Natural Transformation in a Classical-Biotype *Vibrio cholerae* Strain

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ABSTRACT *Vibrio cholerae* causes the gastrointestinal illness cholera, which spreads throughout the globe in large pandemics. The current pandemic is caused by O1 El Tor biotype strains, whereas previous pandemics were caused by O1 classical biotype strains. El Tor *V. cholerae* is noted for its ability to acquire exogenous DNA through chitin-induced natural transformation, which has been exploited for genetic manipulation of El Tor strains in the laboratory. In contrast, the prototypical classical strain O395 lacks this ability, which was suspected to be due to a mutation in the regulatory gene *hapR*. HapR and the regulator TfoX control expression of a third competence regulator, QstR. We found that artificial induction of both TfoX and QstR in the presence of HapR in O395 was required for efficient DNA uptake. However, natural transformation in the classical strain is still orders of magnitude below that of an El Tor strain. O395 expressing HapR could also undergo natural transformation after growth on chitin, which could be increased by artificial induction of TfoX and/or QstR. A plasmid that expresses both TfoX and QstR was created that allowed for consistent DNA uptake in O395 carrying a *hapR* plasmid. This technique was also used to facilitate cotransformation into O395 of unmarked DNA ($\Delta lacZ$, $\Delta flaA$, $\Delta flgG$) for multiplex genome editing by natural transformation (MuGENT). These results demonstrate that the classical biotype O395 strain is functionally capable of DNA uptake, which allows for the rapid genetic manipulation of its genome.

IMPORTANCE Natural transformation (uptake of exogenous DNA) in *Vibrio cholerae* has contributed to the evolution of these human pathogens. Classical biotype *V. cholerae* strains were responsible for the first six cholera pandemics but were replaced by El Tor biotype *V. cholerae* in the current pandemic. This study demonstrates that classical *V. cholerae* is functionally capable of natural transformation, but inactivation of the transformation regulator HapR and inherent levels of transformation that are lower than those of El Tor *V. cholerae* suggest that the classical biotype may be less able to utilize natural transformation for horizontal gene transfer.

KEYWORDS DNA uptake, biotype, cholera, genetic manipulation, natural transformation systems

Vibrio cholerae causes the human diarrheal disease cholera. This disease results from the bacteria colonizing the gastrointestinal tract and expressing cholera toxin, which leads to the characteristic profuse watery diarrhea. Although there are over 200 *V. cholerae* serogroups, documented pandemic cholera since the 1800s has been caused exclusively by the O1 serogroup lineage (which also includes an O1 serogroup strain that had undergone serogroup conversion to O139 [1]). The O1 serogroup is further divided into classical and El Tor biotypes. There have been seven pandemics since 1817. The fifth and sixth pandemics are known to have been caused by the classical O1 biotype, and the first four pandemics are suspected to have also been caused by classical O1 strains

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FIG 1 Regulation of natural competence in El Tor *V. cholerae* by chitin and high cell density. Chitin is sensed by ChiS, which induces the expression of TfoX in a TfoS/TfoR-dependent manner. High cell density (HCD) is sensed via the autoinducers CAI-1 and AI-2 through the LuxO-dependent quorum sensing pathway (LuxO QS path), resulting in induction of HapR expression. TfoX and HapR stimulate the expression of QstR. QstR and TfoX regulate the genes required for natural competence. HapR additionally represses the transcription of the nuclease Dns, which otherwise inhibits natural competence. ChiS (along with SlmA) also regulates chitin utilization, TfoX also regulates chitinases, and QstR also regulates type VI secretion. Another regulator, CytR, is also involved in regulating QstR, transformation, and chitinases, and CRP is involved in regulating TfoX and QstR. We have verified the requirement of the circled portions in natural competence of classical *V. cholerae* O395.

(2). The seventh and current pandemic, which began in 1961, is caused by El Tor biotype O1 strains, and they have completely replaced classical strains as a cause of cholera around the globe. The El Tor biotype is a distinct lineage from the classical biotype and did not originate from a recent common ancestor (3). The El Tor biotype has additional genetic islands and displays different phenotypes from the classical biotype (4).

The *V. cholerae* El Tor biotype is induced for natural competence in the presence of chitin (5), which is found on various surfaces where the bacteria colonize in the aquatic environment (e.g., zooplankton) (Fig. 1). The transmembrane regulatory protein ChiS senses the presence of chitin oligomers and induces the expression of the competence pilus genes, as well as chitin utilization and type VI secretion system (T6SS) genes (6, 7). ChiS controls competence indirectly, via inducing the expression of the regulatory protein TfoX, which is required for transcriptional activation of the *pil* genes that encode the DNA uptake pilus (8, 9). Activation of *tfoX* transcription involves the cAMP receptor protein (CRP) (10). The ChiS-dependent induction of TfoX also involves the regulator TfoS, which activates transcription of the small RNA TfoR that stimulates *tfoX* translation (11, 12). TfoX-dependent induction of *pil* gene transcription may be indirect and requires the activator CytR (10, 13, 14). TfoX also induces the expression of another regulatory protein, QstR, which activates transcription of the *com* genes that facilitate movement of the DNA captured by the pilus across the membrane (15). QstR also activates transcription of the T6SS gene cluster, and thus growth of *V. cholerae* on chitin induces T6SS-dependent killing of neighboring bacteria and subsequent uptake of liberated DNA (16, 17). Transcription of *qstR* also requires the quorum sensing (QS) regulator HapR, which binds to the *qstR* promoter and thus enables T6SS killing and DNA uptake on chitin at high cell density (13, 15, 16, 18).

The ability of El Tor strains to take up exogenous DNA during growth on chitin has been exploited for genetic manipulation of *V. cholerae*. Artificial induction of TfoX bypasses the need for growth on chitin to induce *V. cholerae* competence, and efficient protocols have been developed to allow manipulation of the El Tor *V. cholerae* genome through either growth on chitin or artificial induction of TfoX (19–22). The propensity of the competent cells within a population to continuously take up DNA has allowed for a further expansion of *V. cholerae* genetic manipulation to include multiplex genome editing by natural transformation (MuGENT), in which PCR products with genetic

alleles are cotransformed along with a selectable marker to facilitate allelic exchange of unmarked DNA (23). The chitin- and TfoX-inducible DNA competence program is widespread in *Vibrio* spp., so these techniques have also been adapted to additional species, including *V. parahaemolyticus*, *V. vulnificus*, *V. fischeri*, *V. natriegens*, and *V. campbellii* (24–27).

The *V. cholerae* classical O1 strain O395 has been a workhorse for the cholera research community. It is a sixth pandemic strain originally isolated from a cholera patient in India in 1965, and it has been used extensively in many studies in multiple laboratories, including challenge studies in humans (28). The O395 strain is known to contain a frameshift mutation in *hapR* (29, 30) which makes it defective for HA protease expression and, presumably, HapR-dependent natural transformation. Given our laboratories' investment over the years in genetic manipulation of the O395 genome, we sought to identify conditions in which natural transformation could be induced in this strain. We discovered that overexpression of both TfoX and QstR in the presence of HapR leads to low-level but consistent transformation in O395 and enables MuGENT in this strain, demonstrating that O395 is functionally capable of DNA uptake. Even under these conditions, transformation of O395 is orders of magnitude lower than in an El Tor strain, suggesting underlying differences in transformation efficiency between classical and El Tor *V. cholerae*.

RESULTS

HapR and TfoX are required for transformation in O395. In the past, we failed at inducing chitin-independent transformation in the classical O395 strain by overexpression of TfoX and considered that the inability of O395 to take up DNA was likely due to its lack of functional *hapR*, since HapR is required for expression of the competence regulator QstR in El Tor strains (13; Fig. 1). However, when we initially expressed TfoX from a BADpTfoX plasmid in O395 carrying a plasmid with *hapR* from an El Tor strain, we failed to detect transformation (data not shown). We suspect our initial failure to detect transformants in a *hapR*⁺ O395 overexpressing TfoX was due to the incredibly low efficiency of transformation and inability of the BADp promoter to provide a sufficient amount of TfoX (see below), but it caused us to investigate the requirements for HapR, TfoX, and QstR in O395 transformation.

The *tac* promoter (*tacp*) driving *qstR* transcription from the chromosome (*tacp-qstR*) leads to high-level constitutive QstR expression and T6SS-dependent killing in an El Tor strain (31). We introduced *tacp-qstR* into the O395 genome and investigated the competence of O395 strains with and without chromosomal *tacp-qstR* and carrying *hapR* and/or IPTG (isopropyl- β -D-thiogalactopyranoside)-inducible TfoX plasmids (25; Fig. 2). Under standard chitin-independent competence conditions (see Materials and Methods), the *tacp-qstR* strain carrying either pHapR or pTfoX alone failed to yield detectable transformed colonies; however, the *tacp-qstR* strain carrying both pHapR and pTfoX plasmids resulted in low but consistent transformants (approximately 10^{-5}). This is about 2 orders of magnitude lower transformation efficiency than the El Tor strain A1552 (approximately 10^{-3}) under the same conditions carrying pTfoX (Fig. 2). The O395 wild-type strain (i.e., without *tacp-qstR*) carrying pTfoX and pHapR yielded almost 10-fold lower transformants than the strain with *tacp-qstR*, indicating that HapR- and TfoX-dependent expression of QstR under these conditions is limiting the efficiency of transformation. These results demonstrate that (i) the O395 strain is capable of natural transformation, (ii) it requires HapR and TfoX, and (iii) low induction levels of QstR limit transformation efficiency.

Overexpression of both TfoX and QstR is required for chitin-independent transformation in classical O395, but not El Tor A1552. Given the 10-fold increase in transformation efficiency in a *hapR*⁺ O395 strain expressing TfoX when the *tac* promoter was driving QstR expression from the chromosome, we reasoned that overexpression of both TfoX and QstR from a plasmid would allow for greater transformation efficiency while offering the possibility of curing the strain of TfoX, QstR, and HapR after genetic manipulation. We initially constructed conjugatable plasmids that express

FIG 2 Chitin-independent transformation in O395 requires TfoX and HapR. *V. cholerae* El Tor strain A1552 (ET) and classical strains O395 or KKV3058 (*tacp-qstR*) (CL) carrying plasmids pMMBtfoXsacB (pTfoX) and pKEK739 (pHapR+) or control plasmid pWKS30 (pHapR-) were tested for chitin-independent transformation with SAD033 gDNA (Δ VC1807::Sp) as described (Materials and Methods). *, $P < 0.05$; ***, $P < 0.005$; <d.l., below detection limit.

either TfoX alone (BADpTfoX) or TfoX and QstR (BADpTfoXQstR) from the arabinose-inducible BADp promoter from pBAD24 (32). However, arabinose induction of either BADpTfoX or BADpTfoXQstR in a HapR⁺ O395 strain resulted in no detectable transformants (see Fig. S1 in the supplemental material). Since the *tac* promoter in pTfoX (above) had sufficiently induced TfoX to result in transformation (Fig. 1), we reasoned that replacing the BADp promoter with *lacI-tacp* (i.e., IPTG-inducible) would lead to sufficient expression to induce competence. We therefore generated plasmids *tacpTfoX* and *tacpTfoXQstR* (Fig. S2). These plasmids also contain *sacB* as a counterselectable marker for curing.

O395 with pHapR and additionally carrying either *tacpTfoX* or *tacpTfoXQstR* was induced with IPTG and measured for competence in our standard assay (Fig. 3). The strain expressing both TfoX and QstR yielded transformants, whereas O395 expressing

FIG 3 Chitin-independent transformation induction in O395 requires TfoX and QstR. *V. cholerae* El Tor strain A1552 (ET) and classical strain O395 (CL) carrying plasmids pKEK2284 (pTfoX) or pKEK2285 (pTfoX and pQstR) were tested for chitin-independent transformation with SAD033 gDNA (Δ VC1807::Sp) as described (Materials and Methods). The O395 strains were also carrying pKEK2270 (+pHapR). ***, $P < 0.005$; NS, not significant; <d.l., below detection limit.

FIG 4 Dns inhibits chitin-independent transformation efficiency in O395. *V. cholerae* classical strains O395 (Dns⁺) or KKV3347 (Δ *dns*; Dns⁻) carrying plasmids pKEK2284 (pTfoX) or pKEK2285 (pTfoX and pQstR), and pKEK2270 (pHapR⁺) or control plasmid pWKS30Tc (pHapR⁻), were tested for chitin-independent transformation with SAD033 gDNA (Δ VC1807::Sp) as described (Materials and Methods). **, $P < 0.01$; ***, $P < 0.005$; <d.l., below detection limit.

only TfoX resulted in no detectable transformants. In contrast, the El Tor strain A1552 with either *tacpTfoX* or *tacpTfoXQstR* gave similar amounts of transformants, demonstrating that the induction of TfoX is sufficient for transformation in the El Tor strain but that TfoX induction fails to induce sufficient levels of QstR in the classical O395 strain for transformation under these conditions. Moreover, even under these conditions, transformation levels are more than 10-fold lower in the O395 strain than in the El Tor strain.

As shown in Fig. 2, we detected transformation in O395 with pHapR and pTfoX in the absence of enhanced QstR expression. As shown in Fig. 3, however, there was no detectable transformation in O395 with pHapR and *tacpTfoX* in the absence of enhanced QstR expression. The important difference between these two experiments is the plasmid backbone of the TfoX vector; *tacpTfoX* originates from pBAD24 (32), while the pMMBTfoX plasmid (pTfoX) originates from RSF1010 (33). pBAD24 is a very high copy number vector (34), and pMMBTfoX is a low copy number vector. We conclude that the larger amounts of TfoX induced from pBAD24 may somehow inhibit sufficient levels of QstR expression.

The extracellular nuclease Dns inhibits transformation in O395. The extracellular nuclease Dns inhibits transformation of El Tor strains (35; Fig. 1). HapR represses *dns* transcription, and thus Dns is repressed at high cell density, facilitating DNA uptake. We wondered whether Dns likewise inhibits transformation in O395. We therefore constructed a Δ *dns* O395 strain and measured transformation in this strain expressing HapR, TfoX, and QstR (Fig. 4). Under these conditions, transformation of Δ *dns* O395 was approximately 10-fold higher than transformation of O395 with Dns intact. More strikingly, we did not detect transformants in the O395 strain with *tacpTfoXQstR* in the absence of HapR, but the Δ *dns* strain with *tacpTfoXQstR* was capable of transformation even in the absence of HapR. In contrast, the Δ *dns* strain with *tacpTfoX* and pHapR was not capable of transformation, showing that transformation of this strain is dependent on induction of both TfoX and QstR. This result demonstrates that, like in El Tor strains (35), the extracellular nuclease Dns inhibits DNA uptake in the classical strain and that artificial induction of QstR and removal of Dns eliminates the HapR requirement for transformation.

O395 is capable of chitin-inducible natural transformation. Growth of El Tor *V. cholerae* on chitin induces natural transformation through the ChiS pathway (Fig. 1). To determine if the classical O395 strain is capable of chitin-inducible transformation,

FIG 5 Chitin-dependent transformation in O395 requires HapR; artificial induction of QstR and TfoX enhances transformation. *V. cholerae* El Tor strain E7946 (ET) and classical strain O395 or KKV3058 (*tacp-qstR*) (CL) carrying plasmids pKEK2270 (pHapR+) or control plasmid pWKS30 (pHapR-), and pKEK2284 (pTfoX+) or control plasmid pKEK2254 (pTfoX-), were tested for chitin-dependent transformation with a Δ VC1807::Em PCR product derived from SAD2127 as described (Materials and Methods). *, $P < 0.05$; ****, $P < 0.0001$; <d.l., below detection limit.

O395 or O395 with chromosomal (constitutive) *tacp-qstR* and carrying pHapR and/or *tacpTfoX* was grown on chitin and measured for natural transformation. The El Tor E7946 strain has been used to characterize chitin-dependent transformation due to its high levels of transformation (23) and was used here as a control. E7946 is transformed at high frequency ($\sim 10^{-2}$) under these conditions (Fig. 5). The O395 strain without any plasmids failed to yield any chitin-dependent transformants; however, provision of pHapR resulted in O395 transformants at a low frequency. Chromosomal expression of *qstR* from the *tacp* promoter also resulted in transformants at a slightly but not significantly higher frequency. Provision of pHapR in the *tacp-qstR* strain did not significantly increase transformation frequency further. However, provision of pTfoX in the *tacp-qstR* strain carrying pHapR during growth on chitin gave significantly higher transformants than the O395 strain carrying pHapR alone. This indicates that even in the presence of HapR, insufficient induction of TfoX and/or QstR limits O395 natural transformation. These results demonstrate that the O395 classical strain is functionally capable of chitin-dependent natural transformation, but this capability minimally requires restoration of HapR.

O395 is conducive to MuGENT. The propensity of individual *V. cholerae* cells undergoing natural transformation to continuously take up exogenous DNA is the basis of MuGENT, in which DNA with an antibiotic resistance marker is mixed with a high concentration of unmarked DNA (23). When transformation with the antibiotic marker is selected for, the resulting resistant colonies are screened for cotransformation with the unselected DNA, which transforms at a high rate in this subpopulation. To determine if MuGENT could be performed in O395, we utilized the O395 strain with pHapR and *tacpTfoXQstR*. Under standard assay conditions (see Materials and Methods), we transformed this strain with 200 ng SAD033 (Δ VC1807::Sp) genomic DNA (gDNA) along with 2,000 ng PCR product of unmarked Δ *lacZ*, Δ *flaA*, or Δ *flgG* alleles with 3-kbp flanking regions (Table 1). The resulting Sp^R colonies were then screened by growth on X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) medium to identify white (Lac-) colonies (Δ *lacZ*) or inoculated into motility agar to identify nonmotile strains (Δ *flaA* and Δ *flgG*) (Table 1 and Fig. S3). Strains were confirmed to have the cotransformed alleles by PCR. We found the cotransformation efficiency of unmarked alleles under these conditions to be 6% (7/116; Δ *flaA* strain), 9% (14/150; Δ *flgG* strain), and 7.5% (8/106; Δ *lacZ* strain). These results demonstrate that O395 *V. cholerae* can be genetically manipulated by the MuGENT technique.

TABLE 1 MuGENT cotransformation frequency of O395

Unmarked deletion	$\Delta lacZ$ strain	$\Delta flaA$ strain	$\Delta flgG$ strain
No. of Sp-resistant colonies	106	120	151
No. of colonies with cotransformed deletion	8	7	13
Cotransformation efficiency (%)	7.55	5.83	8.61

DISCUSSION

In this study, we demonstrated that the O1 classical strain O395 is functionally capable of natural transformation. O395 lacks HapR due to a frameshift mutation (removal of a T residue at position 218 bp), and our study shows that this prevents natural transformation. Chitin-dependent transformation was not detectable in O395, but complementation with a functional *hapR* resulted in a low level of chitin-dependent transformants. Additionally, chitin-independent transformation (by artificial induction of TfoX) was not detectable in O395 in the absence of HapR. Thus, the loss of HapR in this strain essentially eliminated the possibility for horizontal gene transfer via natural transformation. HapR mutations are relatively common in *V. cholerae* strains (36). In a survey of 602 clinical and environmental O1/O139 *V. cholerae* strains isolated in China from 1960 to 2007, 228 strains (38%) contained mutations in HapR that rendered them incapable of quorum sensing (QS-) (36); although the published manuscript did not report the biotype for the O1 strains, they are all El Tor (B. Kan and J. Zhu, personal communication). In El Tor *V. cholerae*, mutation of *hapR* also causes a phenotypic switch to a rugose phenotype, which is characterized by expression of copious amounts of the *Vibrio* polysaccharide (VPS) matrix (37), which facilitates biofilm formation and is associated with increased resistance to environmental stresses and protozoan grazing (38–40). In contrast, classical strains do not undergo a phenotypic switch to rugose with *hapR* mutation, and classical *V. cholerae* strains are known to make poor biofilms (30, 41). Thus, the compensatory advantage of *hapR* mutation leading to enhanced environmental persistence of El Tor is missing in the classical biotype.

The central role of HapR in quorum sensing is well established (42), and HapR levels rise in response to high cell density (Fig. 1). HapR binds directly to the *qstR* promoter (15) and is required for QstR expression (8, 13, 15). HapR also binds to the *dns* promoter and represses the expression of this extracellular nuclease (15, 35), and this enhances transformation by preventing degradation of extracellular DNA. We found that artificial induction of QstR, either from the *tacp* promoter in the chromosome or from a plasmid, further enhanced chitin-dependent and -independent transformation of HapR⁺ O395, suggesting that HapR may be unable to fully induce QstR in the classical O1 strain. Also, chitin-independent transformation efficiencies in O395 were increased in the absence of Dns, similar to El Tor O1 strains (35), indicating that HapR-dependent repression of *dns* is a means of enhancing transformation efficiency in classical O1 *V. cholerae*. In fact, we could eliminate the need for HapR through overexpression of QstR (along with TfoX) and simultaneous elimination of Dns (Fig. 4), demonstrating that these are the only relevant activities of HapR in natural transformation of the classical strain. The *qstRp* and *dnsp* sequences of O395 are identical to those in El Tor strains, including the identified HapR binding sites (15), and the Dns protein sequences are 98% identical, so differences in transformation efficiency between the biotypes is not likely to be due to differences in HapR operator sequences or Dns activity.

Chitin-independent transformation in El Tor *V. cholerae* bypasses the need for chitin by artificial induction of TfoX. Normally, chitin oligosaccharides are sensed by transmembrane activators ChiS (6) and TfoS (11) (Fig. 1). ChiS activates transcription of chitin utilization genes (7) and appears to assist TfoS to activate transcription of the small RNA TfoR, which facilitates the translation of the *tfoX* mRNA in an Hfq-dependent manner (11). Artificial induction of TfoX from a plasmid in El Tor strains bypasses this chitin-dependent regulation of TfoX expression. The ability of HapR⁺ O395 to undergo

chitin-dependent transformation, albeit at low efficiency, indicates that this pathway appears to be intact in classical *V. cholerae*. Providing additional amounts of QstR and/or TfoX by artificial induction during chitin-dependent transformation leads to higher levels of transformation in O395, demonstrating that chitin-dependent induction of TfoX and QstR is not optimal in the classical strain. Even with provision of HapR and overexpression of TfoX and QstR, chitin-dependent and -independent transformation of O395 was several orders of magnitude lower than in El Tor strains.

At this point it is not clear what the barrier is to more efficient natural transformation in the classical *V. cholerae* strain, but it is tempting to speculate that an inherent lower transformation efficiency in classical strains may have contributed to their global extinction and replacement with El Tor strains. Variants of *V. cholerae* continually emerge with different genotypic and phenotypic characteristics due to lateral gene transfer events caused by transduction, conjugation, and natural transformation (1, 43). Colonization of chitin surfaces induces El Tor *V. cholerae* to kill neighboring bacteria via its type VI secretion system and to take up the released DNA by natural transformation (5, 17; Fig. 1); the average length of DNA fragment taken up is 50 kbp (44). The conversion of *V. cholerae* O1 serogroup to O139 is suspected to have been caused by natural transformation, because it can be replicated in the laboratory by growth on chitin (45), and even toxigenic conversion may occur by chitin-induced transformation (46). As we have shown here, the superior ability of El Tor *V. cholerae* to undergo chitin-dependent natural transformation in comparison to classical *V. cholerae* (even with HapR restored) would be expected to provide an evolutionary advantage to this biotype.

The plasmids we created to express TfoX, QstR, and HapR have allowed us to consistently manipulate the genome of *V. cholerae* O395 through natural transformation. This affords the possibility to more quickly construct various strains with antibiotic resistance-marked mutations, and with the additional MuGENT technique to construct strains with unmarked mutations. The efficiency of chitin-independent transformation of O395 is lower than in El Tor strains, and the cotransformation frequency of unmarked mutations during MuGENT is also lower, but using these techniques we have routinely obtained mutants of interest. The *tacpTfoXQstR* plasmid also has *sacB* for counterselection (Fig. S2), and we have found that overnight growth on sucrose is sufficient for loss of this plasmid, as well as the pHapR plasmid. The origin of replication of pHapR is derived from the low-copy pSC101 vector (47), so it is not clear why this plasmid, which lacks *sacB*, is lost from *V. cholerae* so rapidly during growth on sucrose medium. These two plasmids allow for efficient genetic manipulation of the O395 genome and subsequent efficient curing of the plasmids. This technique does not replace the traditional method of suicide vector-mediated mutagenesis but reduces the amount of time necessary to construct genetic mutant classical strains by several days. Genetic manipulation of O395 by natural transformation should enhance the study of classical O1 *V. cholerae*.

MATERIALS AND METHODS

Bacterial strains. *Escherichia coli* strains DH5 α and DH5 α pir were used for cloning, and SM10 λ pir was used for conjugation. *V. cholerae* O1 classical strain O395 and El Tor strains A1552 and E7946 were used for all experiments; a complete list of strains is provided in Table 2. Strains were typically grown in Luria broth (LB). Antibiotics were used in the following concentrations: ampicillin (Ap), 100 μ g/ml; streptomycin (Sm), 100 μ g/ml; spectinomycin (Sp), 50 μ g/ml; kanamycin (Kn), 50 μ g/ml; erythromycin (Em), 10 μ g/ml; tetracycline (Tc), 2 μ g/ml (for *V. cholerae*) or 10 μ g/ml (for *E. coli*). When required, isopropyl- β -D-thiogalactopyranoside (IPTG; 0.2 mM) or arabinose (0.2%) was added to the medium.

Plasmid and strain construction. A complete list of primers and plasmids used is provided in Tables 2 and 3. Construction of the TfoX/QstR expression plasmids occurred in several steps. First, *oriT* from pCVD442 (48) was introduced into pBAD24 (32) by *in vivo* assembly (IVA) cloning (49) using primer pairs *oriTF* PstI pBADFC + *oriTR* HindIII pBADFC and pBAD24 PstI FC + pBAD24 HindIIIFC, resulting in pKEK2086. Next, *sacB* was PCR amplified from pKEK229 using primer pairs *sacB* F Sall + *sacB* R FC, digested with Sall and PstI, and ligated into pKEK2086 digested similarly, to create pKEK2254. *qstR* was PCR amplified from pKAS32 *tacp-qstR* (31) with primers *ptac-qstR* F BHI FC + *qstR* R Sall, digested with XmaI and Sall, and ligated into pKEK2254 digested similarly to create pKEK2262, which also has the ribosome binding site (RBS) from the *tacp* promoter. *tfoX* was PCR amplified from O395 gDNA with *tfoX* F NcoI + *tfoX* R XmaI, digested with NcoI and XmaI, and ligated into either pKEK2254 or pKEK2262 that had been digested similarly, to create pKEK2275, which expresses *tfoX*, and pKEK2277, which expresses *tfoX* and *qstR*, from the BADp promoter.

TABLE 2 Strains and plasmids used in this study

Strain or plasmid	Description	Reference
Strains		
DH5 α	<i>E. coli</i>	55
DH5 α λ pir	<i>E. coli</i>	56
SM10 λ pir	<i>E. coli</i>	57
O395	<i>V. cholerae</i> O1 classical wild type	58
A1552	<i>V. cholerae</i> O1 El Tor wild type	59
E7946	<i>V. cholerae</i> O1 El Tor wild type	60
SAD033	E7946 Δ VC1807::Sp	23
SAD034	E7946 Δ VC1807::Kn	23
SAD2127	E7946 Δ VC1807::Erm	23
SAD238	E7946 Δ lacZ	23
KKV598	O395 Δ lacZ	61
KKV2506	O395 Δ flaA Δ lacZ	62
KKV2772	O395 Δ flgG	This study
KKV3058	O395 ptac-qstR	This study
KKV3347	O395 Δ dns	This study
Plasmids		
pWKS30	Low copy vector (pSC101 ori); Amp ^R	47
pWKS30Tc	Low copy vector (pSC101 ori); Tet ^R	50
pBAD24	Expression vector; arabinose-inducible; Amp ^R	32
pMMBtfoX	Amp ^R	27
pMMBsacBtfoX	Kan ^R ; pTfoX	25
pKAS32	Streptomycin-counterselectable suicide vector; Amp ^R	53
pJT88	pKAS32 with ptac-qstR	31
pKEK 229	Sucrose-counterselectable suicide vector; Amp ^R	61
pKEK 739	pWKS30hapR	This study
pKEK2013	Δ flgG in pKEK229	This study
pKEK 2086	pBAD-oriT	This study
pKEK2200	Sucrose-counterselectable suicide vector; Cm ^R	51
pKEK 2254	pBAD-sacB-oriT	This study
pKEK2262	pBAD-qstR-sacB-oriT	This study
pKEK2270	pWKS30TchapR; pHapR	This study
pKEK 2275	pBAD-tfoX-sacB-oriT; BADpTfoX	This study
pKEK 2277	pBAD-tfoX-qstR-sacB-oriT; BADpTfoXQstR	This study
pKEK 2284	ptac-tfoX-sacB-oriT; tacpTfoX	This study
pKEK 2285	ptac-tfoX-qstR-sacB-oriT; tacpTfoXQstR	This study
pKEK2360	Δ dns in pKEK2200	This study

Finally, BADp in pKEK2275 and pKEK2277 was replaced with lacI-lacp by PCR amplification of lacI-lacp from pMMBtfoX (27) with primers lacregion F Apal + lacregion R NcoI and PCR amplification of pKEK2275 and pKEK2277 with primers pBADtfoX F NcoI + pBADtfoX R Apal, and assembly by IVA (49) to form pKEK2284 (tfoX) and pKEK2285 (tfoX qstR; Fig. S2), respectively.

The hapR expression plasmids were created by PCR amplification of hapR from A1552 gDNA with primers HapR3 BHI and HapRU XbaI, which was digested with BamHI and XbaI and ligated into pWKS30 (47) digested similarly to yield pKEK739. The hapR fragment from pKEK739 was digested with NotI and Apal and ligated into pWKS30Tc (50) digested similarly to yield pKEK2270. The Δ flgG deletion strain was synthesized as a gblock (Integrated DNA Technologies) that contained the start codon of flgG fused to the stop codon with 960 bp flanking both sides. This gblock was ligated into pKEK229 to make plasmid pKEK2013. The Δ dns deletion strain was constructed by PCR amplifying upstream and downstream fragments, using primer pairs Del dns frag1 F FC + Del dns frag1 R and Del dns frag2 F + Del dns frag2 R FC, and PCR amplification of pKEK2200 (51) with primer pair universal1 + universal2, followed by IVA assembly to create plasmid pKEK2360. All plasmid constructs were verified by sequencing.

V. cholerae strains with chromosomal manipulations were mostly constructed by sucrose counterselection utilizing pKEK2200, a derivative of pDS132 (52), as described previously (51). tacp-qstR was introduced into the O395 genome by streptomycin counterselection utilizing pKAS32 (31, 53).

Transformation assays. The standard chitin-independent transformation assay was adapted from reference 54. *V. cholerae* strains were inoculated into LB containing 0.2 mM IPTG (for ptac plasmids) or 0.2% arabinose (for pBAD plasmids) and appropriate antibiotic and grown overnight at 30°C to stationary phase on a roller drum. All strains contained either an expression plasmid or the parent vector (as detailed in figure legends) such that identical antibiotics were used in the growth medium for each experiment. Then, 7 μ l of this overnight culture was added to 350 ml 0.5 $\times$ instant ocean sea salts with

TABLE 3 Oligonucleotides used in this study

Name	Sequence
oriTF PstI pBADFC	TAGAGTCGACCTGCAGTTTTGTCCGGTGTGGGTGAAGG
oriTR HindIII pBADFC	CAAAACAGCCAAGCTTAGCCGACCAGGCTTTCCAC
pBAD24 PstIFC	CTGCAGGTCGACTCTAGAGGATC
pBAD24 HindIIIFC	AAGCTTGGCTGTTTTGGCGGA
SacB F Sall	ACTGTAAGTCGACCACATATACCTGCCGTTCACTATTATTAG
SacB R FC	CTTGCATGCCTGCAGGTTATTTGTTAACTGTTAATTGTCCTTGTTC AAG
tfoX F NcoI	GCGCCCATGGTGATTAAGGATCAATGGATATGAATG
tfoX R XmaI	GCGCCCCGGGTTAACGCTGCTGACAACCTTC
Ptac-qstR F BHI FC	CGAGCTCGGTACCCGGGGATCCGCTAAGGAAGCTAAAATGCAACGAG
qstR R Sall	ATATGTGGTCGACTTACAGTAAATTTTGATTAAACCAAGCGATC
lacIregion F ApaI	TTACCAAGGGCCCTCACTGCCCGCTTTCCAGTC
lacIregion R NcoI	ATCACCATGGATTCTGTTTCTGTGTGAAATTGTTATCC
pBAD-tfoX F NcoI	GAAACAGAATCCATGGTGATTAAGGATCAATGGATATG
pBAD-tfoX R ApaI	GCACTGAGGGCCCTTGGTAACGAATCAGACAATTGACGG
Del Dns flank1 F FC	CGAGCTCGGTACCCGGGGATGATGTTTTGGTACAAATCAGCG
Del Dns flank1 R	CATCAACTAGTCATAAAAGACGTAGATAAGTAGGTTTTTATCAG
Del Dns flank2 F	TTATGACTAGTTGATGGGCAGAATCTCACCC
Del Dns flank2 R FC	CTTGCATGCCTGCAGGGCTGAGTAATCACAGCGATTCTACTC
Universal 1	CCGGGTACCGAGCTCGAATTC
Universal 2	CCTGCAGGCATGCAAGCTT
ABD336	ACCCTAAGCGGTTCAATTTTGTGCGATG
ABD399	AACTGATGGCAGAAAAAGCCACTCAG
hapR3 BHI	GCGGATCCGCGACCTCTTGTCTCAGAAATC
hapR U XbaI	GCTCTAGACTAGTTCTTATAGATACACAG
flaA F	CGAGCTCGGTACCCGGCTGAATGATTTCATGAGACGGTTC
flaA R	CTTGCATGCCTGCAGGTCCTCCGCACCAATGTATCG
flgG region F	GTTAACGTATGGCCGGAGTCA
flgG region R	TAGCAAGTTAGGATTGGTAAGCTGA

0.2 mM IPTG or 0.2% arabinose, and 100 ng gDNA (SAD33 or SAD34), and then incubated statically at 30°C for 5 h. For chitin-dependent transformation, 100 ng 6-kbp PCR product of $\Delta VC1807::Em$ from SAD2127 was used in place of gDNA. For MuGENT experiments, 200 ng of gDNA and 2,000 ng of unmarked PCR product was added to the tube prior to incubation. After incubation, 0.5 ml LB was added to each tube, which was then placed in a roller drum at 30°C for 2 h, and then plated on the appropriate antibiotic to determine CFU. For MuGENT experiments utilizing the $\Delta lacZ$ strain, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) was added to plates to allow detection of Lac⁺ colonies.

The plasmids pKEK2270 and pKEK2285 were readily cured from transformed strains by overnight growth at 37°C on LB containing 15% sucrose. pKEK2285 contains the *sacB* gene for counterselection on sucrose, whereas pKEK2270 does not, but strains also readily lost pKEK2270 during overnight growth, leading to virtually all colonies being both Amp^r and Tet^r.

We performed three independent biological replicates per sample. Statistical analysis was performed using Prism 9.0 one-way analysis of variance (ANOVA) with postprocessing Tukey testing.

Motility assays. Strains were grown at 37°C overnight on an agar plate and then inoculated into motility agar (LB 0.3% agar) and incubated at 30°C.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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