus of the CRP-S site. However, since the promoter region containing this motif was not sufficient for transformability we hypothesized that a secondary activator (e.g. QstR) acts upstream the putative CRP-S site.

Then, we localized the transcriptional start site of *comEA* and realized that the putative CRP-S site, centered at -44.5, overlaps with the -35 region of the promoter. These features differ from those described for the CRP-S sites of *H.*

influenzae but do resemble *E. coli* class II CRP-dependent promoters (Cameron & Redfield, 2006; Busby & Ebright, 1997).

Next, we investigated the importance of the putative CRP-S site upstream of *comEA* through site-directed mutagenesis. The conversion of the CRP-S site either in a motif called CRP-Ø or in the canonical CRP binding motif (CRP-N motif; TfoX-independent) did not influence the transformability of *V. cholerae*. However, deletion of the putative CRP-S site severely impaired natural transformation. Surprisingly, the transcription of *comEA* under the mutated CRP sites did not perfectly reflect the transformability of the strains. More precisely, the CRP-Ø motif resulted in higher *comEA* transcript level, which was mirrored by the abundance of the protein. On the contrary, the CRP-N motif caused a severe drop of the *comEA* transcript level, which was in agreement with the absence of the ComEA protein. The complete deletion of the CRP-S site resulted in residual though negligible transcription of *comEA*. Since the putative CRP-S site overlaps with the -35 region, we speculated that the motif CRP-Ø represents a better binding site for the RNA polymerase. On the other hand the transit from the wild type CRP-S site to the consensus of the CRP-N motif might negatively interfere with the interactions between the CRP protein and a secondary activator hypothesized to act upstream the CRP-S site.

Next, we elucidated the contribution of QS to the activation of *comEA*. In the previous section we demonstrated that HapR directly represses *dns* and activates *qstR* (section 3.2). By *in trans* over-expression of either *qstR* or *comEA*, we were able to increase the transformability of *hapRdns* and *qstRdns* double mutant strains. The aim of these experiments was to show that the main contribution of QS to natural competence is truly due to the expression of *comEA* and to the concomitant repression of *dns*.

Finally, we tested the importance of TfoX for the expression of *comEA*. In particular we aimed at understanding whether TfoX is only required for the

expression of *qstR* or if TfoX might also be necessary, together with QstR, for the expression of *comEA.* Thus, we decided to uncouple natural competence induction and the expression of *qstR*. To do so we measured the expression of *comEA* in the absence of *tfoX* induction by *in trans* over-expressing *qstR*. Under these conditions QstR successfully drove the transcription of *comEA* even though *comEA* was not expressed to its full extent.

In summary, this study provided the following insights:

1. The putative CRP-S site upstream of the competence gene *comEA* plays a role in natural competence induction;
2. The overlap between the CRP-S site and the -35 region of the promoter of *comEA* might result in a mechanism of promoter activation similar to that of the *E. coli* class II CRP-dependent promoters;
3. The mutated CRP-Ø site resulted in a higher abundance of ComEA probably because it represents a better binding site for the RNAP.
4. The exchange of the CRP-S motif by a CRP-N site resulted in lower levels of the *comEA* expression;
5. QstR alone activates the expression of *comEA* to a certain level;
6. Besides QstR, TfoX is still necessary for full induction of *comEA*.

We also identified a putative CRP-S site upstream of the start codon of *qstR* and made a model that could explain how TfoX, CRP, and the QS transcription factors (HapR and QstR) establish the activation of the *qstR* and *comEA* promoters. Contrary to the model proposed for *H. influenzae* (Cameron & Redfield, 2008; Chapter 1, Figure 12) we speculate that TfoX is not part of the transcriptional activation machinery but that it directly or indirectly modifies the CRP-cAMP complex to allow its binding to the CRP-S site. Moreover, we suggested that the QS transcriptional regulators HapR and QstR might act as secondary activators that, simultaneously to the binding of CRP, fully activate the promoter of *qstR* and *comEA*.

Transcriptional regulation of *comEA* - a key gene in natural competence for transformation of *Vibrio cholerae*

Lo Scrudato Mirella and Blokesch Melanie

ABSTRACT

The human pathogen *Vibrio cholerae* acquires natural competence in its aquatic ecological niche where it lives in association with the chitinous exoskeleton of zooplankton. The developmental program of natural competence for transformation requires the regulatory pathways of chitin sensing and degradation, quorum sensing (QS) and carbon catabolite repression (CCR). The global regulator of CCR, the cAMP receptor protein (CRP), binds when complexed with cyclic adenosine monophosphate (cAMP) to regulatory DNA elements called CRP sites. Previous studies in another naturally competent bacterium, *Haemophilus influenzae*, demonstrated that CRP binds so called CRP-S sites within the promoter regions of the competence genes *in vitro* and suggested that this binding occurs together with the master regulator of transformation Sxy (homolog is TfoX in *V. cholerae*).

In this study we investigated the transcriptional regulation of *comEA* as an example of the general process that controls competence gene activation in *V. cholerae*. We identified a putative and previously unpredicted CRP-S site at position -44.5 from the transcriptional start site of *comEA*. Deletion of this motif almost abolished the transformability of *V. cholerae* suggesting that it is important for the expression of *comEA*. Site-directed mutagenesis of this site resulted in a lower or higher expression of the *comEA* transcript, which directly correlated with the abundance of the ComEA protein. In addition, we further investigated the role of QstR, the QS- and TfoX- dependent transcriptional regulator, on *comEA* expression. By uncoupling *qstR* expression from competence induction, we showed that *qstR* is sufficient to drive the expression of this gene.

INTRODUCTION

Vibrio cholerae is a Gram-negative bacterium that lives in the aquatic environment in association with the chitinous exoskeleton of zooplankton (Nalin *et al.*, 1979; Pruzzo *et al.*, 2008). Chitin, a polymer of β-1,4-linked N-acetylglucosamine (GlcNAc), is one of the most abundant biopolymers in nature (Gooday, 1990). In addition to its role as nutrient source, chitin also induces natural competence for transformation in *V. cholerae* and in other *Vibrio* species (Meibom *et al.*, 2005; Gulig *et al.*, 2009; Chen *et al.*, 2010; Pollack-Berti *et al.*, 2010).

Natural competence for transformation is a mechanism of horizontal gene transfer (HGT), which is based on the ability of a bacterium to take up free DNA from the environment and to recombine the extracellular DNA with the bacterial genome resulting in natural transformation. In *V. cholerae* chitin leads to the up-regulation of *tfoX* (Meibom *et al.*, 2004), a gene that encodes the master regulator of transformation. Indeed, *tfoX* expression is sufficient to induce natural competence and transformation in *V. cholerae* even in the absence of chitin (Meibom *et al.*, 2005; Lo Scrudato & Blokesch, 2012). The TfoX-homolog, Sxy, was first discovered in *Haemophilus influenzae* (Redfield, 1991; Zulty & Barcak, 1995). In our current working model the components of a type IV pilus (Fullner & Mekalanos, 1999) build up the majority of DNA-uptake machinery, which is then responsible for binding and taking up the extracellular DNA. Once this DNA (or short stretches of it) reaches the periplasmic space, probably due to pilus retraction, ComEA comes into play: the protein binds the DNA in the periplasm, thereby contributing to DNA translocation across the outer membrane. Subsequently ComEA transfers the DNA toward the inner membrane channel ComEC through which it is shuttled into the cytoplasm (Suckow *et al.*, 2011; Lo Scrudato & Blokesch, 2012; Blokesch, 2012b; Seitz & Blokesch, 2013b; Seitz *et al.*, 2014).

In addition to TfoX expression, regulatory pathways of quorum sensing (QS) and carbon catabolite repression (CCR) are also necessary to induce the competence regulon of *V. cholerae*. QS is a process of bacterial communication based on the production and secretion of small molecules called autoinducers (reviewed in Ng & Bassler, 2009). *V. cholerae* produces and secretes at least two different

autoinducers: the intra-species cholera autoinducer 1 (CAI-1) and the universal autoinducer 2 (AI-2) (Miller *et al.*, 2002; Bassler *et al.*, 1997; Xavier & Bassler, 2003). Basically, at high cell density the concentration of autoinducers is sufficient to trigger the expression of HapR, the master regulator of QS, known to regulate virulence repression (Miller *et al*., 2002; Kovacikova & Skorupsky, 2002), biofilms formation (Hammer & Bassler, 2003; Zhu *et al*., 2002; Zhu & Mekalanos, 2003) and natural competence for transformation (Meibom *et al*., 2005; Blokesch & Schoolnik, 2007; Blokesch & Schoolnik, 2008; Antonova *et al.*, 2011; Suchow *et al*., 2011; Lo Scrudato & Blokesch, 2012; Blokesch, 2012b; Lo Scrudato & Blokesch, 2013). In the absence of HapR the extracellular DNA is degraded by the action of the nuclease Dns and consequently, the DNA uptake cannot occur (Blokesch & Schoolnik, 2008; Seper *et al.*, 2011). HapR regulates natural transformation by direct repression of *dns*; furthermore HapR directly drives, concomitantly to TfoX-mediated induction, the expression of *qstR*, the gene coding for the newly identified transcriptional factor QstR (Lo Scrudato & Blokesch, 2013).

The third pathway involved in the regulation of natural competence for transformation is CCR (Meibom *et al.*, 2005; Blokesch, 2012a). This term indicates the mechanism by which in the presence of a preferred carbon source such as glucose the expression of genes necessary for the metabolism of other carbon sources is repressed (reviewed in Deutscher *et al.*, 2006; Deutscher, 2008; Görke & Stülke, 2008). In CCR the major players are the phosphoenolpyruvate-carbohydrate phosphotransferase system (PTS), the adenylate cyclase (CyaA), the metabolite 3',5'-cyclic adenosine monophosphate (cAMP) and the CRP protein. Unsaturated PTS transporters enhance the synthesis of cAMP by the enzyme CyaA. High levels of cAMP trigger the formation of an active complex between CRP and cAMP, which binds the promoters of the target genes (e.g. those genes encoding proteins that are involved in the central metabolism and in the transport of the carbon sources). *Vice versa* when the PTS is saturated cAMP is not produced and CRP-cAMP complex cannot form. Central metabolism and transport of the carbon sources are not the exclusive targets of CCR; cAMP and the CRP protein as well as the PTS components (independently on cAMP) also control biofilms formation (Liang *et al.*, 2007; Fong &

Yildiz, 2008; Houot *et al.*, 2010). With respect to natural competence for transformation the presence of PTS sugars significantly decreases the transformability of *V. cholerae*; moreover knock-out strains for *crp* or for *cyaA* are non-transformable (Blokesch, 2012a).

The role and the function of the CRP protein have been mainly studied in *E. coli* (reviewed in Busby & Ebright, 1999; Harman, 2001). CRP, formerly known as catabolite activator protein (CAP), forms a dimer of two identical subunits. Each CRP subunit contains a N-terminal cAMP binding domain, a C-terminal helix-turn-helix DNA binding motif and in between a flexible hinge region. CRP recognizes and binds 22bp-long symmetrical sequences called CRP sites. Under physiological conditions CRP is likely present either as a free apo-CRP dimer (in the absence of cAMP) or as a dimer bound to two molecules of cAMP, one for each monomer of CRP. *V. cholerae* CRP and *E. coli* CRP (EcCRP) share 81% and 95% identity in the nucleotide sequence and in the amino acid sequence, respectively (Skorupski & Taylor, 1997). As for EcCRP, *V. cholerae* CRP also showed a biphasic dependence on cAMP levels *in vitro*; moreover *V. cholerae* CRP is able to activate the transcription of *E. coli gal* promoters (Mukhopadhyay *et al.*, 1999; Chattopadhyay & Parrack, 2006). These findings strongly suggested that the CRP protein functions similarly in these two bacterial species.

In *H. influenzae* the expression of the competence genes needs not only the master regulator of transformation Sxy (Redfield, 1991; TfoX in *V. cholerae*) but also the CRP-cAMP complex (Chandler, 1992; Dorocicz *et al.*, 1993). The competence regulon of *H. influenzae* consists of genes characterized by the presence of the competence regulatory elements (CRE) (Macfadyen, 2000), which were shown to be CRP binding sites *in vitro* (Redfield *et al.*, 2005). These specific competence-related CRP binding sites were later called CRP-S sites because of their Sxy dependency and to be distinguished from the canonical Sxy-independent CRP-N site (Cameron & Redfield, 2006). Indeed, Cameron and Redfield suggested that, in *H. influenzae* and most likely also in other competent bacteria, the induction of the competence genes is under the control of CRP and Sxy (TfoX) acting in concert at the CRP-S site (Cameron & Redfield, 2006). Based on previously published expression data of *V.*

cholerae (Meibom *et al.*, 2004, 2005) Cameron and Redfield also *in silico* predicted CRP-S and CRP-N consensus motifs for the *Vibrionaceae* family (Cameron & Redfield, 2006).

In this study, we investigated the potential involvement of CRP-S sites in competence regulation in *V. cholerae*. We focused on *comEA* as one of the key element of the competence regulon of *V. cholerae*. This gene was not part of the *in silico* analysis that allowed to predict the CRP-S sites for the *Vibrionaceae* family because *comEA* was not up-regulated in the microarray data used for prediction (Cameron & Redfield, 2006; Meibom *et al.*, 2004). Notably, the *V. cholerae* strain used in this study was defective in QS and therefore not naturally transformable (Meibom *et al.*, 2005). Here, we identified a putative CRP-S site similar to the *in silico* predicted consensus in the putative promoter region of *comEA*. We investigated the importance of this putative CRP-S site upstream of *comEA* using site-directed mutagenesis followed by the analysis of the respective mutants. Our results showed that the transformability of the bacterial strains was impaired when the putative CRP-S site was deleted indicating that this site might have a role in the transcriptional regulation of *comEA.* Furthermore, the role of QstR, as an activator of *comEA*, was further inquired by uncoupling the expression of *qstR* from competence induction. With this strategy we demonstrated that *in trans* over-expression of *qstR* is sufficient to increase the abundance of the *comEA* transcripts.

RESULTS

Narrowing down the promoter region driving the expression of the competence gene *comEA*

The expression of *comEA*, a gene which codes for a periplasmic DNA binding protein is dependent on *a)* the master regulator of transformation, TfoX; *b)* the CRP-cAMP complex; *c)* the transcription factor QstR (Meibom *et al.*, 2005; Blokesch, 2012a; Lo Scrudato & Blokesch, 2013) (Figure 1).

In order to localize the regulatory elements necessary for the transcription of *comEA*, we first mapped the promoter region of this gene. To do so we constructed eight plasmids carrying the competence gene *comEA* preceded by its upstream

region, which we incrementally shortened. The plasmids were originated using direct or inverse PCR and plasmid pBR-[own]*comEA* as template (Table 1). This plasmid contains the *comEA* gene and 900bp of sequence upstream of the start codon, which should include the promoter region. A schematic representation of the upstream region of *comEA* is shown in Figure 2A. All eight constructs were tested for their ability to restore the transformability of the *V. cholerae comEA* minus strain (ΔcomEA) *in trans* after chitin-dependent competence induction.

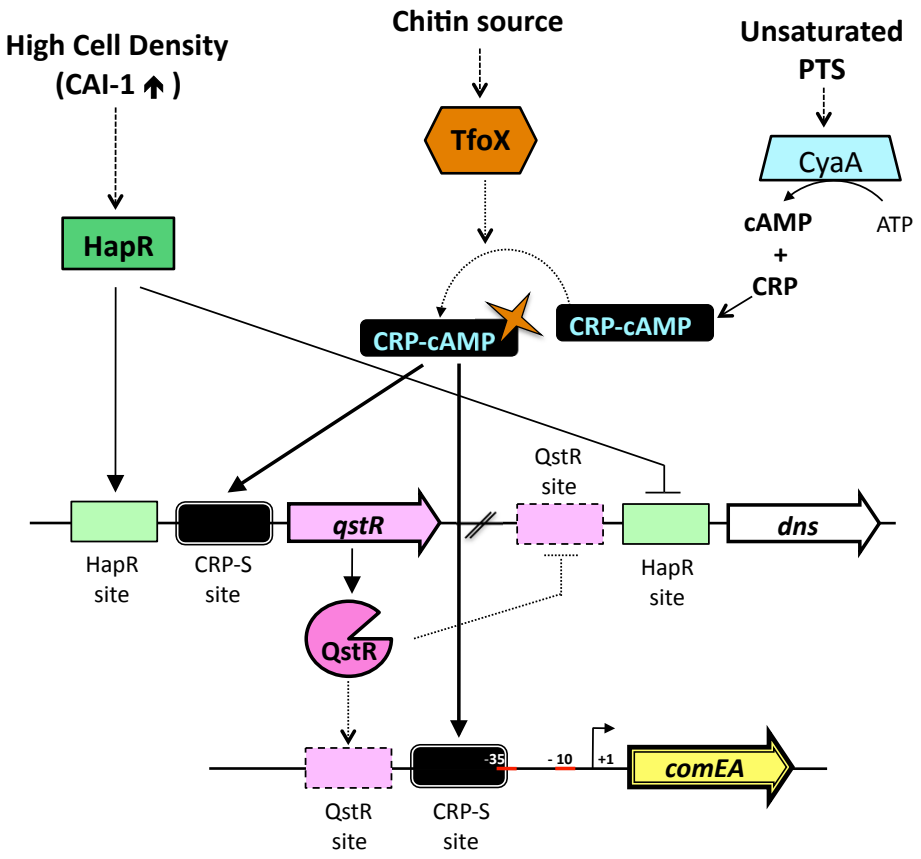
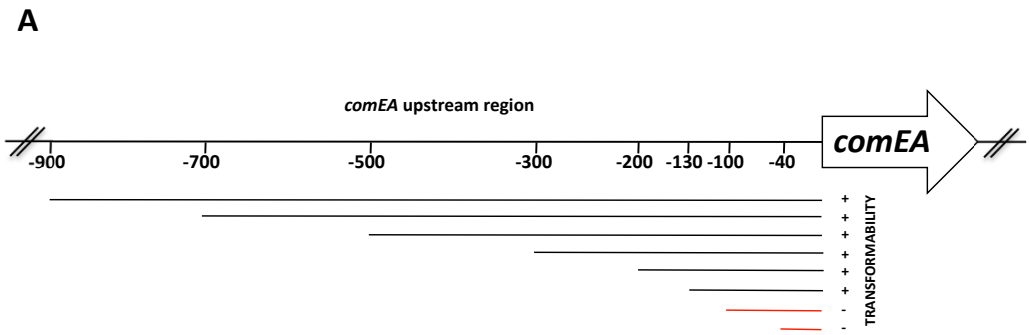
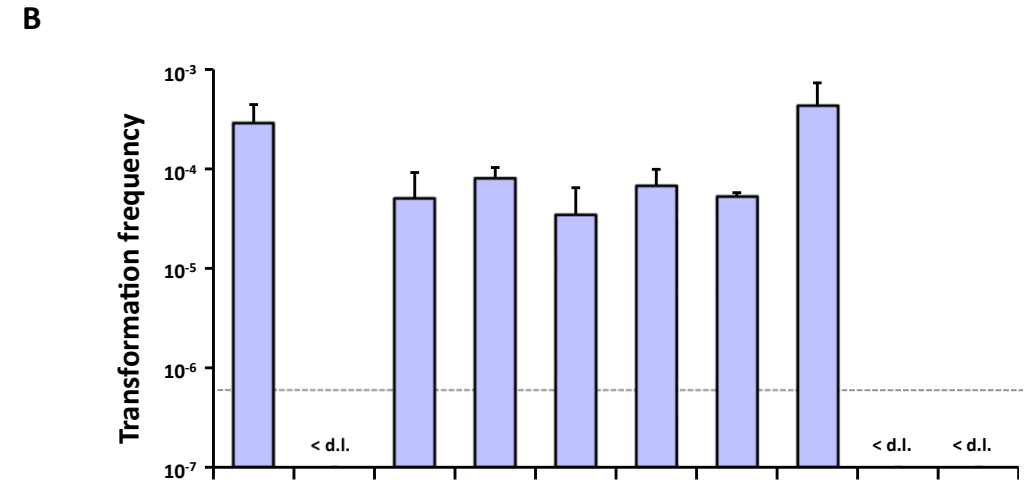


Figure 1. Model describing the transcriptional regulation of the competence gene *comEA* within the network of natural competence for transformation. The competence gene *comEA*, coding for a periplasmic DNA binding protein, is tightly regulated by three different pathways: quorum sensing (QS), chitin-sensing and -degradation, and carbon catabolite repression (CCR). The unsaturated PTS leads to the activation of the enzyme CyaA that synthesizes cAMP, which forms a complex with CRP. Under chitin induction, TfoX is expressed and potentially induces some modifications in the CRP-cAMP complex (directly or indirectly: indicated by the star). The TfoX-modified CRP-cAMP complex specifically binds the competence-related CRP-S sites upstream *qstR* and *comEA*. However, the full expression of those genes requires a QS-dependent secondary activator (HapR and QstR, respectively) that most likely operates in cooperation with the CRP protein. At high cell density the master regulator of QS, HapR, is produced and allows the expression of *qstR* by directly binding its promoter. HapR also directly represses *dns*, a gene coding for an extracellular nuclease. The transcriptional regulator QstR positively regulates the gene *comEA* and most likely contributes to further repression of *dns*.





Strain	WT	ΔcomEA							
plasmid-encoded *comEA* (p[-X]*comEA*)	–	– (p)	+						
length of the *comEA* upstream region (bp)	*n.a.*	–	900	700	500	300	203	134	100	40

Figure 2. Localization of the promoter region of *comEA*. A. Schematic representation of *comEA* with 900bp of its upstream region (not to scale). In order to localize the promoter, the upstream region of *comEA* was incrementally shortened; the numbers indicate the bp upstream of the start codon of the gene; presence (+) or absence (-) of transformability of the strains are indicated on the right of the depicted constructs (according to panel B). **B.** *V. cholerae* strains harboring the plasmid-encoded *comEA* plus the indicated upstream regions were tested for their transformability. The transformation assay was performed in a chitin-dependent manner and the strains' transformation frequencies are shown on the y-axis. WT: wild type *V. cholerae* strain A1552; ΔcomEA: *comEA* minus strain; (-): absence of the plasmid-encoded *comEA*; (p): empty vector: p[-X|*comEA*: plasmid-encoded *comEA* gene preceded by Xbp upstream the start codon. The data are the average of at least three independent experiments and the error bars reflect the standard deviation. <d.l.: below detection limit (average d.l. of strain ΔcomEA/p was 5.17 X 10^{-7} as indicated by the dashed gray line).

The transformation frequencies of the tested strains are represented in Figure 2B. ΔcomEA strains harboring plasmid-encoded *comEA* and at least 134bp of its upstream region were complemented and showed transformation frequencies comparable to the wild type strain A1552 (WT). Neither of the bacterial strains harboring 100bp and the 40bp of the upstream region of *comEA* rescued the

transformability nor did the strain carrying the empty vector (second lane). We concluded that at least 134 nucleotides upstream of the start codon of *comEA* are necessary to drive the expression of the gene.

Prediction of putative promoter elements within the *comEA* upstream region

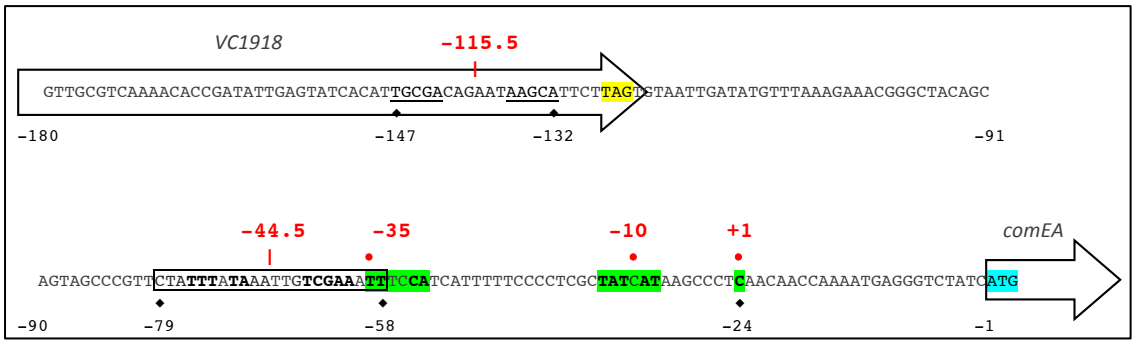
Next, we localized the transcription start site of the *comEA* transcript using 5' RACE (**R**apid **a**mplification of **c**DNA **e**nds). As schematized in Figure 3A we could map the transcription start site at -24bp upstream the start codon of the gene. In *E. coli* the majority of the promoters consists of two conserved hexanucleotides (hexamers), which are located at approximately -35 and -10 from the transcription start site. The consensus of a "typical" promoter of *E. coli* was summarized as follow: TTGACA-(N_{15-19})-TATAAT-(N_{5-7})-start. In this sequence N indicates the number of nucleotides between the -35 and -10 regions (usually 15 to 19bp) and between the -10 region and the transcription start site (usually 5 to 7bp) (Raibaud & Schwartz, 1984). The -35 and -10 regions are specifically recognized and bound by the σ subunit of the RNA polymerase (RNAP) holoenzyme (Campbell *et al.*, 2002) and the most common used σ factor in *E. coli* is σ^{70} (Shultzaberger *et al.*, 2007). Comparing the sequence upstream of the transcription start site of *comEA* with the consensus of the *E. coli* σ^{70}-activated promoter (Shultzaberger *et al.*, 2007) we identified the putative -35 and -10 regions of the *comEA* promoter, **TT**TC**CA**-(N_{16})-**TAT**C**AT**-(N_7)-start, as shown in Figure 3A.

As described above the CRP-S site is hypothesized to represent a CRP binding motif to which the protein binds together with TfoX. We manually screened the upstream region of *comEA* and identified within its promoter region a motif similar to the *in silico* predicted consensus of the *Vibrionaceae* CRP-S site (Figure 3B; Cameron & Redfield, 2006). This putative CRP-S site is 22bp in length and located between -79bp and -58bp upstream of the start codon of *comEA* (Figure 3A).

Investigation of the putative CRP-S site upstream *comEA*

In order to understand the importance of this putative CRP-S site we modified its sequence (CTATTTATAAATTGTCGAAATT) or deleted it entirely using site-directed

A



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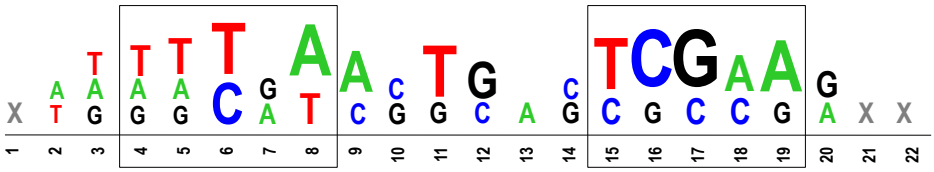

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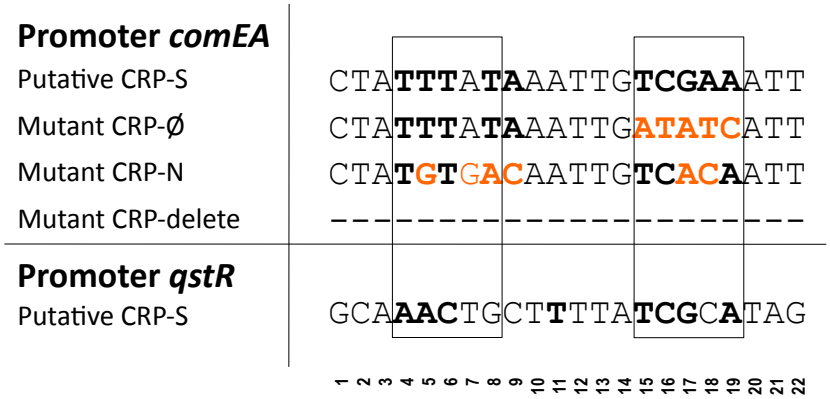


Figure 3. The transcription start site of *comEA* and the putative CRP-S site. A. Sequence of 180bp upstream the start codon of *comEA* (ATG highlighted in blue). Numbers in black and below the nucleotide sequence indicate the position from the start codon of the gene (correspondent to the black diamonds). Red numbers above the nucleotide sequence (correspondent to the red circles) refer to the positions relative to the transcription start site of the gene (which is at position +1 as highlighted in green). The putative -10 and -35 regions are also highlighted in green (nucleotide in bold match the *E. coli* consensus from Shultzaberger *et al.*, 2007). The newly identified CRP-S site is centered at position -44.5 from the transcription start site (sequence surrounded by the black box; highly conserved bases are in bold). Palindrome centered at -115.5 from the transcription start site: motif suggested as potential CRE (= CRP-S) site (Antonova *et al.*, 2012), not necessary to drive the transcription of the gene in this study; TAG highlighted in yellow: stop codon of the preceding gene *VC1918*. **B.** Consensus of the CRP-S site *in silico* predicted for the *Vibrionaceae* family (created based on Cameron & Redfield, 2006). The size of the letters indicates low, medium, and high conserved nucleotides present at each position of the consensus (1 to 22); X: non-conserved nucleotide. **C.** Putative CRP-S sites identified in the upstream region of *comEA* and *qstR* and their site-directed mutagenesis. The most conserved bases (medium and high conserved) of the putative CRP-S site are indicated in bold. Base pair changes made in order to create the CRP-Ø site or the canonical binding motif (CRP-N site; TfoX-independent) are indicated in orange. The entire putative CRP-S site upstream *comEA* was deleted in the construct CRP-delete.

mutagenesis. The sequences of the putative CRP-S site upstream *comEA* as well the site-directly changed CRP sites are represented in Figure 3C. Specifically, we changed the CRP-S site either to a motif considerably different from the CRP-S consensus in the most conserved bases (CTATTTATAAATTGATATCATT), which we named "CRP zero" (CRP-Ø), or to the consensus of the canonical CRP binding site (CTATGTGACAATTGTCACAATT), which works independently of TfoX (CRP-N). We then tested the transformability of *V. cholerae comEA* minus strains (ΔcomEA and ΔcomEA-Tn*tfoX*), carrying the plasmid-encoded *comEA* preceded by its upstream region with, without or with a changed CRP-S site in a chitin-dependent or chitin-independent manner (Figure 4). When natural competence was induced in a chitin-dependent manner the deletion of the CRP-S site (CRP-S-delete) prevented the rescue of the transformability. Interestingly, complementation was still achieved in both ΔcomEA strains carrying the plasmids with the mutated CRP-Ø or CRP-N sites. We then performed the transformation assay in a chitin-independent manner (Lo Scrudato & Blokesch, 2012) by using *V. cholerae* strains harboring an inducible copy of *tfoX* on the chromosome (ΔcomEA-Tn*tfoX*) and the same plasmids described above. Comparable to the results of the chitin-dependent transformation assay ΔcomEA-Tn*tfoX* strains harboring the mutated CRP-Ø or CRP-N sites were both complementable *in trans* with the CRP-Ø site being more efficient. Moreover, the strain lacking the CRP-S site upstream *comEA* (ΔcomEA-Tn*tfoX*/p[CRP-S-delete]*comEA*) showed a residual transformability. The difference between the two assays can be explained as the result of the heterogeneous expression patter around the chitin surfaces (Lo Scrudato & Blokesch, 2012), which leads to an overestimation of the detection limit in the chitin-dependent transformation assay as discussed earlier (Seitz & Blokesch, 2013b). Therefore, our data indicate that the 22bp of the putative CRP-S site are required for natural competence and transformation to occur but that the spacing rather than the exact conserved sequence might be relevant as the mutagenized motifs still supported natural transformation.

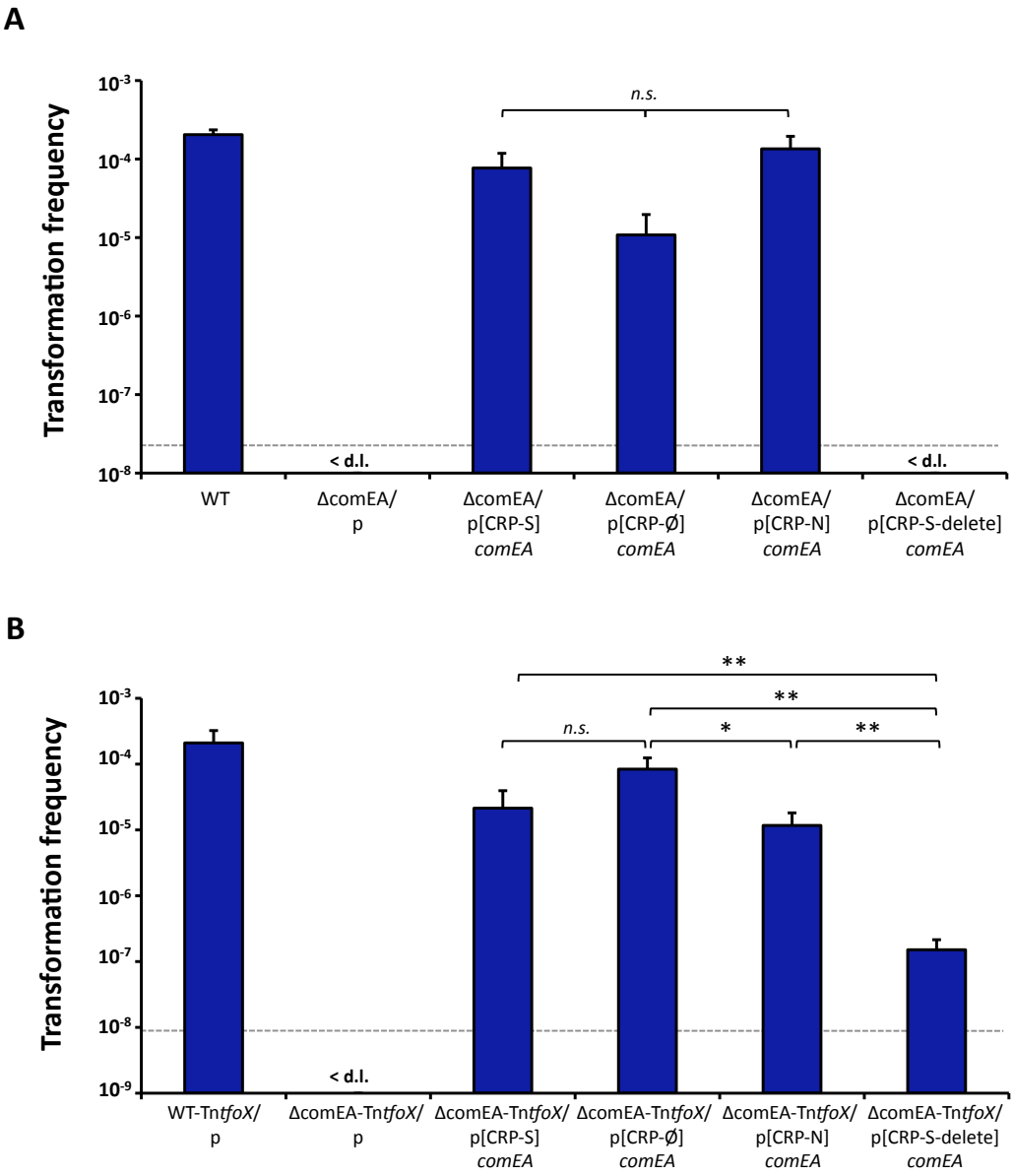


Figure 4. Role of the putative CRP-S site in natural transformation. *V. cholerae comEA* minus strains carrying on a plasmid the WT or mutated CRP-sites upstream the gene *comEA* were tested for their transformability on a chitin surface (**A**) and by artificially inducing *tfoX* in a chitin-independent manner (**B**). WT: wild type *V. cholerae* strain A1552; ΔcomEA: *comEA* minus strain; ΔcomEA-Tn*tfoX*: ΔcomEA strain with an inducible copy of *tfoX* in the chromosome; (p): empty vector; p[CRP-S]*comEA*, p[CRP-Ø]*comEA*, p[CRP-N]*comEA* and p[CRP-S-delete]*comEA*: *comEA* gene on a plasmid preceded by ~ 200bp of upstream region with the putative CRP-S site, the mutated CRP-Ø site, the canonical CRP-N site or the deleted CRP-S site, respectively. The experiments were repeated at least three independent times and the error bars reflect the standard deviation. <d.l.: below detection limit (average d.l. was 2.65 X 10^{-8} for strain ΔcomEA/p (A) and 9.8 X 10^{-9} for ΔcomEA-Tn*tfoX*/p (B) as indicated by the dashed gray line). Statistically significant differences were determined by the Student's *t* tests on log-transformed data. * $P < 0.05$, ** $P < 0.01$, *n.s.* = not significant.

Analyzing the expression of *comEA* preceded by the CRP-S site or its derivatives

Since the deletion of the CRP-S site upstream *comEA* almost abolished the transformation of the bacterial strains we were also interested in investigating the expression of the gene. We extracted the RNA from bacterial cultures grown to high cell density and then performed qRT-PCR to quantify the gene expression. As negative control we grew the wild type strain A1552-Tn*tfoX* (WT-Tn*tfoX*) in the absence of competence induction (-). The relative expression of the indicated genes is shown in Figure 5A. The levels of the *comEA* transcript were higher in strain containing the CRP-Ø motif (ΔcomEA-Tn*tfoX*/p[CRP-Ø]*comEA*) compared to the strains containing all the other constructs. This was in agreement with the higher transformation frequency of strain ΔcomEA-Tn*tfoX*/p[CRP-Ø]*comEA* compared to strain ΔcomEA-Tn*tfoX*/p[CRP-N]*comEA* (Figure 4B). Contrary to the transformability of the strains, the expression of *comEA* was noticeably lower in the strain carrying the CRP-N site (ΔcomEA-Tn*tfoX*/p[CRP-N]*comEA*) compared to the strain carrying the wild type CRP-S site, and it was in the same order of magnitude as the strain containing the deletion of the CRP-S site (ΔcomEA-Tn*tfoX*/p[CRP-delete]*comEA*).

These data prompted us to investigate the protein levels of ComEA. Total proteins were extracted from the same cultures used for RNA extraction and separated in a polyacrylamide gel. ComEA was then detected with protein-specific antibodies (Figure 5B). The relative ComEA protein levels mirrored the transcript abundance within the cells. These data indicate that the site-directly mutated CRP-Ø site results in higher levels of the *comEA* transcript and consequently also in an increased production of the ComEA protein. Moreover, in agreement with the lower *comEA* transcript levels, the protein ComEA was not detectable in the CRP-N-motif mutant.

The artificial expression of *qstR* or *comEA* increases the transformability of *hapR* minus strains in the absence of Dns

Further investigations were also conducted to better understand how Dns and ComEA influence natural competence for transformation within the QS cascade. HapR directly binds to both the 5'-regions of *dns* and *qstR* thereby repressing and activating expression, respectively. QstR in turn induces the expression of *comEA*

TABLE 1. Strains and plasmids used in this study

Strains or plasmids	Genotype*	Reference
***V. cholerae* strains**		
A1552	Wild-type, O1 El Tor Inaba, Rif^R	Yildiz & Schoolnik, 1998
A1552-LacZ-Kan	A1552 strain with *aph* cassette in *lacZ* gene; Rif^R, Kan^R	Marvig & Blokesch, 2010; De Souza Silva & Blokesch, 2010
A1552-Tn*tfoX*	A1552 containing mini-Tn7-*araC*-P_{BAD}-*tfoX*; Rif^R, $Gent^R$	Lo Scrudato & Blokesch, 2012
ΔhapR	A1552ΔVC0583, Rif^R	Meibom *et al.*, 2005
ΔhapR-Tn*tfoX*	A1552ΔhapR containing mini-Tn7-*araC*-P_{BAD}-*tfoX*; Rif^R, $Gent^R$	Lo Scrudato & Blokesch, 2012
ΔcomEA	A1552ΔVC1917 (=A1552*VC1917* in (Ref)), Rif^R	Meibom *et al.*, 2005
ΔcomEA-Tn*tfoX*	A1552ΔcomEA containing mini-Tn7-*araC*-P_{BAD}-*tfoX*; Rif^R, $Gent^R$	Lo Scrudato & Blokesch, 2013
ΔqstR	A1552ΔVC0396, Rif^R	Lo Scrudato & Blokesch, 2013
ΔqstR-Tn*tfoX*	A1552ΔqstR containing mini-Tn7-*araC*-P_{BAD}-*tfoX*; Rif^R, $Gent^R$	Lo Scrudato & Blokesch, 2013
Δdns	A1552ΔVC0470, Rif^R	Blokesch & Schoolnik, 2008
Δdns-Tn*tfoX*	A1552Δdns containing mini-Tn7-*araC*-P_{BAD}-*tfoX*; Rif^R, $Gent^R$	Seitz *et al.*, 2014
ΔhapRΔdns	A1552ΔVC0583ΔVC0470, Rif^R	Blokesch & Schoolnik, 2008
ΔhapRΔdns-Tn*tfoX*	ΔhapRΔdns containing mini-Tn7-*araC*-P_{BAD}-*tfoX*; Rif^R, $Gent^R$	This study
ΔqstRΔdns	A1552ΔVC0396ΔVC0470, Rif^R	This study
ΔqstRΔdns-Tn*tfoX*	ΔqstRΔdns containing mini-Tn7-*araC*-P_{BAD}-*tfoX*; Rif^R, $Gent^R$	This study
Plasmids		
pBR322	Amp^R, Tc^R	Bolivar *et al.*, 1977
pGP704-Sac28	Suicide vector, ori R6K *sacB*, Amp^R	Meibom *et al.*, 2004
pGP704-28-SacB-ΔVC0470	pGP704-Sac28 with a deletion within VC0470 (*dns*)	Blokesch & Schoolnik, 2008
pBAD/Myc-HisA	pBR322-derived expression vector; *araBAD* promoter (P_{BAD}); Amp^R	Invitrogen
pBAD-*comEA*	*comEA* gene cloned into pBAD/Myc-HisA, arabinose inducible; Amp^R	Lo Scrudato & Blokesch, 2013
pBAD-*qstR*	*qstR* gene cloned into pBAD/Myc-HisA, arabinose inducible; Amp^R	Lo Scrudato & Blokesch, 2013
pUX-BF13	*ori*R6K, helper plasmid with Tn*7* transposition function; Amp^R	Bao *et al.*, 1991
pGP704::Tn7	pGP704 with mini-Tn7	Schoolnik lab collection; Nielsen *et al.*, 2010
pGP704-mTn7-*araC-tfoX*	pGP704 with mini-Tn7 carrying *araC* and P_{BAD}-driven *tfoX;* Amp^R	Lo Scrudato & Blokesch, 2012
pBR-Tet_MCSI	pBR322 derivative deleted for Tet promoter and part of *tet*^{R} gene; Amp^R	Lo Scrudato & Blokesch, 2012
pBR-Tet_MCSII	pBR322 derivative deleted for Tet promoter and part of *tet*^{R} gene; new MCS included; Amp^R	Lo Scrudato & Blokesch, 2013
pBR-[own]*comEA*	*comEA* gene preceded by 900 bp of upstream region cloned into pBR-Tet_MCSII; Amp^R	Lo Scrudato & Blokesch, 2013
pBR-[-700]*comEA*	*comEA* gene preceded by 700 bp of upstream region; plasmid generated by inverse PCR of pBR-[own]*comEA*; Amp^R	This study
pBR-[-500]*comEA*	*comEA* gene preceded by 500 bp of upstream region; plasmid generated by inverse PCR of pBR-[own]*comEA*; Amp^R	This study
pBR-[-300]*comEA*	*comEA* gene preceded by 300 bp of upstream region; plasmid generated by inverse PCR of pBR-[own]*comEA*; Amp^R	This study
pBR-[P_{WT}]*comEA*	*comEA* gene preceded by ~ 200 bp of upstream region; plasmid derived from pBR-[own]*comEA* digested with *Aat*II and *Bgl*II and self-ligated; Amp^R	Lo Scrudato & Blokesch, 2013
pBR-[-134]*comEA*	*comEA* gene preceded by 134 bp of upstream region cloned into *Not*I site of pBR-Tet_MCSII; Amp^R	This study
pBR-[-100]*comEA*	*comEA* gene preceded by 100 bp of upstream region; plasmid generated by inverse PCR of pBR-[own]*comEA*; Amp^R	This study
pBR-[-40]*comEA*	*comEA* gene preceded by 40 bp of upstream region; plasmid generated by inverse PCR of pBR-[own]*comEA*; Amp^R	This study
pBR-[-900_CRP-Ø]*comEA*	*comEA* gene preceded by 900 bp of upstream region carrying a mutagenized CRP-S site indicated as CRP-Ø; plasmid generated by inverse PCR of pBR-[own]*comEA*; Amp^R	This study
pBR-[-900_CRP-N]*comEA*	*comEA* gene preceded by 900 bp of upstream region carrying a CRP-N site instead the CRP-S; plasmid generated by inverse PCR of pBR-[own]*comEA*; Amp^R	This study
pBR-[-200_CRP-Ø]*comEA*	*comEA* gene preceded by ~ 200 bp of upstream region carrying a mutagenized CRP-S site indicated as CRP-Ø; plasmid derived from pBR-[-900_CRP-Ø]*comEA* digested with *Aat*II and *Bgl*II and self-ligated; Amp^R	This study
pBR-[-200_CRP-N]*comEA*	*comEA* gene preceded by ~ 200 bp of upstream region carrying a CRP-N site instead the CRP-S; plasmid derived from pBR-[-900_CRP-N]*comEA* digested with *Aat*II and *Bgl*II and self-ligated; AmpR	This study
pBR-[-200_CRP-S-delete]*comEA*	deletion of the putative CRP-S site (from -80 bp to -58bp upstream *comEA*) in pBR-[P_{WT}]*comEA* by inverse PCR; AmpR	This study

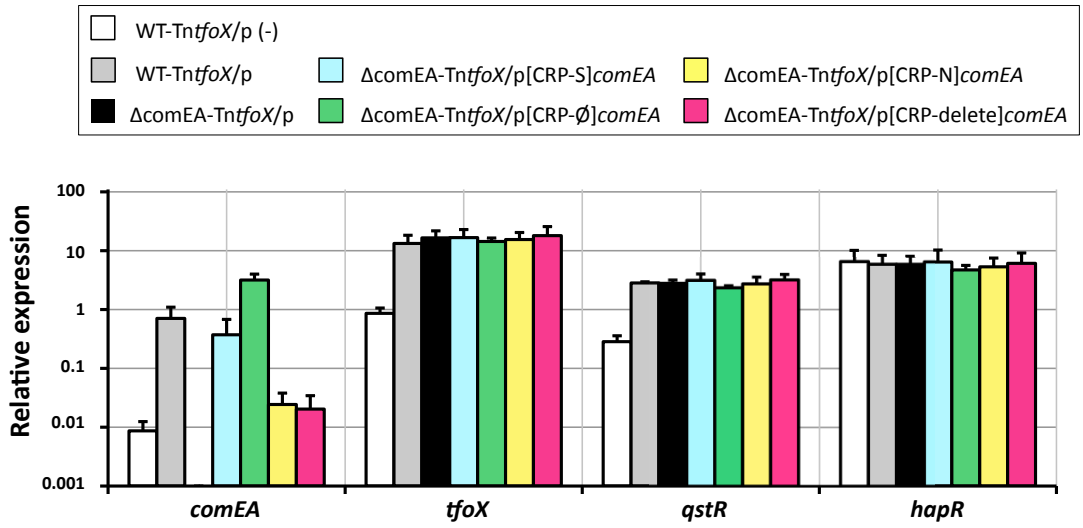
* VC numbers according to Heidelberg *et al.*, 2000.

(directly or indirectly; Figure 1). In our previous study we performed epistasis experiments that showed that *in trans* over-expression of *qstR* and *comEA* rescued the transformability of the *hapR* minus strain, and that over-expression of *comEA* slightly raised the transformation frequency in the absence of *qstR* (Lo Scrudato & Blokesch, 2013). Here, we over-expressed either *qstR* or *comEA in trans* in the double mutant strains ΔhapRΔdns-Tn*tfoX* and ΔqstRΔdns-Tn*tfoX*. The strains, carrying an inducible copy of *tfoX* in the chromosome, were tested for their transformability using the chitin-independent assay. Transformation frequencies are shown in Figure 6. The transformabilities of the strains ΔhapRΔdns-Tn*tfoX* were higher upon *qstR* or *comEA* over-expression. A similar effect was observed for the ΔqstRΔdns-Tn*tfoX* strains (Figure 6). No changes in transformation frequencies were observed in *V. cholerae* wild type (WT-Tn*tfoX*) and of *dns* minus (Δdns-Tn*tfoX*) strains under *qstR* or *comEA* over-expression conditions (see Figure S1). Notably, the transformation frequencies of the strains over-expressing *qstR* were slightly higher than those of the strains harboring the plasmid-encoded *comEA* (Figure 6) confirming that QstR also has a positive effect on other competence genes (e.g. as previously demonstrated for *comEC;* Lo Scrudato & Blokesch, 2013).

Effect of *qstR* expression in the absence of natural competence induction

Finally, we addressed the question whether TfoX regulates *comEA* only indirectly, through its influence on QstR, or whether it also has a role in directly regulating the expression of *comEA*. More precisely, we measured the transcripts of *comEA* in the absence of natural competence induction but with *in trans* over-expression of *qstR* (e.g. by decoupling *qstR* expression from natural competence induction). Wild type strains A1552 carrying either an empty vector (WT/p) or the plasmid-encoded *qstR* (WT/p-*qstR*) were grown in liquid culture containing arabinose to induce *qstR* expression from the plasmid. Importantly, those strains did not contain the arabinose-inducible copy of *tfoX* on their chromosome. RNA was extracted and used to perform the qRT-PCR; the relative expression of the tested genes is shown in Figure 7A. *comEA* was the only tested gene whose expression was induced by *in trans* over-expression of *qstR*. However, the expression of *comEA* was lower than

A



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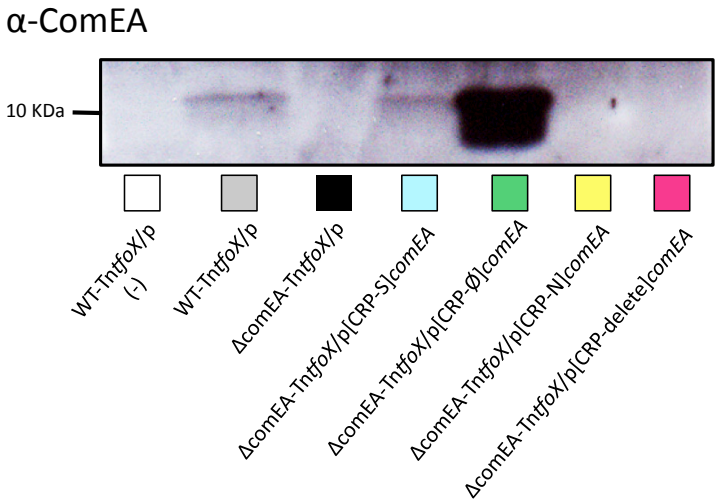
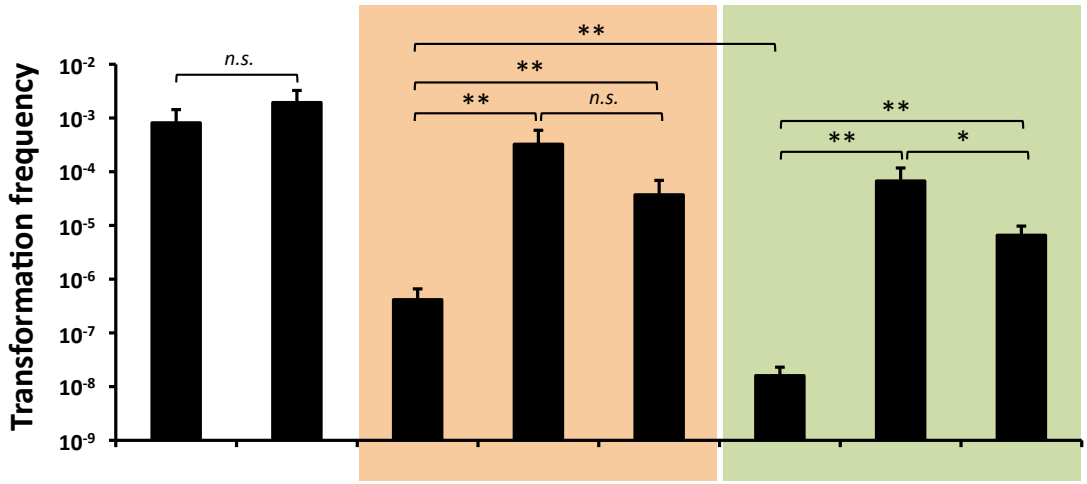


Figure 5. Mutagenesis of the putative CRP-S site alters the transcript and protein levels of *comEA*. A. qRT-PCR data showing the relative expression of *comEA* and as control also the genes *tfoX*, *qstR,* and *hapR* (normalized to *gyrA*) in the indicated bacterial strains. Experiments were repeated three independent times and the data show the averages. Error bars indicate standard deviations. **B.** Western blotting analysis of the ComEA protein (10.97 KDa before transport into the periplasm). Total proteins were extracted from the indicated strains grown until an optical density at 600nm (OD_{600}) ~ 1.5-1.8 and ComEA was subsequently detected with protein specific antibodies. All *V. cholerae* strains harbored the inducible chromosomal copy of *tfoX* (-Tn*tfoX*) and were grown under competence-inducing conditions (except for the negative control strain WT-Tn*tfoX*/p (-), which was grown in the absence of inducer). WT-Tn*tfoX*: wild type *V. cholerae* strain A1552-Tn*tfoX*; ΔcomEA-Tn*tfoX*: *comEA* minus strain; (p): empty vector; p[CRP-S]*comEA*, p[CRP-Ø]*comEA*, p[CRP-N]*comEA* and p[CRP-S-delete]*comEA*: *comEA* gene on a plasmid preceded by ~ 200bp of upstream region with the putative CRP-S site, the mutated CRP-Ø site, the canonical CRP-N site or without any CRP-S site (e.g. deleted), respectively.



Strain	WT-Tn*tfoX*	Δdns-Tn*tfoX*	ΔhapRΔdns-Tn*tfoX*			ΔqstRΔdns-Tn*tfoX*		
p	p	p	p	p-*qstR*	p-*comEA*	p	p-*qstR*	p-*comEA*

Figure 6. The transformability of QS mutants is increased by over-expression of *qstR* or *comEA* in *dns* negative strains. The transformability of *V. cholerae* double mutants either for *hapR* and *dns* or for *qstR* and *dns* were tested under *qstR*- or *comEA*- overexpressing conditions in a chitin-independent assay. The respective transformation frequencies are shown on the y-axis. All strains carried an inducible copy of *tfoX* on the chromosome (-Tn*tfoX*). WT-Tn*tfoX*: wild type *V. cholerae* strain A1552-Tn*tfoX*; Δdns-Tn*tfoX*: *dns* minus strain; ΔhapRΔdns-Tn*tfoX*: *hapR* and *dns* minus strains (orange box); ΔqstRΔdns-Tn*tfoX*: *qstR* and *dns* minus strains (green box); p: empty vector; p-*qstR* and p-*comEA*: plasmid-encoded inducible *qstR* and *comEA*. The data represent the average of at least three biological replicates. Error bars reflect the standard deviation. Statistically significant differences were determined by the Student's *t* tests on log-transformed data. * $P < 0.05$, ** $P < 0.01$, *n.s.* = not significant.

that measured when natural competence was concomitantly induced (average of the relative expression of *comEA* was 0.24 ± 0.05 compared to 0.7 ± 0.38 in WT/p-*qstR* [Figure 7A] and in WT-Tn*tfoX* [Figure 5A], respectively). To exclude that the lower expression was due to a plasmid effect we also measured the expression of *comEA* in a *qstR* minus strain *in trans* complemented by plasmid-encoded *qstR* under competence-inducing conditions (ΔqstR-Tn*tfoX*/p-*qstR*). In this case full expression of *comEA* was observed (Figure S2A). Similar results were obtained when the expression of *comEA* was measured in the *hapR* minus strain *in trans* complemented by *qstR* under competence induction conditions (ΔhapR-Tn*tfoX*/p-*qstR*) (Figure S2B). These data indicate that QstR requires TfoX (or any downstream regulator / effector) not only to regulate its expression but also to fully drive the expression of *comEA*.

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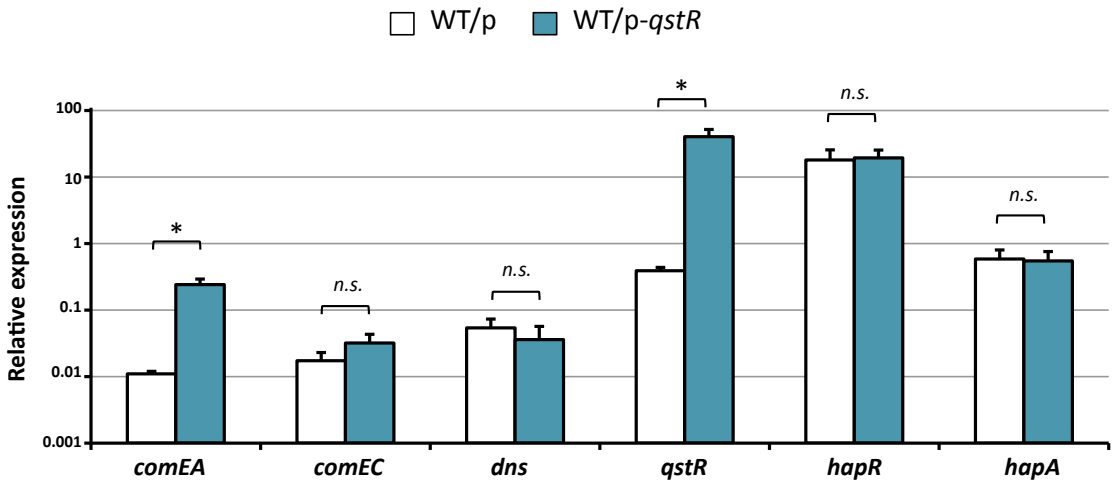

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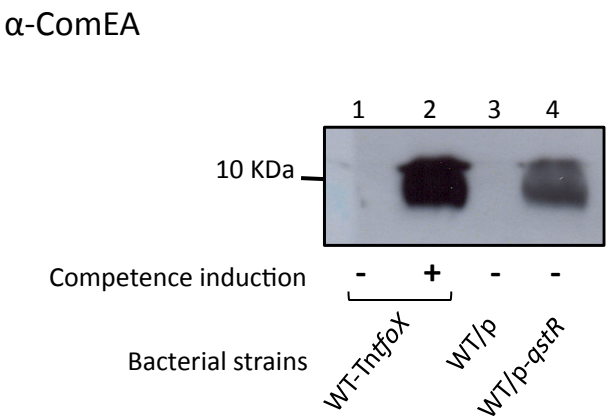


Figure 7. QstR drives the expression of *comEA* in the absence of competence induction. A. qRT-PCR data showing the relative expression of the indicated genes (normalized to *gyrA*) in wild type *V. cholerae* strain A1552 (WT) carrying an empty vector (p) as control or a plasmid encoding *qstR* (induction achieved through arabinose supplementation). Strains were grown to high cell density. Growth medium was supplemented with arabinose to solely induce the expression of *qstR*. Data are the average of three biological replicates. Error bars indicate standard deviations. Statistically significant differences were determined by the Student's *t* tests. * $P < 0.05$, *n.s.* = not significant. **B.** Detection of the ComEA (10.97 KDa before transport into the periplasm) by western blot analysis using protein specific antibodies. Total proteins were extracted from the indicated strains grown until stationary phase (OD_{600} ~ 3.0). WT-Tn*tfoX*: wild type *V. cholerae* strains A1552-Tn*tfoX*; WT: wild type *V. cholerae* A1552; (-) or (+): absence or presence of competence induction; (p): empty vector; p-*qstR*: *qstR* carrying plasmid. Strains indicated in lanes 3 and 4 were grown in the presence of 0.02% arabinose to induce the P_{BAD} promoter on the plasmids.

We then investigated the production of the protein ComEA in order to evaluate if the transcript levels would also reflect the protein levels. Apart from the strains used to measure the transcripts of *comEA*, we also included wild type strains harboring an inducible copy of *tfoX* on the chromosome and grew the strains under both competence non-inducing and competence-inducing conditions.

Total protein extracts were prepared from bacterial strains having reached the high cell density state (OD_{600} ~ 3.0; note the higher OD_{600} values used here compared to Figure 5B in order to increase the possibility to detect the ComEA protein). As shown in Figure 7B the ComEA protein was detected in the absence of competence induction only when *qstR* was over-expressed, which is in agreement with the measured mRNA levels of *comEA* and demonstrated once more that changes at the transcript levels are mirrored by the abundance of the protein. This result strengthened the evidence that QstR *per se* is able to drive the expression of *comEA* in absence of competence induction. However, the protein was more abundant in the wild type strain under competence inducing conditions (WT-Tn*tfoX* (+), lane 2) suggesting that full ComEA expression requires more than the QstR protein alone.

DISCUSSION

Using *comEA* as a representative gene of the competence regulon, this study contributes to the understanding of the regulatory network responsible for the activation of the competence genes. We localized the promoter region of *comEA* within the 134bp upstream the start codon of the gene and demonstrated that the deletion of the newly identified CRP-S site has a negative effect on natural transformability.

In bacteria gene transcription starts only after 1) binding of the RNA polymerase (RNAP) holoenzyme to the promoter region and 2) formation of an open complex of the DNA. Promoters that are non-constitutively active (e.g. those of the competence genes) are considered “defective” due to the need of one or several activating protein(s) (e.g. such as the CRP protein) that directly interact with the RNAP and assist the holoenzyme in the steps preceding the transcriptional initiation (Lee *et al.*, 2012). Here, a shortened 5’-region upstream *comEA* of only 100bp, although it contained the CRP-S site, was not sufficient to drive *comEA* expression (Figure 2 and 4). These findings suggest that either a secondary activator (if not CRP itself) binds the upstream region of the CRP-S site or that the certain mRNA structure is defined by this stretch of DNA and that it is therefore required for expression. However, the existence of a secondary activator is also supported by the occurrence

of rare transformants in the strain deleted for the CRP-S site; we hypothesized that such a secondary activator might weakly assist the binding of the RNAP to the promoter even in the absence of CRP-S-mediated binding of CRP.

We suggest that the transcription factor QstR, which is only necessary for the expression of *comEA* and *comEC* (Lo Scrudato & Blokesch, 2013), represents this secondary activator involved in the activation of *comEA*. Indeed, QstR might regulate the expression of *comEA* by interacting with the RNAP and in cooperation with the CRP protein, which by itself recognizes the CRP-S motif upstream *comEA*. Interestingly, we also identified a putative CRP-S site upstream *qstR* (Figure 3C). In this case, the protein HapR, known to bind the *qstR* promoter between -130 and -111bp upstream the start codon (Lo Scrudato & Blokesch, 2013), likely acts as a secondary activator. Other competence genes might not require a secondary activator as those genes are not co-regulated by the QS pathway (Lo Scrudato & Blokesch, 2012).

CRP-mediated activation at the CRP-S site is expected to be different from that at the canonical CRP binding site (CRP-N site; Sxy/TfoX-independent); however the interaction of the RNAP with the DNA could be similar at both promoters. This is strongly indicated by the sequence of the -10 and -35 regions identified upstream the transcription start point of the *comEA* gene (Figure 3A). Those sequences resemble binding sites for the most common sigma factor σ^{70} (Shultzaberger *et al.*, 2007) as also previously observed for the competence promoter regions in *H. influenzae* (Cameron & Redfield, 2008). CRP-dependent (CAP-dependent) promoters have been classified into class I and II based on i) the position of the CRP binding site relative to the transcription start site; ii) the type of interactions between CRP protein and RNAP (reviewed in Busby & Ebright, 1997; Busby & Ebright, 1999; Lee *et al.*, 2012). The CRP-S sites of *H. influenzae* resemble class I promoters because they are localized upstream of the -35 region and centered at -61.5 from the transcription start site (Redfield *et al.*, 2005; Cameron & Redfield, 2006; Cameron & Redfield, 2008). These features greatly differ from those of the putative CRP-S site identified in this study. Notably, the location of the putative CRP-S site of *comEA*, centered at -44.5 and partially overlapping with the -35 region, resembles the class II CRP-

dependent promoters, which also overlap with the -35 region. Indeed, this location indicates that the protein CRP might be in close association with the RNAP. Furthermore, class I and II promoters are activated by a CRP dimer. Moreover, a third class of CRP-dependent promoters exists in *E. coli*, which is characterized by the presence of two activators, one acting in a class I manner and the other acting in a class II manner (e.g. two dimers of CRP or a dimer of CRP and a secondary different activator) (Busby & Ebright, 1999). It is tempting to speculate that the *comEA* promoter could also be considered as a class III CRP-dependent promoter if a secondary activator would indeed bind upstream the putative CRP-S site.

Another difference between *H. influenzae* and *V. cholerae*'s CRP-S sites is the structure of the sequence. *H. influenzae* has CRP-S sites that are symmetrical motifs (Macfadyen, 2000; Redfield *et al.*, 2005); on the contrary the *Vibrionaceae* CRP-S consensus (Cameron & Redfield, 2006) as well as the putative CRP-S sites upstream *comEA* and *qstR* are asymmetric. Notably, asymmetric CRP-S sites have also been predicted for genes induced by *in trans* expression of *sxy* in *E. coli* (Sinha *et al.*, 2009). Therefore asymmetric CRP-S sites might be common in other species rather than being an exception and imply that each monomer of the CRP dimer should interact with the DNA asymmetrically. The characteristics of the CRP-S sites and the features discussed above suggest that CRP interacts with the DNA in a different manner in the two species of *H. influenzae* and *V. cholerae*; we speculate that in *V. cholerae* the CRP protein, as a dimer and in a complex with the cAMP, requires some structural modifications to shape and adapt its conformation to the CRP-S site prior to bind this DNA motif.

We did not identify other motifs resembling the CRP-S site within the putative promoter region of *comEA.* The palindromic sequence centered at -115.5 from the transcription start point (Figure 3A), was indicated as a potential CRE element (competence regulatory element; Redfield *et al.*, 2005; former nomenclature for CRP-S sites) by Antonova *et al*. (Antonova *et al.*, 2012) in spite of the lack of similarity with the *in silico* predicted CRP-S consensus (Cameron & Redfield, 2006). Although we cannot exclude that this palindrome is indeed a CRP binding motif, this site is not required for the transcription of *comEA*, as it was not

part of the construct that *in trans* complemented the respective knock out strain (e.g. construct [-134]*comEA*, Figure 2B). Furthermore, the localization of this putative CRP-S site within the open reading frame of the adjacent gene (*VC1918*) also questions its functionality with respect to the regulation of *comEA*.

In *E. coli* changing of the non-perfect CRP-N site of the galactose operon (*gal*) into the perfect consensus sequence resulted in a tighter binding of the CRP-cAMP complex to this DNA sequence *in vitro*. Furthermore, the expression of the *lacZ* gene driven by this mutagenized *gal* promoter was hardly repressed *in vivo* when glucose was added to the growth medium. This indicates that the CRP-cAMP complex was almost "glued" at the CRP-N consensus site (Gaston *et al.*, 1988; Gaston *et al.*, 1989). Moreover, the binding of the CRP protein to such a modified *gal* promoter (containing the CRP-N consensus) was much less dependent on the cAMP levels (Gaston *et al.*, 1989). Indeed, Gaston *et al.* showed that none of the *E. coli* promoters perfectly matched the CRP-N consensus and they concluded that "such a site, whilst ensuring high levels of transcription initiation, would be useless in mediating fluctuations in transcriptional activity in response to changes in environmental stress" (Gaston *et al.*, 1988). In our study, we observed a decreased expression of *comEA* when the gene was preceded by a motif that perfectly matched the *in silico* predicted CRP-N consensus sequence. However, the conversion of a CRP-S site into a "perfect" CRP-N site might dramatically change the dynamics of the *comEA* promoter activation especially if compared to the somewhat milder change of a "non-perfect" CRP-N site into a "perfect" (based on the predicted consensus sequence) CRP-N motif. Indeed, as the CRP-S site upstream *comEA* partially overlaps with the -35 region we suggest that the CRP-S to CRP-N motif exchange might negatively affect the interaction of the RNAP complex with the DNA and thus cause a drop in *comEA* expression. In addition, the CRP-N consensus might also negatively affect the interaction between the CRP protein and the secondary activator due to a too tight binding of the CRP-cAMP complex to the DNA.

When we looked at the protein levels the ComEA protein was undetectable by western blot analysis in a strain carrying the CRP-N construct. However, in the same strain such a low expression of *comEA* was sufficient to drive transformation

under competence-inducing conditions (Figures 4B and 5). Similar results were obtained when the expression of *comEA*, under the control of a pBAD promoter, was induced with increasing amounts of arabinose (Blokesch, unpublished). This suggests that the amount of protein ComEA has to reach a certain threshold to efficiently bind the DNA in the periplasmic space but that ComEA might not be limiting in the cells (at least for the uptake of short DNA fragments as assayed in our study).

E. coli and *V. cholerae* share the same CRP-N consensus (Cameron & Redfield, 2006), which is in accordance with the high percentage of identity of the two CRP proteins as mentioned above. Interestingly, when we tested the expression of a CRP-N driven *gfp* construct ([P_{comEA}_CRP-N]-*gfp*), we did not detect any fluorescent signals in both species reinforcing the idea that a CRP-N motif replacing the existing CRP-S site has a negative effect on the expression of *comEA* (data not shown). Opposite results were observed for the strains carrying the CRP-Ø mutation. Despite the fact that this mutation affects the most conserved bases of the CRP-S consensus (right part of the motif) the *comEA* gene was expressed at higher levels, which correlated well with increased levels of the ComEA protein and higher transformation frequencies (Figure 4 and 5). Accordingly, a transcriptional reporter fusion [P_{comEA}_CRP-Ø]-*gfp* resulted in an increased green fluorescent signal (data not shown). We therefore speculate that the CRP-Ø mutation might: 1) be a better binding site for the RNAP; 2) allow a better binding of the CRP protein at this modified CRP-S site; 3) improve the interactions between the CRP protein and the RNAP complex and/or between CRP and the secondary activator. Remarkably, when strains were grown in the absence of competence induction, the relative expression of *comEA* under the control of the CRP-Ø motif was 4-times higher than that driven by the CRP-S motif (0.067 compared to 0.014; preliminary data; experiment only performed once). Nevertheless this expression was still far lower compared to that of the strain carrying the CRP-Ø motif but grown under competence-inducing condition (3.173 ± 0.827, Figure 5A). These data support the hypothesis that CRP-Ø motif results in a better binding site for the RNAP and that *comEA* is therefore transcribed at low levels even in the absence of competence induction; however, full expression of *comEA* still requires TfoX.

The mechanism by which TfoX induces the expression of the competence genes is still unknown. In the competence regulon of *H. influenzae*, Redfield and colleagues proposed that Sxy (TfoX ortholog) binds the DNA upstream the CRP-S site and functions as co-activator of the CRP-cAMP complex (Cameron & Redfield, 2008). Since TfoX has no sequence resembling a DNA binding motif, we propose an alternative function for this master regulator of transformation: TfoX might be an enzyme that directly (e.g. chemical modification(s) of some amino acids of CRP) or indirectly (e.g. synthesis of a cofactor that binds CRP) modifies the CRP-cAMP complex to adapt and shape its conformation to the CRP-S sites. Moreover, because QstR only partially drove the expression of *comEA* in the absence of *tfoX*-induction (Figure 7), TfoX-mediated modifications of the CRP-cAMP complex might be also necessary to allow an interaction between CRP and the secondary activator.

This study suggests that the putative CRP-S site of the promoter of *comEA* is a competence-specific CRP binding site and it is the first study to draft the mechanism of activation at *V. cholerae*'s CRP-S-containing promoters. Further studies are necessary to characterize the CRP-S sites in the promoters of other competence genes and to generalize the mechanism of their activation.

We conclude with a model describing the link of TfoX, CRP and the QS transcription factors HapR and QstR in the regulation of *qstR* and *comEA* (Figure 1). Unsaturated PTSs trigger the production of cAMP and thus the formation of the CRP-cAMP complex. Furthermore, upon growth on chitin the protein TfoX is produced and enzymatically modifies the CRP-cAMP complex (or alternatively synthesizes a cofactor that might play a role in this regulatory network). This modified complex specifically binds the DNA at the competence-related CRP-S sites. Still, full activation of the promoter of *qstR* and *comEA* only occurs at high cell density through the concomitant binding of the QS secondary activators, HapR and QstR, respectively.

MATERIALS AND METHODS

Bacterial strains and plasmids

Vibrio cholerae strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains DH5α (Yanisch-Perron *et al.*, 1985) and One Shot PIR1 or PIR2

(Invitrogen) were employed as hosts for cloning. *E. coli* strain S17-1λpir (Simon *et al.* 1983) was used as mating donor for plasmid transfer between *E. coli* and *V. cholerae*.

Media and growth conditions

V. cholerae and *E. coli* strains were grown at 30 and 37°C respectively. Overnight cultures were grown in LB medium under aerobic conditions. For *V. cholerae* static growth in defined artificial seawater medium (DASW) containing chitin flakes (Meibom *et al.*, 2005; Marvig & Blokesch, 2010) and supplemented with vitamins (MEM, Gibco) was used. To induce natural competence by artificially expression of *tfoX*, *V. cholerae* strains were grown in LB medium with the addition of 0.02% arabinose. The same growth conditions were used for *in trans* over-expression of the plasmid-encoded genes *comEA* and *qstR*. Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar plates were used to counter-select *E. coli* strains after triparental mating with *V. cholerae* strains. The TCBS agar plates were prepared following the manufacturer's instructions (Fluka). NaCl-free LB medium containing 6 % sucrose was prepared for sucrose-based *sacB*-counter-selection. Antibiotics were added to the growth media for plasmid maintenance or selection of transformants/transconjugants at concentrations of 50 or 100 µg/ml for ampicillin, 75 µg/ml for kanamycin, and at 50 µg/ml for gentamicin.

Construction of *V. cholerae* mutant strains

The gene *dns* (VC0470) was deleted from the parental strains ΔqstR and ΔqstR-Tn*tfoX* using the plasmid pGP704-28-SacB-ΔVC0470 (Blokesch & Schoolnik, 2008) based on the disruption method previously described (Meibom *et al.*, 2005).

Construction of plasmids

Most of the plasmids carrying the gene *comEA* preceded by a certain length of its upstream region were made by inverse PCR using the plasmid pBR-[own]*comEA* (Lo Scrudato & Blokesch, 2013) as template and the primers listed in Table S1. Site directed mutagenesis of the putative CRP-S site was done by inverse PCR of plasmid pBR-[own]*comEA* with the primers carrying the envisioned mutations to generate

the constructs pBR-[-900_CRP-Ø]*comEA* and pBR-[-900_CRP-N]*comEA*. The latter were then digested with *Aat*II and *Bgl*II and self-ligated to obtain the shorter versions pBR-[CRP-Ø]*comEA* and pBR-[CRP-N]*comEA* containing ~ 200bp DNA region upstream the start codon of *comEA*. The plasmid pBR-[-134]*comEA* was constructed by cloning the PCR-amplified insert [-134]*comEA* into *Nco*I site of pBR-Tet_MCSII (Lo Scrudato & Blokesch, 2013). The CRP-S site was completely deleted from the upstream region of *comEA* by inverse PCR of plasmid pBR-[P_{WT}]*comEA* (Lo Scrudato & Blokesch, 2013) with the primers F-CRP-site-delet and R-CRP-site-delet.

Natural transformation assay (chitin-dependent and chitin-independent)

Natural transformation assays were performed as previously described growing *V. cholerae* strains on chitin flakes (Marvig & Blokesch, 2010) or in LB medium supplemented with arabinose to express an inducible chromosomal copy of *tfoX* (Lo Scrudato & Blokesch, 2012). The chitin transformation assay shown in Figure 2 was performed by replacing the growth medium with a fresh one prior to add the genomic DNA to the bacterial culture. The chitin transformation assay shown in Figure 4A resulted in a better detection limit because the growth medium was not changed. Statistical analyses of transformation data were done on log-transformed data (Keene, 1995) using a two-tailed Student's *t* test.

SDS-PAGE and Western blotting

Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) and then subjected to western blotting as previously described (Lo Scrudato & Blokesch, 2012). Primary antibody against ComEA (GP 1248; see below) and secondary antibody goat anti-rabbit horseradish peroxidase (HRP) (Sigma-Aldrich, Switzerland) were diluted at 1:5'000 and 1:20'000, respectively. Luminescence signals were produced and detected by Western Lightning®-ECL (PerkinElmer) and chemiluminescence-detecting films (Amersham Hyperfilm ECL, GE Healthcare).

Generation of the antibodies against ComEA

Rabbit-derived anti-ComEA antibodies were raised against synthetic peptides and produced by Eurogentec (Belgium). The antibody was tested in western blot analysis against the respective *comEA* knock-out strain to exclude potential cross-reactions with proteins migrating towards the same position as the target protein.

Quantitative reverse transcription PCR (qRT-PCR)

V. cholerae strains were grown in LB medium supplemented with 0.02% arabinose to induce the *qstR* gene. Cells were harvested after reaching an OD_{600} ~ 2.0. RNA preparation, reverse transcription and qPCR were performed as previously described (Lo Scrudato & Blokesch, 2013).

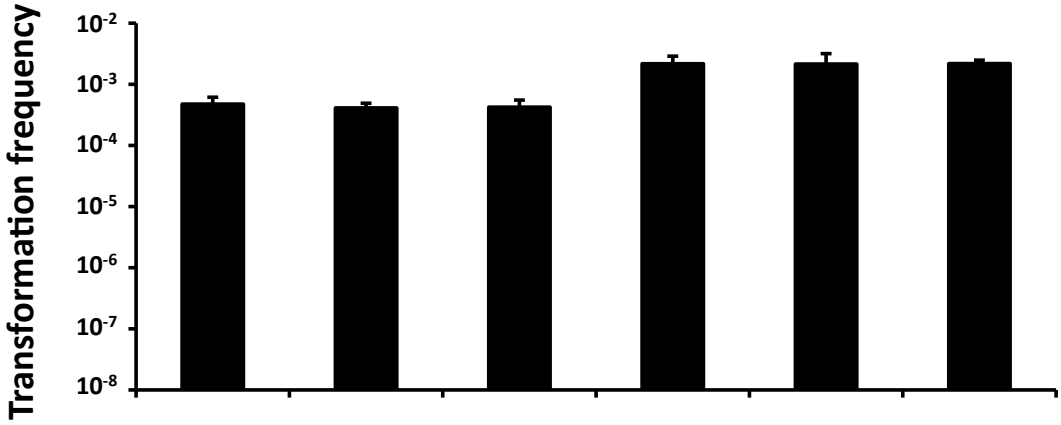
5' Rapid Amplification of cDNA Ends (5'RACE)

V. cholerae wild type strain A1552-Tn*tfoX* was grown in LB medium supplemented with 0.02% arabinose to induce competence by artificially expression of *tfoX*. Cell harvest and RNA preparation were performed as previously described (Lo Scrudato & Blokesch, 2013). 5'/3' RACE Kit 2nd Generation (Roche) was used to identify the transcription start of the gene *comEA*. All the steps were done following manufacturer's protocol if not otherwise stated. Two µg of total RNA and the gene-specific primer comEA_284_rev were used to synthesize the first strand cDNA of *comEA*. The latter was purified using the High Pure PCR Product Purification Kit (Roche). After Poly(A) tailing of the 3' end, the first strand cDNA of *comEA* was PCR amplified with the gene-specific primer comEA_217_rev and oligo dT-Anchor primer. The PCR products were visualized in agarose gel and purified. The double stranded cDNA of *comEA* was further PCR amplified with primers F-EcoRI_Anchor_P and R-BamHI_comEA_217. The PCR products were then cloned into the *Eco*RI/*Bam*HI sites of the plasmid pBR-Tet_MCSI (Lo Scrudato & Blokesch, 2012). To determine the transcription start point of *comEA*, fifteen of those plasmids were sequenced. 12 out of those 15 sequences indicated the C at position -24bp upstream the start codon as transcriptional start site.

SUPPLEMENTARY DATA

TABLE S1. Primers used in this study

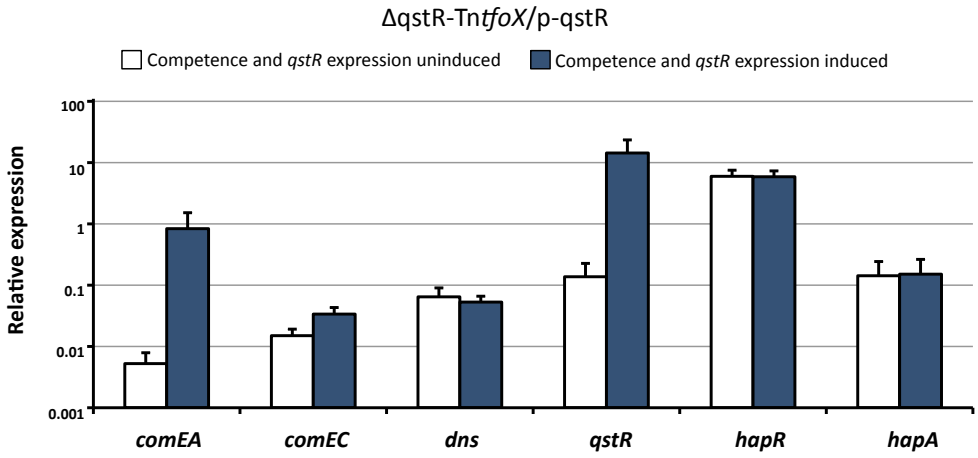
Primer name	Sequence (given in 5' to 3' direction)	Comments
Rev[VC1917]-NotI	GCGGCCGCGAGCTCTAGAGGTTTCTTAG	Inverse PCR plasmids: pBR-[-700]*comEA*, pBR-[-500]*comEA*, pBR-[-300]*comEA*, pBR-[-100]*comEA*, pBR-[-40]*comEA*
Fwd[VC1917]-700	AGAGCTCGCGGCCGCAGGTGTTAACCACTCCTGCGGTAC	Inverse PCR pBR-[-700]*comEA*
Fwd[VC1917]-500	AGAGCTCGCGGCCGCCAACAAGCACTTGAACTGGGTAAC	Inverse PCR pBR-[-500]*comEA*
Fwd[VC1917]-300	AGAGCTCGCGGCCGCTATCGTTGTGATTGAGTTGAGC	Inverse PCR pBR-[-300]*comEA*
VC1917-134-NotI	GCGGCGGCCGCATTCTTAGTGTAATTGATATG	PCR [-134]*comEA*,
pBR-TET_MCS after	ATCATGCGCACCCGTGGCCAGGACCC	
Fwd[VC1917]-100	AGAGCTCGCGGCCGCGGGCTACAGCAGTAGCCCGTTC	Inverse PCR pBR[-100]*comEA*
Fwd[VC1917]-40	AGAGCTCGCGGCCGCCGCTATCATAAGCCCTCAACAAC	Inverse PCR pBR[-40]*comEA*
CrpS-EcoRV-fwd	AATTGATATCATTTCCATCATTTTTCCCCTCGCTATC	Inverse PCR pBR-[-900_CRP-Ø]*comEA*
CrpS-EcoRV-rev	GGAAATGATATCAATTTATAAATAGAACGGGCTACTGC	
CrpS->N-fwd-MfeI	TGTGACAATTGTCACAATTTCCATCATTTTTCCCCTCGC	Inverse PCR pBR-[-900_CRP-N]*comEA*
CrpS->N-rev-MfeI	TGTGACAATTGTCACATAGAACGGGCTACTGCTGTAGC	
F-CRP-site-delet	TCCATCATTTTTCCCCTCGCTATCATAAGCCCTC	Inverse PCR pBR-[CRP-S-delete]*comEA*
R-CRP-site-delet	GGAAAAATGATGGAAACGGGCTACTGCTGTAGCCCGTTTC	
gyrA-157-fwd	AATGTGCTGGGCAACGACTG	qRT-PCR for *gyrA* transcription (Lo Scrudato & Blokesch, 2012)
gyrA_332_bwd	GAGCCAAAGTTACCTTGGCC	
comEA_50_fwd	CGACATTACCGTTACTGGCC	qRT-PCR for *comEA* transcription (Lo Scrudato & Blokesch, 2012)
comEA_224_bwd	CCGTTGGCTTCTCGATAATCG	
comEC_1029_fwd	GGTCGCGATTGTTCTCTACC	qRT-PCR for *comEC* transcription (Lo Scrudato & Blokesch, 2012)
comEC_1186_bwd	CCAAATTGTACAGAACTGCCG	
dns_276_fwd	GCAATGCTGGCAACAAGGTG	qRT-PCR for *dns* transcription (Lo Scrudato & Blokesch, 2012)
dns_443_bwd	CCATAGGTGACGCCATCAAC	
VC0396_188_fwd	GCCTGATTCGCCAGCAATTG	qRT-PCR *qstR* transcription (Lo Scrudato & Blokesch, 2012)
VC0396_356_bwd	CCAAGACCGTGGGCAATAAAG	qRT-PCR for *qstR* transcription
hapR-230-fwd	CCAACTTCTTGACCGATCAC	qRT-PCR for *hapR* transcription (Lo Scrudato & Blokesch, 2013)
hapR-399-bwd	GGTGGAAACAAACAGTGGCC	
hapA_175_fwd	ACGGTACAGTTGCCGAATGG	qRT-PCR for *hapA* transcription (Lo Scrudato & Blokesch, 2013)
hapA_358_bwd	GCTGGCTTTCAATGTCAGGG	
comEA_284_rev	CGCACTGTCGCTTCACCAATCC	5'RACE: synthesis of first strand cDNA of *comEA*
comEA_217_rev	CTTCTCGATAATCGACAATGGCCTGAGC	5'RACE: PCR amplification of Poly(A) cDNA *comEA*
oligo dT-Anchor primer (Roche)	GACCACGCGTATCGATGTCGAC	
F-EcoRI_Anchor_P	CCAAGAATTCGACCACGCGTATCGATGTCGAC	5'RACE: PCR fragment of Poly(A) cDNA *comEA* cloned into plasmid pBR-Tet_MCSI
R-BamHI_comEA_217	CCAAGGATCCCTTCTCGATAATCGACAATGGCCTGAGC	



Strain	WT-Tn*tfoX*			Δdns-Tn*tfoX*		
plasmid	p	p-*qstR*	p-*comEA*	p	p-*qstR*	p-*comEA*

Figure S1. Chitin-independent transformation assay of *V. cholerae* strains. Transformation frequencies of wild type strain A1552-Tn-*tfoX* (WT-Tn*tfoX*) or the *dns* minus strains (Δdns-Tn*tfoX*). Each strain carried an empty vector (p) or *qstR* (p-*qstR*) and *comEA* (p-*comEA*) on a plasmid. All cells were grown under competence-inducing conditions. The average of at least three biological replicates is indicated. Error bars reflect the standard deviation.

A



B

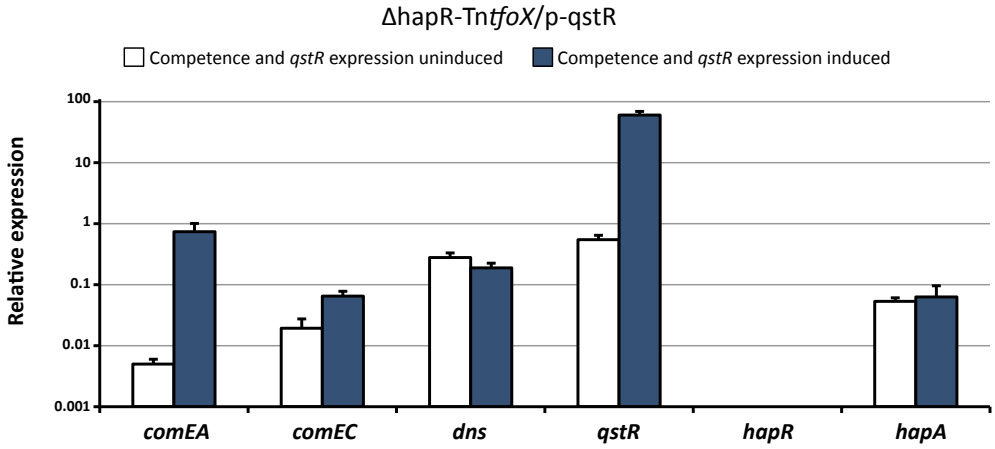


Figure S2. Expression of *comEA* in *qstR* and in *hapR* minus strains complemented *in trans* by *qstR*. qRT-PCR data showing the relative expression (normalized to *gyrA*) of the indicated genes in a *V. cholerae qstR* minus strain (ΔqstR-Tn*tfoX*; **A**) or in a *hapR* minus strain (ΔhapR-Tn*tfoX*; **B**) (both carrying the inducible copy of *tfoX* on the chromosome (-Tn*tfoX*) and an inducible copy of *qstR* on a plasmid). Strains were grown under competence non-inducing / *qstR* non-expressing conditions (without arabinose) or under competence- and *qstR*- inducing conditions (+ 0.02% arabinose). Data are the average of three biological replicates. Error bars indicate standard deviations.

Chapter 4.

Conclusion and Perspectives

The association of *V. cholerae* with the zooplankton is the key event of the development of natural competence. The chitinous exoskeleton of zooplankton represents a nutritious surface allowing biofilm formation and bacterial multiplication. Moreover, the signalling cascade that leads to chitin utilization is also responsible for the expression of the competence genes. However, *V. cholerae* cells are only ready to take up the extracellular DNA upon they 'sense' the presence of each other through the QS pathway.

In addition to positively regulate natural competence, QS is involved in the regulation of other *V. cholerae*'s cellular mechanisms, such as biofilm formation and virulence induction, both repressed at high cell density (HCD). The dependency of cellular mechanisms from cell density is *per se* the result of an evolutionary process in order to optimize the bacterial adaptation to different environments. Thus the selective pressure has favored some cellular mechanisms at HCD and some others at low cell density (LCD). For example in the human intestine, during the early stage of the infection, the condition of LCD favors the production of biofilm and virulence factors in order to colonize and infect the host cells.

In the aquatic environment, QS optimizes the success of natural transformation *1)* allowing the DNA uptake only at HCD (when the availability of extracellular DNA should be higher); and *2)* selecting the extracellular DNA that is taken up. In fact the intra-species autoinducer CAI-1 allows the DNA uptake process only in the presence of DNA belonging to the same and closely-related species, thus ensuring that this DNA carries homologous regions necessary to recombine with the bacterial chromosome. If free-swimming bacteria would initiate natural competence, the chances of taking up extracellular DNA of the same species or of close relatives would be very low compared to surface-attached bacteria. Hence, on the one hand chitin surfaces are the perfect ecological niche for the establishment of the bacterial

communities and for the initial development of the competence state in *V. cholerae*, but on the other hand QS pathway maximizes the efficiency of natural transformation and consequently the evolution of *V. cholerae*. Moreover, although QS is thought to allow bacteria to act as an unison, this is not the case for the induction of natural competence in *V. cholerae*, most likely because QS signalling molecules as well the oligomers of the inducer chitin are non-homogeneously distributed on the surface of the zooplankton.

The findings achieved during this thesis contributed extensively to the understanding on how natural competence is regulated in *V. cholerae*; in particular our main findings were:

- The decryption of the mechanisms by which QS determines the fate of the extracellular DNA;
- The discovery of the crosstalk between the regulatory pathways of QS and chitin-sensing / -degradation;
- The construction of the first model describing the promoter regulatory elements necessary for the expression of the competence genes.

As the chitinous exoskeleton of zooplankton represents the main habitat of *V. cholerae*, natural competence for transformation might be the mechanism of HGT with the major impact on the evolution of this bacterium. Indeed, further insights into the molecular mechanisms driving natural competence and transformation will be of prime importance to better understand bacterial evolution. In particular, it will be significant to investigate the DNA target(s) of the QstR protein, to disclose the exact role of TfoX and to inquire the cAMP-dependency of the CRP protein. The future perspectives of this thesis are briefly mentioned below.

Investigation of the DNA targets of QstR

Although QstR was clearly required for the transcription of the competence genes *comEA* and *comEC* we could not observe any QstR binding to the promoter of both genes *in vitro*. This failure to bind these two promoters could have several reasons:

1) another intermediate regulator could be involved; 2) the purified QstR protein was inactive or misfolded *in vitro*; or 3) QstR might require a cofactor for DNA binding. ChIP (Chromatin Immunoprecipitation) coupled to qPCR (quantitative PCR) would therefore be a better approach than using the purified protein, as in ChIP QstR would be directly captured from *V. cholerae* cells thereby avoiding any *in vitro* artifacts. Thus, further studies will be required to show whether QstR indeed binds the promoters of *comEA* and *comEC*.

ChIP-qPCR could also be used to investigate whether QstR binds the promoter of the gene *dns*. Our data indicated that HapR directly represses this gene by binding to its promoter region; however, a further repression was observed when natural competence was induced (Section 3.2, Figure 5) and we therefore speculate that QstR, in addition to HapR, might contribute to the repression of *dns*. ChIP might conclusively show if QstR directly binds the dns promoter. Moreover, a ChIP-Seq approach could also lead to discovery of other QstR target genes.

Function of TfoX

The role of TfoX and the signalling cascade downstream of this protein remains obscure. In our model the TfoX protein is depicted as the factor that directly or indirectly modifies the CRP-cAMP complex to allow binding to the competence-related CRP sites (Section 3.3, Figure 1). In order to validate this model it will be necessary to investigate whether TfoX physically interacts with CRP. Co-immunoprecipitation (Co-IP) experiments or two-hybrid systems could be used to answer this question. Moreover, in our model we expect that TfoX does not bind the DNA as opposed to the mechanism proposed for *H. influenzae* (Cameron & Redfield, 2008; Chapter 1, Figure 12). Thus, the next step is to investigate whether TfoX binds the promoter region of the competence genes or does not. This aim might be achieved by ChIP-seq comparing the data from wild type strains under competence non-inducing and competence-inducing conditions.

Elucidation of the cAMP dependency of the CRP protein

The role of cAMP as cofactor of the CRP protein was not fully investigated in our

study. However, we confirmed that cAMP is important for natural competence to occur (Blokesch, 2012), as transformation was abolished in an adenylate cyclase mutant. Moreoever, in the absence of CpdA, the enzyme that degrades cAMP, the transformation frequency was increased (Section 3.1, Table 2).

Our idea was to study the link between the cAMP and the activation of the competence genes by site-directly mutating the *crp* gene. Thus, we generated a CRP* variant of the CRP protein. This variant, initially studied in *E. coli*, harbors the substitution of two amino acids at position 128 and 129 (falsely annotated in the literature as T127L/S128A) within the cAMP binding region. The conformation of CRP* therefore resembles the CRP-cAMP complex rendering the protein constitutively active even in the absence of cAMP (Krueger *et al*., 1998; Chu *et al*., 2001). Since the CRP proteins of *V. cholerae* and *E. coli* share 95% amino acid sequence identity (Skorupski & Taylor, 1997) we expected that the same mutations would result in a similar conformational change (Annexes, Figures A1-A2).

Our data showed that CRP* is functional and able to contribute to the activation of natural competence (Figure A1, strains at 22h). Moreover we investigated whether the function of the CRP* variant was also comparable to that of the wild type protein at lower cell density. To do so we tested the transformability of the bacterial strains by adding the extracellular DNA to the cells at an earlier time point (e.g. after 8 hours of growth on chitin flakes). The results of this experiment showed that the transformability of the bacterial strain harboring the CRP* variant proved less efficient than that of the strain harboring the wild type protein (Figure A1, strains at 8h). We hypothesized that *1)* CRP* is not independent on cAMP in *V. cholerae*, but indeed this variant is more sensitive to the intracellular levels of the cAMP (that is most likely lower at 8h); *2)* CRP* is independent on cAMP but it is more sensitive to the presence of a factor/cofactor that has to accumulate within the cells.

The activity of the proteins CRP and CRP* was very recently investigated in *V. cholerae* and quantified using the luciferase reporter construct. In the absence of cAMP the production of the light was observed only in the strain carrying the variant CRP* suggesting that the protein is active even in the absence of cAMP (Wang *et al*.,

2013). However, a direct comparison between this study and our data is not feasible because the expression of the competence genes also require TfoX in addition to CRP. it is possible that the CRP* variant is impaired for its interaction with TfoX or with a factor that is downstream TfoX. Nonetheless, our data are not conclusive and further experiments are required. For example it will be useful to investigate the expression of the competence genes driven by the CRP* variant and to do a comparison with genes that are also dependent on CRP but that are not part of the competence regulon (TfoX independent).

Annexes

a)

```
VcCRP       MVLGKPQTDPTLEWFLSHCHIHKYPSKSTLIHAGEKAETLYYIVKGSVAVLIKDEEGKEM
EcCRP       MVLGKPQTDPTLEWFLSHCHIHKYPSKSTLIHQGEKAETLYYIVKGSVAVLIKDEEGKEM
            ****************************** ************************** 
            1                                                        60

VcCRP       ILSYLNQGDFIGELGLFEEGQERTAWVRAKTPCEVAEISFKKFRQLIQVNPDILMRLSGQ
EcCRP       ILSYLNQGDFIGELGLFEEGQERSAWVRAKTACEVAEISYKKFRQLIQVNPDILMRLSAQ
            ********************** ******* ******* ***************** * 
           61                                                        120

VcCRP       MARRLQVTSQKVGDLAFLDVTGRIAQTLLNLARQPDAMTHPDGMQIKITRQEIGQIVGCS
EcCRP       MARRLQVTSEKVGNLAFLDVTGRIAQTLLNLAKQPDAMTHPDGMQIKITRQEIGQIVGCS
            ********* *** ***************** **************************
          121                                                        180

VcCRP       RETVGRILKMLEEQNLISAHGKTIVVYGTR
EcCRP       RETVGRILKMLEDQNLISAHGKTIVVYGTR
            ************ ***************** 
          181                          210
```

b)

```
VcCRP       MARRLQVTSQKVGD
VcCRP*      MARRLQVLAQKVGD
            121          134
```

Figure A1. Sequence alignments of the CRP protein. a). Alignment of the amino acid sequences of the CRP proteins from *V. cholerae* (VcCRP) and *E. coli* (EcCRP). (*) identical amino acid in the two bacterial species. **b)** The CRP* variant holds a two amino acidic substitution at positions 128 and 129 (highlighted in red), which is falsely annotated in the literature as T127L/S128A.

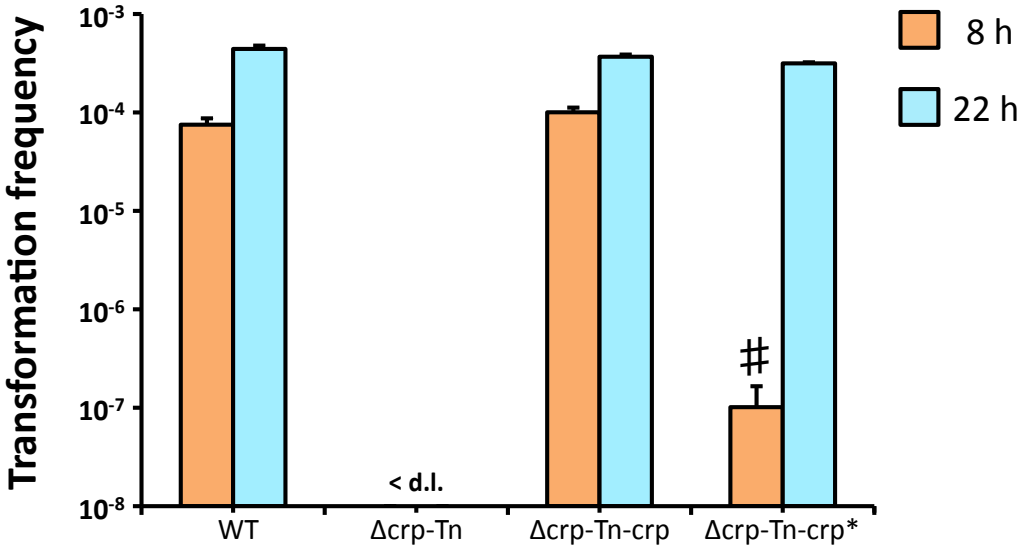


Figure A2. The CRP* variant is functional with respect to natural competence for transformation. The transformability of the indicated *V. cholerae* strains was tested on chitin surfaces. The extracellular DNA was added after 8 h and 22 h of static growth at 30°C in artificial sea water medium. The respective transformation frequencies (TF) are shown on the y-axis. WT: wild type strain A1552; Δcrp-Tn: *crp* minus strain carrying an empty transposon on the chromosome; Δcrp-Tn-crp and Δcrp-Tn-crp*: *crp* minus strain carrying the wild type *crp* and the variant crp* on the transposon. The data are the average of four biological replicates. < d.l.: under detection limit. Average d.l. of *V. cholerae* strain Δcrp-Tn (8 h samples) was 2.37 x 10^{-7}. (#): TF was under d.l. in three out of four experiments and the d.l. was used to calculate the TF. Error bars reflect the standard deviation.

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Curriculum Vitae

Mirella Lo Scrudato
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Biotechnologist graduate
Background: molecular biology, microbiology, biochemistry

EDUCATION AND RESEARCH EXPERIENCE

Dec 2009 – Feb 2014	**Swiss Federal Institute of Technology Lausanne (EPFL)**, Lausanne, Switzerland PhD candidate in Life Sciences, Biotechnology and Bioengineering program, Advisor: Prof. Melanie Blokesch Thesis: The regulatory circuit of natural competence for transformation in the human pathogen *Vibrio cholerae*
Nov 2006 – Nov 2009	**University of Palermo (UNIPA),** Palermo, Italy M.Sc. in Biotechnology for Industries and Scientific Research, *magna cum laude*
Oct 2007- July 2008	**Institute of Life Technologies, University of Applied Sciences HES-SO Valais,** Sion, Switzerland Master Project Advisor: Prof. Sergio Schmid Thesis: Development of an Expression Platform for the Production of Recombinant Peptides
Sept 2001 – Oct 2006	**University of Palermo (UNIPA),** Palermo, Italy B.Sc. in Biotechnology, Biomedical Curriculum, *magna cum laude*
Oct 2005 – July 2006	**Department of Experimental Oncology and Clinical Application, University of Palermo (UNIPA),** Palermo, Italy Bachelor Project Advisor: Prof. Ida Pucci-Minafra Thesis: Serum Levels of Matrix Metalloproteases in Patients with Breast Cancer in Antiblastic Treatment

PEER-REVIEWED PUBLICATIONS

1. **Lo Scrudato M** and Blokesch M (2013). A transcriptional regulator linking quorum sensing and chitin induction to render *Vibrio cholerae* naturally transformable. *Nucleic Acids Res.* 41(6): 3644-58.

2. **Lo Scrudato M** and Blokesch M (2012). The regulatory Network of Natural competence and Transformation of *Vibrio cholerae*. *PLos Genet.* 8(6): e1002778.

UNPUBLISHED WORK

1. **Lo Scrudato M** and Blokesch M. Transcriptional regulation of *comEA* - a key gene in natural competence for transformation of *Vibrio cholerae*. Manuscript to be submitted.

SELECTED CONFERENCE COMMUNICATIONS

- **Lo Scrudato M** and Blokesch M. Quorum sensing drives DNA uptake in naturally competent *Vibrio cholerae*. FEMS 2013, 5th Congress of European Microbiologists, Leipzig, Germany, July 21-25, 2013. Poster presentation.

- **Lo Scrudato M** and Blokesch M. Quorum sensing tightly regulates the DNA uptake in naturally competent *Vibrio cholerae* cells. 71st Annual Assembly of the Swiss Society for Microbiology, Interlaken, Switzerland, June 26-27, 2013. Oral presentation.

- **Lo Scrudato M** and Blokesch M. A regulatory element involved in natural competence gene expression in *Vibrio cholerae*. Bacteria, Archea & Phages, Cold Spring Harbor Laboratory (CSHL), Cold Spring Harbor, New York, August 21-25, 2012. Poster presentation.

- **Lo Scrudato M** and Blokesch M. Natural competence of *Vibrio cholerae* is subject to three regulatory pathways. The Fourth Conference on the Biology of Vibrios, Santiago de Compostela, Spain, November 1-4, 2011. Poster presentation.

- **Lo Scrudato M**, Suckow G, Blokesch M. Natural competence of *Vibrio cholerae* – New insights into the regulatory process. 69th Annual Assembly of the Swiss Society for Microbiology, ETH Zurich, Switzerland, June 24-25, 2010. Poster presentation.

TECHNICAL SKILLS

- Bacterial cell culture, genetics and manipulation
- Fluorescence and light microscopy
- Flow cytometry
- DNA and RNA isolation
- Transcriptional analysis by quantitative reverse transcription PCR (qRT-PCR)
- Protein expression, purification (column chromatography) and detection methods (SDS-PAGE, Zymography, Protein silver staining, Western Blot)
- Statistical analysis (Student's *t* test)
- Electrophoretic Mobility Shift Assay (EMSA)
- Chromatin immunoprecipitation coupled to detection by qPCR (ChIP-qPCR).

EXTRA CULLICULAR INTERESTS

Tennis, dance, cooking, chess.

LANGUAGES

Italian (mother tongue), English (Fluent), French (Intermediate).