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MicroReview

Competence and natural transformation in vibrios

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Summary

Natural transformation is a major mechanism of horizontal gene transfer in bacteria. By incorporating exogenous DNA elements into chromosomes, bacteria are able to acquire new traits that can enhance their fitness in different environments. Within the past decade, numerous studies have revealed that natural transformation is prevalent among members of the *Vibrionaceae*, including the pathogen *Vibrio cholerae*. Four environmental factors: (i) nutrient limitation, (ii) availability of extracellular nucleosides, (iii) high cell density and (iv) the presence of chitin, promote genetic competence and natural transformation in *Vibrio cholerae* by co-ordinating expression of the regulators CRP, CytR, HapR and TfoX respectively. Studies of other *Vibrionaceae* members highlight the general importance of natural transformation within this bacterial family.

Introduction

Many bacterial species can become competent to take up environmental DNA. While DNA transported across the cytoplasmic membrane by the competence machinery may serve as a source of nutrients or as a template for repairing chromosomal damage, it can also be incorporated onto the chromosome by homologous recombination. This latter phenomenon, referred to as natural transformation, is a prime example of horizontal gene transfer, which, along with conjugation and transduction, can result in the emergence of new traits (Chen *et al.*, 2005). Recently, various members of the *Vibrionaceae* family have been shown to be naturally competent to take up DNA.

The *Vibrionaceae* family consists of a remarkably diverse set of Gram-negative bacteria. In general, *Vibrionaceae* members are readily isolated from aqueous environments ranging from freshwater to marine conditions and are easily cultured. Pathogenic *Vibrionaceae* members, particularly *Vibrio cholerae*, have significantly impacted human health, both historically and currently, and, as a result, have garnered a great deal of attention from the biomedical community (Piarroux and Faucher, 2012; Marin *et al.*, 2013). However, numerous ecological studies of this diverse family of bacteria have revealed that they often form non-pathogenic, and in many cases beneficial relationships with eukaryotes (Huq *et al.*, 1983; Visick and Ruby, 2006; Senderovich *et al.*, 2010).

Many features of the competence machinery and its regulation in *Vibrionaceae* members are similar to those of archetypical systems described for Gram-negative bacteria. In general, uptake of environmental DNA requires a complex apparatus that first binds the DNA at the cell surface and then delivers it through the membrane to the cytoplasm (Dubnau, 1999; Seitz and Blokesch, 2013). In *Neisseria* species, such as *Neisseria meningitidis* and *Neisseria gonorrhoeae*, the outer membrane secretin pore PilQ allows double-stranded DNA to enter into the periplasm (Lång *et al.*, 2009). With the help of a pseudo-pilus encoded in part by *pilE*, the periplasmic protein ComEA binds the DNA and directs it to the inner-membrane channel ComEC (Fig. 1, Wolfgang *et al.*, 2002). The minor pilin ComP also contributes to natural transformation by serving as a DNA receptor that recognizes species-specific DNA uptake sequences (DUS) (Aas *et al.*, 2002; Cehovin *et al.*, 2013). One strand of the DNA enters the cytoplasm through ComEC, while the complement strand is degraded (Fig. 1, Chen *et al.*, 2005). Once inside the cytoplasm, this DNA may be integrated into the chromosome through homologous recombination (Fig. 1). In *Haemophilus influenzae*, another Gram-negative bacterium that serves as a model system for natural transformation, the secretin that allows the entry of double-stranded DNA is called ComE; the crucial subunit of the pseudo-pilus involved in DNA

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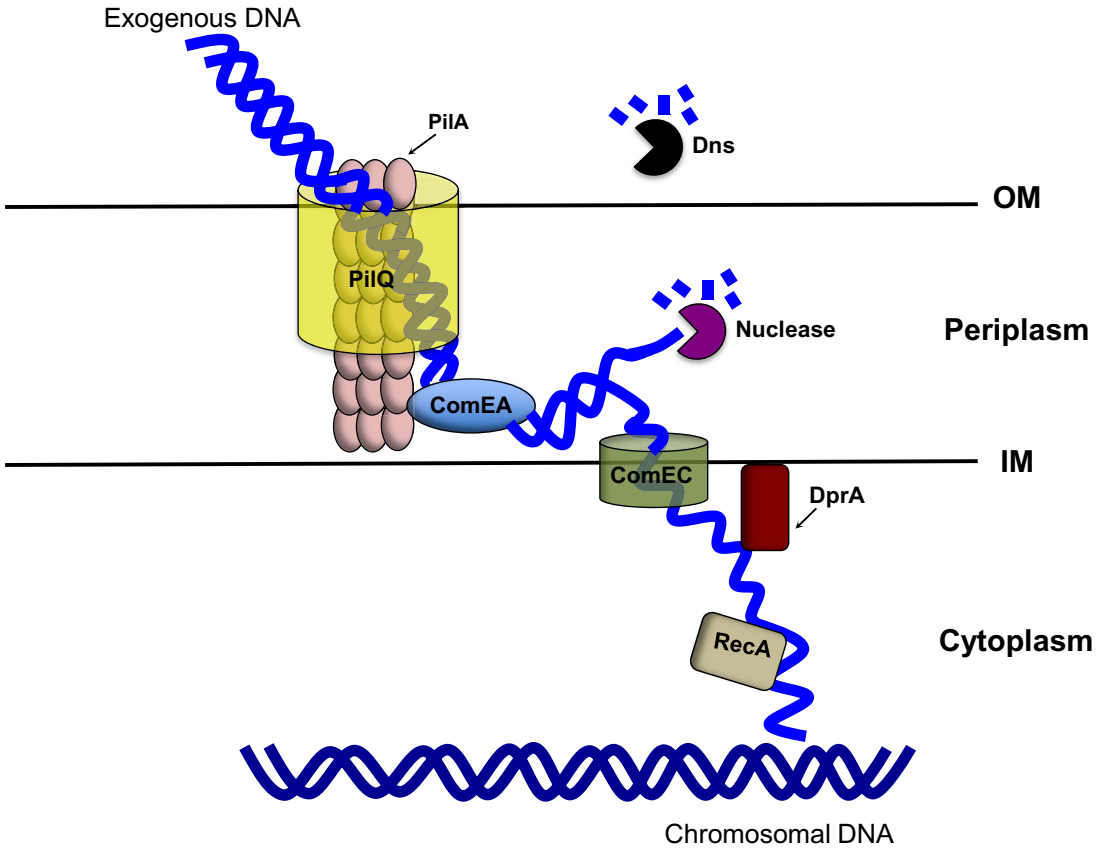

Fig. 1. The natural transformation machinery in *V. cholerae*. Double-stranded DNA enters the periplasm by means of the secretin pore PilQ located within the outer membrane. The pseudo-pilus (PilA represents a subunit) helps the DNA bind to the periplasmic protein ComEA, which directs the DNA to the inner-membrane channel ComEC. Other components of a type IV pili system (not shown) may contribute to this process, but their involvement in natural transformation remains unknown. The extracellular DNA that fails to enter the periplasm is degraded by extracellular deoxyribonuclease Dns. One strand of the DNA enters the cytoplasm through ComEC, while the complement strand is degraded. The internalized single-stranded DNA is shielded from nuclease attack by the DNA protecting protein DprA and incorporated into chromosome by the recombinase RecA. IM, inner membrane; OM, outer membrane.

uptake is PilA; and the counterparts of ComEA and ComEC are ComE1 and Rec2 respectively (MacFadyen *et al*., 2001; Chen and Dubnau, 2004; Mell *et al*., 2012). *Vibrionaceae* members such as *V. cholerae* possess homologues of PilQ, PilA, ComEA and ComEC, which play crucial roles in the uptake of exogenous DNA (Fig. 1, Lo Scrudato and Blokesch, 2012). To our knowledge, no homologue of ComP has been reported for *H. influence* or members of the *Vibrionaceae*. In addition, for most Gram-negative bacteria, whether DNA uptake is achieved by a type IV pilus or a pseudo-pilus remains generally unknown.

While the primary components of the competence machinery are conserved among bacteria, the regulatory networks that govern their expression vary tremendously to accommodate differences in lifestyles. For instance, *Streptococcus pneumoniae* and *Bacillus subtilis*, which have served as model organisms for natural transformation in Gram-positive bacteria, use the cell–cell form of communication known as quorum sensing to regulate the expression of competence genes (Solomon and Grossman, 1996). In *H. influenzae*, nutrient deprivation, rather than quorum sensing, controls competence (Chandler, 1992; Dorocicz *et al*., 1993). Finally, both *N. meningitidis* and *N. gonorrhoeae* are constitutively competent (Biswas *et al*., 1977).

The complex regulatory network that controls competence in *Vibrionaceae* members exhibits some features that are reminiscent of the model systems listed above. We begin this MicroReview with a description of the environmental signals that stimulate the competence pathway in *V. cholerae*. We then describe the complex regulatory mechanisms that control TfoX, which is a critical transcription factor that controls competence in *Vibrionaceae* as well as in other bacteria. This MicroReview also highlights the numerous studies of natural transformation in *Vibrionaceae* members other than *V. cholerae*. Finally, we discuss the role of natural transformation and, more generally, that of horizontal gene transfer on the diversity of the *Vibrionaceae* family and their corresponding impacts on human health.

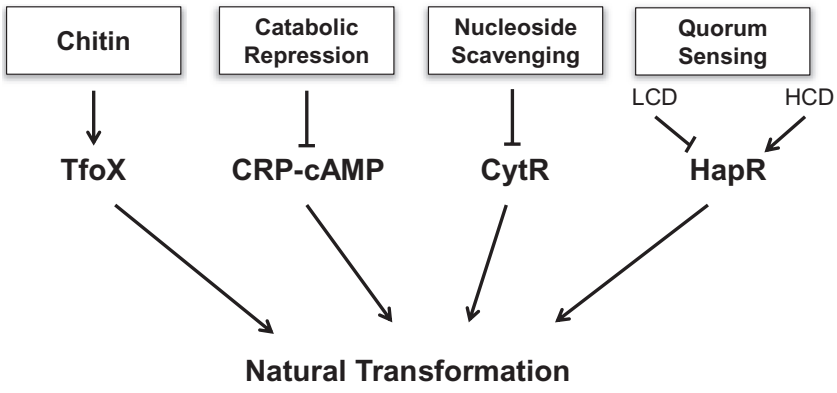

Fig. 2. The current model of the regulatory network governing competence in *V. cholerae*. The four known environmental stimuli are chitin, quorum sensing, and the availability of carbon sources and extracellular nucleosides. The model does not include information concerning cross-talk between signalling cascades. The lines connecting components of the signalling cascade do not indicate direct interaction. LCD, low cell density; HCD, high cell density.

Environmental inputs

Numerous studies have shown that various environmental and physiological factors impact competence and natural transformation in *V. cholerae* (Meibom *et al.*, 2005; Antonova and Hammer, 2011; Antonova *et al.*, 2012; Blokesch, 2012; Lo Scrudato and Blokesch, 2012). The current model of the regulatory network governing competence in *V. cholerae* consists of four stimuli: chitin, quorum sensing, and the availability of carbon sources and extracellular nucleosides (Fig. 2).

Chitin

In 2005, it was first reported that *V. cholerae* becomes competent for natural transformation in the presence of chitin (Meibom *et al.*, 2005). This result was independent of whether the chitin source was synthetically derived or in its natural state as crustacean exoskeletons (e.g. crab-shell tiles). To our knowledge, chitin has never been directly tested for its involvement in natural transformation in *H. influenzae*, *N. gonorrhoeae* or *N. meningitidis* (Biswas *et al.*, 1977; Smith, 1980). However, given the absence of chitin in the natural habitats of these three bacterial species, chitin is unlikely to play any significant role in their transition to a genetically competent state.

Chitin, which is composed of chains of β-1,4-linked *N*-acetylglucosamine (GlcNAc) residues, is the second most abundant biopolymer found in nature (Keyhani and Roseman, 1999; Hunt *et al.*, 2008). Chitin can be found throughout all kingdoms, and its presence is widespread in marine environments, ranging from the cell walls of certain green algae to the exoskeletons of crustaceans (Keyhani and Roseman, 1999; Hunt *et al.*, 2008). The majority of chitin in the aquatic biosphere is recycled by chitinolytic bacteria, including members of the *Vibrionaceae* family (Keyhani and Roseman, 1999; Meibom *et al.*, 2004; Hunt *et al.*, 2008). Chitinolytic bacteria use chitin as a source of both carbon and nitrogen, through a complex process that involves the initial detection of chitin, attachment to a chitinaceous surface and degradation of chitin (Keyhani and Roseman, 1999; Li and Roseman, 2004; Hunt *et al.*, 2008). By means of extracellular, secreted chitinases, chitin is cleaved into oligosaccharides fragments, $(GlcNAc)_n$, which translocate into the periplasm and bind to a high-affinity, chitin oligosaccharide-binding protein (CBP) (Fig. 3). Binding of $(GlcNAc)_n$ to CBP activates a two-component sensor kinase named ChiS (Fig. 3). Signalling by ChiS, in turn, leads to production of chitinolytic enzymes, which break down $(GlcNAc)_n$ into monomers that are channelled into the central metabolism as fructose-6-phosphate, acetate and ammonium (Li and Roseman, 2004; Hunt *et al.*, 2008). In addition to providing a source of carbon and nitrogen, chitin also influences many aspects of *Vibrio* physiology, including chemotaxis, biofilm formation and pathogenicity (Amako *et al.*, 1987; Bassler *et al.*, 1989; Watnick *et al.*, 1999; Kirn *et al.*, 2005; Reguera and Kolter, 2005; Pruzzo *et al.*, 2008; Mandel *et al.*, 2012). Consistent with these effects on physiology, growth in chitin results in significant, global changes in gene expression (Meibom *et al.*, 2004).

What is the role of chitin in activating the competence pathway in *V. cholerae*? One intriguing possibility is that chitin and its oligosaccharide derivatives signal the presence of a nearby host. Within this context, competent individuals may acquire from neighbouring bacterial cells genetic elements for host interaction. In this manner, the chitin signal would result not only in enhanced genetic diversity but also the acquisition of host specificity factors. Consistent with this model, chitin is highly abundant in zooplankton and exoskeletons of shellfishes, which are two animal reservoirs that play crucial roles in transmission of *V. cholerae* (Sack *et al.*, 2004). Another possibility is that chitin functions as a signal for *Vibrionaceae* to access an alternative nutrient source in particular environments, as previously implicated in the genetic competence-induction programme in *V. cholerae* (Meibom *et al.*, 2005). The resulting DNA uptake, in turn, may provide the *Vibrion-*

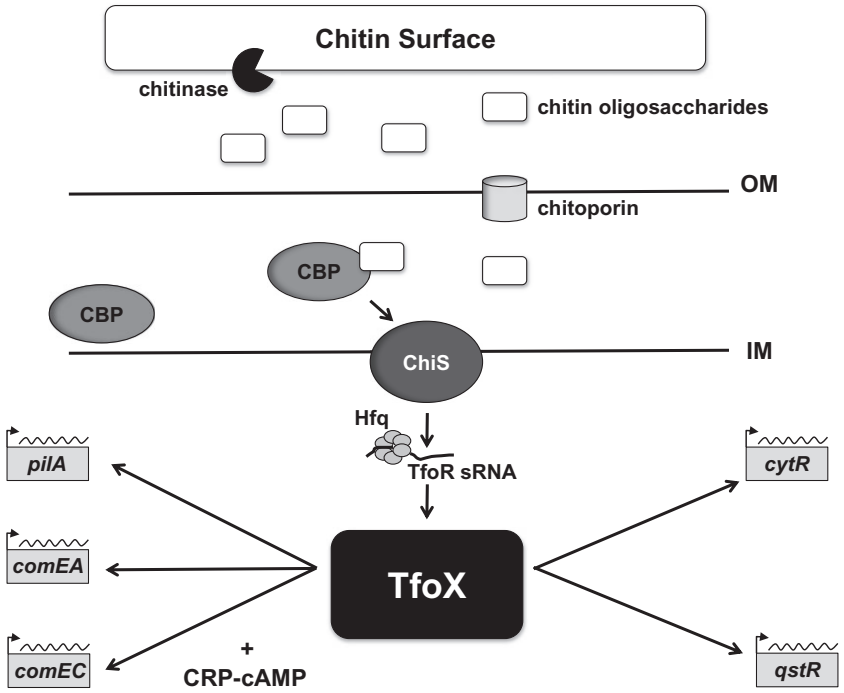

Fig. 3. Chitin-dependent signalling pathways for natural transformation in *V. cholerae*. Chitin is degraded by extracellular chitinases into oligosaccharides fragments, which enter the periplasm through a chitoporin. With the help of CBP (chitin oligosaccharides binding protein), the sensor kinase ChiS senses the presence of chitin oligosaccharides and activates the TfoR sRNA. Via the RNA chaperone Hfq, TfoR initiates translation of TfoX, the master regulator of competence in *Vibrionaceae*. Consequently, TfoX upregulates the expression of numerous competence genes (e.g. *pilA*, *comEA* and *comEC*) and of two genes encoding transcription factors (*cytR* and *qstR*). Regulation of the competence genes by TfoX also requires the cAMP–CRP complex. The lines connecting components of the signalling cascade represent positive regulation, but not direct interaction. IM, inner membrane; OM, outer membrane.

aceae members with an extra nutrient resource. Interestingly, chitin, which is crucial for natural transformation in *V. cholerae* (Meibom *et al*., 2005), is also one of the substrates on which *V. cholerae* develops biofilms (Watnick *et al*., 1999), which enable *V. cholerae* to survive stressful environments (Alam *et al*., 2007). In addition, exogenous DNA, which provides genetic material for natural transformation, is also prevalent in biofilm formation (Seper *et al*., 2011). Future studies that focus on chitin signalling in *V. cholerae* are required to elucidate its link to natural transformation.

Quorum sensing

A second regulatory system controlling competence in *V. cholerae* is quorum sensing, which is a process of cell–cell communication that allows bacteria to co-ordinate gene expression according to population density (Ng and Bassler, 2009). All *Vibrionaceae* members produce and detect chemical signalling molecules called autoinducers (AIs). *V. cholerae* produces two AIs: CAI-1, which is restricted to certain *Vibrionaceae* members, and AI-2, an interspecies autoinducer produced by many bacteria (Bassler *et al*., 1997; Zhu and Mekalanos, 2003). At low cell density, i.e. when CAI-1 and AI-2 levels are low, their unbound cognate receptors CqsS and LuxP/Q complex, respectively, behave as kinases and initiate a phosphorylation cascade via LuxU that phosphorylates the response regulator LuxO (Fig. 4). Phosphorylated LuxO activates the transcription of four small RNAs, called Qrrs (quorum regulatory RNAs), which, via the RNA chaperone Hfq, post-transcriptionally repress HapR, the master regulator of quorum sensing (Lenz *et al*., 2004; Bardill *et al*., 2011; Bardill and Hammer, 2012). In contrast, at high cell density, when CAI-1 and AI-1 levels are high, binding of the AIs to their cognate receptors reverses the phosphate flow in the LuxU–LuxO phosphorelay, resulting in production of HapR. The role of HapR in controlling virulence and surface-attachment genes important *in vivo* during association with a human host has been well described (Zhu *et al*., 2002; Hammer and Bassler, 2003).

HapR plays a crucial role in modulating chitin-induced natural competence in *V. cholerae* (Fig. 4). Deletion of *hapR*, which abolishes many phenotypes controlled by quorum sensing, also eliminates DNA uptake. Natural transformation can be restored by introducing *hapR in trans*, suggesting that HapR is required for this process (Meibom *et al*., 2005). Accordingly, both transformation frequency and *comEA* expression are affected by AI levels, with CAI-1 eliciting a stronger response than AI-2 (Antonova and Hammer, 2011; Suckow *et al*., 2011). In addition, within a mixed-species biofilm, *V. cholerae* cells can become competent in response to AIs that are produced from other *Vibrio* spp. located within the biofilm, suggesting that quorum sensing may facilitate DNA exchange among members of the genus (Antonova and Hammer, 2011). Such interspecies HGT has yet to be demonstrated under laboratory conditions, and detecting low-frequency events will probably be difficult. Interestingly, a homologue of ComP, which dictates DNA sequence

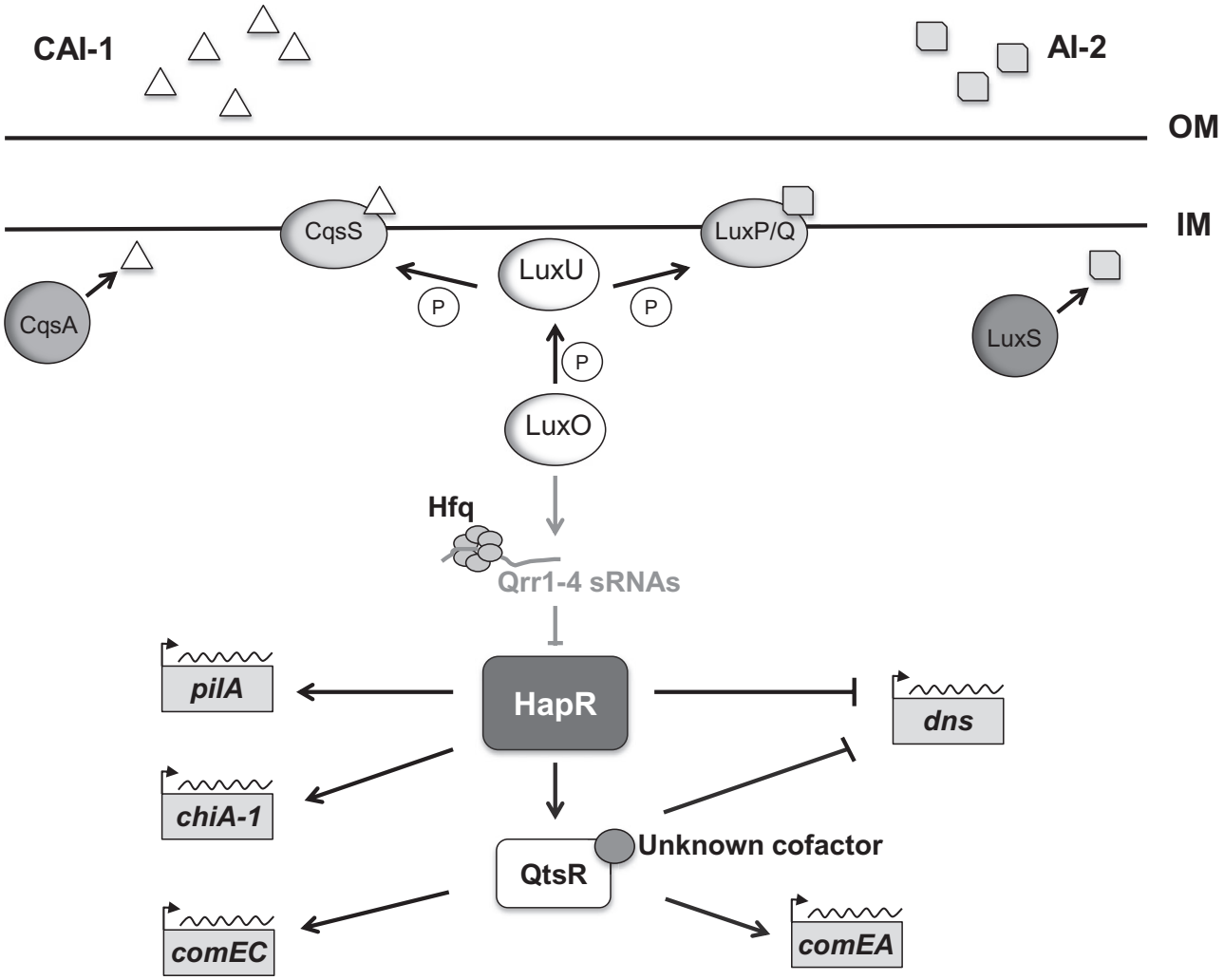

Fig. 4. Quorum sensing-dependent pathways for natural transformation in *V. cholerae*. At high cell density (depicted here), CAI-1 and AI-2 autoinducers bind to their cognate histidine kinase receptors (CqsS and LuxP/Q respectively), which shuttle phosphate from the response regulator LuxO. In the unphosphorylated form, LuxO is unable to transcribe the Qrr1–4 sRNAs (grey), and, despite the presence of the RNA chaperone Hfq, the quorum-sensing master regulator HapR is produced. HapR positively regulates transcription of *pilA* and *chiA-1* while repressing transcription of *dns*. By upregulating expression of the transcription factor QstR, HapR also indirectly exerts positive effect on transcription of *comEA* and *comEC*. An unknown cofactor was also implicated in the transcriptional regulation by QstR. The lines connecting components of the signalling cascade represent positive or negative regulation, but not direct interaction. Grey lines and factors indicate inactive pathways and factors not present at high cell density. IM, inner membrane; OM, outer membrane.

specificity in *N. meningitidis* (Cehovin *et al.*, 2013), is absent from the genomes of *Vibrio* spp. In addition, *Vibrio* spp. do not use a typical generalized DUS to recognize species-specific DNA during natural transformation, which is contrary to the case in *H. influenzae*, which also lacks a ComP homologue but still relies on DUS for sequence specificity (Suckow *et al.*, 2011; Mell *et al.*, 2012). Quorum sensing, which does not regulate competence in *Neisseria* spp., may provide *Vibrionaceae* members with a ComP/DUS-independent, yet species-specific mechanism to prevent the general uptake and genomic incorporation of exogenous DNA from unrelated bacterial species (Suckow *et al.*, 2011).

Progress has been made in elucidating the mechanism by which HapR controls competence. At high cell density, HapR repression of *dns* (Fig. 4), which encodes an extracellular nuclease (Fig. 1), is believed to allow for sufficient single-stranded DNA in the periplasm for transport into the cytoplasm (Meibom *et al.*, 2005; Blokesch and Schoolnik, 2008). Transcript abundance of *dns* is higher in a Δ*hapR* mutant than in a Δ*luxO* mutant that constitutively expresses *hapR* (Blokesch and Schoolnik, 2008; Lo Scrudato and Blokesch, 2012), and a Δ*dns* mutant is 'hyper-transformable', with transformation frequencies two orders of magnitude higher than a wild-type strain (Blokesch and Schoolnik, 2008). Therefore, it was sug-

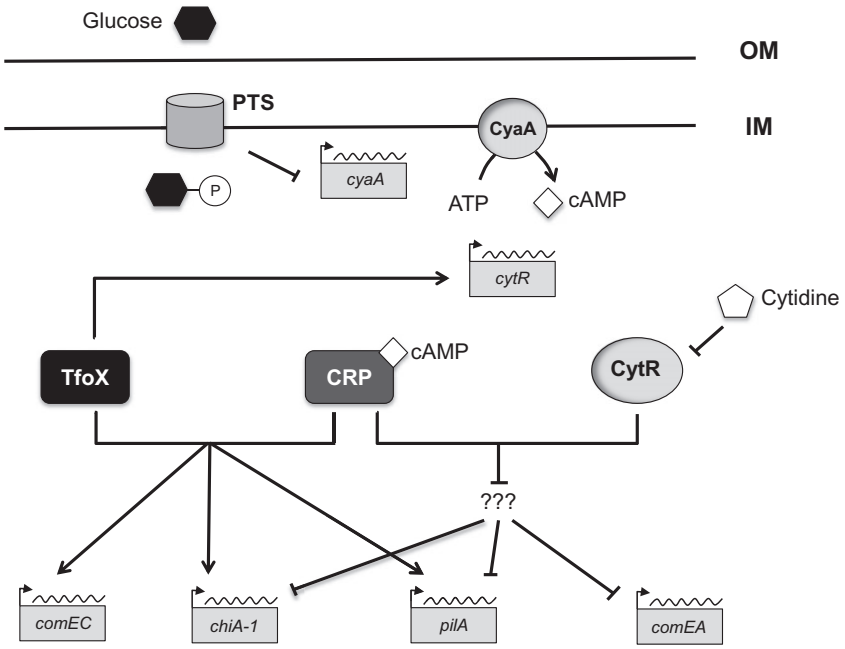

Fig. 5. Impact of external carbon-source and nucleosides on natural transformation pathways in *V. cholerae*. In the absence of a preferred carbon source, CyaA increases the intracellular concentration of cAMP. The cAMP–CRP complex interacts with TfoX to activate transcription of multiple genes involved in natural transformation (*pilA*, *comEC* and *dprA*) and in chitin metabolism (*chiA-1*). When pyrimidine levels are low, the nucleoside scavenging cytidine repressor CytR interacts with CRP to anti-activate putative repressors of *comEA*, *pilA* and *chiA-1*, resulting in upregulation of these genes. The model is over-simplified, and it remains unknown whether *comEA*, *pilA* and *chiA-1* share a common repressor that is regulated by CytR–CRP. The lines connecting components of the signalling cascade represent positive or negative regulation, but not direct interaction. IM, inner membrane; OM, outer membrane.

gested that the non-transformability of a Δ*hapR* mutant is partly due to the failure to repress *dns*, causing constant degradation of extracellular DNA. Consistent with this model, the transformation frequency of a Δ*hapR* mutant can be restored to wild-type levels by deleting *dns* (Blokesch and Schoolnik, 2008). Finally, HapR appears to directly bind to the *dns* promoter to prevent transcription (Lo Scrudato and Blokesch, 2013), despite the lack of a conventional HapR binding site (Tsou *et al*., 2009). These studies suggest that quorum signalling permits Dns-mediated degradation of extracellular DNA at low cell density but represses Dns at high cell density, thereby preserving DNA for natural transformation.

HapR also positively regulates expression of *comEA* and *comEC* (Fig. 4), which encode components of the competence machinery important for DNA uptake (Fig. 1, Lo Scrudato and Blokesch, 2013). However, regulation of these genes by HapR appears indirect, as HapR does not directly bind to the promoters of *comEA* or *comEC* (Lo Scrudato and Blokesch, 2013). Instead, a LuxR-type transcription regulator named QstR, whose expression requires HapR (Fig. 4), was recently reported to be involved in the intermediate step (Lo Scrudato and Blokesch, 2013).

Additional evidence indicates that HapR also activates *pilA* and *chiA-1*, which encode a component of the pseudopilus and a chitinase respectively (Fig. 4, Meibom *et al*., 2005; Antonova *et al*., 2012). A Δ*hapR* mutant shows significantly decreased expression of *pilA* and *chiA-1* when compared with the wild-type strain C6706 (Antonova *et al*., 2012). These results are similar to HapR control of *comEC* and *comEA*, further highlighting the important role that HapR plays in regulating competence in strain C6706. Surprisingly, similar studies of A1552, another pathogenic isolate of *V. cholerae*, show HapR-independent expression of these genes (Lo Scrudato and Blokesch, 2013). Studies of additional *V. cholerae* clinical and environmental isolates are required to determine the general role of HapR and quorum sensing in natural transformation. Since HapR also regulates many target genes via synthesis of the intracellular second messenger cyclic-di-GMP (Galperin, 2004; Waters *et al*., 2008), the contribution of HapR to natural competence likely includes direct and indirect control of competence genes.

Availability of carbon sources

Extracellular DNA taken up by bacteria may also be used as a food source during times of nutrient starvation (Redfield, 1993; Finkel and Kolter, 2001; Palchevskiy and Finkel, 2009). When resources are abundant, bacteria, like *Escherichia coli*, utilize preferred carbon sources such as glucose while repressing genes involved in catabolism of less favoured substrates. However, when preferred carbon sources are unavailable, genes are expressed to internalize and metabolize alternative carbon sources. Carbon catabolite repression (CCR) controls carbon source utilization in this manner. CCR in Gram-negative bacteria is mediated by the secondary messenger cyclic adenosine monophosphate (cAMP) and the transcriptional activator cAMP receptor protein (CRP). When glucose is absent, intracellular concentrations of cAMP increase (Fig. 5, Bruckner and Titgemeyer, 2002; Deutscher, 2008). cAMP allosterically binds to CRP, and the resulting CRP–cAMP

complex binds to and activates promoters by recruiting RNA polymerase (Tagami and Aiba, 1998; Busby and Ebright, 1999). In addition to controlling expression of genes involved in catabolism of sugar substrates, CCR also regulates genes important for virulence in many pathogens such as *S. pneumoniae*, *Salmonella enterica*, *Staphylococcus aureus* and *V. cholerae* (Skorupski and Taylor, 1997; Iyer *et al*., 2005; Seidl *et al*., 2006; Teplitski *et al*., 2006).

A role of CCR in inducing natural competence in *V. cholerae* was demonstrated by the inhibition of natural transformation by glucose (Fig. 5, Meibom *et al*., 2005). A separate study showed that a Δ*crp* mutant, as well as a Δ*cya* mutant that was incapable of synthesizing cAMP, were recalcitrant to transformation either on chitinaceous surfaces or in liquid culture (Blokesch, 2012), which was consistent with previous reports in *H. influenzae* (Chandler, 1992; Dorocicz *et al*., 1993). The Δ*cya* and Δ*crp* mutations also significantly decreased expression of competence genes *pilA*, *pilM* and *comEA*, albeit not completely abolishing the expression of these genes like in *H. influenzae* and *E. coli* (Blokesch, 2012; Cameron and Redfield, 2008). On the other hand, natural transformation increased in a mutant unable to degrade intercellular cAMP, which provides strong evidence that CCR is involved in controlling numerous competence genes (Blokesch, 2012; Lo Scrudato and Blokesch, 2012). Interestingly, CRP mutants have also been shown to repress *hapR* expression by reducing CAI-1 synthesis (Liang *et al*., 2008), thus indirectly modulating natural competence via quorum sensing as well.

Extracellular nucleosides

The intricate response to nutrient starvation mediated by CRP typically involves other regulatory factors that enable bacteria to utilize not only various sugars but also nucleic acids. In *E. coli*, many genes responsible for scavenging and metabolizing extracellular pyrimidine nucleosides are negatively regulated by the cytidine repressor CytR (Fig. 5, Valentin-Hansen *et al*., 1996). When free nucleosides are scarce, CytR binds with CRP and acts as an 'anti-activator' of the promoters of specific CRP-activated genes involved in nucleoside transport and breakdown. However, in the presence of pyrimidines, cytidine enters the cell and binds allosterically to CytR. Conformational changes in CytR when in complex with cytidine hamper association with CRP and, as a result, eliminate CytR-dependent anti-activation of CRP-regulated genes. CRP can then activate expression of these promoters by recruiting RNA polymerase (Barbier *et al*., 1997). Thus, genes required for nucleoside uptake and metabolism are only expressed in the presence of the nucleosides themselves.

Availability of nucleotides such as purine was also shown to play an important role in regulating natural competence in *H. influenzae* (MacFadyen *et al*., 2001; Sinha *et al*., 2013). However, the *H. influenzae* genome lacks a CytR homologue, and a functionally similar protein, PurR, was found not to be responsible for purine-mediated repression of competence (Sinha *et al*., 2013). Interestingly, *cytR* was originally identified as a *V. cholerae* gene regulated by TfoX (Fig. 5, Meibom *et al*., 2005). However, its specific role in natural competence was revealed only recently, when a Δ*cytR* mutant was shown to be non-transformable and displayed decreased expression of *comEA*, *chiA-1* and *pilA* (Fig. 5, Antonova *et al*., 2012). The presence of nucleosides, namely cytidine, also inhibited both transformation and *comEA* expression directly, suggesting a regulatory mechanism similar to that proposed in *H. influenzae* (Antonova *et al*., 2012). Mutants containing CytR variants unable to bind to CRP were impaired for transformation, suggesting that the CytR–CRP protein–protein interactions important for nucleoside scavenging in *E. coli* are also vital to activating natural competence genes in *V. cholerae* (Antonova *et al*., 2012). Although the exact role of nucleotide scavenging in natural competence has yet to be determined, these results provide evidence that natural competence in the *Vibrionaceae* may be used not only for HGT but also for nutrient acquisition.

TfoX: an integral regulator of natural transformation

The *tfoX* gene was originally identified among other *V. cholerae* genes that display elevated expression levels in response to chitin (Meibom *et al*., 2004). TfoX is required for chitin-dependent natural transformation in *V. cholerae* (Meibom *et al*., 2005). When constitutively expressed, TfoX can induce genetic competence in the absence of chitin (Meibom *et al*., 2005). A homologue of TfoX named Sxy plays a central role in controlling genetic competence in *H. influenzae* (Williams *et al*., 1994; Zulty and Barcak, 1995; Redfield *et al*., 2005), *Aggregatibacter actinomycetemcomitans* (Bhattacharjee *et al*., 2007), *Actinobacillus pleuropneumoniae* (Bossé *et al*., 2009) and *Actinobacillus suis* (Sinha *et al*., 2013). *E. coli* also possesses a homologue of Sxy, but expression of *E. coli* Sxy is insufficient to induce natural transformation in this organism, despite activating expression of homologues of competence genes that are used by other bacterial species (Cameron and Redfield, 2006; Sinha *et al*., 2009; Sinha and Redfield, 2012).

Chitin, in the form of either crab shells or oligosaccharides, enhances the production of TfoX, at both transcriptional and post-transcriptional levels (Fig. 3) (Yamamoto *et al*., 2010). Genetic studies in *V. cholerae* have shown that chitin is sensed by the hybrid sensor kinase ChiS,

which in turn promotes production of a small regulatory RNA named TfoR (Fig. 3, Li and Roseman, 2004; Meibom *et al*., 2004; Yamamoto *et al*., 2010; Lo Scrudato and Blokesch, 2012). The signalling pathway initiated by ChiS that leads to *tfoR* expression remains unclear. TfoR is predicted to base pair with the 5′-untranslated region (5′-UTR) of the *tfoX* transcript, thus preventing formation of an inhibitory secondary structure that sequesters the predicted Shine–Dalgarno sequence for *tfoX* (Fig. 3, Yamamoto *et al*., 2011). Translational activation of *tfoX* by TfoR also requires the RNA chaperone Hfq (Fig. 3), which is often a crucial player in *trans*-encoded small RNA-mediated regulation (Vogel and Luisi, 2011; Yamamoto *et al*., 2011). A transcriptional repressor element was also identified within the *tfoX* promoter in *V. cholerae* (Yamamoto *et al*., 2010). However, in contrast to the strong translational regulation described above, the transcriptional derepression of *tfoX* by chitin and $(GlcNAc)_n$ is moderate, and the transcription factor involved in this process remains unknown (Yamamoto *et al*., 2010). In *H. influenzae*, which does not possess a TfoR homologue, transcription of *sxy* is strongly induced by the CRP–cAMP complex (Zulty and Barcak, 1995). Whether CRP–cAMP affects *tfoX* transcription in *V. cholerae* remains unknown at this time. Future studies of *tfoX* regulation will greatly help our understanding of how the *Vibrionaceae* process the external chitin signal to become genetically competent.

TfoX activates the expression of many genes involved in chitin degradation and chitin-induced competence in *V. cholerae* (Meibom *et al*., 2005). Members of the chitin-dependent TfoX regulon include the *pilQ*, *pilA*, *comEA* and *comEC* genes (Fig. 3), whose gene products play essential roles in the uptake and transport of exogenous DNA (Fig. 1, Fullner and Mekalanos, 1999; Meibom *et al*., 2004; Chen *et al*., 2005; Hamilton and Dillard, 2006; Claverys *et al*., 2009; Lo Scrudato and Blokesch, 2012). In addition, expression of a gene encoding a homologue of DprA, which plays a crucial role in the integration of exogenous DNA, was reported to be elevated in response to induction of *tfoX* expression (Lo Scrudato and Blokesch, 2012). In *H. influenzae*, Sxy is proposed to direct the CRP–cAMP complex to a non-canonical CRP (CRP-S) site (TGCGA-N_6-TCGCA) within the promoter regions of competence genes (Cameron and Redfield, 2006; Cameron and Redfield, 2008). These studies suggested that Sxy may serve as an accessory factor to CRP, rather than a primary transcription factor, in promoting expression of competence genes (Redfield *et al*., 2005; Cameron and Redfield, 2006; Sinha *et al*., 2012). This model is consistent with the lack of an obvious DNA-binding domain in Sxy. The demonstration that cAMP, presumably in complex formation with CRP, is required for chitin-dependent induction of *comEA* and *pilA* in *V. cholerae* further highlights that TfoX may also serve as an accessory factor (Fig. 4, Lo Scrudato and Blokesch, 2012). Consistent with this hypothesis, potential CRP-S sites are located within the promoter regions of *comEA* and *pilA* (Antonova *et al*., 2012). A common motif 5′-ACTCG(A/C)AA was identified in most of the 19 TfoX-induced promoter elements by means of a phylogenetic footprint analysis (Cameron and Redfield, 2006). At this time, whether TfoX binds DNA in complex with CRP has not yet been directly examined by experimental approaches.

Recently, TfoX was shown to positively regulate the expression of two transcription factors: CytR and QstR (Fig. 3, Meibom *et al*., 2005; Lo Scrudato and Blokesch, 2013). As described in the previous section, CytR is the nucleoside scavenging cytidine repressor that represses expression of *comEA*, *pilA* and *chiA-1* in *V. cholerae* (Fig. 5, Antonova *et al*., 2012). TfoX also activates transcription of *qstR* (Fig. 3), which encodes a transcription factor essential for *comEA* and *comEC* expression (Fig. 4, Lo Scrudato and Blokesch, 2013). Activation of QstR also requires the major regulator of quorum sensing, HapR (Fig. 4, Lo Scrudato and Blokesch, 2013), which accounts for the subset of TfoX-regulated genes that are also controlled by quorum sensing (Lo Scrudato and Blokesch, 2012). Together, these studies underscore the complex roles of TfoX in co-ordinating expression of the genes required for competence in *V. cholerae*.

Natural transformation in other *Vibrio* species

Recently, homologues of TfoX were identified in all sequenced *Vibrionaceae* members (Pollack-Berti *et al*., 2010). This observation, combined with the highly conserved ability of *Vibrionaceae* members to utilize chitin as a nutrient (Hunt *et al*., 2008), strongly support the notion that chitin-induced natural transformation is a shared trait among the *Vibrionaceae*. While the presence of chitin and chitin-derived oligosaccharides does induce natural competence in various *Vibrio* spp., differences between these organisms have been detected and are highlighted here.

Vibrio fischeri

Vibrio fischeri is a bioluminescent bacterium commonly found in free-living form within marine environments, as well as in symbiotic relationships with marine animals, such as the Hawaiian bobtail squid *Euprymna scolopes* (Visick and Ruby, 2006; Miyashiro and Ruby, 2012). When grown in the presence of chitohexaose $(GlcNAc)_6$, *V. fischeri* can incorporate exogenous DNA into its chromosome via natural transformation (Pollack-Berti *et al*., 2010). *V. fischeri* also possesses *tfoX*, which is a homologue of the *V. cholerae tfoX* gene and similarly required for the $(GlcNAc)_6$-dependent natural transformation in *V. fischeri*.

When multiple copies of *tfoX* are present (i.e. on a plasmid), *tfoX* is capable of inducing genetic competence in *V. fischeri* independently of $(GlcNAc)_6$ (Pollack-Berti *et al.*, 2010). A paralogue of *tfoX* named *tfoY* was also identified in the *V. fischeri* genome and shown to play a role in $(GlcNAc)_6$-dependent natural transformation. Intriguingly, *tfoX in trans* restores $(GlcNAc)_6$-induced genetic competence in a Δ*tfoY* mutant, while *tfoY* in multi-copy fails to compensate for the loss of *tfoX* or result in $(GlcNAc)_6$-dependent transformation, suggesting functional differences between the two homologues (Pollack-Berti *et al.*, 2010). In the simplest model to explain these observations, TfoY serves to positively regulate TfoX in a pathway of chitin-induced natural competence. Since TfoY orthologues were also identified in other *Vibrio* spp., including *V. cholerae* (Pollack-Berti *et al.*, 2010), studying the regulation of *tfoY* expression in response to chitin as well as its functions in chitin-induced natural competence will add valuable insights to our understanding of the gene transfer processes.

Remarkably, polymeric chitin in the form of crab-shell tiles, which can induce natural competence in *V. cholerae* (Meibom *et al.*, 2005; Antonova and Hammer, 2011; Antonova *et al.*, 2012), fails to do so in *V. fischeri* (Pollack-Berti *et al.*, 2010). Even in the presence of $(GlcNAc)_6$, *V. fischeri* exhibits significantly lower levels of natural transformation than *V. cholerae* (Pollack-Berti *et al.*, 2010). The underlying causes of these discrepancies are unknown. Perhaps in *V. fischeri*, the chitin-dependent competence cascade (e.g. production or activities of TfoX) is under more stringent regulatory control. Alternatively, *V. fischeri* may have higher extracellular DNase activity than *V. cholerae*. These scenarios may not be mutually exclusive and additional investigation is required.

Recently, in *V. fischeri*, the *N*-acetyl-D-glucosamine (GlcNAc) transcriptional repressor NagC was shown to negatively regulate the expression of numerous chitin- and GlcNAc-utilization genes, including *VF_2139*, which is predicted to encode the chitin oligosaccharide-binding protein (CBP) (Miyashrio *et al.*, 2011). CBP was proposed to play an essential part in the ChiS-mediated chitin-sensing pathways in *V. cholerae*, which eventually leads to chitin-dependent natural transformation (Li and Roseman, 2004; Meibom *et al.*, 2005). It is therefore conceivable that NagC may also be involved in regulating genetic competence in *V. fischeri*. On the other hand, NagC-mediated gene regulation responds to extracellular GlcNAc (and its phosphorylated form *N*-acetyl-D-glucosamine-6-phosphate) (Miyashrio *et al.*, 2011), which does not induce natural competence in *V. fischeri* or *V. cholerae* (Pollack-Berti *et al.*, 2010; Yamamoto *et al.*, 2010). Taken together, these observations suggest that any potential role of NagC in chitin-responsive natural transformation in *V. fischeri* is likely to be complex. Intriguingly, chitin oligosaccharides produced by *E. scolopes* also serve as chemotactic signals for *V. fischeri* to facilitate host colonization (Mandel *et al.*, 2012). Whether the level of chitin oligosaccharides encountered by *V. fischeri* cells during colonization is sufficient to induce the natural competence pathway has yet to be determined.

Vibrio vulnificus

Vibrio vulnificus is another member of the *Vibrionaceae* family present in marine environments, such as estuaries, and is an opportunistic human pathogen that causes primary septicaemia and wound infection (Jones and Oliver, 2009). Like *V. cholerae*, *V. vulnificus* becomes naturally competent while growing on chitin and can both take up and incorporate exogenous DNA (Gulig *et al.*, 2009). Chitin disaccharides $(GlcNAc)_2$, but not GlcNAc, can also induce natural competence in *V. vulnificus* (Neiman *et al.*, 2011). The frequency of chitin-induced transformation varies among different strains. Interestingly, transformation frequency can be significantly improved by exposing biofilms comprised of different isolates to strain-specific lytic phage, which presumably leads to increased amount of extracellular DNA from cell lysis (Neiman *et al.*, 2011). In this study, *V. vulnificus*, when exposed to chitin, was able to take up and incorporate within its chromosome exogenous genomic DNA containing a complete *cps* loci that encodes capsular polysaccharide, which is a virulence factor. As a result of this transformation, the tested *V. vulnificus* strain underwent carbotype (capsule type) conversion. Diversity of carbotypes among *V. vulnificus* strains is thought to help this organism evade a host's immune system. Based on these results, it was proposed that chitin-induced transformation could potentially play an important role in the evolution of *V. vulnificus* (Neiman *et al.*, 2011).

Vibrio parahaemolyticus

Vibrio parahaemolyticus is a halophilic bacterium found in brackish seawater and can cause gastrointestinal illness in humans (Chen *et al.*, 2011). In 1990, a *V. parahaemolyticus* strain was experimentally demonstrated to pick up exogenous plasmid DNA (Frischer *et al.*, 1990). Furthermore, a more recent study reported that chitin (in the form of crab shells) enables *V. parahaemolyticus* to pick up and incorporate exogenous linear DNA, which has been adapted as a genetic tool to generate specific mutations (Chen *et al.*, 2010).

Environmental and clinical implications

Although still under intense debate, numerous studies suggest that bacteria, including *Vibrionaceae* members,

have evolved the competence pathway to aid in three major processes: nutrition, DNA repair and horizontal gene transfer (HGT) (Redfield, 1993; Solomon and Grossman, 1996; Dubnau, 1999). A particularly popular model is that natural transformation functions as a common mechanism for HGT (Paul *et al*., 1991; Dubnau, 1999). Natural competence is linked to enhanced genetic diversity, improved fitness and, in some cases, increased virulence in *Vibrio* spp. Due to the abundance of the *Vibrionaceae* in a wide range of aquatic ecosystems, and their close association with various marine and freshwater plants and animals, natural competence in the *Vibrionaceae* is expected to have significant environmental, ecological and clinical implications. A previous study designed to mimic aquatic reservoirs demonstrated that, through chitin-induced natural transformation, a *V. cholerae* strain can acquire a gene cluster (Blokesch and Schoolnik, 2007), which effectively converts the recipient into a different serogroup (Bik *et al*., 1995; Mooi and Bik, 1997) that is known for its heightened fitness, virulence and central role in the 1992 cholera epidemic (Albert *et al*., 1997). *V. cholerae* strains lacking the cholera toxin (*ctxAB*) genes required to cause cholera, can also acquire DNA encoding *ctxAB* via chitin-induced natural competence (Udden *et al*., 2008). The potential involvement of chitin-induced natural transformation in enhancing virulence was also shown in *V. vulnificus* (Neiman *et al*., 2011). In the latter two cases (*V. cholerae* acquiring cholera toxin genes and *V. vulnificus* obtaining virulence genes), presence of the species and strain-specific lytic phages greatly enhanced transformation frequencies. In particular, exposure of the *Vibrio* cultures to DNase exhibited negative effects on the transformation efficiency (Udden *et al*., 2008; Neiman *et al*., 2011). These results suggest that phage could release cellular DNA that could contribute to natural transformation. Such bacteriolytic mechanisms are used by other naturally competent bacterial species, such *S. pneumoniae* and *B. subtilis*, in the provision of donor DNA (Claverys and Håvarstein, 2007; Wei and Håvarstein, 2012). In contrast, *N. gonorrhoeae* uses a type IV secretion system encoded in the gonococcal genetic island to secret DNA outside the cells, and mutation in this system reduces the ability of a *N. gonorrhoeae* strain to act as a donor in transformation. Together, these studies highlight the various strategies employed by bacteria to acquire DNA from exogenous sources.

Importantly, the impact of natural transformation is unlikely to be solely limited to enhancing the overall pathogenicity of *Vibrio* spp. For instance, chitin-induced natural transformation was shown to facilitate the transfer of gene clusters encoding different metabolic functions (mannose and diglucosamine utilization) (Miller *et al*., 2007). Since *Vibrionaceae* members are well known for their contribution in the carbon and nitrogen cycle (Criminger *et al*., 2007; Anorsti, 2011), mutations in metabolic genes as a result of natural transformation could affect the overall metabolite levels in the aquatic environments.

Outlook

Natural competence in *Vibrionaceae* and its ecological and clinical impact have been attracting increasing amount of interest in the scientific community in the recent years. There are still many unanswered questions regarding the underlying molecular mechanisms of natural competence, as well as the physiological (both short-term and long-term) ramifications of taking up exogenous DNA. As more environmental and genetic factors controlling the regulation of natural competence have been uncovered, it will certainly improve our understanding of this important biological phenomenon and provide valuable insight in the microbial adaptation to environmental cues.

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