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REVIEW ARTICLE

Cues and regulatory pathways involved in natural competence and transformation in pathogenic and environmental Gram-negative bacteria

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Abstract

Bacterial genomics is flourishing, as whole-genome sequencing has become affordable, readily available and rapid. As a result, it has become clear how frequently horizontal gene transfer (HGT) occurs in bacteria. The potential implications are highly significant because HGT contributes to several processes, including the spread of antibiotic-resistance cassettes, the distribution of toxin-encoding phages and the transfer of pathogenicity islands. Three modes of HGT are recognized in bacteria: conjugation, transduction and natural transformation. In contrast to the first two mechanisms, natural competence for transformation does not rely on mobile genetic elements but is driven solely by a developmental programme in the acceptor bacterium. Once the bacterium becomes competent, it is able to take up DNA from the environment and to incorporate the newly acquired DNA into its own chromosome. The initiation and duration of competence differ significantly among bacteria. In this review, we outline the latest data on representative naturally transformable Gram-negative bacteria and how their competence windows differ. We also summarize how environmental cues contribute to the initiation of competence in a subset of naturally transformable Gram-negative bacteria and how the complexity of the niche might dictate the fine-tuning of the competence window.

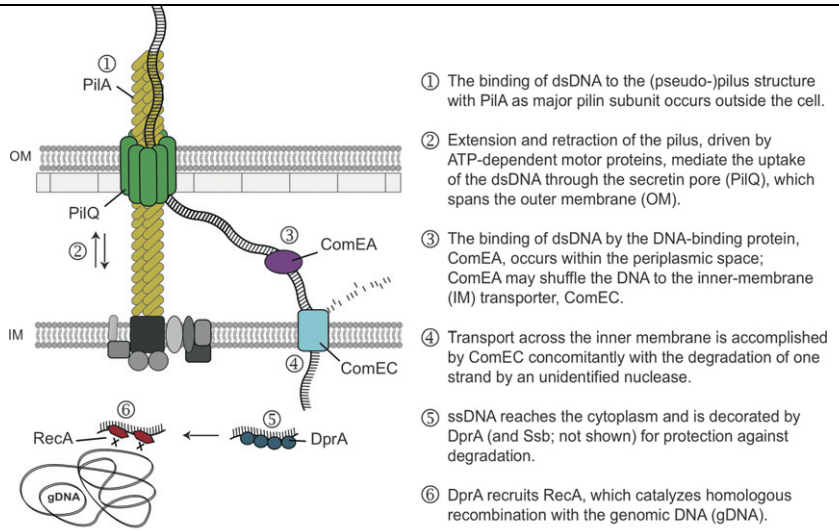
Introduction

In 2011, the spread of food-borne gastroenteritis in Europe, most notably in Germany, was widely reported. The pathogen caused severe bloody diarrhoea, often accompanied by haemolytic uremic syndrome. The causative agent of the outbreak was *Escherichia coli* serotype O104:H4. The strain was unique in that it combined the 'potentials of two different pathogens: Shiga-toxin-producing *E. coli* and enteroaggregative *E. coli*' (Bielaszewska *et al.*, 2011). Multiple isolates of this outbreak were fully sequenced for molecular epidemiological analysis (Rasko *et al.*, 2011; Grad *et al.*, 2012). Such whole-genome sequencing (WGS) studies are extremely helpful in analysing single-nucleotide polymorphisms (SNPs) and allow us to understand patterns of genetic diversity, which can help in the identification of transmission routes. Furthermore, WGS sheds light on the acquisition of horizontally acquired genetic elements. Horizontal gene transfer (HGT) is important in rendering harmless bacteria into major human pathogens, as exemplified by the commensal bacterium *E. coli* mentioned above. Indeed, Rasko *et al.* (2011) concluded from their WGS findings that 'horizontal genetic exchange allowed for the emergence of the highly virulent Shiga-toxin–producing enteroaggregative *E. coli* O104:H4 strain that caused the German outbreak'. Recent reviews on this topic concluded that there is substantial evidence that HGT contributes to the acquisition, integration and maintenance of pathogenicity islands (PAIs) in bacterial genomes (Hacker *et al.*, 1997, 2003; Dobrindt *et al.*, 2004, 2010; Ahmed *et al.*, 2008). In general, it is widely accepted that HGT significantly impacts the evolution of bacterial genomes and their adaptation to new environments (a nonexhaustive list of reviews on this topic published within this century would be Eisen, 2000; Ochman *et al.*, 2000; Boucher *et al.*, 2001;

Koonin *et al.*, 2001; Boucher *et al.*, 2003; Thomas & Nielsen, 2005; Davidsen *et al.*, 2007; Cohan & Koeppel, 2008; Koonin & Wolf, 2008; Ambur *et al.*, 2009; Popa & Dagan, 2011; Stokes & Gillings, 2011; Wiedenbeck & Cohan, 2011).

HGT in bacteria occurs through three main mechanisms: transduction, conjugation, and natural transformation. The latter mechanism is based on the uptake of free DNA from the environment and therefore does not rely on mobile genetic elements such as phages and plasmids; instead, it is solely encoded by the acceptor bacterium. Natural competence is the developmental state of the bacterium in which it is able to take up external DNA and to recombine this DNA into the chromosome, thereby undergoing natural transformation. Natural competence and transformation are common to a wide variety of bacterial species and have been extensively reviewed (Lorenz & Wackernagel, 1994; Dubnau, 1999; Chen & Dubnau, 2004). The composition and dynamics of the DNA-uptake complexes (Box 1) have also been the focus of several excellent recent reviews (Averhoff & Friedrich, 2003; Allemand & Maier, 2009; Claverys *et al.*, 2009; Burton & Dubnau, 2010; Allemand *et al.*, 2012). However, less is known about the initiation of competence, particularly in Gram-negative bacteria. It has become clear in recent years that there are major differences in the regulatory network involved in competence induction in Gram-negative bacteria. Thus, the present review aims to provide an overview of how environmental signals drive natural competence and transformation in these organisms. Differences and similarities in the conditions that induce competence in Gram-positive bacteria (a topic that has been recently reviewed by Claverys *et al.*, 2006) are highlighted throughout the text.

Finally, although this review emphasizes on pathogens (with or without a niche outside of humans), a plethora of nonpathogenic bacteria are known to be naturally transformable. The rationale behind our focus on pathogens is based on two factors: (1) Research on HGT has long been influenced by medically relevant questions, such as how virulence factors are acquired by pathogens and how antibiotic-resistance genes spread among microorganisms. In fact, the discovery of natural transformation in bacteria was based on the seminal work by Frederick Griffith. Griffith was interested in understanding the difference between virulent and nonvirulent strains of *Streptococcus pneumoniae* and how such strains can interconvert (Griffith, 1928). A more recent example would be the opportunistic pathogen *Porphyromonas gingivalis*. *Porphyromonas gingivalis* belongs to the phylum bacteroidetes and contributes to periodontal disease upon stable colonization of the human oral cavity. Recent data demonstrated that natural competence and transformation is the major driving force behind DNA exchange in this organism (Tribble *et al.*, 2012). The resulting genetic variability is most likely the reason for *P. gingivalis*' survival in the human host and its evasion from human immune defences (Tribble *et al.*, 2012). (2) The second reason that pathogenic bacteria are sometimes considered more appropriate for the study of HGT is the somewhat biased sequencing effort. Whereas often only one or very few whole-genome sequences of nonpathogenic bacteria are available, a trend towards sequencing many different



The model is simplified for the sake of clarity and is based on homology to predicted components that might be involved in DNA uptake in other naturally competent bacteria, as recently reviewed for Gram-positive and Gram-negative bacteria (Chen & Dubnau, 2004, Allemand & Maier, 2009, Claverys et al., 2009, Burton & Dubnau, 2010, Allemand et al., 2012).

Box 1 . A hypothetical mode for DNA update in *V. cholerae*

isolates of human pathogens has recently emerged (Tettelin *et al.*, 2005; Croucher *et al.*, 2011; Mutreja *et al.*, 2011). The use of information from multiple isolates facilitates the identification of horizontally acquired regions and the underlying regulatory circuits driving HGT.

Caught in competence – constitutively competent Gram-negative bacteria

Helicobacter pylori responds to DNA damage

Helicobacter pylori is an extremely successful human pathogen. This bacterium colonizes the gastric epithelia of more than half of the world's population (Suerbaum & Michetti, 2002) and has probably done so since humans migrated out of Africa roughly 60 000 years ago (Falush *et al.*, 2003; Linz *et al.*, 2007). *Helicobacter pylori* is also one of the most diverse bacterial species (Achtman *et al.*, 1999). This bacterium's diversity has been studied for almost two decades, and it is clear that every person infected by *H. pylori* carries a unique strain (Langenberg *et al.*, 1986; Majewski & Goodwin, 1988; Achtman *et al.*, 1999). Extremely frequent recombination events are key to this extraordinary genetic diversity (Suerbaum *et al.*, 1998; Kersulyte *et al.*, 1999; Falush *et al.*, 2001; Morelli *et al.*, 2010). This high recombination frequency and frequent horizontal flow of genetic material in *H. pylori* is directly linked with its ability to undergo natural transformation, the topic of this review. However, in terms of natural competence, *H. pylori* differs from other Gram-negative bacteria in two important respects: the mechanism of the DNA-uptake process and the constitutive competence state of the bacterium.

Most naturally competent bacteria rely on type IV pilus-related DNA-uptake machineries (Hobbs & Mattick, 1993 and for recent review see Chen & Dubnau, 2004; Allemand & Maier, 2009; Burton & Dubnau, 2010; Box 1). In *H. pylori,* however, the components involved in the translocation of transforming DNA resemble type IV secretion systems (T4SS; Hofreuter *et al.*, 1998, 2000; Karnholz *et al.*, 2006), such as the archetypical VirB/VirD4 system of *Agrobacterium tumefaciens* (for recent review see Alvarez-Martinez & Christie, 2009). Indeed, many bacteria use similar systems to export both protein and DNA substrates. In addition to this competence-related T4SS, known as the *comB* system, many *H. pylori* strains harbour a 'classical' T4SS encoded on a PAI. This system is used to inject the virulence factor CagA into gastric epithelial cells (Odenbreit *et al.*, 2000). These two T4SS are independent of each other. Moreover, the Cag-PAI is fully dispensable in natural transformation (Hofreuter *et al.*, 2000; Israel *et al.*, 2000). Interestingly enough, another naturally competent ε-proteobacterium, *Campylobacter jejuni*, does not seem to share this dependency on a transformation-related T4SS; instead DNA uptake and transformation is dependent on genes encoding components of a putative type II secretion or type IV pilus-like system (Wiesner *et al.*, 2003). More information on natural competence of *C. jejuni* has been recently reviewed by Young *et al.* (2007). And although DNA uptake in *H. pylori* is exceptional, recent studies indicate that certain steps might be conserved in DNA transport. Whereas the uptake of DNA into the periplasm by a T4SS is specific to *H. pylori*, transport across the inner membrane involves homologous proteins found in both naturally competent Gram-negative and Gram-positive bacteria (Stingl *et al.*, 2010; Box 1).

DNA uptake is not the only 'exception that proves the rule' in *H. pylori*. An even more striking difference to most other naturally transformable bacteria occurs at the regulatory level of competence. In contrast to those bacteria, *H. pylori* does not limit its competence window in response to environmental stimuli. Instead, this bacterium is naturally competent during all growth phases (Israel *et al.*, 2000; Baltrus & Guillemin, 2006). Because transformation frequencies specifically peak within certain growth phases during *in vitro* growth (Baltrus & Guillemin, 2006), a regulatory system must exist to at least fine-tune DNA uptake. The mediators involved in this process remain unknown. The only stimulus experimentally proven to increase natural transformation in *H. pylori* is

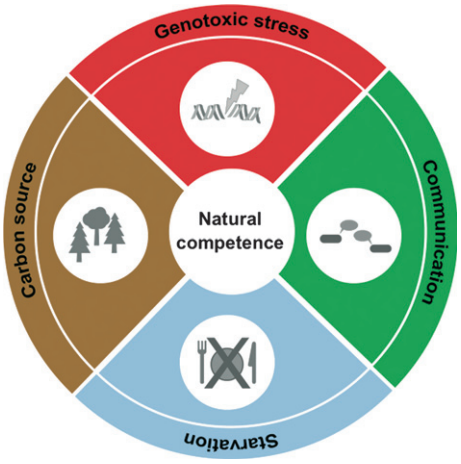


Fig. 1. The most prominent environmental cues involved in competence induction or the fine-tuning of competence. These signals include genotoxic stresses causing DNA damage, such as UV light or certain antibiotics (in red); bacterial cell-cell communication systems (e.g. quorum-sensing; in green); the starvation of preferred carbon sources, leading to the accumulation of the intracellular secondary messenger cAMP (carbon catabolite repression; in blue); and the presence or absence of certain carbon sources (such as the GlcNAc polymer chitin for *Vibrio cholerae*). Details are provided in the text.

DNA damage (Dorer *et al.*, 2010; Fig. 1). Indeed, Dorer *et al.* (2010) showed that mutations in a DNA repair protein, as well as treatment with the DNA-damaging agent ciprofloxacin, led to the upregulation of transcription of many genes involved in DNA uptake and to increased recombination. Consequently, transformation frequencies increased four to fivefold. Among the upregulated genes was a homologue of a T4 phage-derived lysozyme that had previously been characterized for its lytic activity (Marsich *et al.*, 2002). Therefore, DNA damage might lead not only to increased DNA uptake but also to the enhanced lysis of neighbouring cells and ultimately to high levels of natural transformation (for recent review see Dorer *et al.*, 2011). The induction of competence upon DNA damage, coupled with fratricide, has also been observed in the Gram-positive bacterium *S. pneumoniae* (Guiral *et al.*, 2005; Havarstein *et al.*, 2006; Prudhomme *et al.*, 2006). Although the two bacterial species are only distantly related, they share another characteristic: *S. pneumoniae* and *H. pylori* both lack a bona fide SOS response system. Competence induction in response to DNA damage might thus represent a product of convergent evolution in distantly related bacteria that lack an SOS response system (Dorer *et al.*, 2010). This same condition holds for the Gram-negative pathogen *Legionella pneumophila*. In this organism natural competence is also induced upon antibiotics- and UV-induced DNA damage (Charpentier *et al.*, 2011).

Some naturally competent bacteria are biased towards the uptake of genetic material derived from closely related neighbours. Various strategies have evolved to this end (Fig. 2). *Helicobacter pylori* is believed to be able to take up any kind of DNA, independent of its source, but recent results indicated that incoming DNA can be controlled by restriction-modification (R-M) systems (Aras *et al.*, 2002; Humbert & Salama, 2008; Humbert *et al.*, 2011). R-M systems are based on restriction endonucleases (REases), which cleave DNA at specific sequence motifs, and corresponding DNA methyltransferases, which inhibit the DNA cleavage of self DNA by methylation of the respective recognition sites. This mechanism recognizes and degrades foreign genetic material (Fig. 2). Indeed, R-M systems were traditionally associated with protection against bacteriophages or conjugative plasmids (for review see Kessler & Manta, 1990). It is not entirely clear how the REases destroy unmethylated DNA to reduce transformation frequencies in *H. pylori* (Humbert *et al.*, 2011) as current models suggest that only single-stranded DNA enters the cytoplasm (as reviewed by Chen & Dubnau, 2004; Allemand & Maier, 2009; Burton & Dubnau, 2010; Allemand *et al.*, 2012; Box 1). Thus, it was concluded in a recent review: 'Because restriction enzymes prefer double-stranded DNA, they likely act prior to unwinding and perhaps extracellularly' (Dorer *et al.*, 2011). The idea that such foreign species-derived DNA degradation could occur outside the cells might very well be possible as DNA damage-induced lysis of neighbouring cells would not only release these cells' DNA but also their REases.

Nuclease-mediated degradation of extracellular DNA has also been described for nontransformable variants of *C. jejuni* (Gaasbeek *et al.*, 2009, 2010). More specifically, these authors showed that acquisition of prophage-encoded endonucleases lead to an inhibition of natural transformability of *C. jejuni* (Gaasbeek *et al.*, 2009, 2010). This is in excellent agreement with an earlier publication on another naturally competent Gram-negative bacterium, *Vibrio cholerae*: in this study Blokesch and Schoolnik demonstrated that an extracellular nuclease Dns degrades external and potentially transforming DNA thus inhibiting transformation of this organism (Blokesch & Schoolnik, 2008). However, repression of nuclease production at high cell density occurs via a quorum-sensing regulatory circuit and thus still allows natural transformation to occur in *V. cholerae* (see below; Blokesch & Schoolnik, 2008; Lo Scrudato & Blokesch, 2012).

The competence-induced uptake and secretion of DNA – the unique case of *Neisseria*

Neisseria spp. have been known to be naturally competent for more than half a century (Alexander & Redman, 1953; Catlin & Cunningham, 1961). Recent research on the natural competence and transformation of *Neisseria* has primarily focused on the human pathogens *Neisseria meningitidis* and *Neisseria gonorrhoeae*. These species are very similar with respect to competence regulation and to their DNA-uptake mechanisms (Koomey, 1998). Thus, many findings derived from one organism can readily be applied to the other species.

Like *H. pylori*, *Neisseriae* are competent for natural transformation during all growth phases (Sparling, 1966). Environmental stimuli or secreted factor responsible for the induction or enhancement of competence and transformation have not been identified to date, except for a minor effect of growth temperature (Sparling, 1966). No further studies have been conducted on growth temperature, most likely because it only reflects elevated metabolic activity. Natural transformation peaked at 37 °C for *N. gonorrhoeae* (Sparling, 1966), which corresponds to the constant ambient temperature of its niche, which is the mucosal tissue of the human urogenital tract. As for *H. pylori*, no environmental reservoir is known for *N. gonorrhoeae*. Within the human host, the immune system puts *Neisseriae* under strong selective pressure. Thus, the extensive horizontal flow of genetic material mediated

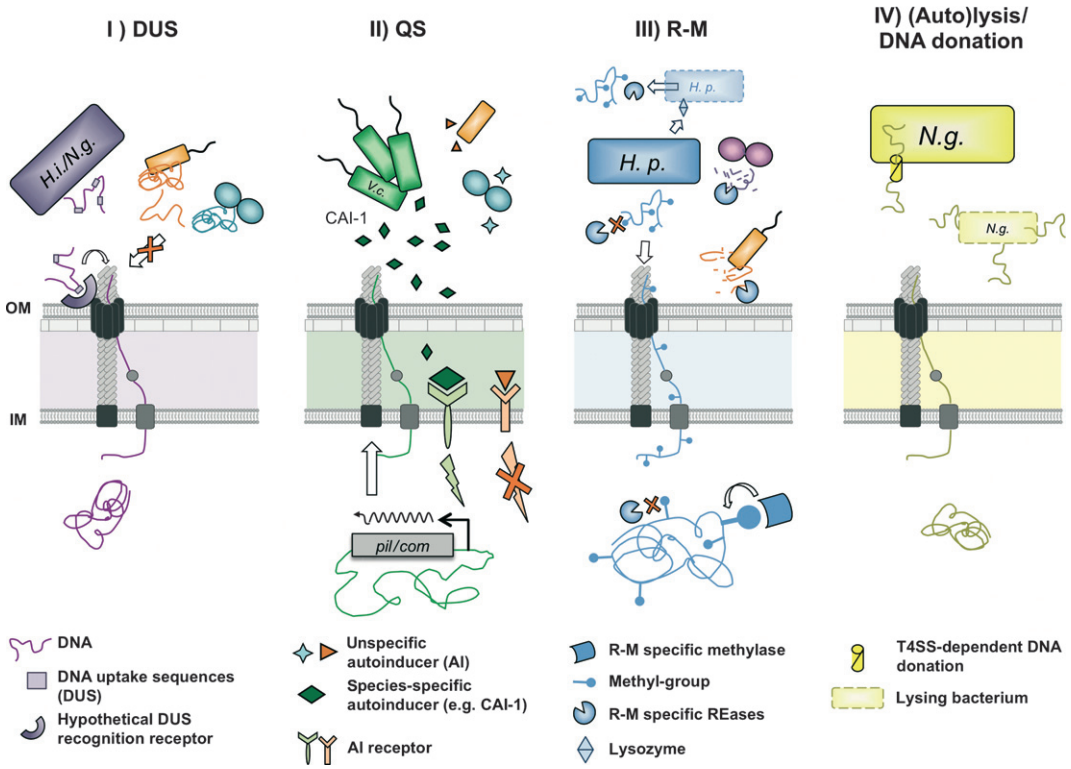


Fig. 2. Different strategies have evolved to enhance the probability of integrating species-specific DNA. Certain Gram-negative bacteria, such as *Haemophilus influenzae* and *Neisseria gonorrhoeae,* are notably fastidious about the kind of DNA that they absorb. To sort species-specific from nonspecies-specific DNA, these organisms have evolved specific DNA uptake sequences (DUS) that are overrepresented in their genomes and recognized by naturally competent members of the same species through as-yet-unknown receptor proteins. These receptors are most likely surface-exposed and initiate the ingestion of species-specific DNA into the cytoplasm of the bacterium (I). An alternative strategy to increase the chances of taking up species-specific DNA is to link the regulation of natural competence and transformation to species-specific autoinducer synthesis and recognition (II). The involvement of a 'competence pheromone' is a common strategy of Gram-positive bacteria and has also been recently suggested for the Gram-negative bacterium *Vibrio cholerae*. Other bacteria, such as *Helicobacter pylori,* have evolved specific restriction-modification (R-M) systems, which assure that species-nonspecific DNA is degraded and thus cannot recombine with the chromosome. The exact mechanism is so far unknown for this mode of restricting nonrelated DNA. One possible mechanism could be that the restriction enzymes (REases) are released from lysed bacteria (together with the DNA) and degrade species-nonspecific dsDNA extracellularly. Finally, as in the Gram-positive bacterium *Streptococcus pneumoniae*, certain naturally competent Gram-negative bacteria initiate the donation of DNA from their siblings. This process may be mediated either by autolysis, by the intentional killing of a subpopulation (e.g. the lysis of neighbouring cells) or by the active secretion of DNA through a T4SS, as in a subset of *Neisseria gonorrhoeae* strains.

by natural transformation might provide an important means of adaptation. Indeed, natural transformation might contribute significantly to the high genomic diversity of *Neisseria* (Viscidi & Demma, 2003; Hanage *et al.*, 2005; Maiden, 2008). Researchers provided evidence to support extensive interspecies recombination and intergeneric transfer of DNA in the late 1990s (Feil *et al.*, 1996, 1999; Kroll *et al.*, 1998). Only recently have genotyping techniques made it possible to determine the extent of diversity in *Neisseria* (for recent review see Maiden, 2008).

As described earlier, *H. pylori* actively secretes a lysozyme-like protein to increase the concentration of naked DNA in the environment (Dorer *et al.*, 2010). *Neisseria gonorrhoeae* facilitates the release of DNA by two different mechanisms. In this organism, DNA is either donated to siblings via autolysis or actively exported via a T4SS (Hamilton *et al.*, 2005; Hamilton & Dillard, 2006; Fig. 2). Although the T4SS involved in DNA secretion is found only in a subset of *N. gonorrhoeae* strains (Dillard & Seifert, 2001) and does not contribute to DNA secretion in *N. meningitidis* (Woodhams *et al.*, 2012), all investigated *Neisseria* strains can undergo autolysis. Little is known about the regulation of either of these processes or whether they are driven by environmental stimuli; however, both mechanisms promote genetic transfer within bacterial communities and might be beneficial for genome maintenance (Fig. 2).

Whereas *H. pylori* limits interspecies transformation via intracellular R-M systems, *Neisseria* spp. discriminate between self and foreign DNA at the level of the

DNA-uptake machinery. In fact, the finding that *N. meningitidis* and *N. gonorrhoeae* preferentially take up DNA from closely related species is one of the strongest arguments supporting the 'DNA for repair' hypothesis. More precisely, for *Neisseria* spp., as well as for *Haemophilus influenzae*, species-specific uptake of genetic material is dependent on so-called DNA-uptake sequences (DUS; Goodman & Scocca, 1988; Elkins *et al.*, 1991), which are conserved short DNA sequence motifs (Danner *et al.*, 1980; Fitzmaurice *et al.*, 1984; Elkins *et al.*, 1991; Aas *et al.*, 2002). DUS motifs may serve as a binding target for a still unidentified and hypothetical receptor protein localized on the outside of DUS-containing Gram-negative bacteria (Fig. 2). Furthermore, DUS motifs are highly overrepresented in the bacterial genomes. Early estimates of roughly one DUS motif per kb of DNA (Goodman & Scocca, 1991) were validated when the first two *Neisseria* genomes were made publicly available [GenBank numbers AE002098 (Tettelin *et al.*, 2000) and AE004969]. Interestingly, further analysis revealed a strong bias in DUS frequencies towards genome maintenance genes (Davidsen *et al.*, 2004). DUS motifs provide yet another mechanism to support species-specificity in transformation (Fig. 2) and may play a role in 'recombinational repair' (Michod *et al.*, 2008).

Recent findings, however, call into question both the nature and function of DUS (Ambur *et al.*, 2007; Duffin & Seifert, 2010). DNA uptake can occur in the absence of DUS, and the effect of DUS on transformation frequencies varies significantly among strains of *N. gonorrhoeae* (Duffin & Seifert, 2010). Because the effect of DUS on natural transformation efficiency was not proportional to its effect on DNA uptake, the authors proposed an additional role for DUS downstream of the DNA-uptake process (Duffin & Seifert, 2010). In addition, Duffin & Seifert (2012) demonstrated that DUS sequences can enhance the somewhat less efficient transformation by single-stranded DNA and that this phenomenon shows strand preference. The identification of a human gene fragment in the genome of *N. gonorrhoeae* provides further evidence for DUS-independent DNA uptake (Anderson & Seifert, 2011). This finding highlights the evolutionary potential of *N. gonorrhoeae* and the importance of HGT in host-pathogen interactions.

Competence induction based on starvation signals

Haemophilus influenzae requires a nutrient downshift to induce competence

Haemophilus influenzae is yet another Gram-negative bacterium in which natural competence and transformation has been studied for many years. As in the *Neisseriaceae* and *H. pylori,* the primary ecological niche of this organism is the human body, specifically, the upper respiratory tract. *Haemophilus influenzae* also causes ear infections and meningitis in children and respiratory disease in the elderly, as well as in patients with cystic fibrosis and AIDS.

The environmental signal that triggers the onset of natural competence in *H. influenzae* is not known; however, because 'the human respiratory tract is a hostile environment for bacteria', and 'nutrients and energy sources are limited' (Murphy & Brauer, 2011), it is not surprising that a link between starvation for preferred carbon sources and competence induction has been discovered *in vitro* for this organism. Indeed, in the laboratory, *H. influenzae* becomes naturally transformable when the culture reaches stationary phase (Redfield, 1991) or when the cells are transferred from rich to starvation medium (Herriott *et al.*, 1970). The addition of the secondary messenger cyclic adenosine monophosphate (cAMP) to exponentially growing bacteria also induces competence (Wise *et al.*, 1973). As a consequence it was shown that cAMP receptor protein (CRP) and adenylate cyclase (CyaA) are essential for the development of natural transformation in this organism (Chandler, 1992; Dorocicz *et al.*, 1993).

Recent work by Rosemary Redfield's group and others have provided further insight into the induction of competence in *H. influenzae*. These researchers identified the gene *sxy* (Redfield, 1991; homologue in other competent bacteria is *tfoX*), which is essential for natural competence. Furthermore, Sxy induced constitutive competence upon overexpression from a multi-copy plasmid (Williams *et al.*, 1994). The expression of *sxy/tfoX* as an early competence gene was increased upon the addition of cAMP, a phenomenon consistent with the increase in transformability (Zulty & Barcak, 1995). Sxy may act as a positive regulator of late competence-specific genes; however, the mechanism of this positive regulation has long been debated because Sxy lacks recognizable DNA-binding motifs (Macfadyen, 2000).

The competence regulon in *H. influenzae* was identified in 2005 using microarray expression profiling (Redfield *et al.*, 2005). The regulon consisted of 25 genes in 13 transcriptional units, each containing a characteristic promoter-associated 22-bp element, the competence regulatory element (CRE; Karudapuram & Barcak, 1997; Macfadyen, 2000; Redfield *et al.*, 2005). In these studies, competence-induced transcription was again strongly dependent on cAMP and the competence regulator Sxy. Based on this information, it was proposed that Sxy might act as an accessory factor, directing CRP to CRE sites and/or stabilizing the transcription-initiation complex (Macfadyen, 2000; Redfield *et al.*, 2005). The CRE elements were later renamed as CRP-S sites, describing a binding site for CRP; these sites, unlike canonical

CRP-binding sites (CPR-N), are dependent on Sxy (Cameron & Redfield, 2006, 2008). What is the exact nature of the link between cAMP/CRP, Sxy and competence induction? Although there is no definitive answer at present, the current model (based on work by the Redfield laboratory) is as follows: (1) CRP and cAMP are required to strongly induce the transcription of *sxy* (Cameron *et al.*, 2008) via the binding of the cAMP/CRP complex to a CRP-binding site in the *sxy* promoter region (Zulty & Barcak, 1995; Cameron *et al.*, 2008). (2) A stem loop structure is formed within the 5′ UTR of the *sxy* mRNA, which is involved in controlling both the amount and translation efficiency of the *sxy* mRNA (Cameron *et al.*, 2008). The authors of this study suggested that the latter was caused by the sequestration of the SD site by the mRNA stem loop structure, which limited the binding of ribosomes. Thus, the increase in Sxy protein following the transfer of the *H. influenzae* cells to starvation medium might occur because the secondary structure of the *sxy* mRNA has 'the potential to play a sensory role' (Cameron *et al.*, 2008). Such a sensory function might be based on the speed of transcription; ribosomes may bind before the 5′ UTR stem loop is properly folded, 'thus making the progress of RNA polymerase a potential transducer of nutritional signals' (Cameron *et al.*, 2008). Alternatively, the 5′ UTR of the *sxy* mRNA might be directly involved in regulation, perhaps by a riboswitch-like mechanism. This hypothesis is supported by the recently described riboswitch, which was identified in association with a *sxy* homologue in *V. cholerae* (*tfoX*^{GEMM}, Sudarsan *et al.*, 2008; details below). In conclusion, cAMP and CRP, as the major players in carbon-catabolite repression (CCR), are important in the natural transformation of *H. influenzae* because they are involved in the transcription of both early- (*sxy*) and late- (e.g. *comA*) competence genes. This feature couples the nutritional state of the bacterium to the induction of competence.

Apart from its cAMP-dependent competence induction, *H. influenzae* has a preference for species-specific DNA. The species-specificity of the DNA is discriminated at the level of the DNA-uptake process through the recognition of specific DUS sequences, as described above for *Neisseria* species (Danner *et al.*, 1980; Fitzmaurice *et al.*, 1984; Smith *et al.*, 1999).

Environmental cues involved in competence induction in bacteria commonly found outside a host

Natural transformation in *Pseudomonadaceae*

In contrast to the highly adapted human pathogens discussed earlier, the pseudomonads are an extremely versatile group of environmental bacteria that live predominantly in soil, sediment, and water, occurring less commonly as opportunistic human pathogens (for recent review on *Pseudomonas* taxonomy and isolation of type strains see Peix *et al.*, 2009). Many *Pseudomonas* spp. undergo natural transformation, including *P. mendocina*, *P. alcaligenes*, *P. pseudoalcaligenes*, *P. fluorescencs* and *P. stutzeri* (Carlson *et al.*, 1983; Demaneche *et al.*, 2001); to date, there is no evidence for natural competence of *P. aeruginosa*. Thus, most of the research on natural competence has focused on the ubiquitous soil bacterium *P. stutzeri*. This bacterium has a notably complex metabolism, which allows it not only to grow on a wide variety of carbon sources but also to degrade environmental pollutants and to fix nitrogen (as recently reviewed by Lalucat *et al.*, 2006).

Most of the research on the regulation and mechanism of natural transformation in *P. stutzeri* was performed in Wilfried Wackernagel's laboratory, and a seminal review, 'Bacterial Gene Transfer by Natural Genetic Transformation in the Environment', was published by Lorenz & Wackernagel (1994). This group showed, for example, that as with most other Gram-negative bacteria, the transformability of *P. stutzeri* correlates with the expression of type IV pilus structural or assembly genes (Graupner *et al.*, 2000), indicating that DNA uptake in *P. stutzeri* might be similar to the mechanism proposed for other Gram-negative bacteria (as reviewed by Chen & Dubnau, 2004 and described in Box 1). There are, however, several interesting differences. For example and in contrast to *H. influenzae*, transformation by single-stranded DNA (ssDNA) is efficient in *P. stutzeri*, reaching up to 5% compared with double-stranded DNA (dsDNA; Meier *et al.*, 2002). Notably, transformation with ssDNA was dependent on the same components identified for dsDNA, indicating that both substrates follow identical routes into the cell (Meier *et al.*, 2002). Furthermore, and again in contrast to *N. gonorrhoeae* or *H. influenzae*, DNA uptake was not dependent on the presence of DNA-uptake motifs. However, early competition experiments with either homologous or heterologous DNA suggested that *P. stutzeri* might still discriminate between self and foreign DNA at an early step of transformation through an unknown mechanism (Carlson *et al.*, 1983; Lorenz & Wackernagel, 1990). It was also shown that homology-facilitated illegitimate recombination is much less efficient in *P. stutzeri* than in other naturally transformable bacteria, such as *S. pneumoniae* or *Acinetobacter* (Meier & Wackernagel, 2003). Finally, DNA restriction has also been proposed as a barrier to natural transformation in *P. stutzeri* (Berndt *et al.*, 2003). All of these factors result in a limited exchange of heterologous DNA (Fig. 2), which has important implications for the spread

of alleles in populations of different *Pseudomonas* species and strains.

Transformation levels not only varied significantly among strains of *P. stutzeri* (Lorenz & Sikorski, 2000; Sikorski *et al.*, 2002), but they also varied across growth phases (Carlson *et al.*, 1983; Lorenz & Wackernagel, 1990). Specifically, transformation frequencies for growth in rich media peaked at the onset of the stationary phase, when nutrients start to become limited (Lorenz & Wackernagel, 1990). Thus, to mimic the natural habitat of this bacterium and to study nutrient situation and its effect on competence induction in *P. stutzeri*, Lorenz & Wackernagel (1991) tested growth and transformation on soil-extract-containing medium. These authors demonstrated that nutrients in those extracts were limited, allowing for the growth of only one or two generations. By supplementing the soil media with different combinations of carbon sources, phosphate and ammonium, these researchers determined the effect of each nutrient on growth and transformation. Limiting N, C or P stimulated transformation; this effect was most pronounced in soil extracts spiked with pyruvate and phosphate but lacking additional ammonium (Lorenz & Wackernagel, 1991). The underlying regulatory mechanism remains unknown.

Soil microcosm experiments demonstrated that conditions supporting the efficient transformation of *P. stutzeri* are readily encountered in the environment (Sikorski *et al.*, 1998). The same phenomenon was observed in *P. fluorescens.* Interestingly, transformation frequencies were significantly higher in nonsterile soil samples than in gamma-irradiated microcosms, possibly 'due to the presence of an organic compound in soil that is in part destroyed by soil sterilization' (Demaneche *et al.*, 2001). Even more strikingly, none of the *in vitro* conditions tested induced competence in *P. fluorescens* (Demaneche *et al.*, 2001), illustrating once more that most of the environmental signals that foster natural competence and transformation have not yet been identified. Thus, many bacteria used in the laboratory might have the potential for competence, although the trigger has not yet been elucidated.

Competence of *Acinetobacter* – is a nutrient boost required?

Another well-studied, naturally competent, Gram-negative bacterium is *Acinetobacter*. *Acinetobacter* spp. belong to the γ-proteobacteria, order *Pseudomonadales*. *Acinetobacter* strains are abundant in soil and water (Warskow & Juni, 1972), where they have been determined to make up at least 0.001% of the total culturable aerobic bacterial population (Baumann, 1968). *Acinetobacter* strains, however, are also frequently isolated from patients and have recently 'emerged from an organism of questionable pathogenicity to an infectious agent of importance to hospitals worldwide' (Munoz-Price & Weinstein, 2008). More importantly, this bacterium readily acquires resistance to antibiotics, another trait linked with HGT.

With respect to natural competence and transformation, most studies have been performed on *Acinetobacter baylyi* BD413 (Carr *et al.*, 2003; representative strains of this species were earlier named *Acinetobacter calcoaceticus* BD4, *A. calcoaceticus* BD413, *A. calcoaceticus* ADP1, and *Acinetobacter* spp. strain ADP1; Young *et al.*, 2005). The transformation efficiency of *A. baylyi* BD413 (formerly named *A. calcoaceticus* BD413) was shown to be notably high, ranging from 0.1% to 0.7% of all cells in an autotrophy/prototrophy transformation assay (Juni & Janik, 1969). Furthermore, this bacterium is not fastidious about the source of DNA; no species-specificity was observed with respect to DNA uptake (Lorenz *et al.*, 1992; Palmen *et al.*, 1993; Palmen & Hellingwerf, 1997).

Acinetobacter baylyi BD413 is always transformable, though with large variations in efficiency throughout the growth cycle (Cruze *et al.*, 1979). Reports on the time of peak transformability are conflicting. The initial study suggested that 'a peak of competency is found as the culture enters the stationary phase' (Juni, 1978) and that transformation was less efficient in the exponential phase (Juni & Janik, 1969). This finding was confirmed by others who found that the appearance of transformants was maximal at the end of the exponential growth phase for the soil bacteria *Bacillus subtilis* and *A. baylyi* BD413 (Lorenz *et al.*, 1991). These authors also demonstrated that there was no active DNA release by competent *Acinetobacter* cells. Other groups provided evidence that the highest transformation frequencies occurred early in the exponential phase, which was consistent with rapid growth (Cruze *et al.*, 1979). The latter finding was also confirmed by several groups (Palmen *et al.*, 1993, 1994; Porstendörfer *et al.*, 2000), who showed that natural transformation was maximally induced after the dilution of an overnight culture into fresh medium or 'after an increase in nutrient availability, preceded by cessation of growth' (Palmen *et al.*, 1994). In an earlier review, these contradictory findings were resolved with the conclusion that competence 'reaches its maximum during early to late log phase in *A. calcoaceticus*' (Lorenz & Wackernagel, 1994). Based on the results of a study on early competence induction, Palmen *et al.* (1994) concluded that 'the biological function of natural transformation in *A. calcoaceticus* is not to provide the cell with nutrients' and that 'the regulation of competence development as a function of the stringency of carbon, nitrogen, and phosphate limitation was just opposite to what one would expect if the 'nutrient supply hypothesis' would apply'.

Beate Averhoff and collaborators demonstrated that the transcription of at least two of the competence genes, *comP* and *comB*, did not correlate with the transformation pattern (Herzberg *et al.*, 2000; Porstendörfer *et al.*, 2000). Instead *comP* expression peaked in the late stationary phase, a finding that was also confirmed at the protein level (Porstendörfer *et al.*, 2000). *ComB* transcriptional activation also turned out as growth phase dependent: expression slightly peaked after dilution of the bacteria in fresh medium followed by an immediate decreased of *comB* transcription. Within mid-exponential phase expression of *comB* resumed up to a constant and high level of *comB* transcription in late stationary phase (Herzberg *et al.*, 2000). Based on these data Herzberg *et al.* (2000) concluded that the DNA machinery is already present in late stationary phase; diluting the cells into fresh medium then provides the required energy to allow efficient DNA uptake and consequently transformation. This conclusion is consistent with other reports on competence induction and transformation (Palmen *et al.*, 1994; Porstendörfer *et al.*, 2000) and the finding that transformation of *A. baylyi* BD413 is not inhibited by the protein synthesis inhibitor chloramphenicol (Palmen & Hellingwerf, 1997). The group of B. Averhoff has also conducted work on the natural transformation of an unusual Gram-negative bacterium, the thermophile *Thermus thermophilus*. This bacterium is a record-holder with respect to natural transformability (Koyama *et al.*, 1986) and a detailed review on *T. thermophilus* transformation was recently published (Averhoff, 2009). The impact and basis of natural competence and transformation for survival of *Thermus* in and adaptation to hot environments has recently been analysed (Omelchenko *et al.*, 2005; Averhoff & Müller, 2010).

Based on these *in vitro* studies described earlier, it is difficult to determine the environmental signals that could trigger competence in *Acinetobacter*. Nielsen *et al.* (1997) performed soil microcosm studies ('*in situ*') and observed the induction of competence by nutrient upshifts, as well as a transformation-enhancing effect of phosphate. These authors concluded that 'poorly transformable *A. calcoaceticus* cells can be induced to undergo natural transformation with chromosomal DNA in soil' and that 'their level of competence is influenced by their metabolic state' (Nielsen *et al.*, 1997). Such transformability was maintained for several hours after induction. A recent *in situ* study confirmed that *A. baylyi* entered competence soon after the inoculation of leaves, as evaluated by the re-isolation of bacteria from the leaves and *in vitro* provision of transforming DNA (Pontiroli *et al.*, 2009). Furthermore, these authors directly visualized transformants *in situ* using *gfp* reporter fusion and transforming DNA derived from transgenic plants. They concluded that the phytosphere might constitute a hotspot for host/pathogen HGT (Pontiroli *et al.*, 2009). In summary, the induction of transformability of *Acinetobacter* by an upshift in nutrients may behave in a manner contrary to what was observed for many other naturally transformable bacteria in which competence and transformation are often linked with starvation signals.

Natural transformation in aquatic *Vibrio* spp., with a focus on *V. cholerae*

Vibrio cholerae is a human pathogen and the causative agent of cholera; however, this bacterium's main niches are aquatic environments, such as rivers, estuaries, and coastal regions. Within these environments, *V. cholerae* is often found in association with chitinous zooplankton or their molts (Colwell, 1996; Lipp *et al.*, 2002; Pruzzo *et al.*, 2008). This association results from the ability of *V. cholerae* and other aquatic *Vibrio* species to degrade the chitinous exoskeletons of zooplankton and use them as a carbon and nitrogen source (for review see Keyhani & Roseman, 1999). Because the abundance of plankton changes significantly between plankton blooms, *Vibrio* species regulate gene expression in accordance with nutrient availability. Whether such environmental changes also influence competence induction is discussed below.

HGT within the species *V. cholerae*

Comparative genomic hybridization experiments performed by John Mekalanos's group revealed a high degree of conservation among *V. cholerae* strains (Dziejman *et al.*, 2002). This study was based on microarray hybridization experiments and compared the strains' genomes to the first sequenced strain of *V. cholerae,* which was a pandemic O1 El Tor strain (N16961; Heidelberg *et al.*, 2000). Only presence/absence data about genes and operons were collected; no information about novel genetic material was retrievable with this experimental approach. The authors of this study identified a limited number of genomic islands that were specific to the seventh pandemic strain and absent in earlier isolates, such as the representative strains of the classical biotype (the causative agent of the sixth and, likely earlier, cholera pandemics; Dziejman *et al.*, 2002). Furthermore, this initial study focused mainly on patient isolates and only investigated representatives of the serogroups O1 and O139. A follow-up study by Dziejman *et al.* broadened this analysis to include pathogenic non-O1 and non-O139 *V. cholerae* isolates. These strains were 'quite divergent' from the *V. cholerae* representatives belonging to the O1 and O139 serogroups (Dziejman *et al.*, 2005).

Technologically advanced, large-scale WGS studies made it possible to generate a clearer picture of SNPs and

horizontally acquired genetic material; these data enabled the construction of phylogenetic trees from which to derive information about the evolutionary relationships among strains. For example, recent WGS studies on *V. cholerae* suggested that several transcontinental transmission events have occurred (Mutreja *et al.*, 2011), significantly contributing to the understanding of the recent emergence of this pathogen in an enormous cholera outbreak in Haiti (Chin *et al.*, 2010; Hendriksen *et al.*, 2011; Mutreja *et al.*, 2011). Rita Colwell and collaborators also used WGS to understand the genetic diversity of 23 different *V. cholerae* strains isolated over the past 98 years. These authors identified 73 genomic islands (containing five or more ORFs) in their study and thus provided strong evidence for 'extensive lateral gene transfer in *V. cholerae*' (Chun *et al.*, 2009).

Natural competence has only recently been described in *V. cholerae* (Meibom *et al.*, 2005). The authors demonstrated that during growth on chitinous surfaces – a common environmental niche for this bacterium (Lipp *et al.*, 2002), *V. cholerae* can take up naked DNA and recombine it into its genome (Meibom *et al.*, 2005). As a mechanism of HGT, natural competence enables *V. cholerae* to acquire new genes, including those that specify pathogenic features. Indeed, Blokesch and Schoolnik demonstrated experimentally that the O1-to-O139 serogroup conversion could occur in a single step through a natural transformation-mediated exchange of the large O-antigen cluster (> 32 kb exchanged for > 42 kb; Blokesch & Schoolnik, 2007). The transformants displayed all phenotypes associated with the acquired gene cluster, including the changed O-antigen chain of the LPS and the capsular material surrounding the cells (Blokesch & Schoolnik, 2007). A stretch of homologous DNA located within the O-antigen cluster of many different *V. cholerae* strains (the IS1358 element) enabled transformants with only partial exchange of the cluster (only the upstream or downstream region of IS1358) to be obtained (Blokesch & Schoolnik, 2007). In this way, natural transformation may contribute to a 'reshuffling' of O-antigen genes and thus to the creation of new serogroups of *V. cholerae*. That serogroup conversion of *V. cholerae* occurs frequently in nature was also supported by comparative genomics (Chun *et al.*, 2009). A study notably similar to that conducted by Blokesch & Schoolnik (2007) was performed 4 years later, and the authors demonstrated a carbotype conversion of *Vibrio vulnificus* based on chitin-induced transformation (Neiman *et al.*, 2011). In this case, the transformation event led to the exchange of a capsular polysaccharide locus in this organism (Neiman *et al.*, 2011). Udden *et al.* (2008) used a similar experimental approach to show that chitin-induced natural competence of *V. cholerae* may be responsible for the spread of cholera-toxin prophages, most notably from non-O1/non-O139 strains. These authors provided evidence that strains that were unable to produce infectious cholera-toxin phage particles could still undergo horizontal transfer of the prophage using natural transformation (Udden *et al.*, 2008). Apart from these virulence-associated gene clusters, the transfer of genes involved in sugar metabolism via chitin-induced natural transformation has also been demonstrated for *V. cholerae* isolates from the Californian coast (Miller *et al.*, 2007). Thus, natural competence and transformation of *V. cholerae* provide a newly recognized mechanism by which this organism can effectively acquire new genes, thereby adapting to changing environmental conditions. Ways in which the natural competence programme is regulated in *V. cholerae* are summarized below.

Competence induction in some bacteria involves more than just one signal – the three interlinked regulatory pathways of *V. cholerae*

Sensing the carbon source chitin and colonizing chitinous surfaces

The resolution of the genome sequence of *V. cholerae* (Heidelberg *et al.*, 2000) provided the starting point for the investigation of regulatory circuits using microarray expression profiling. This approach enabled the identification of genes that were specifically activated during *V. cholerae*'s association with chitinous crab-shell fragments or purified soluble chitin oligosaccharides, the so-called chitin-utilization programme (Meibom *et al.*, 2004). Genes that specifically responded to chitin oligosaccharides included those genes predicted to encode components for the biogenesis and function of a type IV pilus (Fullner & Mekalanos, 1999; Meibom *et al.*, 2004), although this pilus has not yet been visualized. Components of type IV pilus complexes in naturally competent bacteria are hypothesized to participate in the transport of DNA through the outer membrane and the peptidoglycan (reviewed by Chen & Dubnau, 2004; Box 1), but 'it is currently unclear how the components interact to generate movement of the incoming DNA through the cell envelope' (Allemand & Maier, 2009). The chitin-dependent induction of type IV pilus-encoding genes cohered with natural competence and transformation, demonstrating for the first time that *V. cholerae* is a naturally transformable bacterium (Meibom *et al.*, 2005).

But how is the chitinous surface sensed, and how is the signal transmitted to induce gene-specific expression? In this context, the induction of genes involved in chitin degradation is strictly dependent on a unique two-component signalling system composed of a chitin hybrid

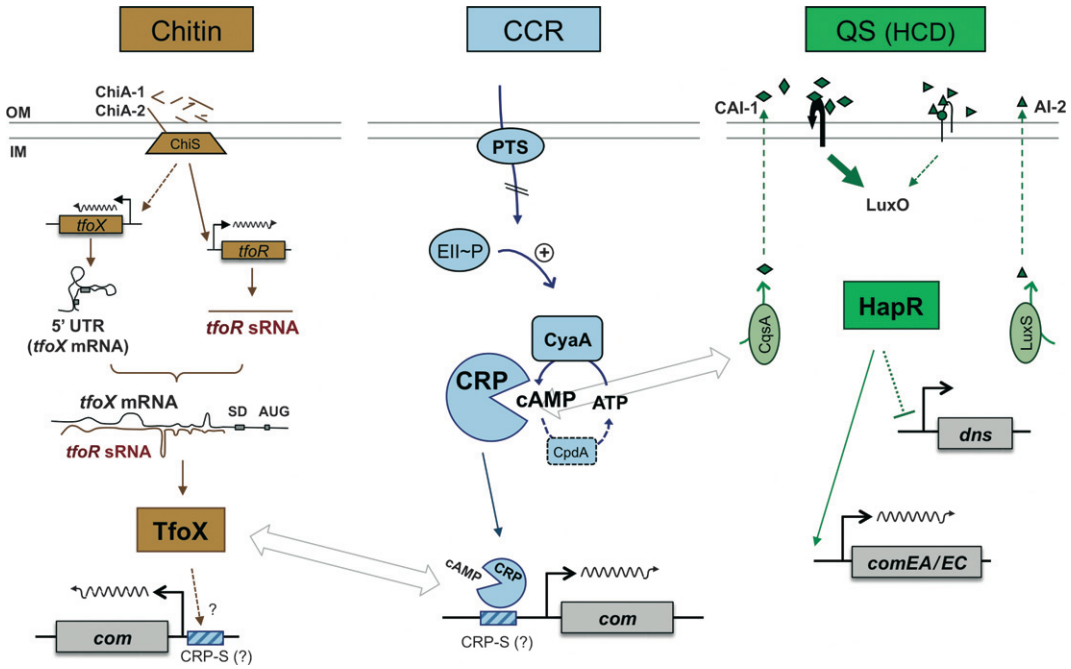


Fig. 3. A complex, threefold regulatory network is involved in the natural transformation of *Vibrio cholerae*. As shown in Fig. 1, the induction of natural competence and transformation in *V. cholerae* is linked with three of the common environmental cues: (1) the chitinous surface as the sole carbon source for this bacterium and other *Vibrio* spp. (brown pathway); (2) CCR and the induction of competence after starvation for preferred PTS-transported sugars (blue pathways); and (3) cell-to-cell communication, such as that exerted by the quorum-sensing systems (green pathway). Left – the chitin pathway. Extracellular chitinase (e.g. ChiA-1 and ChiA-2 directly secreted by *V. cholerae* or chitinases from other chitinolytic aquatic bacteria, which are abundant in this environment) degrades the insoluble *N*-acetylglucosamine (GlcNAc) polymer chitin and releases diacetylchitobiose units as the major product (together with some triacetylchitotriose). These chitin oligomers are recognized by a sensory histidine kinase, ChiS, and a signal transduction pathway subsequently leads to the induction of *tfoX* and *tfoR* expression. *tfoR* encodes for a small regulatory RNA, which enhances the translation of the *tfoX* mRNA by freeing the Shine-Dalgarno (SD) site, as well as the start codon (AUG) from a secondary stem loop structure formed by the 5′ UTR of the *tfoX* mRNA. TfoX, as the major regulator of transformation, is involved in the induction of competence genes. This induction most likely involves pairing with the cAMP-CRP complex (grey double arrow), as TfoX itself does not contain any obvious DNA-binding domain. Middle –CCR. In the absence of preferred PTS-transported sugars (such as glucose or the chitin monomer GlcNAc), the PTS systems are not saturated, and the phosphoryl group is kept by enzyme II (EII). EII–P is a direct activator of the cAMP-producing enzyme CyaA. Accumulating cAMP, together with its binding protein (CRP), leads to the induction of the competence genes. This complex might interact with TfoX to allow for the binding of cAMP-CRP to competence-specific CRP-S sites. Right – high cell density, as measured by QS, is required for competence induction and transformation. *V. cholerae* produces two main autoinducers: CAI-1 and autoinducer 2 (AI-2). At high cell density (HCD), the binding of the autoinducer to the respective receptors initiates a signalling cascade (simplified), which conclusively results in the production of the major regulator of QS, HapR. HapR acts as repressor of the extracellular nuclease gene *dns* and as a positive regulator of the competence genes *comEA* and *comEC*. The encoded proteins of all three genes are expected to be in direct contact with transforming DNA; these proteins either induce the degradation of external DNA at low cell density (Dns) or are part of the DNA-uptake machinery (ComEA and ComEC; see Box 1). Thus, QS fine-tunes the fate of the surrounding DNA. References on which the scheme is based on are mentioned in the text.

sensor/kinase ChiS (Li & Roseman, 2004) and a thus far unidentified response regulator tentatively named ChiR (Fig. 3). Li & Roseman (2004) speculated that ChiS works in conjunction with an *N*,*N*′-diacetylchitobiose ABC-type transporter and that ChiS is activated upon the transport of the chitin degradation product $(GlcNAc)_2$. The connection between ChiS and the expression of the type IV pilus-encoding genes became obvious when the latter genes were not upregulated in a *V. cholerae chiS* mutant during growth on chitin (Meibom *et al.*, 2004). Noteworthy, a *chiS* mutant is not naturally transformable under chitin-dependent competence-inducing conditions (M. Blokesch, unpublished data).

The *V. cholerae* chitin utilization programme also indicated that a gene encoding a protein with similarity to the transformation regulators of other naturally competent bacteria (TfoX/Sxy) was induced during growth on chitin (Meibom *et al.*, 2004). TfoX was later shown to be essential for natural transformation of *V. cholerae* (Meibom *et al.*, 2005). There is substantial evidence that TfoX is downstream of ChiS in the chitin-dependent signalling cascade (Fig. 3). Expression of *tfoX in trans* or *in cis*

(Meibom *et al.*, 2005; Lo Scrudato & Blokesch, 2012) relieved the requirement for chitin to induce the pilus-specific genes and other genes involved in DNA uptake (Box 1), henceforth referred to as competence genes. Furthermore, the transformation-negative phenotype of a *chiS* mutant can be overcome by chitin-independent and low-level expression of *tfoX* (Lo Scrudato & Blokesch, 2012), which restores natural transformability in this strain (M. Blokesch, unpublished data).

Haruo Watanabe's group demonstrated another link between chitin-dependent transformation and TfoX production in *V. cholerae*. These researchers first confirmed the transcriptional data of Meibom *et al.* (2004): in contrast to $(GlcNAc)_n$ oligomers ($n \geq 2$), the chitin monomer GlcNAc is not sufficient to induce *tfoX* expression (Yamamoto *et al.*, 2010). In addition, Watanabe *et al.* discovered a translational component of chitin-induced TfoX production (Yamamoto *et al.*, 2010). The latter finding was supported by the discovery of a chitin-induced small RNA (named TfoR), which positively contributes to the translation of the *tfoX* mRNA (Yamamoto *et al.*, 2011; Fig. 3). These authors showed *in vitro* that the 102-nucleotide sRNA TfoR activates the translation of the *tfoX* mRNA, most likely by freeing the SD motif and the start codon from a secondary structure formed by the 5′ UTR of the *tfoX* mRNA in the absence of TfoR. This mechanism mimics the analogous mechanism in *H. influenzae* (Cameron *et al.*, 2008): a 5′ stem loop structure formed within the *tfoX/sxy* mRNA is significant in initiating translation, but the release of this stem loop by base pairing with the sRNA TfoR seems to be unique to *V. cholerae* and, possibly, other *Vibrio* spp. (Yamamoto *et al.*, 2011).

CCR and the requirement for cAMP and CRP in *V. cholerae*

When chitin-induced natural competence was first described, the authors demonstrated that glucose prevented natural transformation. They hypothesized therefore that CCR might play a role in competence induction (Meibom *et al.*, 2005; Figs 1 and 3). *Vibrio cholerae* takes advantage of the abundance of zooplankton in its natural environment. Zooplankton have chitinous exoskeletons, which provide a nutritious surface that can serve as the sole carbon source for *V. cholerae* and can induce natural competence (Fig. 1). Therefore, the link between CCR and chitin-induced natural competence was further investigated (Blokesch, 2012). In this study, the hypothesis that CCR is involved in the regulation of natural competence was further strengthened by showing that not only glucose but also other phosphoenolpyruvate : carbohydrate phosphotransferase system (PTS)-transported sugars, including the chitin monomer GlcNAc, interfere with natural transformation (Blokesch, 2012). Such carbon sources are known to counteract against the intracellular accumulation of the secondary messenger cAMP (for review see Deutscher *et al.*, 2006). The author of this study also demonstrated that the lack of cAMP or of the CRP changes at least three levels of chitin-dependent natural competence induction in *V. cholerae*: chitin surface colonization, chitin degradation/metabolism and competence gene expression (Blokesch, 2012). Indeed, with respect to chitin metabolism, *V. cholerae* strains deficient in CCR showed significantly lower expression of *chiA-2* (Blokesch, 2012). *ChiA-2* and *chiA-1* encode two redundant extracellular chitinases, and at least one of these chitinases is required for growth on chitin (Meibom *et al.*, 2004). The lack of expression of such chitin degradation enzymes might explain why *V. cholerae* mutants that are defective for CCR, such as a *crp* minus strain, are unable to grow with GlcNAc oligomers as the sole carbon source and are unable to colonize the chitinous surface (Blokesch, 2012).

The expression of the competence genes also being directly dependent on CCR was further proven by uncoupling natural competence induction from chitin surface colonization and chitin metabolism (Blokesch, 2012; Lo Scrudato & Blokesch, 2012). Using different chitin-independent competence-inducing conditions and different readouts (reverse transcription followed by PCR and transcriptional reporter fusions for Blokesch, 2012 and Lo Scrudato & Blokesch, 2012, respectively), a change in competence gene expression became obvious when comparing wild-type *V. cholerae* with CCR-deficient strains. Furthermore, a *V. cholerae* strain that lacked the cAMP-degrading enzyme cAMP phosphodiesterase (*cpdA*) showed enhanced frequencies of natural transformation (Lo Scrudato & Blokesch, 2012). Based on these and other findings, Lo Scrudato & Blokesch (2012) concluded that an imbalanced intracellular cAMP pool affects competence induction at the transcriptional level. How these two regulatory circuits – chitin-dependent induction of TfoX and CCR – are connected is not fully understood. However, a current hypothesis is that the transformation regulator TfoX can act only in conjunction with CRP. This speculation is based on the suggested CRP-Sxy interaction as described earlier for *H. influenzae*. Indeed, based on published expression data for *V. cholerae* grown on chitin surfaces or *tfoX* overexpression (Meibom *et al.*, 2004, 2005), competence-specific CRP-S sites have also been predicted *in silico* by Cameron and Redfield for *V. cholerae* (Cameron & Redfield, 2006). It might be interesting to note that cAMP-CRP also positively regulate the expression of the integron-specific integrase gene in *V. cholerae* (Baharoglu *et al.*, 2012), which might avoid

the need to homologously recombine the incoming transforming DNA into the chromosome; instead, the newly acquired DNA could be integrated into the gene-capturing integron island (Cambray *et al.*, 2010).

Bacterial communication is key for competence induction in *V. cholerae*

The first suggestion of the involvement of quorum sensing (QS) in natural competence and transformation (Fig. 3) was based on the finding that *V. cholerae* was transformable in a more efficient manner after an extended growth period on chitin surfaces, which is in accordance with increased cell densities (Meibom *et al.*, 2005). Blokesch & Schoolnik (2008) followed up on this finding and described the connection between QS and natural competence and transformation in further detail. They demonstrated that the main regulator of QS, HapR, which is only produced at high cell density, represses the gene encoding the extracellular nuclease Dns (Blokesch & Schoolnik, 2008; Fig. 3). This repression is an essential step in the regulation of natural transformation because the nuclease Dns degrades surrounding DNA, thereby destroying any potential transforming material (Blokesch & Schoolnik, 2008). Recently, other researchers also confirmed the HapR-dependent down-regulation of *dns* (Seper *et al.*, 2011).

HapR also acting as a positive regulator of at least a subset of competence genes, such as the periplasmic DNA-binding protein encoding gene *comEA*, became obvious early on (Meibom *et al.*, 2005; Blokesch & Schoolnik, 2008). This speculation was initially based on microarray expression data (Meibom *et al.*, 2005) and *V. cholerae hapR/dns* double knockout mutants being transformable at an *c.* 100-fold lower frequency than a *dns* single knockout mutant, even though the degradation of extracellular DNA was fully abolished (Blokesch & Schoolnik, 2008). The expression of *comEA* being indeed regulated in a QS-dependent manner was nicely demonstrated using transcriptional fusion with a luciferase-based readout (Antonova & Hammer, 2011) and also more recently by detecting *comEA* expression using transcriptional fusions with fluorescent proteins (Lo Scrudato & Blokesch, 2012). The latter study significantly extended this finding by not only monitoring the expression of *comEA* but also other competence genes, such as *pilA* (Lo Scrudato & Blokesch, 2012; Fig. 3). Furthermore, Lo Scrudato and Blokesch investigated a plethora of competence genes with respect to any potential QS-dependent regulation. The results of this study were remarkable because they provided good evidence for only a small subset of competence genes of *V. cholerae* being coregulated by TfoX and QS (Lo Scrudato & Blokesch, 2012). More importantly, the genes that are indeed linked with QS (*comEA* and *comEC*) are all extremely relevant for transformation because the encoded proteins are hypothesized to directly interact with the transforming DNA (Box 1). Indeed, Dns degrades the DNA at low cell density, whereas ComEA and ComEC are produced at high cell density and present major components of the DNA-uptake machinery (Box 1; Suckow *et al.*, 2011), thereby directly interacting with the incoming DNA in *V. cholerae.* Thus, in conclusion, QS acts as a switch from extracellular DNA degradation to DNA uptake in *V. cholerae* (Lo Scrudato & Blokesch, 2012).

Based on HapR being involved in the natural competence and transformation of *V. cholerae*, several research groups became interested in the question of how the autoinducers of *V. cholerae* contribute to this regulatory circuit. Based on work by Bonnie Bassler and collaborators, two main autoinducers have been identified and have been allocated a specific role in *V. cholerae*. Cholera autoinducer 1 (CAI-1), a (S)-3-hydroxytridecan-4-one (Higgins *et al.*, 2007), is thought to play a role as an intraspecies communication agent. By contrast, AI-2, a furanosyl borate diester (Chen *et al.*, 2002), serves as a 'universal signal' in bacteria as this molecule is produced and sensed by many bacteria and therefore allows interspecies communication (Xavier & Bassler, 2003). Comparing *V. cholerae* mutants devoid of the capacity to synthesize CAI-1, AI-2 or both autoinducers, the group of Melanie Blokesch showed that in the absence of CAI-1, natural transformation is (almost) completely abolished in experiments mimicking the natural chitinous environment (Suckow *et al.*, 2011) and that transformation was consistently undetectable under homogenous competence-inducing conditions (Lo Scrudato & Blokesch, 2012). Furthermore, the authors of these studies were never able to detect any transformants in the absence of both autoinducers (CAI-1 and AI-2), a phenotype that mirrors *hapR* minus strains (Suckow *et al.*, 2011; Lo Scrudato & Blokesch, 2012). Not surprisingly no HapR protein was detectable in CAI-1/AI-2 synthase-deficient cells (Lo Scrudato & Blokesch, 2012). This transformation-negative phenotype of autoinducer-deficient *V. cholerae* cells was not restorable by cross-feeding of solely AI-2 from co-cultured bacteria, in contrast to the efficient restoration of transformation by cross-fed CAI-1 (Suckow *et al.*, 2011). Suckow *et al.* therefore concluded that the intraspecies autoinducer CAI-1 might be similar to competence pheromones described in Gram-positive bacteria and that such a regulation might increase the chances of taking up species-specific DNA (Fig. 2). However, even though another study illustrated the same tendency, it did not show such a strict CAI-1-dependency. More specifically, Antonova & Hammer (2011) concluded in their study

that '*Vibrio*-specific CAI-1 appears to play a major role and interspecies AI-2 a minor role, suggesting that induction of DNA uptake may not be restricted exclusively to a response to autoinducers produced by *Vibrio* species, but that HGT may also be promoted by AI-2 derived from non-*Vibrio* members of a biofilm'. These authors also showed that strains lacking both autoinducers (CAI-1 and AI-2) were readily transformable and thereby significantly above the detection level of a nontransformable *hapR* mutant strain (Antonova & Hammer, 2011). Based on these data, it can be hypothesized that the discrepancy in autoinducer dependency in these different *V. cholerae* strains is not due to a different connection between the QS regulator HapR and natural transformation but most likely reflects a difference in the QS circuitries. Further studies will be required to confirm or refute this hypothesis.

The induction of competence in *V. cholerae* compared with the other naturally transformable bacteria

Based on the results of CAI-1 dependency for natural transformation in *V. cholerae*, CAI-1 can be considered a competence pheromone (Suckow *et al.*, 2011; Lo Scrudato & Blokesch, 2012). Indeed, this molecule might perform a function similar to a function proposed for Gram-positive bacteria (for a seminal review on competence induction in Gram-positive bacteria see Claverys *et al.*, 2006). For example, competence induction in *S. pneumoniae* is not solely dependent on the cell density and thereby on the passive accumulation of the competence-stimulating peptide (CSP), a competence pheromone. Instead, the production of CSP varies with changing environmental conditions (Claverys *et al.*, 2000, 2006). Claverys *et al.* showed that DNA-damaging antibiotics induce the expression of the competence regulon in a CSP-dependent manner in *S. pneumoniae*. These authors argued that CSP serves as a stress signal and can therefore be considered an alarmone rather than a quorum-sensing effector (Prudhomme *et al.*, 2006). Prudhomme *et al.* (2006) therefore concluded that 'CSP could thus play a crucial role in generating genetic diversity under stress conditions for a species that seems unable to rely on inducible mutagenic repair (such as the SOS response)'. The hypothesis that CAI-1 in *V. cholerae* is similar to competence pheromones is supported by the fact that the synthesis of CAI-1 by the synthase CqsA is also not constitutive in *V. cholerae*. In fact, Liang *et al.* (2007, 2008) demonstrated that CCR is involved in the post-transcriptional regulation of *cqsA* expression in *V. cholerae*, providing another connection among the three regulatory pathways that drive natural competence and transformation in *V. cholerae*. This process also connects a process indicative of preferred carbon source starvation, or CCR, to the induction of competence. This link might reflect a similarity to the function of natural transformation in *S. pneumoniae* 'as a rescue process' (Prudhomme *et al.*, 2006).

In a recent review by Ng and Bassler, the authors wrote 'One oddity is that homologues of *cqsA* and *cqsS*, called *lqsA* and *lqsS* respectively, exist in the distantly related bacterium *L. pneumophila*. LqsA produces 3-hydroxypentadecan-4-one; a molecule with a longer hydrocarbon chain than CAI-1 (Spirig *et al.*, 2008)' (Ng & Bassler, 2009). Interestingly, a recent analysis of six fully sequenced *L. pneumophila* genomes suggested that the genomes were highly dynamic, as a result of extensive HGT and recombination (Gomez-Valero *et al.*, 2011). Furthermore, '*L. pneumophila* ranks as the prokaryote with the widest variety of eukaryotic-like proteins', all of which may have been acquired from the host by HGT (Cazalet *et al.*, 2004). At this point, it is tempting to speculate that α-hydroxyketone signalling molecules are commonly involved in the regulation of natural competence. Thus, it will be interesting to see whether the *Legionella* autoinducer-1 (LAI-1; Spirig *et al.*, 2008) is also required for natural competence and transformation in this bacterium. It had long been appreciated that *L. pneumophila* is naturally transformable (Stone & Kwaik, 1999), and recent studies have provided evidence that competence might fulfil a similar function in this organism as in *S. pneumoniae*, replacing the absent SOS response (Charpentier *et al.*, 2011).

The induction of natural competence in noncholera *Vibrio* species

Studies in the 1990s had already suggested that 'marine *Vibrio* spp.' were naturally transformable (Frischer *et al.*, 1990, 1993; Jeffrey *et al.*, 1990; Paul *et al.*, 1992). These aquatic isolates were assigned to the genus *Vibrio* and were most similar to *V. campbelli* based on the biochemical features described in Bergey's manual; however, follow-up studies on this phenomenon provided evidence that the isolates belonged to the genus *Pseudomonas,* a finding based on 16S-rRNA gene analysis (Frischer *et al.*, 1996). Thus, the natural transformability of *Vibrio* spp. had not been demonstrated before Meibom *et al.* (2005) described chitin-induced natural transformation in *V. cholerae*.

Following the first studies on natural competence in *V. cholerae* (Meibom *et al.*, 2005; Blokesch & Schoolnik, 2007, 2008), other researchers have found that *V. cholerae* is not the only representative of the genus *Vibrio* to be naturally transformable; natural transformation has also been demonstrated in *V. vulnificus* (Gulig *et al.*, 2009),

Vibrio parahaemolyticus (Chen *et al.*, 2010) and *Vibrio fischeri* (Pollack-Berti *et al.*, 2010). The first two studies used a modified version of the natural transformation protocol initially described for *V. cholerae* (Meibom *et al.*, 2005; Blokesch & Schoolnik, 2007) to modify the bacteria genetically (Gulig *et al.*, 2009; Chen *et al.*, 2010). Because this protocol is based on competence induction on chitinous surfaces, a common theme of chitin-based induction seems clear. Indeed, it was experimentally shown that many other species of the genus *Vibrio* grow on chitin and that most of them contain proteins homologous to those identified in the *V. cholerae* chitin utilization programme (Meibom *et al.*, 2004 and as summarized in Hunt *et al.*, 2008). Thus, the chitinous surface may be a common niche for *Vibrio* spp., and chitin-induced natural competence may be widespread.

This finding was further emphasized by the group of Ned Ruby, who described the induction of natural competence in the symbiotic *Vibrio*, *V. fischeri* (Pollack-Berti *et al.*, 2010). These researchers provided evidence that as in *V. cholerae*, natural competence in *V. fischeri* is induced in the presence of chitin oligomers and in a TfoX-dependent manner. Interestingly, Pollack-Berti *et al.* identified a second TfoX-like protein, named TfoY, in *V. fischeri*. Indeed, all fully sequenced *Vibrionaceae* contain these two paralogues of TfoX (Pollack-Berti *et al.*, 2010). The authors of this study also demonstrated that the two paralogs were unable to compensate fully for the loss of the counterpart and accordingly that they influence natural transformation in distinct manners. Interestingly, the corresponding TfoY counterpart of *V. cholerae* had been discovered earlier during a search for RNA motifs and riboswitches in bacteria because it contains a Genes for the Environment, for Membranes, and for Motility (GEMM) motif (Weinberg *et al.*, 2007; Sudarsan *et al.*, 2008). In this context, Weinberg *et al.* described that based on the conserved domain database (CDD), *V. cholerae* contains two genes belonging to the COG3070 family (encompassing TfoX-domains). As TfoY (named $tfoX^{GEMM}$ in Weinberg *et al.*, 2007) but not *tfoX* contained this structured GEMM motif, the authors concluded 'in *V. cholerae* and related bacteria, GEMM might participate in chitin-induced competence, or even regulate competence in environments not containing elevated chitin concentrations'. In line with the latter hypothesis, TfoY does not play an obvious role in chitin-induced natural competence of *V. cholerae*. Indeed, a *V. cholerae* knockout strain of *tfoY* is fully transformable during growth on chitinous surfaces and artificial expression of *tfoY* does not render *V. cholerae* naturally competent (M. Blokesch, unpublished data).

In conclusion, the regulation of natural competence and transformation in *Vibrio* spp. seems to be consistent with respect to chitin-dependent induction and the requirement for TfoX. The QS circuits, by contrast, differ among these organisms, and not all *Vibrio* spp. contain a CqsA/CqsS system (for recent review see Milton, 2006). Additionally, not all *Vibrio* spp. that do contain a CqsA/CqsS system produce the same autoinducer. For example, work by Bonnie Bassler's group demonstrated that the Vh-CAI-1 autoinducer of *Vibrio harveyi* differs slightly from its counterpart in *V. cholerae* in that the former contains an 8-carbon tail instead of a 10-carbon tail (Ng *et al.*, 2011). An 8-carbon tail CAI-1 was also present in spent supernatants of other *Vibrio* spp., including *V. parahaemolyticus*, *Vibrio alginolyticus*, *Vibrio anguillarum* and *Vibrio furnissii* (Ng *et al.*, 2011). The authors of this study concluded that 'different *Vibrio* species display unique production and detection profiles for the CAI-1 family of molecules'. One could hypothesize that 'the forces that drove these two species [= *V. cholerae* and *V. harveyi*] to evolve different signalling specificities' (Ng *et al.*, 2011) might be linked with natural transformation and species-specificity of the system (Fig. 2). Further studies will be required to elucidate the connection between QS and the natural transformability in noncholera *Vibrios* and especially in those *Vibrios* that do not contain CqsA/CqsS systems.

The induction of natural transformation in plant pathogenic bacteria

Ralstonia solanacearum – a large host range plant pathogen takes up DNA

Ralstonia solanacearum is a representative bacterium of the β-proteobacteria class, family *Burkholderiaceae*. Most importantly, *R. solanacearum* is a major plant pathogen 'because of its aggressiveness, large host range, broad geographical distribution and long persistence in soil and water environments' as recently reviewed (Genin, 2010). After the genome of *R. solanacearum* was sequenced (Salanoubat *et al.*, 2002 and reviewed in Genin & Boucher, 2004), analysis revealed some interesting features. First, as in *V. cholerae* (Heidelberg *et al.*, 2000), the genome of *R. solanacearum* is split into two chromosomes of unequal sizes (3.7 and 2.1 megabases). Second, the genome displayed a mosaic structure with respect to the G+C content throughout several kilobases of both chromosomes (7% of the total genomes differed). Together with the fact that the encoded genes differed in codon usage from the rest of the bacterium, this difference was indicative of HGT (Genin & Boucher, 2004). The authors of this review concluded 'that [these regions] could play an important role in the rapid adaptation of the bacterium to the change of ecological niche' (Genin & Bou-

cher, 2004). Because *R. solanacearum* is naturally transformable, this mode of HGT might have contributed significantly to the mosaic structure of the genome. Indeed, two recent studies provided evidence that large regions of DNA (from 30 to 90 kb and up to 80 kb, respectively) can be integrated into the recipient's genome (Coupat *et al.*, 2008; Coupat-Goutaland *et al.*, 2011).

With respect to the regulation of natural competence in *R. solanacearum*, transformation frequencies peaked in the middle of the exponential phase and dropped quickly afterwards (Bertolla *et al.*, 1997). Furthermore, as is the case for many other naturally competent bacteria, growth on minimal medium reflecting limiting growth conditions permitted higher transformation efficiencies than those observed in rich medium (Bertolla *et al.*, 1997). Bertolla *et al.* (1999) also demonstrated that natural transformation occurs *in situ* (e.g. *in planta*). During cell-host interaction, natural transformation was most efficient while *R. solanacearum* cells were multiplying within the plant vessels (Bertolla *et al.*, 1999).

Ralstonia solanacearum also harbours more than one quorum-sensing system (Whitehead *et al.*, 2001). Specifically, this species contains a LysR-type regulator, PhcA, which regulates the production of extracellular polysaccharide (EPS) and extracellular enzymes (Brumbley *et al.*, 1993). PhcA is subject to regulation by the two-component system, which responds to the autoinducer 3-hydroxypalmitic acid methyl ester (3OH PAME; Clough *et al.*, 1997; Flavier *et al.*, 1997). This autoinducer is most likely synthesized by the synthase PhcB, which exhibits homology to small-molecule SAM-dependent methyltransferases (Flavier *et al.*, 1997). Interestingly, the CAI-1 synthase CqsA of *V. cholerae* also uses SAM as a substrate (as do many other autoinducer synthases; Wei *et al.*, 2011), and, at first glance, CAI-1 does show similarities to the 3OH PAME of *R. solanacearum*. Evidence that, as in *V. cholerae*, this quorum-sensing system is also involved in the regulation of natural transformation was generated by a recent study by Kang *et al.*, who demonstrated that the gene encoding the major pilin subunit of a type IV pilus, *pilA*, is essential for natural transformation of *R. solanacearum* and is regulated in a cell density-dependent manner. Specifically, these authors demonstrated that the expression of *pilA* decreases at high cell density and that this downregulation is dependent on the major regulator of QS, PhcA (Kang *et al.*, 2002).

Another plant pathogen, *Xylella fastidiosa*, was only recently discovered to be naturally transformable

Another plant pathogen, *X. fastidiosa*, has also been identified as being naturally transformable (Kung & Almeida, 2011). This bacterium belongs to the class γ-proteobacteria, family *Xanthomonadaceae*. The lifestyle of *X. fastidiosa* has recently been reviewed and the authors mentioned that 'strains of *X. fastidiosa* have been associated with a large number of diseases, many causing great economic losses' (Chatterjee *et al.*, 2008). This bacterium causes disease in many plants, including grapes and citrus fruits. Transmission of this bacterium is mediated by insects, such as sharpshooters (Chatterjee *et al.*, 2008). The ability to exchange genes horizontally may benefit *X. fastidiosa* bacteria and 'potentially permit them to explore a wider variety of host plants' (Kung & Almeida, 2011).

The information regarding when and how natural competence in *Xylella* is induced is still limited; however, Kung & Almeida (2011) demonstrated that cell growth affects the number of transformants, and transformants were obtained more efficiently if the transforming DNA was provided upon entry into exponential phase, with the efficiency declining significantly afterwards. These authors also demonstrated that methylated plasmid DNA served better as transforming DNA than its unmethylated counterpart, suggesting that R-M systems might play a role in the transformation of *X. fastidiosa* (Kung & Almeida, 2011) comparable with what was demonstrated for *H. pylori* (as described above; Humbert & Salama, 2008; Humbert *et al.*, 2011). Finally, the authors observed a medium-specificity of natural transformation because transformation occurred only in nutrient-limited medium (Kung & Almeida, 2011). Thus, the induction of natural competence seems to be linked with the nutritional status of the cell in *X. fastidiosa*. Chitin has been proposed as a carbon source for *X. fastidiosa* upon entry into insect vectors (e.g. leafhoppers), and chitinolytic activity has been demonstrated *in vitro* (Killiny *et al.*, 2010). Thus, it is tempting to speculate that a co-regulation between chitin utilization and CCR, as described earlier for *V. cholerae*, might also be involved in competence induction in *X. fastidiosa*.

Evolution in an evolutionary system – what benefits does natural transformation bring to an organism?

The question of why natural transformation exists remains the subject of ongoing debate. Three hypotheses are frequently discussed: 'DNA for food', 'DNA for repair', and 'DNA for evolution'. These hypotheses are not necessarily mutually exclusive, but they are not equally supported by the data. This topic has recently been extensively reviewed elsewhere (Michod *et al.*, 2008; Vos, 2009); however, due to the strong link between the induction of natural competence by environmental signals

and the role transformation may play in bacteria, we summarize some of the major arguments below.

DNA for food

The role of DNA as a source of nutrients was initially proposed by Redfield (2001). She wrote, 'the simplest model thus might be that the nutrients that transformation reliably provides pay the evolutionary bills (and have been responsible for the evolution of its regulation), and as a bonus the cell gets the occasional benefits of recombination and repair' (Redfield, 1993b). Among others, the main arguments put forward by Redfield for a role of transformation as 'Genes for Breakfast' were as follows: (1) DNA as food provides a direct short-term advantage, whereas a role of novel genes in evolution might be selected only in the long run. (2) *H. influenzae* and *B. subtilis* react to nutritional limitations when inducing competence (Redfield, 1993a, b). Notably, this is not the case for *S. pneumoniae*. In this species, nutrient starvation has never been observed to induce competence (Claverys *et al.*, 2006); instead, during growth in rich medium the bacteria acquire competence in early log phase and maintain it only over a short period of time or differentially said 'competence is induced in times of feast rather than famine' (Claverys & Havarstein, 2007). (3) Neither *H. influenzae* nor *B. subtilis* induce competence upon DNA damage. (4) Unrelated DNA can be used as a nutrient source, especially because it could not recombine homologously into the chromosome. (5) The addition of ribonucleotide monophosphates, most notably AMP and GMP, to competence-inducing starvation medium reduced the natural transformation in *H. influenzae* by more than two orders of magnitude and significantly reduced the expression of competence genes in this organism (MacFadyen *et al.*, 2001). These authors argued that the depletion of purines within the cell induces competence and that the incoming DNA could subsequently replenish the purine pool. Interestingly, this effect was not observable for desoxyribonucleotide monophosphates, triphosphates, or the free bases (MacFadyen *et al.*, 2001). (6) The poor-quality DNA derived from dead cells might not be suitable for transformation-mediated evolution (Redfield, 1988; Redfield *et al.*, 1997). But certain competent bacteria kill their (non-or not yet competent) siblings within the population, whereas other bacteria actively donate DNA through a T4SS (Hamilton *et al.*, 2005; Hamilton & Dillard, 2006) or through a currently unknown mechanism (Stewart *et al.*, 1983), suggesting that not all transforming DNA is of poor quality.

Recent studies on *E. coli* support Redfield's work. Finkel & Kolter (2001) provided evidence that *E. coli* can grow with DNA as the sole source of carbon and energy; however, this ability was disrupted in *E. coli* mutants lacking genes that encode for potential competence-related proteins. The authors of this and a follow-up study identified eight genes encoding proteins that are between 12% and 74% identical to the *H. influenzae* counterparts involved in natural competence (Finkel & Kolter, 2001; Palchevskiy & Finkel, 2006). In the absence of these genes, most of these strains encountered a stationary phase competition defect during co-culture with the parental wild-type bacteria. The authors of this study concluded that taking up DNA for nutritional purposes, 'particularly when that DNA is heterologous and less likely to recombine onto the chromosome' (Finkel & Kolter, 2001; Palchevskiy & Finkel, 2006), might confer a significant advantage even over the acquisition of a beneficial gene by HGT. However, because most of the homologous proteins identified in *E. coli* and other proteobacteria (Palchevskiy & Finkel, 2006) resemble the type IV pilus part of the DNA-uptake machinery, the question arises as to whether such DNA indeed reaches the cytoplasm as linear ssDNA, as occurs in naturally competent bacteria. Alternatively, the type IV pilus-like structure may assist in recruiting free dsDNA into the periplasm and thus facilitate transport across the outer membrane through the secretin PilQ/HofQ. DNA might be further degraded in the periplasm into nucleosides, which are subsequently taken up into the cytoplasm by specific nucleoside transporters to serve as a source of carbon and energy. Thus, whereas the first part of this process would resemble natural competence-induced DNA uptake and involve type IV pilus-like protein components, DNA transport across the inner membrane with concomitant degradation of one strand might not be identical in 'nutritional competence' (Palchevskiy & Finkel, 2006). Indeed, DNA uptake is a 2-step process in naturally competent *H. pylori* (Stingl *et al.*, 2010); however, as discussed earlier, *H. pylori* uses a T4SS, not a type IV pilus-like structure, to shuffle the DNA across the outer membrane. But there are good indications that this 2-step DNA-uptake process also holds true for other Gram-negative bacteria, as demonstrated in a recent review (Kru ger & Stingl, 2011). Furthermore, *V. cholerae* regulates the competence genes required for DNA movement across the outer membrane differentially from the competence genes whose products are involved in DNA translocation across the periplasmic space and the inner membrane (Lo Scrudato & Blokesch, 2012).

Other findings also oppose the 'DNA for food' hypothesis. For example, David Dubnau stated in a review from 1999 that *B. subtilis* possesses a powerful extracellular nuclease and adequate uptake systems for DNA degradation products (Dubnau, 1999). Extracellular nucleases have also been described for *Vibrio cholerae* (Newland

et al., 1985; Focareta & Manning, 1987). Most notably, the main nuclease in this organism, Dns, is oppositely regulated from the competence genes that are directly involved in DNA uptake (Blokesch & Schoolnik, 2008; Lo Scrudato & Blokesch, 2012), and Dns is a major inhibitor of natural transformation in *V. cholerae* because it degrades the transforming material around the cell (Blokesch & Schoolnik, 2008). A role of this nuclease in the utilization of DNA as a nutrient source has been suggested (Blokesch & Schoolnik, 2008) and was experimentally supported with respect to the utilization of DNA as a phosphate source (Seper *et al.*, 2011). Interestingly, the crystal structure of a concentrative nucleoside transporter of *V. cholerae* (NupC) has recently been solved. This protein uses a sodium-ion gradient for nucleoside transport across the inner membrane (Johnson *et al.*, 2012) and may be involved in the uptake of nucleotides released by the extracellular nuclease Dns.

Another important point that undermines the DNA for food hypothesis is the energy associated with DNA uptake itself. As mentioned earlier and discussed in detail elsewhere (Chen & Dubnau, 2004; Allemand & Maier, 2009; Burton & Dubnau, 2010; Allemand *et al.*, 2012), the DNA-uptake machinery is most likely a multiprotein complex (Box 1). Although the composition and mode of action of this complex has not fully been elucidated, the complex is assumed to resemble type IV pili (with the exception of *H. pylori*, as discussed earlier). Such type IV pilus-like structures (or shortened forms, also known as pseudopili; Pugsley, 1993; Chen & Dubnau, 2004) allow for the transport of DNA across the peptidoglycan layer and/or the outer membrane of Gram-positive and Gram-negative bacteria, respectively. An inner membrane channel subsequently allows translocation of the DNA across the inner membrane; this structure is probably conserved across all naturally transformable bacteria (Draskovic & Dubnau, 2005; Stingl *et al.*, 2010; Suckow *et al.*, 2011). Consistent with the resemblance of the components, the forces generated by type IV pilus retraction and DNA uptake were in the same range for both systems and represent some of the strongest linear motors characterized to date (Merz *et al.*, 2000; Maier *et al.*, 2002, 2004). As recently reviewed by Berenike Maier and others (Maier, 2005; Allemand & Maier, 2009; Allemand *et al.*, 2012) such 'directed DNA translocation is often energetically unfavourable and requires an active process that uses energy, namely the action of molecular motors' (Allemand *et al.*, 2012). Thus, the question arises as to whether the use of transforming DNA as an energy source would be able to supply enough energy to compensate for the consuming uptake process and still provide sufficient extra energy to be more cost-effective than the *de novo* synthesis of nucleotides. Furthermore, incoming ssDNA is protected against degradation in naturally competent bacteria by competence-specific proteins such as DprA (Mortier-Barriere *et al.*, 2007). This mechanism is also more consistent with a role of the transforming DNA in DNA-repair processes or in the donation of new alleles/genes.

DNA for repair

Arguments for the repair hypothesis are principally based on the following facts:

The ability to take up DNA in some Gram-negative, naturally transformable bacteria is highly biased towards genetic material from the same or closely related species. Various strategies have evolved to this end (Fig. 2). For example, *N. gonorrhoeae* and *H. influenzae* discriminate between self and foreign DNA through the recognition of DUS that are overrepresented in their own genomes (Danner *et al.*, 1980; Fitzmaurice *et al.*, 1984; Elkins *et al.*, 1991). *Vibrio cholerae,* in contrast, does not discriminate between self and foreign DNA at the level of the DNA uptake (Suckow *et al.*, 2011). Because competence induction in this organism is tightly linked with an accumulation of the species-specific autoinducer CAI-1 (Suckow *et al.*, 2011; Lo Scrudato & Blokesch, 2012), species-specific DNA is highly likely to reach the cytosol. In contrast, *H. pylori* does not display any preference for species-specific DNA. This assumption is based on the fact that competence is constitutive in this organism and that DUS-dependent sorting does not occur at the level of the DNA-uptake machinery. However, recent data has indicated that a mechanism based on R-M systems might control DNA uptake in *H. pylori*, as explained above (Aras *et al.*, 2002; Humbert & Salama, 2008; Humbert *et al.*, 2011). This system may also ensure the species-specificity of transforming DNA by recognizing and degrading foreign genetic material, and protecting the genome from foreign DNA (Fig. 2; Aras *et al.*, 2002; Humbert & Salama, 2008; Humbert *et al.*, 2011). Another source for species-specific DNA may be fratricide. More precisely, bacterial fratricide is associated with natural competence of *S. pneumoniae* (Guiral *et al.*, 2005; Havarstein *et al.*, 2006 and recent review by Claverys & Havarstein, 2007) and probably also in *H. pylori* (Dorer *et al.*, 2010; Fig. 2). In this context, bacterial cells of the same population are killed to provide transforming DNA. Based on several examples of the intentional killing of bacterial siblings, Gilmore & Haas (2005) concluded that 'the selective lysis of siblings by a subpopulation of bacterial cells appears to be a highly evolved and complex process'.

Based on these important points, the idea that natural transformation serves as a mechanism of DNA repair

seems sound. In this scenario, the uptake of DNA from closely related organisms would facilitate the maintenance of genomic integrity. Evidence from experiments on the naturally competent Gram-positive bacterium *B. subtilis* supports this hypothesis (Michod *et al.*, 1988; Wojciechowski *et al.*, 1989), but recent findings on the two Gram-negative bacteria, *H. pylori* and *L. pneumophila*, with nonsense mutations in their DNA-uptake systems, did not support the repair hypothesis (Dorer *et al.*, 2010; Charpentier *et al.*, 2011). In these studies the bacterial strains incapable of DNA uptake showed no increased sensitivity to genotoxic agents (Dorer *et al.*, 2010; Charpentier *et al.*, 2011).

DNA for evolution

Finally, natural transformation might enable rapid evolution when diversity may be beneficial; these circumstances include such stresses as high population densities, DNA damage, abundance or a lack of certain carbon sources and/or starvation. All of these conditions are known to induce natural competence in at least a subset of naturally transformable bacteria, as described earlier and illustrated in Fig. 1. A recent study on the long-term *in vitro* passage of *H. pylori* (*c.* 1000 generations) supported an evolutionary advantage of natural transformation because competent bacteria increased their fitness faster than those unable to take up external DNA (Baltrus *et al.*, 2008). Another recent study investigated the occurrence of multi-drug-resistant (MDR) strains based on recombination following DNA uptake as part of the natural transformation process of *A. baylyi* (Perron *et al.*, 2012). The authors demonstrated that in the presence of recombination, resistance genes were readily exchanged, and MDR strains were obtained within fewer generations (Perron *et al.*, 2012).

However, as noted by Perron *et al.* and as described in depth in a recent review on this topic (MacLean *et al.*, 2010), it is important that 'evolution experiments offer a useful approach to uncover the factors determining the evolution of resistance, but most experiments have studied clonal populations without any contribution of recombination'. Furthermore, the 'benefits of recombination are context-dependent', and experimental setups are crucial to the outcome of such experiments. Such context dependency was also highlighted in a recent study by Engelmoer & Rozen (2011). These authors emphasized once more the biased setup of most experimental studies, which only examine benefits dependent on the acquisition of DNA as part of natural transformation. However, because natural competence is a developmental programme and often induces other processes apart from DNA uptake (e.g. fratricide in *S. pneumoniae* or competence-dependent growth arrest, as seen in *B. subtilis*), benefits arising from these processes might have been overlooked in the past (Engelmoer & Rozen, 2011). In this study, the authors investigated the natural competence of *S. pneumoniae* and confirmed that transformation is beneficial in a 'DNA for repair' scenario upon treating cells with DNA-damaging agents (Engelmoer & Rozen, 2011), but they also provided evidence that competence is beneficial in withstanding other kinds of stresses and that these benefits do not rely on transformation (Engelmoer & Rozen, 2011). The authors concluded that their findings were in line with 'Claverys' hypothesis (Claverys *et al.*, 2006) that competence but not necessarily transformation may act as a general process to relieve stress' (Engelmoer & Rozen, 2011).

In summary, we conclude that there are many benefits of natural competence and transformation, and there might be no single reason that transformation is maintained in bacteria. The fact that numerous bacteria are known or predicted to be naturally transformable is a strong indication of the importance of this mode of HGT.

Concluding remarks

Regulation of the competence window may have co-evolved as an ability to respond to environmental cues

Summarizing the information on competence induction presented here, we conclude that natural competence occurs constitutively in certain Gram-negative bacteria but is tightly regulated and transient in others. But still the question of 'who's competent and when', which was initially asked by Solomon & Grossman (1996), cannot yet be fully answered. Interestingly, several recent studies examine how natural competence and transformation are evolutionarily maintained over time (Johnsen *et al.*, 2009; Maughan & Redfield, 2009). The question of why some naturally competent bacteria are competent only in a brief, finely tuned time window has not yet been

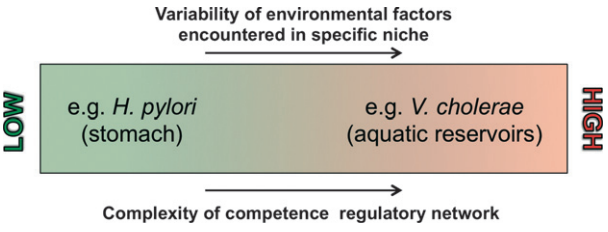


Fig. 4. The direct correlation between the variation encountered in an organism's niche and the complexity of the regulatory network driving competence induction. For details, see the text.

conclusively addressed. An examination of the environments of naturally competent bacteria might yield evidence as to why some of these organisms have tighter competence windows than others (Fig. 4). For example, *H. pylori,* as a gastric pathogen, solely colonizes the epithelium of the human stomach, and no other reservoir has been reported. *Helicobacter pylori* does not need to cope with sudden and/or dramatic changes in its environment (Fig. 4). Instead, such parameters as pH or nutrient availability are likely to fluctuate only within a narrow range. However, during long-term infection, environmental alterations might include the ageing of the host and concomitant changes in immune status or a secondary infection with another *H. pylori* strain. In this context, natural transformation can lead to the rapid spread of beneficial mutations and thus provides a means for adaption. Because *H. pylori* is constitutively competent, it maintains high genetic plasticity during infection. Therefore, the bacteria are constantly able to fine-tune interactions with their human hosts, as suggested in a recent review on the evolution and phenotypic diversification of *H. pylori* (Suerbaum & Josenhans, 2007).

The dynamics might differ significantly in naturally transformable bacteria that frequently encounter dramatic fluctuations in their environment (Fig. 4). A good example is *V. cholerae*, an aquatic and naturally transformable bacterium. In its environmental niche, this organism can be found as free-living bacterium in a planktonic state or as part of a sessile biofilm community (Yildiz & Visick, 2009). Furthermore, *V. cholerae* is well known for its ability to interact with phyto- and zooplankton, of which the mucilaginous sheath and the exoskeleton, respectively, can serve as carbon and energy sources (reviewed by Lipp *et al.*, 2002). The abundance of such carbon sources, however, can fluctuate significantly, and climate changes and human behaviour are thought to contribute significantly to plankton blooms and *V. cholerae* abundance (Colwell, 1996; Lobitz *et al.*, 2000; Lipp *et al.*, 2002; Pruzzo *et al.*, 2008; Fig. 4). Furthermore, selection pressure from environmental cholera bacteriophages, which are also thought to play a significant role in cholera seasonality and the emergence of new clones (Faruque *et al.*, 2005), as well as pressure from protozoan grazers (Matz *et al.*, 2005; Erken *et al.*, 2011), may vary in time and space. Thus, the adjustment between famine and feast and between growth and growth arrest including the ability to enter the state of natural competence must be fine-tuned (Fig. 4). Further studies will be required to address the potential disadvantages of constitutive competence and transformation under changing environmental conditions; such weaknesses could explain why the competence window is adjusted according to the bacterium's lifestyle.

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