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Natural transformation of *Vibrio fischeri* requires *tfoX* and *tfoY*

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Summary

Recent evidence has indicated that natural genetic transformation occurs in *Vibrio cholerae,* and that it requires both induction by chitin oligosaccharides, like chitohexaose, and expression of a putative regulatory gene designated *tfoX*. Using sequence and phylogenetic analyses we have found two *tfoX* paralogues in all sequenced genomes of the genus *Vibrio*. Like *V. cholerae*, when grown in chitohexaose, cells of *V. fischeri* are able to take up and incorporate exogenous DNA. Chitohexaose-independent transformation by *V. fischeri* was observed when *tfoX* was present in multicopy. The second *tfoX* paralogue, designated *tfoY,* is also required for efficient transformation in *V. fischeri*, but is not functionally identical to *tfoX*. Natural transformation of *V. fischeri* facilitates rapid transfer of mutations across strains, and provides a highly useful tool for experimental genetic manipulation in this species. The presence of chitin-induced competence in several vibrios highlights the potential for a conserved mechanism of genetic exchange across this family of environmentally important marine bacteria.

Introduction

Vibrio fischeri can be isolated from several niches in the marine environment including chitin surfaces, and as a part of a specific, beneficial relationship with the Hawaiian bobtail squid, *Euprymna scolopes* (Ruby and Lee, 1998). In their role as bioluminescent symbionts, a dense population of up to 10^9 *V. fischeri* cells is housed within a specialized light-emitting organ of the squid host (Nyholm and McFall-Ngai, 2004). The association is long-term and dynamic: the squid will maintain the polyclonal symbiont population (Wollenberg and Ruby, 2009) throughout its life, which can last up to a year. An important part of the persistent nature of this relationship is that *V. fischeri* cells experience a daily cycle of expulsion of the light-organ contents and re-growth of the remaining population (Nyholm and McFall-Ngai, 1998). This periodic behaviour, while providing the host with a fresh inoculum of symbionts each day, exerts a continuous selective pressure on the *V. fischeri* population. These characteristics of the light-organ environment reveal a potential for both genetic exchange and allelic fixation.

Natural competence is a form of bacterial genetic exchange that can be dependent on the presence of inducing conditions in the environment (Chen and Dubnau, 2004). Recently, it has been reported that both *Vibrio cholerae* and *Vibrio vulnificus* can be transformed when grown on chitinaceous surfaces such as shrimp shells or crab-shell tiles (Meibom *et al*., 2005; Udden *et al*., 2008; Gulig *et al*., 2009). Thus, chitin-induced competence may be a shared trait of marine vibrios that is related to their capability to utilize this common nutrient (Hunt *et al*., 2008). The discovery of chitin-induced competence in these two vibrio species, and evidence that chitin is present in the squid–vibrio symbiosis (Wier *et al*., 2010), led us to ask whether *V. fischeri* shares this capability.

Disruption of the *V. cholerae tfoX* gene ($tfoX^{VC}$; VC_1153), encoding a putative regulator of competence, abolished detectable transformation (Meibom *et al*., 2005). BLAST searches of the *V. fischeri* ES114 genome revealed two putative $tfoX^{VC}$ homologues, which we have named $tfoX^{VF}$ (VF_0896) and $tfoY^{VF}$ (VF_1573) (Fig. 1). In this study we sought to determine whether, like *V. cholerae* and *V. vulnificus*, *V. fischeri* cells grown in the presence of chitin derivatives are genetically competent for transformation. We further investigated whether the *V. fischeri* homologue of $tfoX^{VC}$ is required for transformation in culture. Observing the sequence similarity to $tfoX^{VF}$, we also asked whether $tfoY^{VF}$ was required for transformation, and whether the two *V. fischeri tfoX* homologues were functionally redundant. Finally, given the value of natural transformation as a tool for genetic manipulation, we investigated methods for increasing the frequency and convenience of transformation in *V. fischeri*.

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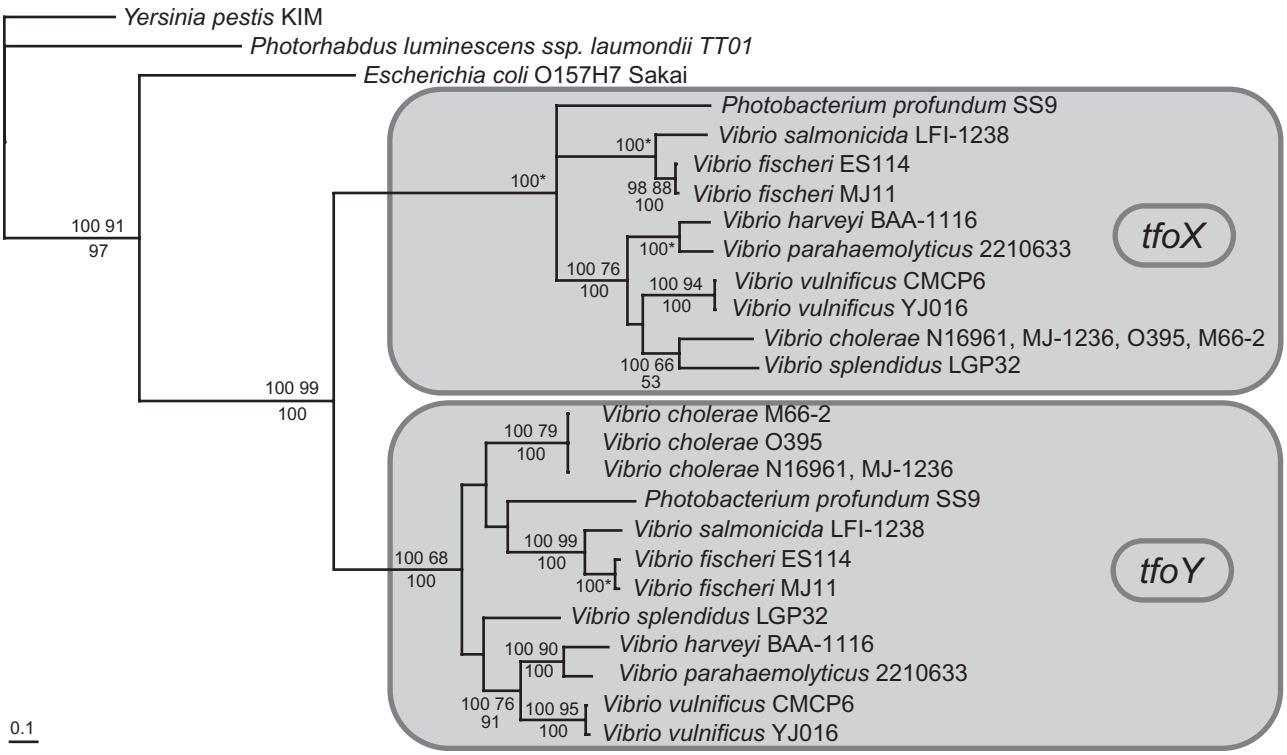

Fig. 1. Two paralogous *tfoX*-like sequences exist in all fully sequenced *Vibrionaceae*. Phylogenetic reconstruction of *tfoX* homologues found in 13 *Vibrionaceae* and two Gram-negative out-groups, rooted with *Yersinia pestis* KIM. The two shaded clades contain two groups of closely related *tfoX*-like sequence homologues found in all *Vibrionaceae*. One clade includes only previously identified *tfoX*-like sequence homologues (*tfoX*), while the other contains another, previously unidentified group of *tfoX*-like sequence homologues (*tfoY*). Identical nodes obtained from three reconstruction methods [Bayesian, maximum likelihood (ML) and maximum parsimony (MP)] with support > 50% are identified by three numbers: (top left) posterior probabilities from Bayesian analysis, multiplied by 100; (top right) bootstrap percentage from 500 likelihood pseudoreplicates; (bottom) bootstrap percentage from 1000 parsimony pseudoreplicates. The bar indicates 0.1 expected change per site; the asterisk (*) indicates that all methods give identical support values of 100.

Results

Two paralogous, tfoX*-like loci are present in the genomes of all sequenced* Vibrionaceae

A BLASTp alignment using the translated protein sequence of *tfoX*^{VC} identified two likely matches from the *V. fischeri* ES114 genome. The TfoX^{VC} sequence was a close match to a translation of VF_0896 (*tfoX*^{VF}), and a more distant match to a translation of VF_1573 (*tfoY*^{VF}). Alignment of predicted TfoX^{VF} and TfoY^{VF} sequences shows 32% identity and 55% similarity. Surprisingly, BLASTn searching using *tfoX*^{VF} found two sequence homologues in each *Vibrionaceae* genome (Table S1). In each genome, one sequence was more similar to *tfoX*^{VF}, whereas the other shared more similarity with *tfoY*^{VF}. We hypothesized that the two similar, but distinct genes were evidence of a duplication event in the ancestral *Vibrionaceae* lineage, leading to paralogous sequences in all extant vibrio species. Phylogenetic analyses of the multiple alignment of all *tfoX* and *tfoY* homologues from the *Vibrionaceae* confirmed this hypothesis; two sister groups, corresponding to extant *tfoX*- and *tfoY*-like sequences, had strong statistical support in all reconstructions (Fig. 1). Alignment of the *tfoX* and *tfoY* gene neighbourhoods of *V. fischeri*, *V. cholerae* and *V. vulnificus* – organisms that exhibit chitin-induced competence – revealed two local sets of ORFs that appear conserved (Fig. 2).

Transformation occurs in the presence of chitin oligosaccharides

To determine the extent to which *V. fischeri* is capable of uptake and incorporation of exogenous DNA, we asked whether a chromosome-encoded chloramphenicol-resistance marker inserted within the *ainS* gene (VF_1037) could be transformed into wild-type *V. fischeri*. Cells provided with both DNA and soluble chitin oligosaccharides (in the form of chitohexaose) produced putative transformants with an average efficiency of ~10^{-7}, a level that is > 800 times the limit of detection (Table 1). *Vibrio fischeri* did not produce detectable transformants when provided with either polymeric chitin (*i.e.* crab-shell tiles) or the chitin monomer GlcNAc instead of chitohexaose

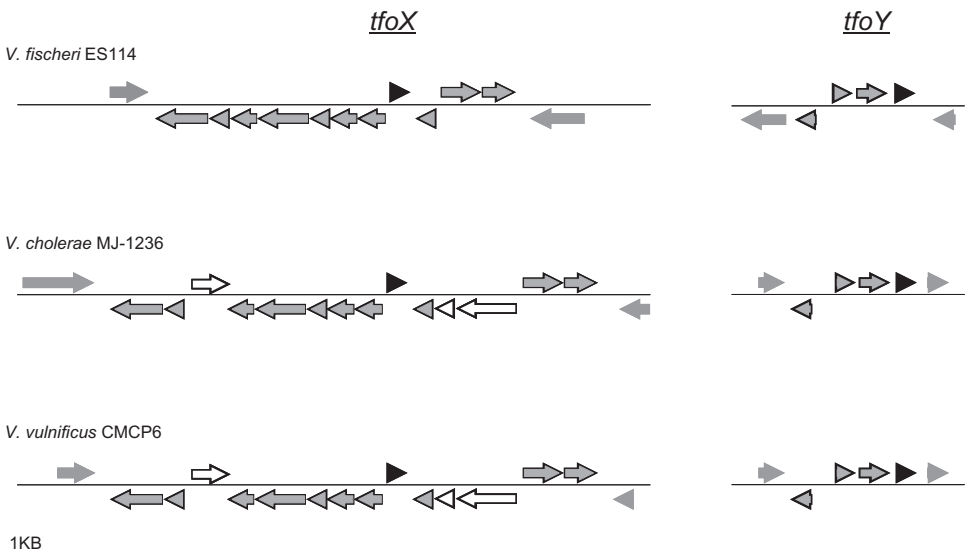

Fig. 2. Loci surrounding the *tfoX* paralogues of *V. fischeri, V. cholerae* and *V. vulnificus*. Schematic representations of orthologous genomic regions of three *Vibrio* species. Solid black arrows indicate *tfoX* or *tfoY*. Grey arrows indicate ORFs found in the *tfoX* or *tfoY* loci of all completed *Vibrio* genomes. White arrows indicate ORFs found in all completed *Vibrio* genomes except *V. fischeri* strains ES114 and MJ11. Unlined grey arrows indicate ORFs flanking the conserved *tfoX* or *tfoY* loci.

(Table 1). The frequency of spontaneous Cm resistance was below the limit of detection ($< 1 \times 10^{-10}$), as estimated in the same medium, either in the absence of added DNA, or with DNase-treated DNA.

Putative transformants were selected by Cm resistance, and further analysed by a PCR screen with primers specific to the *ainS*::Cm^R region (Table S2). The appearance of PCR products of the predicted size and sequence indicated transformation had occurred in all 10 of 10 putative transformants (Fig. S1). Because an *ainS* mutant is non-bioluminescent in culture, recombination of the *ainS* mutant allele into the chromosome was further confirmed in all of these transformants by the loss of luminescence (Fig. S2).

Table 1. Effect of carbon source on transformation frequency of ES114 by chromosomal DNA[a].

Medium[b]	Plasmid	Mean transformation frequency[c]	Range
+GlcNAc	–	< 0.1[d]	–
+Crab shell	–	< 0.1[d]	–
+$(GlcNAc)_6$	–	80	2–130
+$(GlcNAc)_6$	pMulTfoX	1500	400–3500
+$(GlcNAc)_6$	pLosTfoX	430[e]	340–520
+GlcNAc	pMulTfoX	65	2–170
+GlcNAc	pLosTfoX	260	4–520
+Glcn	pMulTfoX	360	7–710
+Glcn	pLosTfoX	520	100–950

a. In the absence of added DNA, transformation frequency was $< 1 \times 10^{-10}$.
b. Grown in minimal media (MM) containing either chitohexaose $(GlcNAc)_6$, *N*-acetylglucosamine (GlcNAc) or glucosamine (Glcn).
c. Average of two experiments ($\times 10^9$).
d. Below detection limit, 1×10^{-10}.
e. Antibiotics not added to maintain pLosTfoX.

Vibrio fischeri tfoX *is required for normal transformation*

To determine whether *V. fischeri tfoX* plays a role in transformation, we made an internal deletion of 78% of the gene, creating strain AGP200 (Table 2). The deletion was confirmed by PCR (Fig. S3). Analysis of AGP200 indicated that this strain was transformation defective: no transformants were detected under conditions where the wild-type was competent (Table 3). The transformation defect of AGP200 could be genetically complemented by supplying *tfoX^{VF} in trans* on the plasmid pMulTfoX. The pMulTfoX plasmid carries the *tfoX^{VF}* gene on the plasmid vector pVSV104, which is maintained in *V. fischeri* with ~10 copies per genome (Dunn *et al*., 2006).

Presence of tfoX^{VF} *in multicopy confers chitin-independent competence*

Carriage of pMulTfoX not only restored competence to a *tfoX^{VF}* mutant, but also increased its frequency of transformation by 50- to 80-fold over that of the wild-type (Table 3). This transformation enhancement in the presence of multiple copies of *tfoX^{VF}* also occurred in wild-type. In addition, when *V. fischeri* carried *tfoX^{VF}* on this multicopy plasmid, transformation became independent of chitin oligosaccharides. That is, growth on two

Table 2. Bacterial strains and plasmids used.

Strain or plasmid	Relevant characteristics	Source or reference
Bacterial strain		
V. fischeri		
ES114	Wild-type isolate from *E. scolopes* light organ	Boettcher and Ruby (1990)
ESR1	Spontaneous Rf^R derivative of ES114	Graf *et al.* (1994)
AGP200	ES114 *tfoX* gene partially deleted	This study
BF3	ES114 *hadA* gene inactivated by mini-Tn*10* insertion; Cm^R	Feliciano (2000)
CL21	ES114 *ainS* gene partially deleted and replaced by Cm^R marker	Lupp *et al.* (2003)
JRM100	ES114 with mini-Tn*7* insertion; Em^R	McCann *et al.* (2003)
JRM200	ES114 with mini-Tn*7* insertion; Cm^R	McCann *et al.* (2003)
KV618	ESR1 *rpoN*::Tn*luxAB* Δ*luxA::erm;* Rf^R	Wolfe *et al.* (2004)
NL1	ES114 with *tfoY* gene inactivated by mini-Tn*5* insertion; Em^R	Lyell *et al.* (2008)
Plasmid		
pEVS79	*V. fischeri* suicide cloning vector, Cm^R, Tc^R	Stabb and Ruby (2002)
pEVS104	Conjugative plasmid with *tra* and *trb* genes	Stabb and Ruby (2002)
pEVS122	R6Kγ *oriV, oriT*$_{RP4}$ Em^R, *lacZα, cosN, loxP, incD*	Dunn *et al.* (2005)
pLosTfoX	995 bp of *V. fischeri* ES114 DNA containing the *tfoX*^{VF} ORF cloned into pEVS79	This study
pMU106	Allelic exchange vector carrying partially deleted *ainS* ORF replaced by Cm^R marker	Lupp *et al.* (2003)
pMulTfoX	995 bp of *V. fischeri* ES114 DNA containing the *tfoX*^{VF} ORF cloned into pVSV104; Km^R	This study
pMulTfoY	930 bp of *V. fischeri* ES114 DNA containing the *tfoY*^{VF} ORF cloned into pVSV104; Km^R	This study
pUBRtfoX	pEVS122 carrying the *tfoX* deletion construct.	This study
pVSV104	*V. fischeri* stable cloning vector, Km^R	Dunn *et al.* (2006)

Rf^R Rifampicin resistance; Cm^R, chloramphenicol resistance; Em^R, erythromycin resistance; Tc^R, tetracycline resistance; Km^R, kanamycin resistance.

compounds that normally did not induce competence (GlcNAc or Glcn) by wild-type *V. fischeri* produced transformants when pMulTfoX was present (Table 1), but not when the cells carried the vector plasmid (pVSV104) alone (data not shown).

This enhanced transformation suggested that providing *tfoX*^{VF} in multicopy to *V. fischeri* would enable the exploitation of natural transformation as a genetic tool for *V. fischeri* researchers. To further refine this method, the *tfoX*^{VF} complementation fragment was inserted into the vector backbone of plasmid pEVS79, which contains an origin that can be mobilized into *V. fischeri* cells, but that is not stably maintained without selection (Stabb and Ruby, 2002). The resulting plasmid, pLosTfoX, when introduced into *V. fischeri* ES114, also conferred an increased transformation frequency that was chitoligosaccharide-independent; however, the plasmid had to be maintained by antibiotic selection, at least until donor DNA was added (Table 1). Conveniently, after overnight recovery in non-selective medium, 19% of these transformants had lost the pLosTfoX plasmid as judged by the absence of both antibiotic resistance to chloramphenicol, and a PCR product when using primers targeting the oriT region of the plasmid (data not shown).

Table 3. Transformation of Δ*tfoX*^{VF} and *tfoY*^{VF}::Tn5 strains[a].

Recipient strain	Plasmid	Mean transformation frequency[b]	Range
ES114 (wild-type)	pVSV104 (vector)	54	2–120
	pMulTfoX	12 000	7700–16 000
	pMulTfoY	< 0.1[c]	–
AGP200 (Δ*tfoX*^{VF})	pVSV104 (vector)	< 0.1[c]	–
	pMulTfoX	4 400	190–8600
	pMulTfoY	< 0.1[c]	–
NL1 (*tfoY*^{VF}::Tn5)	pVSV104 (vector)	< 0.1[c]	–
	pMulTfoX	21 000	350–41 000
	pMulTfoY	1	0.1–2

a. Grown in minimal media with chitohexaose $(GlcNAc)_6$.
b. Average of two experiments ($\times 10^9$).
c. Below detection limit, 1×10^{-10}.

Vibrio fischeri *cells can be transformed by chromosomal, plasmid or linear forms of DNA*

Smaller fragments of DNA, such as plasmids or PCR products, are conveniently obtained and contain, by weight, more copies of a given genetic marker than chromosomal DNA. We asked whether such fragments of DNA carrying a Cm-resistance cassette (Cm^R) would transform more efficiently than chromosomal DNA when competent cells were provided with an equivalent weight of DNA in one of these three forms: (i) chromosomal DNA isolated from *V. fischeri* strain CL21 (containing a chromosomal replacement of *ainS* with the Cm^R); (ii) plasmid pMU106 (Table 2), originally used to make the CL21 replacement mutant, and isolated from an *E. coli* host; or (iii) PCR DNA that was produced by amplification of the region surrounding the *ainS* mutation, using CL21 chromosomal DNA as the template. To control for the possible presence of residual chromosomal DNA in the PCR-DNA condition, a no-polymerase PCR reaction was run in parallel. No transformants were observed

Table 4. Influence of the form of extracellular DNA on transformation frequency[a].

DNA form	Estimated extent of flanking homology (bp)	Mean transformation frequency[b]	Range
Chromosomal	$> 10^4$	23 000	12 000–33 000
Plasmid	10^3	22 000	16 000–28 000
PCR product	10^2to10^3	530	460–610

a. *V. fischeri* strain ES114 carrying pMulTfoX was grown in minimal media (MM) with $(GlcNAc)_6$.
b. Transformation frequency when equal weights of DNA were added; average of two experiments ($\times 10^9$).

when cells were provided with the products of the no-polymerase PCR control reaction. While transformants were observed with each of the three forms of donor DNA, transformation with either chromosomal or plasmid DNA gave the highest frequency. Similarly, PCR-generated DNA was ~40-fold less effective when compared on the basis of the number of transformants/copy of Cm^R marker added (Table 4). This lower frequency may result from either the shorter stretch of homologous flanking DNA ($< 10^3$ base pairs) present in the PCR product or, perhaps, the absence of methylation or other modification of the PCR product.

Natural transformation can be used to transfer mutations to new strain backgrounds

Existing mutations at several sites in the genome can be experimentally transferred into new *V. fischeri* strain backgrounds by transformation (Table 5). As anticipated, chromosomal DNA isolated from various mutant strains transformed at similar frequencies. For instance, there was no detectable difference in transformation frequency at the same site on the chromosome, regardless of the antibiotic-resistance cassette being transferred. While most of these transfers of marked DNA into wild-type cells simply re-created existing strains, we also performed transformations that created new mutant derivatives. Specifically, we transformed the Cm^R cassette from CL21 into Em-resistant JRM100 (Table 5), creating a novel strain that carried antibiotic-resistance cassettes at two separate sites. Similarly, transformation using donor DNA from the *rpoN*::Tn*luxAB*(Cm^R) Δ*luxA*::*erm* strain, KV618 (Table 2), into wild-type *V. fischeri*, and selection for Cm-resistance, resulted in the transfer of the *rpoN* mutation from a non-isogenic *V. fischeri* strain background (ESR1), into a wild-type strain (ES114). In the latter case, putative transformants were immotile on soft agar plates, consistent with the phenotype of the *rpoN* mutation; however, unlike the donor KV618, the transformants were still luminescent, like the recipient ES114 background (data not shown).

tfoX^{VF} *and* tfoY^{VF} *contribute to competence, but are not identical in function*

To determine whether the paralogue *tfoY^{VF}* also influences DNA uptake and incorporation, a transposon–insertion mutant of *tfoY^{VF}*, NL1 (Table 2), was used as the recipient strain in the transformation assay. Like the *tfoX^{VF}* mutant, NL1 yielded no detectable transformants, even in the presence of chitohexaose (Table 3). The transformation defect of NL1 was partially complemented by supplying *tfoY^{VF} in trans* on pMulTfoY. These findings suggest that both *tfoX^{VF}* and *tfoY^{VF}* are required for producing detectable levels of transformation in culture and, while they may be functionally redundant, they are unable to fully compensate for the loss of the other. If the functions of the two genes were identical, we would predict that the *tfoX^{VF}* mutant could be complemented by additional copies of *tfoY^{VF}* supplied *in trans*, and vice versa. In fact, while complementation of NL1 (*tfoY^{VF}*::Tn*5*) with pMulTfoX restored transformation, complementation of AGP200 (Δ*tfoX^{VF}*) with pMulTfoY did not. Furthermore, carriage of pMulTfoY in a wild-type background did not result in the chitoligosaccharide-independent transformation conferred by pMulTfoX, and instead appeared to decrease the frequency observed in wild-type cells. Thus, the influences of *tfoX^{VF}* and *tfoY^{VF}* on transformation competency are distinct.

Discussion

Vibrio fischeri cells appear capable of at least three forms of genetic transfer. First, they can mobilize and maintain DNA using a plasmid-borne conjugation system (Dunn *et al*., 2005); this ability to exchange both small and large plasmids may contribute to the large number and diversity of extrachromosomal elements they can carry (Boettcher and Ruby, 1994). In addition, an inspection of the completed genomes of two strains of *V. fischeri* provides evidence of several phage incorporation events (Ruby, 2005; Mandel, 2008), suggesting transduction as another

Table 5. Transformation frequency of chromosomal different markers between *V. fischeri* strains[a].

Donor strain	Recipient strain	Transformation frequency[b]
CL21	ES114	490
CL21	JRM100	900
JRM100	ES114	150
JRM200	ES114	170
BF3	ES114	80
KV618	ES114	100

a. Recipient strains (Table 2) were grown in minimal medium containing $(GlcNAc)_6$.
b. Representative experiment, values are the number of transformants per 10^9 cells.

potential mechanism for genetic exchange. Transduction is believed to be a central element in the ecology and virulence of *V. cholerae* (Jensen *et al*., 2006), and lytic phage have been shown to increase the frequency of *V. cholerae* transformation by releasing host cell DNA into the environment (Udden *et al*., 2008). However, there is little information about the role of phage and transduction in *V. fischeri*. In the work presented here, we show evidence of a third mechanism of genetic exchange in *V. fischeri*: transformation resulting from genetic competence.

Natural competence can be mediated by environmental signals, and is facilitated by the induction of specific structural and enzymatic proteins (Solomon and Grossman, 1996). When these factors are present, bacterial proteins bind extracellular DNA and transport it into the cell. These nucleic acids can then be either catabolized to serve as nutrients (Palchevskiy and Finkel, 2006) or, if the DNA is sufficiently similar to its own, incorporated into the cell's genome by homologous recombination (Hamilton and Dillard, 2006). While the function(s) of competence and/or transformation in ecologically relevant settings remains poorly understood for most bacteria, the machinery by which extracellular DNA is mobilized into another genome appears to be relatively conserved among Gram-negative bacteria (Chen and Dubnau, 2004). Nevertheless, the regulation of this process is often tailored to a given species' unique lifestyle.

Transformation was first demonstrated in the genus *Vibrio* in 2005, when chitin-induced competence and chromosomal incorporation of extracellular DNA was reported in *V. cholerae* (Meibom *et al*., 2005). This role for chitin suggested that chitinous structures are important sites not only for growth, but also for horizontal gene transfer. The report also showed that a regulatory protein encoded by *tfoX* was required for efficient transformation by *V. cholerae*. Soon after this discovery, expression studies of cultured *V. fischeri* cells grown on acetylchitobiose, the soluble dimeric subunit of chitin, indicated that this nutrient caused an upregulation of not only the genes of the chitin utilization program but also several putative competence genes, such as *pilA* and *comE* (A. Schaefer and E. Ruby, unpubl. data.). We show here that *V. fischeri* becomes genetically competent in a *tfoX*-dependent manner when grown in the presence of chitin oligosaccharides (Tables 1 and 3). As in *V. cholerae*, this induction was specific and did not occur when other carbon sources, including the chitin monomer *N*-acetylglucosamine (GlcNAc), were provided (Meibom *et al*., 2005). However, unlike *V. cholerae*, transformation by *V. fischeri* cells was not detectable induced by the presence of chitin in the form of crab shell tiles (Table 1), perhaps due to their relatively poor attachment and growth on this insoluble substrate. More recently, chitin-dependent induction of competence has also been demonstrated in *V. vulnificus*, although the contribution of this bacterium's *tfoX* gene to this process was not examined (Gulig *et al*., 2009). Taken together, these results make a strong argument that chitin is an important factor in the ecological genetics of the *Vibrionaceae*.

The role of TfoX in transformation has been studied not only in *V. cholerae*, but also in *Haemophilus influenzae*, *Aggregatibacter (Actinobacillus) actinomycetemcomitans* and *Escherichia coli* (Williams *et al*., 1994; Zulty and Barcak, 1995; Bhattacharjee *et al*., 2007; Sinha *et al*., 2009). In each case, TfoX (or the homologous Sxy) is thought to promote transcription of competence-related genes by assisting the cyclic-AMP receptor protein (CRP) in binding to Sxy-dependent CRP (CRP-S) sites within their promoter region (Sinha *et al*., 2009). In addition, the presence of the *tfoX/sxy* gene in multicopy, either overexpressed or under the control of its native promoter, results in increased, constitutive or, in the case of *V. cholerae*, chitin-independent competence (Williams *et al*., 1994; Meibom *et al*., 2005; Bhattacharjee *et al*., 2007).

The rate of transformation we report for *V. fischeri* in culture is ~100-fold lower than that observed in *V. cholerae* (Meibom *et al*., 2005). These data suggest that *V. fischeri* has either a more stringent competence regulation, or a higher level of extracellular DNase activity (Blokesch and Schoolnik, 2008). This lower transformation frequency of cells grown with soluble chitin oligosaccharides could explain the absence of observed transformation of *V. fischeri* grown on crab shell tiles. Nevertheless, the increased transformation frequency conferred by the carriage of multiple copies of $tfoX^{VF}$ provides a new technique for rapidly introducing mutations into *V. fischeri* (Table 4), probably through *recA*-mediated homologous recombination (Dunn *et al*., 2005). This approach not only adds a new method to the genetic repertoire of researchers working on *V. fischeri*, but also suggests that, given the widespread distribution of *tfoX* genes (Fig. 1), the introduction of multiple copies of *tfoX* homologues into other *Vibrionaceae* may facilitate the discovery of natural competence in these species as well.

We also report that a novel gene and putative paralogue of $tfoX^{VF}$, designated $tfoY^{VF}$, is required for normal levels of transformation in culture. These loci, $tfoX^{VF}$ and $tfoY^{VF}$, resemble each other in sequence and appear to converge in their influence on transformation under similar experimental conditions. However, they are unable to compensate fully for the loss of the other and may have distinct influences on transformation frequency (Table 3). Transcriptional studies in *V. fischeri* have also shown different regulation patterns for the two genes. For instance, in the light organ both genes are differentially regulated over the course of the day, and their transcription appears sequential: $tfoX^{VF}$ peaks first, with $tfoY^{VF}$ continuing an upward trajectory of expression (Wier *et al*., 2010). Like its

V. cholerae orthologue, the *tfoY*^{VF} gene of *V. fischeri* contains a putative riboswitch motif upstream of the start site (Weinberg *et al*., 2007; Sudarsan *et al*., 2008), while *tfoX*^{VF} does not. This difference further suggests that *tfoX*^{VF} and *tfoY*^{VF} have distinct cellular inputs for their activation.

The presence of *tfoX* homologues in all sequenced *Vibrionaceae* (Fig. 1), combined with a common capability to utilize chitin as a nutrient (Hunt *et al*., 2008), indicates that chitin-induced transformation is likely a shared-derived trait of this family of marine bacteria. Given the different ecologies of *V. cholerae, V. vulnificus and V. fischeri,* it is intriguing that they each respond to the presence of chitin oligosaccharides by inducing competence. Perhaps this common response to chitin reflects a shared life stage in seawater, or the common presence of chitin in association with a host. One of the best understood natural environments of *Vibrio* species is the light-organ symbiosis of *V. fischeri* (Nyholm and McFall-Ngai, 2004). Whether the uptake of DNA results in a fitness advantage for competent *V. fischeri* in the light organ, by providing an additional nutrient source and/or as a means of generating further diversity within the polyclonal population (Wollenberg and Ruby, 2009), is a direction for future studies. To date, we have observed no colonization defect for either a *tfoX*^{VF} or a *tfoY*^{VF} mutant within the first 48 h of symbiosis (data not shown). However, recent evidence indicates that *rscS,* a gene that is essential for colonization of the squid light organ, may have been acquired horizontally (Mandel *et al*., 2009), perhaps by transformation during the evolution of *V. fischeri*. Thus, over evolutionary time scales, there may be a direct connection between the capacity for genetic competence and symbiotic fitness. Further investigation may reveal how this mechanism of genetic exchange has been tailored to the individual lifestyles of diverse vibrios, from pathogens to beneficial symbionts.

Experimental procedures

Bacteria and culturing techniques

The bacterial strains and plasmids used in this study are described in Table 2. *Vibrio fischeri* srains were grown at 28°C in either a defined seawater minimal medium (MM) [0.33 mM β-glycerophosphate, 300 mM NaCl, 50 mM $MgSO_4$, 10 mM $CaCl_2$, 10 mM KCl, 0.01 mM $FeSO_4$ and 100 mM Tris-HCl (pH 7.5)], Luria–Bertani salt medium (LBS) (Dunlap, 1989), or a seawater-based tryptone (SWT) medium [5 g Bacto-tryptone (Difco), 3 g yeast extract, 3 ml glycerol, 700 ml filtered Instant Ocean (Aquarium Systems, Inc, Mentor, OH), and 300 ml distilled water] (Boettcher and Ruby, 1990). *Escherichia coli* strains used in construction of plasmids were grown at 37°C in either LB medium (Miller, 1992) or brain-heart infusion medium (BHI; BD, Sparks, MD). Media were solidified with 1.5% agar as needed. When added to LBS, chloramphenicol (Cm), erythromycin (Em) and kanamycin (Km) were used at concentrations of 5, 5 and 100 μg ml^{-1} respectively. When added to LB, Cm and Km were used at concentrations of 25 and 50 μg ml^{-1} respectively. When added to BHI, Em was used at a concentration of 150 μg ml^{-1}.

Sequence collection

The amino acid sequence for the *tfoX*-like ORF in *V. fischeri* ES114 (VF_0896) was used to query GenBank using the BLASTp algorithm (Altschul *et al*., 1997). The results of this search revealed that all large chromosomes of fully assembled *Vibrionaceae* genomes encode two protein sequences with similarity to the query sequence. The nucleotide sequences for both loci in *V. fischeri* ES114 (VF_0896 and VF_1573) were used to query GenBank using the BLASTn algorithm; from these searches, a collection of *tfoX*-like sequences in the 13 fully assembled *Vibrionaceae* (and three out-group) species were tabulated (Table S1).

Phylogenetic analyses

Nucleotide sequences were aligned using the default pairwise and multiple alignment parameters in ClustalX 2.0.11 (Larkin *et al*., 2007). Phylogenetic reconstructions were completed using three methods: maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference. Maximum parsimony reconstructions were performed by treating gaps as missing, searching heuristically using simple addition, tree–bisection reconnection for swaps, and swapping on best only with 1000 repetitions. For ML and Bayesian reconstructions, likelihood scores of 56 potential evolutionary models were tested using the Akaike information criteria (AIC) as implemented in Modeltest 3.7 (Posada and Buckley, 2004); based on AIC, a general time-reversible model with a gamma-shape parameter (GTR+Γ) was used. Maximum likelihood reconstructions were performed by treating gaps as missing, searching heuristically using simple addition, sub-tree pruning and regrafting (Morrison, 2007) for swaps, and swapping on best only with 1000 repetitions as implemented by PAUP*4.0b10 (Swofford, 2003). Bayesian inference was performed by invoking rates = gamma and nst = 6 settings (GTR+Γ model) and temp = 0.15 (to insure an appropriate amount of chain swapping) in the software package MrBayes3.1.2 (Ronquist and Huelsenbeck, 2003). Confidence in the topology of the ML and MP reconstructions was statistically assessed using either 100 bootstrap pseudoreplicates with the above search parameters. To test the Bayesian inference, an appropriately stationary posterior probability distribution was sampled every 200 generations; a stationary distribution was defined as having an average standard deviation of split frequencies between two chains in a Metropolis-coupled Markov chain Monte Carlo (MCMCMC) run < 0.01 for ~70–90% of samples (~500 000–2 000 000 total generations; 2501 of 10001 trees discarded as 'burn-in'). Consensus trees drawn from the sample distribution generated by MCMCMC were used for the assessment of the posterior probabilities of all clades. *Y. pestis* strain KIM (Table S1) was used to root all reconstructions.

DNA

Chromosomal DNA was purified using the MasterPure DNA Purification kit (Epicentre, Madison, WI, USA) and served as a template both for amplifying the regions that flanked genes targeted for deletion, and for direct transfer of chromosomal markers between strains. Plasmid DNA was isolated using a QIAprep spin miniprep kit (Qiagen, Valencia, CA, USA). PCR primers (Table S2) were purchased from either IDT (Coralville, IA, USA) or the UW Biotechnology Center (Madison, WI, USA). PCR fragments used for transformation were amplified with Pfx50 DNA polymerase (Invitrogen, Carlsbad, CA, USA) and purified with Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA).

Luminescence in culture

To determine the luminescence characteristics of *V. fischeri* wild-type and mutant strains, 2 ml of SWT was inoculated to an optical density at 600 nm (OD) of about 0.05 with cells that had been pre-grown overnight in LBS. Cultures were maintained at 28°C with shaking, and sampled at various times during growth to measure both luminescence and OD. Maximum specific luminescence (luminescence/OD) values were averaged for measurements made between OD 0.2 and 3.0. Growth rates were also determined during these experiments by observing the change in OD as a function of time.

Transformation of V. fischeri

To determine the frequency of transformation in liquid culture, cells were grown to mid-log phase in MM supplemented with different sources of carbon. These sources included: soluble chitin oligosaccharides, provided in the form of chitohexaose ($[GlcNAc]_6$; 2 μM) (Associates of Cape Cod, East Falmouth; MA, USA); or *N*-acetylglucosamine (GlcNAc; 10 μM) or glucosamine (Glcn; 10 μM) (Sigma, St Louis, MO, USA). Approximately 48 μg of DNA was added per ml of culture, which was incubated at 25°C for 30 min, and the cells were then diluted into LBS for a recovery period of between 4 and 14 h. These transformation reactions were plated onto LBS with the appropriate antibiotic selection. Antibiotic-resistant colonies that arose were considered putative transformants. Serial dilutions plated in parallel without antibiotic were used to determine the total number of cells placed on the selective plates. The frequency of transformation was calculated as the proportion of transformants arising from the total cell population. Because there was no difference in the growth rates of the wild-type and any of the transformants made in this study (data not shown), the length of recovery is unlikely to change the calculation of transformation efficiency. In any given experiment, the frequency of transformation could vary by as much as an order of magnitude; therefore, we report both the mean and range of frequencies observed. Transformation on crab-shell tiles was performed as previously described (Meibom *et al*., 2005). Briefly, *V. fischeri* cells were grown statically for 24 h in MM containing a crab-shell tile. The supernatant was then removed, and the tiles were resuspended in MM containing chromosomal DNA, and incubated for an additional 24 h. The tiles were removed and placed in LBS overnight for recovery before plating on selective and non-selective media as described above.

Verification of recombination in putative transformants

To confirm the introduction of a mutant copy of the *ainS* gene (VF_1037) into the recipient, chromosomal DNA isolated from putative transformants was used as the template in PCR reactions. Primer CamRCheckRev1 (Table S2) targeted the transformed Cm^R cassette, while primer ainSCheckRev1 targeted the region outside the *ainS* gene on the recipient's chromosome. To further confirm the identity of the putative transformants, we took advantage of the *ainS* defect in luminescence. The luminescence of candidate transformants grown in SWT was measured as a function of cell density; the presence of the wild-type *ainS* allele resulted in detectable luminescence, whereas a mutant allele did not.

Construction of $tfoX^{VF}$ *and* $tfoY^{VF}$ *mutants*

To generate the Δ*tfoX* mutant allele, approximately 2.0 kb of DNA upstream, and including a portion, of the $tfoX^{VF}$ gene (VF_0896) was PCR-amplified using primers tfoXUSFor and tfoXUSRev (Table S2). An approximately 1.6 kb DNA fragment containing part of the $tfoX^{VF}$ gene, as well as sequence downstream of the stop codon, was PCR-amplified using tfoXDSFor and tfoXDSRev (Table S2). These upstream and downstream fragments were fused by an engineered restriction site, resulting in a 492 bp internal deletion. The deletion leaves a 50 bp *N*-terminal sequence and an 82 bp *C*-terminal sequence intact, carried on the pUBRtfoX plasmid (Table 2). The mutant construct was introduced into ES114 via triparental mating, and incorporation of the deletion was confirmed by amplification with PCR primers flanking the $tfoX^{VF}$ gene (tfoXconfirmFor4 and tfoXconfirmRev; Table S2). The $tfoY^{VF}$ mutant NL1 contains a mini-Tn*5* transposon insertion in VF_1573, and was obtained from a previously described transposon-mutant library (Lyell *et al*., 2008).

Construction of $tfoX^{VF}$ *and* $tfoY^{VF}$ *complementation plasmids*

To supply the $tfoX^{VF}$ gene *in trans*, approximately 979 bp of DNA containing the $tfoX^{VF}$ gene and 150 bp of upstream sequence were PCR amplified using primers tfoXcompFor and tfoXcompRev (Table 2). The resulting product was cloned as a KpnI/SacI fragment into the complementation vector pVSV104 (Table S1), the origin of which is stably maintained in *V. fischeri* without selection. To supply the $tfoY^{VF}$ gene *in trans*, approximately 930 bp of DNA containing the $tfoY^{VF}$ gene and 233 bp of upstream sequence was PCR-amplified using primers tfoYcompFor and tfoYcompRev. The resulting product was similarly cloned into pVSV104.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. PCR screen confirming *ainS* gene replacement. Following transformation of Δ*ainS* chromosomal DNA into wild-type recipient cells, 10 putative transformants were analysed by PCR for evidence of gene replacement of intact *ainS* with Δ*ainS::*Cm^R. The primers used were ainScheckRev1 and camRcheckRev1 (Table S2), which will amplify a product only if the Cm^R cassette is within *ainS*. Lanes 1, 12 and 13: ladder (1KB DNA Ladder, Promega). Lanes 2–11, 14 and 15: PCR fragments. ES114 (wild-type) (lane 2), CL21 (Δ*ainS)* (lane 3), 10 putative transformants (lanes 4–11, 14 and 15). The predicted position (~1500 bp) of the PCR product containing an insertion of the Cm^R cassette into *ainS* is indicated by an arrow.

Fig. S2. Luminescence screen confirming *ainS* gene replacement. Following transformation of Δ*ainS* chromosomal DNA into wild-type recipient cells, the luminescence of ten transformants (1–10) was measured to confirm gene replacement of the intact *ainS* (bright in culture) with Δ*ainS::*Cm^R (dark in culture) phenotype. The luminescence of the transformants was compared with ES114 (wild-type) and CL21 (Δ*ainS*) over a range of OD measurements.

Fig. S3. PCR analysis confirming the $tfoX^{VF}$ internal deletion. The replacement of the intact *V. fischeri tfoX* gene with Δ*tfoX* was confirmed by PCR using primers tfoXconfirmFor4 and tfoXconfirmRev1 (Table S2). The intact $tfoX^{VF}$ gene yields a ~3400 bp PCR product; Δ*tfoX* yields a ~2900 bp PCR product. 1 kb DNA Ladder, Promega (lane 1); AGP200 (Δ*tfoX*) (lane 2); ES114 (wild-type) (lane 3).

Table S1. Loci used for phylogenetic reconstruction.

Table S2. Oligonucleotides used in this study.

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