

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

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## 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

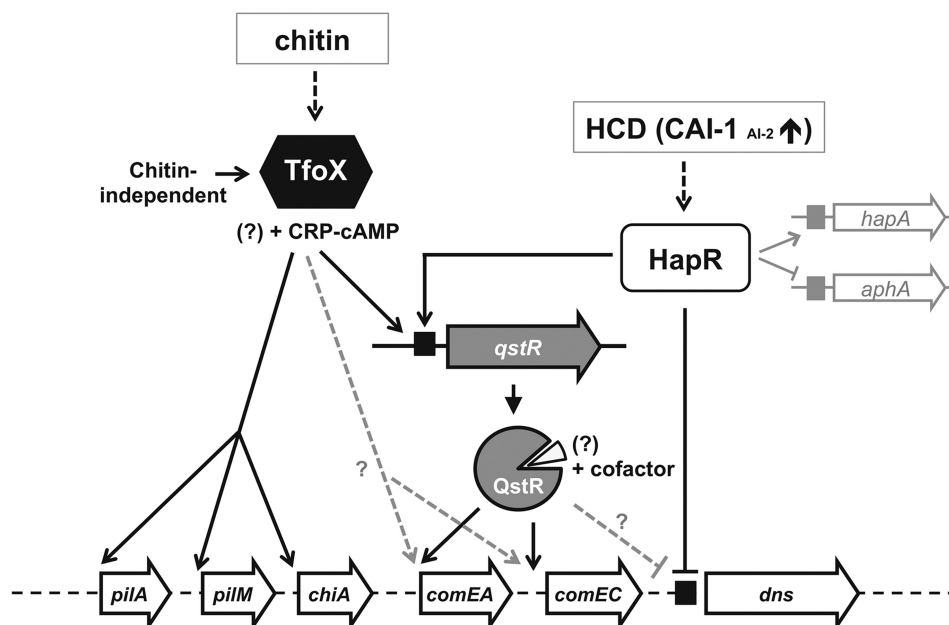
The bacterium *Vibrio cholerae* is a facultative pathogen and the causative agent of cholera. Cholera is far from extinction and is even considered a re-emerging disease (1). *V. cholerae* commonly occurs in aquatic ecosystems,

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 artificial 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 Scudato 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  $\alpha$ -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  $\alpha$ -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 DH5 $\alpha$  (43) and S17-1 $\lambda$ pir (44) were used for cloning and as a donor strain in bacterial mating experiments, respectively. *E. coli* Origami 2<sup>TM</sup> (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<sub>Tet</sub>, 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 *NotI* 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 *AatII* and *BglII* to shorten the upstream region of *comEA*. Self-ligation resulted in the plasmid pBR-[P<sub>w</sub>]*comEA*, carrying *comEA* and 200 bp of its upstream region (Table 1). Site-directed mutagenesis using inverse PCR on plasmid pBR-[P<sub>w</sub>]*comEA* generated the plasmids pBR-[P<sub>mut L</sub>]*comEA*, pBR-[P<sub>mut R</sub>]*comEA* and pBR-[P<sub>mut L/R</sub>]*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 <sup>a</sup>	Reference
Strains ( <i>V. cholerae</i> )		
A1552	Wild-type (WT), O1 El Tor Inaba, Rif <sup>R</sup>	(36)
A1552-LacZ-Kan	A1552 strain with <i>aph</i> cassette in <i>lacZ</i> gene; Rif <sup>R</sup> , Kan <sup>R</sup>	(37,38)
A1552-TntfoX	A1552 containing mini-Tn7- <i>araC</i> -P <sub>BAD</sub> - <i>tfoX</i> ; Rif <sup>R</sup> , Gent <sup>R</sup>	(8)
ΔhapR	A1552ΔVC0583, Rif <sup>R</sup>	(2)
ΔhapR-TntfoX	A1552ΔhapR containing mini-Tn7- <i>araC</i> -P <sub>BAD</sub> - <i>tfoX</i> ; Rif <sup>R</sup> , Gent <sup>R</sup>	(8)
ΔcomEA	A1552ΔVC1917 [= A1552VC1917 in (2)], Rif <sup>R</sup>	(2)
ΔcomEA-TntfoX	A1552ΔcomEA containing mini-Tn7- <i>araC</i> -P <sub>BAD</sub> - <i>tfoX</i> ; Rif <sup>R</sup> , Gent <sup>R</sup>	This study
ΔqstR	A1552ΔVC0396, Rif <sup>R</sup>	This study
ΔqstR-TntfoX	A1552ΔqstR containing mini-Tn7- <i>araC</i> -P <sub>BAD</sub> - <i>tfoX</i> ; Rif <sup>R</sup> , Gent <sup>R</sup>	This study
Plasmids		
pBR322	Amp <sup>R</sup> , Tc <sup>R</sup>	(39)
pGP704-Sac28	Suicide vector, ori R6K <i>sacB</i> , Amp <sup>R</sup>	(14)
pGP704-28-SacB-ΔqstR	pGP704-Sac28 with a gene fragment resulting in a 402-bp deletion (incl. stop codon) within VC0396 ( <i>qstR</i> )	This study
pBAD/Myc-HisA	pBR322-derived expression vector; <i>araBAD</i> promoter (P <sub>BAD</sub> ); Amp <sup>R</sup>	Invitrogen
pBAD- <i>comEA</i>	<i>comEA</i> gene cloned into pBAD/Myc-HisA, arabinose inducible; Amp <sup>R</sup>	This study
pBAD- <i>hapR</i>	<i>hapR</i> gene cloned into pBAD/Myc-HisA, arabinose inducible; Amp <sup>R</sup>	This study
pBAD- <i>hapR</i> -N-Strep	<i>hapR</i> gene preceded by sequence encoding <i>Strep</i> -tagII <sup>®</sup> cloned into pBAD/Myc-HisA, arabinose inducible; Amp <sup>R</sup>	This study
pBAD- <i>qstR</i>	<i>qstR</i> gene cloned into pBAD/Myc-HisA, arabinose inducible; Amp <sup>R</sup>	This study
pUX-BF13	<i>oriR6K</i> , helper plasmid with Tn7 transposition function; Amp <sup>R</sup>	(40)
pGP704::Tn7	pGP704 with mini-Tn7	Schoolnik laboratory collection; (41)
pGP704-mTn7- <i>araC-tfoX</i>	pGP704 with mini-Tn7 carrying <i>araC</i> and P <sub>BAD</sub> -driven <i>tfoX</i> ; Amp <sup>R</sup>	(8)
pBR-Tet_MCSI	pBR322 derivative deleted for Tet promoter and part of <i>tet</i> <sup>R</sup> gene; Amp <sup>R</sup>	(8)
pBR-Tet_MCSII	pBR322 derivative deleted for Tet promoter and part of <i>tet</i> <sup>R</sup> gene; new MCS included; Amp <sup>R</sup>	This study
pBR-[own] <i>comEA</i>	<i>comEA</i> gene preceded by 900 bp of upstream region cloned into pBR-Tet_MCSII; Amp <sup>R</sup>	This study
pBR-[P <sub>WT</sub> ] <i>comEA</i>	<i>comEA</i> gene preceded by 200 bp of upstream region; Amp <sup>R</sup>	This study
pBR-[P <sub>mut L</sub> ] <i>comEA</i>	plasmid generated by inverse PCR of pBR-[P <sub>WT</sub> ] <i>comEA</i> ; mutated within the left part of the putative HapR binding site upstream <i>comEA</i> ; Amp <sup>R</sup>	This study
pBR-[P <sub>mut R</sub> ] <i>comEA</i>	plasmid generated by inverse PCR of pBR-[P <sub>WT</sub> ] <i>comEA</i> ; mutated within the right part of the putative HapR binding site upstream <i>comEA</i> ; Amp <sup>R</sup>	This study
pBR-[P <sub>mut L/R</sub> ] <i>comEA</i>	plasmid generated by inverse PCR of pBR-[P <sub>WT</sub> ] <i>comEA</i> ; mutated within the left and right part of the putative HapR binding site upstream <i>comEA</i> ; Amp <sup>R</sup>	This study
pBR-[P <sub>dns</sub> -100-50]	DNA sequence corresponding to the -100 to -50 bp region upstream of <i>dns</i> was inserted into pBR-Tet_MCSI by inverse PCR; Amp <sup>R</sup>	This study
pBR-[P <sub>dns</sub> -50-1]	DNA sequence corresponding to the -50 to -1 bp region upstream of <i>dns</i> was inserted into pBR-Tet_MCSI by inverse PCR; Amp <sup>R</sup>	This study
pBR-[P <sub>qstR</sub> -150-120]	DNA sequence corresponding to the -150 to -120 bp region upstream of <i>qstR</i> was inserted into pBR-Tet_MCSI by inverse PCR; Amp <sup>R</sup>	This study
pBR-[P <sub>qstR</sub> -150-102]	DNA sequence corresponding to the -150 to -102 bp region upstream of <i>qstR</i> was inserted into pBR-Tet_MCSI by inverse PCR; Amp <sup>R</sup>	This study
pBR-[P <sub>qstR</sub> -150-102] <sub>mut_AgeI</sub>	plasmid generated by inverse PCR of pBR-[P <sub>qstR</sub> -150-102]; site-directly mutated in two bases as indicated in Figure 6D thereby creating an <i>AgeI</i> restriction enzyme recognition site; Amp <sup>R</sup>	This study

<sup>a</sup>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 *NcoI* and *EcoRI*-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<sup>®</sup> 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<sub>dns</sub>-100-50], pBR-[P<sub>dns</sub>-50-1], pBR-[P<sub>qstR</sub>-150-120] and pBR-[P<sub>qstR</sub>-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<sub>qstR</sub>-150-102]\_mut\_AgeI, carrying mutations in the promoter region -150 to -102 upstream of the start codon of *qstR* (P<sub>qstR</sub>; see Figure 6D), was constructed through inverse PCR using the oligonucleotides PVC0396\_AgeI\_fw and PVC0396\_AgeI\_bw and the plasmid pBR-[P<sub>qstR</sub>-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 Scudato 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 ( $\alpha$ -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<sup>PLUS</sup> western blotting substrate (Roche, Rotkreuz, Switzerland) or Western Lightning<sup>®</sup>-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<sup>TM</sup> 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<sup>®</sup> 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<sup>®</sup>-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<sup>TM</sup> 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<sub>2</sub> 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<sub>BAD</sub> promoter in this system) (8). The cell harvest, RNA preparation, and reverse transcription followed by quantitative PCR (qRT-PCR) (LightCycler<sup>®</sup> 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  $\Delta$ hapR-TntfoX 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<sub>WT</sub>]*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<sub>mut L</sub>]*comEA*, pBR-[P<sub>mut R</sub>]*comEA* and pBR-[P<sub>mut L/R</sub>]*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

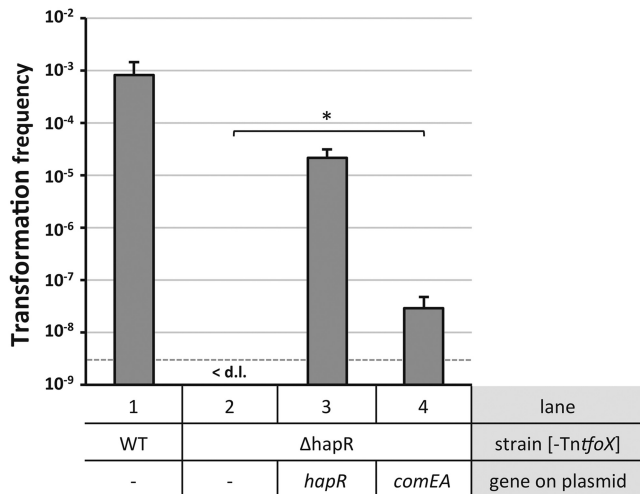
better (Supplementary Figure S1C). The most likely explanation for this difference in complementation efficiency is that the plasmids used in this study were derivatives of pBR322, which changes copy number under different growth conditions/growth rates (47). These results demonstrate that the putative 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  $\Delta$ hapR-TntfoX 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<sup>TM</sup> (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<sup>®</sup> Sepharose column (see ‘Material and Methods’ section for details; Supplementary Figure S2C) and stored at  $-20^{\circ}\text{C}$ .

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<sub>*comEA*</sub>)-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  $\sim 600$  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-*TntfoX* (WT-*TntfoX*, lane 1) or a *hapR* minus variant ( $\Delta$ hapR-*TntfoX*, 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  $\Delta$ hapR-*TntfoX* was  $2.9 \times 10^{-9}$ , as indicated with a dashed grey line). Statistically significant differences were determined using Student's *t*-test. \* $P < 0.05$ ; for strain  $\Delta$ hapR-*TntfoX*, 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  $\alpha$ -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

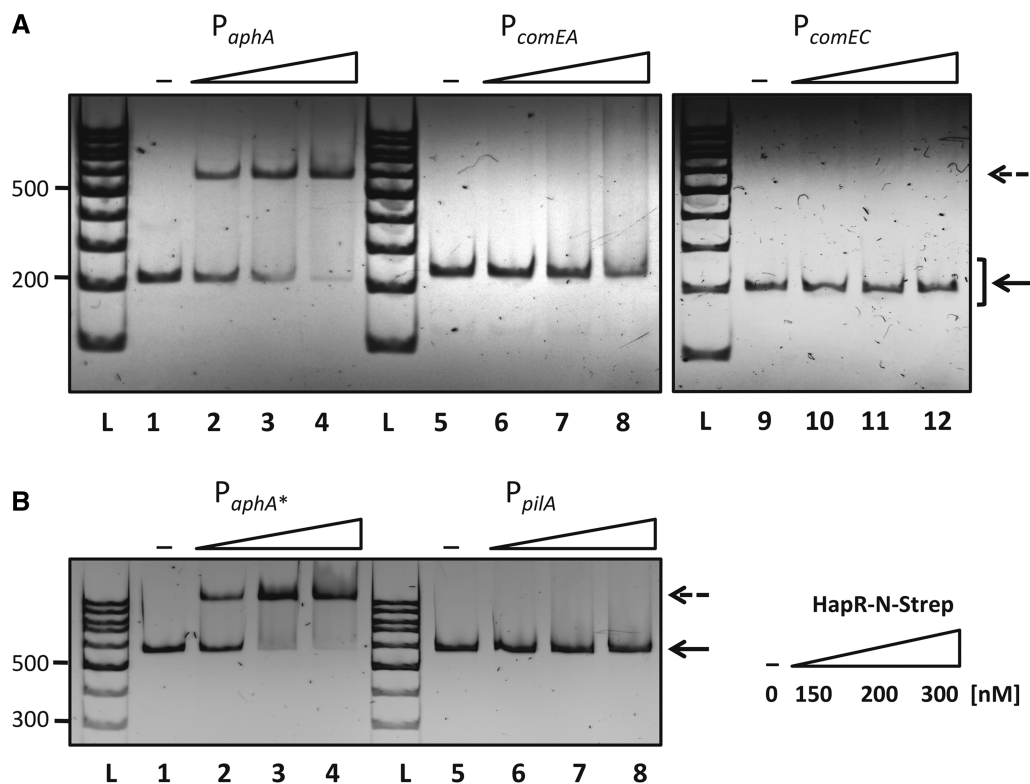
HapR-dependent mobility shifts, and the results are indicated in the table on the left of Figure 4B. Apart from the outermost fragments (fragments A, G and H), all other probes remained bound to the HapR protein (Figure 4B). These fragments contained either the DNA sequence from –100 to –50 bp and/or 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-*TntfoX*. 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  $\Delta qstR$  genetic background (Table 2). The transformation frequency in this setting (e.g.  $\Delta qstR/pBAD-comEA$ ) was  $\sim 10$ -fold higher than in strain  $\Delta hapR/pBAD-comEA$  (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*

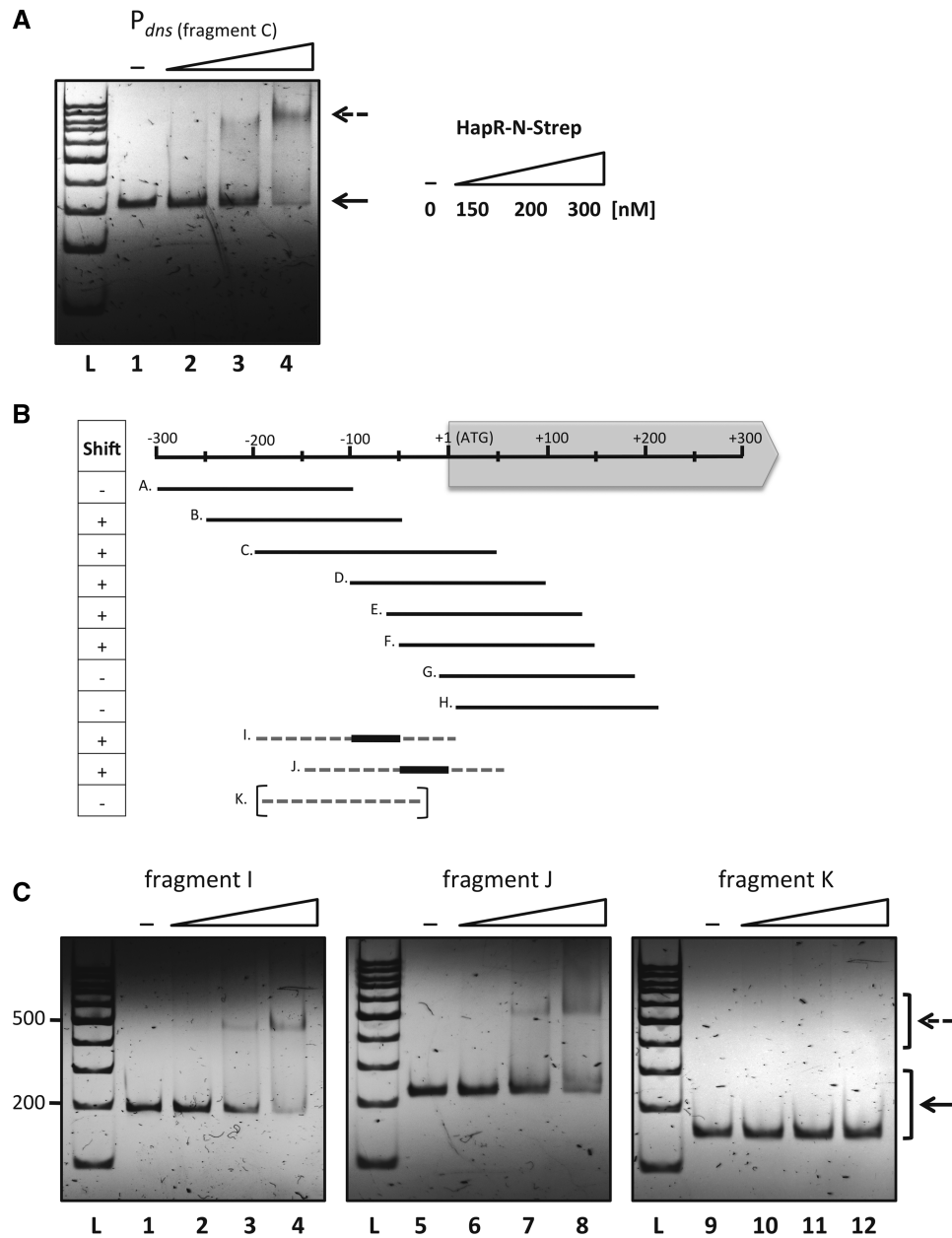
from a plasmid in the *hapR* minus strain. Indeed, the exogenous expression of *qstR in trans* in the  $\Delta hapR-TntfoX$  strain rescued natural transformation to the same extent as the *hapR* gene itself (Table 2). We therefore propose that HapR directly or indirectly influences *qstR* expression 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*

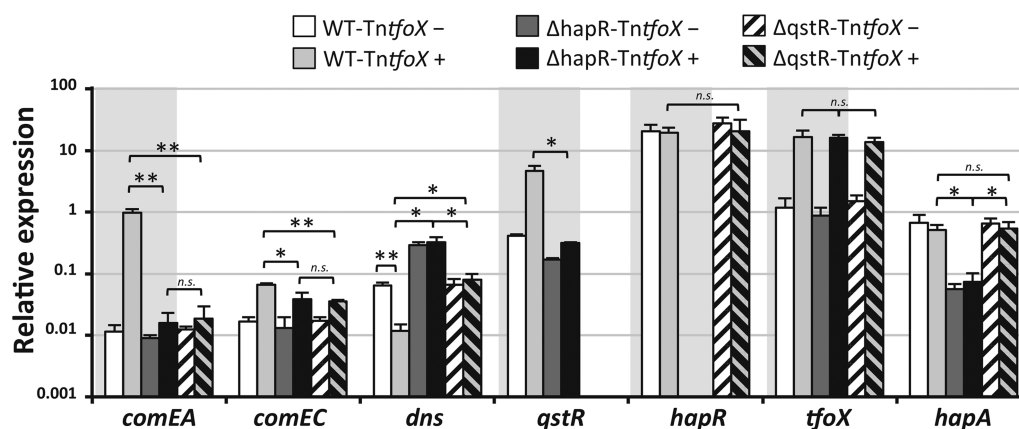




**Figure 4.** The HapR protein binds to the *dns* upstream region. (Panel A) Binding of HapR to the upstream region of *dns* results in a shifted DNA fragment. Lanes 1–4: EMSA of the *dns* promoter fragment covering the region –203 to +48 bp with respect to the “ATG” start codon (corresponding to fragment 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-*TntfoX* and its *hapR* or *qstR* knockout derivatives ( $\Delta$ *hapR*-*TntfoX* and  $\Delta$ *qstR*-*TntfoX*). All three strains were grown under competence non-inducing (*TntfoX* -) and competence-inducing (*TntfoX*+) 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*

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

<sup>x</sup>Average of at least three independent experiments.

<sup>y</sup>Statistically significant differences between transformation data indicated with the same characters were determined using Student's *t*-tests.

\*\**P* < 0.01.

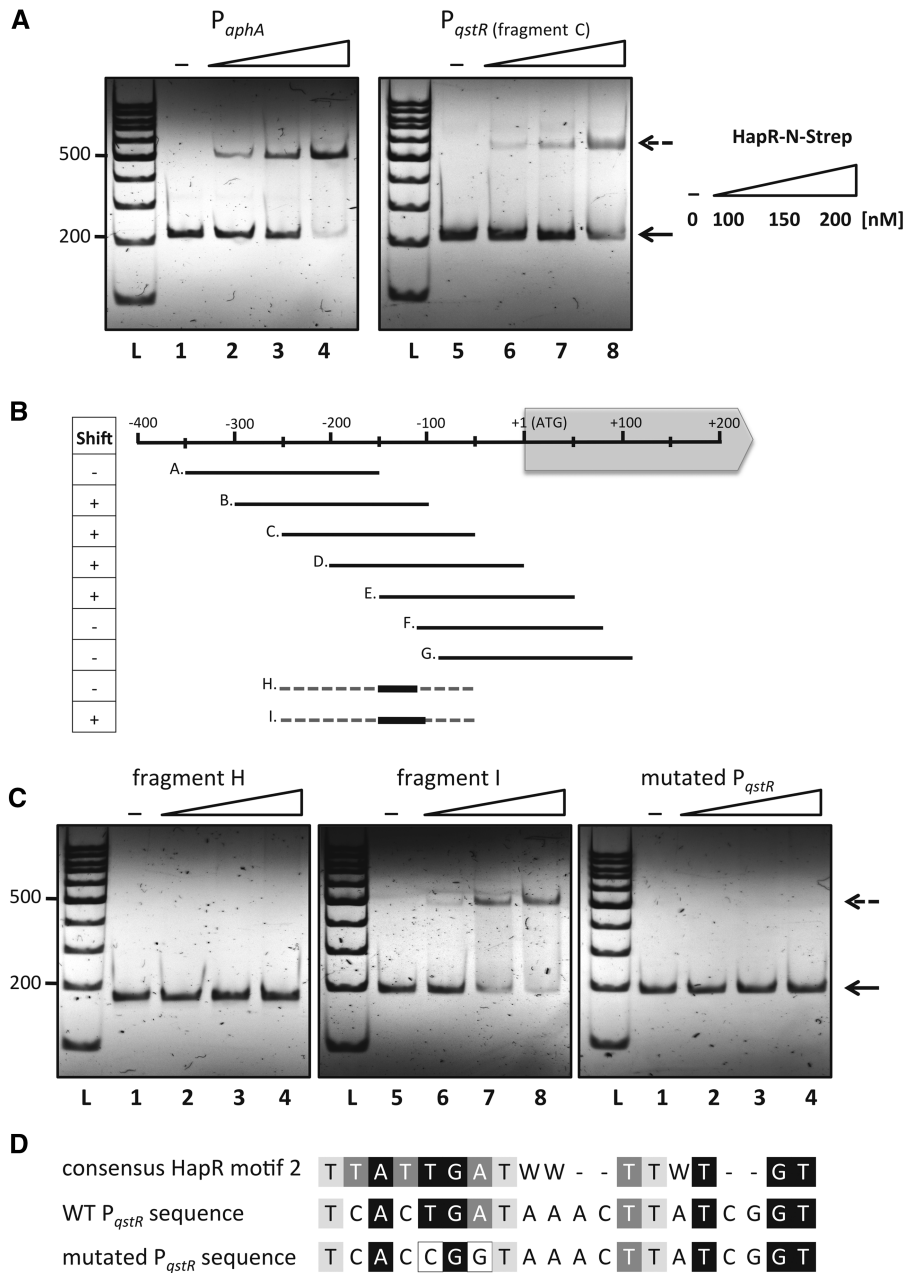
n.s. = not significantly different.

<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 ( $\sim 200$  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 *TntfoX* (8) the genetic backgrounds of these strains were either wild-type (A1552-*TntfoX*), *hapR*-negative ( $\Delta$ *hapR*-*TntfoX*) or *qstR*-negative ( $\Delta$ *qstR*-*TntfoX*) (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* (VVI\_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 ( $\Delta$ qstR-TntfoX) 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 ( $\Delta$ hapR-TntfoX) showed a statistically significant increase of  $\sim 2$ -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.

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