Quorum Sensing Contributes to Natural Transformation of *Vibrio cholerae* in a Species-Specific Manner[▽]

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Received 26 May 2011/Accepted 7 July 2011

Although it is a human pathogen, *Vibrio cholerae* is a regular member of aquatic habitats, such as coastal regions and estuaries. Within these environments, *V. cholerae* often takes advantage of the abundance of zooplankton and their chitinous molts as a nutritious surface on which the bacteria can form biofilms. Chitin also induces the developmental program of natural competence for transformation in several species of the genus *Vibrio*. In this study, we show that *V. cholerae* does not distinguish between species-specific and non-species-specific DNA at the level of DNA uptake. This is in contrast to what has been shown for other Gram-negative bacteria, such as *Neisseria gonorrhoeae* and *Haemophilus influenzae*. However, species specificity with respect to natural transformation still occurs in *V. cholerae*. This is based on a positive correlation between quorum sensing and natural transformation. Using mutant-strain analysis, cross-feeding experiments, and synthetic cholera autoinducer-1 (CAI-1), we provide strong evidence that the species-specific signaling molecule CAI-1 plays a major role in natural competence for transformation. We suggest that CAI-1 can be considered a competence pheromone.

The bacterium *Vibrio cholerae* is a facultative pathogen and the causative agent of cholera. The potential spread and devastation of cholera outbreaks can currently be seen in the Haiti epidemics, and several mathematical models have been developed to predict the epidemic's further development (2, 5, 54). This clearly indicates that cholera is far from extinct and can instead be considered a reemerging disease (41).

V. cholerae commonly lives in aquatic ecosystems, its true habitat, where it intimately associates with zooplankton and their chitinous exoskeleton (35). Chitin induces natural competence for transformation, a mode of horizontal gene transfer in this organism (38). During transformation, the bacterium can import and recombine DNA from the environment, thus rendering the organism naturally transformed.

The purpose of natural competence for transformation has been intensively discussed (48). While the obvious role of using DNA as a nutrient source was initially suggested and recently also experimentally proven for starved *Escherichia coli* cells (19, 46), several facts challenge this hypothesis. For example, the presence of cytoplasmic proteins that protect the incoming DNA against degradation (4, 42) highlight the importance of keeping the DNA intact. Second, the existence of complex and energy-consuming uptake machineries supports the now widely accepted role that natural competence for transformation is a mechanism for DNA repair and evolution.

Another important argument contradicting the “DNA solely for food” hypothesis is that two Gram-negative bacteria, *Neisseria gonorrhoeae* and *Haemophilus influenzae*, are very fastidious about their source of DNA in that they take up genetic material only from their own species (51, 52). This species

specificity is based on the presence of short DNA uptake signal sequences (DUS; 10 to 12 bp) that are overrepresented in their respective genomes (13, 18, 20). The molecular mechanism of DUS recognition is not known. However, Elkins et al. showed that linearized plasmid DNA was taken up only in a DNase I-resistant state if the plasmid contained the 10-bp DUS sequence GCCGTCTGAA (18). Based on this and other studies, it has been suggested that sequence-specific binding of the DUS occurs at the level of a receptor protein (9) that is located on the bacterial surface. This interaction is required for DNA uptake across the outer membrane (23). The statement that the naturally competent bacteria *N. gonorrhoeae* and *H. influenzae* “have specific receptors that recognize their own DUS and allow them to take up only DNA from their own species” is already included in textbooks (58).

A recent study discovered a conserved 9-bp sequence in *V. cholerae* as part of the formerly described JUMPstart sequence, which is located at the junction point of the O-antigen gene cluster (28); this 9-bp sequence is highly similar to the DUS of *H. influenzae* (21). However, the copy number of this sequence was only 23 and was therefore in the range of any random nonamer (expected number = 25). This contradicts the general DUS feature that requires overrepresentation in the genome, such as in *H. influenzae* (1,471 copies of the DUS [50]). Gonzalez-Fraga et al. therefore concluded that this sequence contributes solely to the enhanced uptake of the O-antigen gene region (21), which had been experimentally shown to be horizontally transferable (7). We therefore hypothesize that *V. cholerae* does not have a typical generalized DUS and that it makes use of another mechanism to increase the likelihood of the uptake of species-specific DNA: the regulation of genes involved in natural transformation upon accumulation of the species- and genus-specific autoinducer cholera autoinducer-1 (CAI-1).

Bacteria communicate with each other using a process called quorum sensing (QS). They produce small molecules, desig-

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[▽] Published ahead of print on 22 July 2011.

FIG. 1. Model of how quorum sensing contributes to natural transformation. In *V. cholerae*, two autoinducers are produced by the synthases CqsA and LuxS: cholera autoinducer-1 (CAI-1) and autoinducer 2 (AI-2), respectively. These autoinducers are sensed by their respective receptors CqsS and LuxPQ. The latter are involved in a phosphotransfer relay (dotted arrows [simplified]) and act as kinases at low cell density (LCD) and as phosphatases at high cell density (HCD). Depending on the resulting phosphorylation state of LuxO, the synthesis of HapR protein may be posttranscriptionally inhibited at LCD (indirectly; shown by the dashed line), whereas this inhibition is absent at HCD. HapR positively contributes to natural competence and transformation by activating the competence gene *comEA* and by repressing the extracellular nuclease gene *dns*. The smaller font size of “LuxS,” “AI-2,” and “LuxPQ” indicates the lower significance of this quorum-sensing system toward HapR-regulated processes as described here and elsewhere (43). Strain A1552ΔhapR (✧) is comparable to this LCD state. Strain A1552ΔluxO (★) is similar to an HCD state because it lacks the posttranscriptional inhibition of HapR synthesis. Abbreviations: $\sim\text{P}$, phosphorylated; $\text{de}\sim\text{P}$, dephosphorylated. This scheme is based on findings in references 40, 43, and 59.

nated autoinducers, and secrete them into the environment. By sensing the abundance of these molecules, bacteria can estimate the cell density of the community and adjust cellular functions accordingly. For *V. cholerae*, two major autoinducers have been identified, mainly by Bonnie Bassler and collaborators: CAI-1 (40), an (S)-3-hydroxytridecan-4-one molecule (27) that is suggested to be used for intraspecific and interspecific communication (system 1), and autoinducer 2 (AI-2), a furanosyl borate diester used more universally as an interspecies communication agent (system 2) (43, 59). CAI-1 and AI-2 are produced in *V. cholerae* by the synthases CqsA and LuxS, respectively, and are sensed by their respective receptors, CqsS and LuxPQ. As illustrated in Fig. 1, both systems signal indirectly to LuxO and then via small RNAs toward the repression or the release of repression of HapR synthesis (33). HapR is the major regulator of QS in *V. cholerae* and accumulates at a high cell density (Fig. 1). A variety of cellular processes are regulated by HapR, such as the repression of virulence gene expression, biofilm formation, and induction of hemagglutinin/protease (HA/protease) gene expression (24, 30, 32, 40, 63, 64). Due to the species specificity of CAI-1 and due to the fact that the virulence cascade is downregulated at a high cell density (e.g., high concentration of extracellular autoinducers), it was suggested that CAI-1 could be a therapeutic agent used to prevent or treat cholera infections (27). Duan and March developed this idea further by creating *csqA*-carrying commensal *E. coli* strains that could serve as probiotics (15, 16).

It is difficult to imagine how *V. cholerae* can discriminate between different autoinducers if the signal merges into the same signal transduction pathway (Fig. 1). A recent study on *Vibrio harveyi*, a close relative of *V. cholerae* with a similar QS circuit, shed light on this counterintuitive fact; the study showed that different combinations of autoinducers indicate members in the vicinity, determine the amount of LuxR pro-

duced in the cell (the HapR homolog in *V. harveyi*), and reflect these changes by various gene expression patterns (55).

HapR also plays a major role in natural competence for transformation in *V. cholerae* because it downregulates transcription of the extracellular nuclease gene *dns* and contributes to the full induction of *comEA* expression (Fig. 1) (6, 38; M. Lo Scrudato and M. Blokesch, unpublished data). ComEA is essential for natural transformation and is suggested to be a periplasmic DNA-binding protein involved in the DNA uptake process (38). We are only at the beginning of understanding the DNA uptake machinery of *V. cholerae* and therefore propose a simplified model (based on recent reviews of other naturally competent bacteria [1, 9, 10, 12, 23]), as follows. (i) If only species-specific DNA is recognized and bound by a specific receptor, this recognition should occur outside the cells. (ii) DNA enters the periplasm by crossing the outer membrane via the secretin PilQ. *pilQ* and 11 other genes encode proteins involved in the biosynthesis and assembly of a type IV pilus (Tfp), and all 12 genes have been shown to be induced by chitin (39). (iii) A protein channel homologous to ComEC of *Bacillus subtilis* shuttles the transforming DNA from the periplasm into the cytoplasm. A “DNA internalization-related competence protein, ComEC/Rec2,” has been annotated for *V. cholerae* El Tor O1 serogroup strain N16961 (25; GenBank accession no. AAF95027.1) and for other *V. cholerae* strains, such as the classical serogroup O1 strain O395 (the J. Craig Venter Institute; GenBank accession no. ABQ20037.1). (iv) As for other naturally competent bacteria, it is most likely single-stranded DNA (ssDNA) that reaches the cytoplasm, where it is decorated by DprA to avoid degradation and to promote loading of RecA (4, 42). RecA is required for homologous recombination. Based on this model, we have established an assay to test DNA uptake. We provide evidence that *V. cholerae* does not distinguish between species-specific and non-species-specific

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype ^a	Reference or source
<i>V. cholerae</i> strains		
A1552	Wild type, O1 El Tor Inaba, Rif ^r	61
A1552-LacZ-Kan	A1552 strain with <i>aph</i> cassette in <i>lacZ</i> gene; Rif ^r Kan ^r	14, 37
A1552Δdns	A1552 ΔVC0470	6
A1552ΔhapR	A1552 ΔVC0583	38
A1552ΔhapRΔdns	A1552 ΔVC0583 ΔVC0470	6
A1552ΔhapRΔdnsΔcomEA	A1552 ΔVC0583 ΔVC0470 ΔVC1917	This study
A1552ΔluxO	A1552 ΔVC1021	38
A1552ΔluxS	A1552 ΔVC0557	45
A1552ΔcqsA	A1552 ΔVCA0523	This study
A1552ΔcqsAΔdns	A1552 ΔVCA0523 ΔVC0470	This study
A1552ΔcqsAΔluxS	A1552 ΔVCA0523 ΔVC0557	This study
A1552ΔcqsAΔluxSΔdns	A1552 ΔVCA0523 ΔVC0557 ΔVC0470	This study
A1552ΔcqsAΔluxSΔdnsΔcomEA	A1552 ΔVCA0523 ΔVC0557 ΔVC0470 ΔVC1917	This study
A1552ΔcomEA	A1552 ΔVC1917	38
A1552ΔcomEAΔdns	A1552 ΔVC1917 ΔVC0470	This study
A1552ΔcomEAΔcqsA	A1552 ΔVC1917 ΔVCA0523	This study
A1552ΔcomEAΔluxS	A1552 ΔVC1917 ΔVC0557	This study
A1552ΔcomEAΔcqsAΔluxS	A1552 ΔVC1917 ΔVCA0523 ΔVC0557	This study
A1552ΔcomEC	A1552 ΔVC1879	This study
A1552ΔpilQ	A1552 ΔVC2630	38
A1552ΔrecA	A1552 ΔVC0543	This study
A1552ΔdprA	A1552 ΔVC0048	This study
A1552ΔcomECΔdprA	A1552 ΔVC1879 ΔVC0048	This study
A1552ΔcomECΔdns	A1552 ΔVC1879 ΔVC0470	This study
A1552ΔpilQΔdns	A1552 ΔVC2630ΔVC0470	This study
A1552“ <i>str</i> ”	A1552 containing <i>str</i> from strain N16961	38
A1552ΔcqsAΔluxS“ <i>str</i> ”	A1552ΔcqsAΔluxS containing <i>str</i> from strain N16961	This study
Plasmids		
pGP704-Sac28	Suicide vector, <i>ori</i> R6K <i>sacB</i> Amp ^r	39
pGP704-28-SacB-ΔVCA0523	pGP704-Sac28ΔVCA0523	This study
pGP704-28SacB-ΔVC0470	pGP704-Sac28ΔVC0470	6
p28-VC1917	pGP704-Sac28ΔVC1917	38
pGP704-28-SacB-ΔcomEC	pGP704-Sac28ΔVC1879	This study
pBR322	Amp ^r Tc ^r	8
pBR-[Tet+own]-cqsA	PCR fragment containing promoter region and <i>cqsA</i> gene ([own]-cqsA) cloned into EcoRV/BamHI site of pBR322, Amp ^r	This study
pBR-[own]-cqsA	Promoter <i>P_{tet}</i> deleted from pBR-[Tet+own]-cqsA by inverse PCR; Amp ^r	This study
pBAD/Myc-HisA	pBR322-derived expression vector; <i>araBAD</i> promoter (<i>P_{BAD}</i>)	Invitrogen
pBAD- <i>tfoX</i> -stop	<i>VC1153</i> (<i>tfoX</i>) in pBAD/Myc-HisA without tag; arabinose inducible	This study

^a VC numbers are according to reference 25.

DNA at the level of the DNA uptake process. However, species specificity mediated by QS contributes to natural competence for transformation.

MATERIALS AND METHODS

Bacterial strains and plasmids. *V. cholerae* strains and plasmids used in this study are listed in Table 1. Primer sequences for plasmid constructions are shown in Table 2. *E. coli* strains DH5α (60) and TOP10 (Invitrogen) were used as hosts for cloning purposes. *E. coli* strain S17-1λpir (49) served as a mating donor strain for plasmid transfers between *E. coli* and *V. cholerae*. *E. coli* BL21(DE3) (53) and *B. subtilis* 168 (62) were used to test DNA uptake specificity.

Media and growth conditions. Overnight cultures were cultivated in LB medium under aerobic conditions. Cultures for *tfoX* overexpression (see below) were also performed in LB medium. For sucrose-based *sacB* counterselection, NaCl-free LB medium containing 6% sucrose was used. Thiosulfate citrate bile sucrose (TCBS) agar with or without antibiotic addition was prepared by following the manufacturer's instructions (Fluka). Defined artificial seawater medium (DASW) supplemented with vitamins (38) was used for the natural-transformation experiments. LB agar plates contained 1.5% agar and were supplemented with antibiotics if required. The final concentrations of antibiotics were 75 μg/ml, 100 μg/ml, 100 μg/ml, and 50 to 100 μg/ml for kanamycin, rifampin, streptomycin, and ampicillin, respectively.

Construction of *Vibrio cholerae* strains. Deletion of the gene *cqsA* in the parental strain A1552 and its derivatives was achieved using the gene disruption method described earlier and based on the counterselectable plasmid pGP704-Sac28 (39). Oligonucleotides are indicated in Table 2. The *V. cholerae* strain A1552ΔcqsAΔluxS“*str*” was generated by artificially transforming strain A1552ΔcqsAΔluxS with the plasmid pBAD-*tfoX*-stop, coculturing it with the *E. coli* strain DH5α/pBR-[Tet+own]-cqsA (Table 1), and adding donor genomic DNA (gDNA) from strain N16961 (25). After selection for streptomycin-resistant transformants, the A1552ΔcqsAΔluxS“*str*” strain was formed upon removal of the pBAD-*tfoX*-stop plasmid. This strain was used in mixed-community experiments (see Table 3) and allowed the differentiation of the autoinducer-producing strain from the autoinducer-sensing strain.

Construction of pBR-[Tet+own]-cqsA and pBR-[own]-cqsA. The *cqsA* gene was amplified with primers cqsA[own]up-EcoRV and cqsA-down-BamHI (Table 2) using gDNA from *V. cholerae* strain A1552 as a template. The EcoRV- and BamHI-digested PCR product was cloned into the equally digested plasmid pBR322 to generate pBR-[Tet+own]-cqsA. An inverse PCR on pBR-[Tet+own]-cqsA using primers Inv-pBR-flp-fwd and Inv-pBR-flp-bwd was performed to generate pBR-[own]-cqsA, which lacks the constitutive promoter *P_{tet}*.

Standard natural-transformation protocol using chitin flakes. Unless otherwise indicated, natural-transformation frequencies were determined using chitin flakes as a carbon source and an inducer of natural competence according to a previously published protocol (37). Transformation frequencies were log trans-

TABLE 2. Primers used in this study

Primer name	Comment(s)	Sequence (5' to 3')
KO-VCA0523-SacI#1	Δ cqsA strain constructions	CGCGAGCTCAACTTCCTGATTTTATTTCAGAAC
KO-VCA0523#2	Δ cqsA strain constructions	AGGTTTTGGCTTAAGGATGCGCCTGAGCATTGGCGTAGCG
KO-VCA0523#3	Δ cqsA strain constructions	GGCGCATCCTTAAGCCAAAACCTTTGCTTATCGCGCAGGAGC
KO-VCA0523-NcoI#4	Δ cqsA strain constructions	CGCCCATGGTAGTTGACCGCATCAGAGCAAACC
cqsA[own]up-EcoRV	PCR fragment [own]-cqsA	CTCGATATCAACTTTGTTGCTGGGTC
cqsA-down-BamHI	PCR fragment [own]-cqsA	CTCGGATCCCTACCTGCAACTCCAAGTTG
Inv-pBR-flp-fwd	Inverse PCR of pBR-[Tet+own]-cqsA	AACCATTAGGTTATGCCGGTACTGCCGGGCCTC
Inv-pBR-flp-bwd	Inverse PCR of pBR-[Tet+own]-cqsA	GCATAACCTAATGGTTTCTTAGACGTCAGGTGGC
VC1153-up-NcoI	Cloning of pBAD- <i>tfoX</i> -stop	GCGCCATGGTGATTAAGGATCAATGG
VC1153-down-Stop-EcoRI	Cloning of pBAD- <i>tfoX</i> -stop	CGCGAATTCTAACGCTTTAACTTAACGCTGCTGACAAC
LacZ-missing-fw	Specific for <i>V. cholerae</i> A1552 and <i>lacZ</i> ⁺ derivatives of it; #1 in Fig. 2	GCCGACTTTCGAATGATCCACAATGGG
LacZ-missing-bw	Specific for <i>V. cholerae</i> A1552 and <i>lacZ</i> ⁺ derivatives of it; #2 in Fig. 2	CCCTCGCTATCCCATTTGGAAATGCC
Kan-start-inwards	<i>V. cholerae</i> A1552-LacZ-Kan specific; #3 in Fig. 2	ATGAGCCATATTCAACGGGAAACGTC
Kan-end-inwards	<i>V. cholerae</i> A1552-LacZ-Kan specific; #4 in Fig. 2	TTAGAAAACTCATCGAGCATCAAATG
T7RNA-pol-750 up	<i>E. coli</i> BL21(DE3) specific; #5 in Fig. 2	GCACGGGTTGCGATAGCCTCAGC
T7RNA-Pol#3	<i>E. coli</i> BL21(DE3) specific; #6 in Fig. 2	ATGAACACGATTAACATCGCTAAGAACGACTTCTC
SpoIIIE-fw	<i>B. subtilis</i> 168 specific; #7 in Fig. 2	GGTAGTCGGGCAAACGTTTATCTATTGTTCCG
SpoIIIE-bw	<i>B. subtilis</i> 168 specific; #8 in Fig. 2	GCTAACCTTCACTCCGACATCAGGATATACTTC

formed (31) and subjected to statistics using a two-tailed Student *t* test. Auto-inducer cross-feeding between different *V. cholerae* strains was done by growing two strains together as a mixed culture and inoculating them onto the same chitin flake sample to gain mixed biofilms.

Transformation experiments on crab shell surfaces. Chitin-induced natural transformability of *V. cholerae* strains A1552 and A1552 Δ cqsA Δ luxS was additionally tested on crab shell fragments. Minor modifications from the original protocol (38) were made. Donor gDNA was derived from strain A1552-LacZ-Kan (37), and the chitin-attached bacteria were mechanically released from the crab shell fragments within the culture medium. Transformation frequencies were calculated as described previously (38). Synthetic cholera autoinducer-1 [(S)-3-hydroxytridecan-4-one; Ecole Polytechnique Fédérale de Lausanne (EPFL) synthetic platform (Lausanne, Switzerland)] was added to the respective samples at both the time of inoculation and after the growth medium was changed (24 h postinoculation). The final concentration of synthetic CAI-1 was 5 μ M. Experiments were performed four times (CAI-1-free samples) and six times (CAI-1-supplemented strain A1552 Δ cqsA Δ luxS).

Transformation in the absence of chitin by artificial overexpression of *tfoX*. The *V. cholerae* wild-type strain A1552 and its derivatives were electroporated to take up plasmid pBAD-*tfoX*-stop. Transformed strains were used for chitin-independent natural-transformation experiments as described previously (38). Minor variations from the original protocol include the usage of the newly created plasmid pBAD-*tfoX*-stop (resulting in untagged TfoX protein), lowering of the ampicillin concentration to 50 μ g/ml, and inducing *tfoX* expression with 0.02% arabinose.

To test the cross-feeding ability of CAI-1-producing *E. coli* strains, strain DH5 α /pBR322 and DH5 α /pBR-[Tet+own]-cqsA were cocultured with *V. cholerae* strains harboring plasmid pBAD-*tfoX*-stop (induced or uninduced, respectively). When cultures reached an optical density at 600 nm (OD₆₀₀) of ~0.8 to 1.0, 0.5-ml aliquots were supplemented with 2 μ g/ml donor gDNA derived from strain A1552-LacZ-Kan. After a static incubation period at 37°C for 2 h, the cultures were shaken aerobically for 3 h. The undiluted and diluted samples were plated on selective (75 μ g/ml kanamycin to detect transformants; 100 μ g/ml rifampin to distinguish *V. cholerae* from *E. coli*) and plain LB agar plates. Transformation frequencies were calculated as the ratio of the number of transformants to the total number of CFU of *V. cholerae* cells.

Specificity of the DNA uptake process. Two approaches were used to study the uptake of DNA into a DNase-resistant state (e.g., at least into the periplasmic space). In approach 1, for DNA uptake on chitin flakes, cells of strains A1552, A1552 Δ comEC, A1552 Δ pilQ, A1552 Δ recA, and A1552 Δ dprA (Table 1) were induced for natural competence by growing them on chitin flakes (37). A total of 2 μ g donor gDNA of either the *V. cholerae* strain A1552-LacZ-Kan, *E. coli* strain BL21(DE3), or *B. subtilis* strain 168 was added 22 h after inoculation. After 24 h of incubation, the cells were detached from the chitin surface by extensive

vortexing (~30 s) and pelleted by centrifugation. The bacteria were resuspended in phosphate-buffered saline (PBS) buffer containing 10 mM MgCl₂ and treated with 10 units DNase I (Roche) for 15 min at 37°C. Excess nuclease was removed by 3 rounds of washing before the bacterial pellet was resuspended in PBS buffer. These bacteria served as a template in a whole-cell duplex PCR. The primer pair LacZ-missing-fw and -bw (Table 2) was used at a 10-fold-lower concentration and served as a control for the total amount of acceptor cells released from the chitin flakes' surface. The second primer pair used in the duplex PCR was Kan-start-inwards/Kan-end-inwards, T7RNA-pol-750 up/T7RNA-Pol#3, and SpoIIIE-fw/SpoIIIE-bw to detect DNA uptake of donor gDNA derived from *V. cholerae*, *E. coli*, and *B. subtilis*, respectively (Table 2). In approach 2, for a more quantitative measurement of the DNA uptake process, cultures of *V. cholerae* strains harboring the plasmid pBAD-*tfoX*-stop were grown as described above. Aliquots taken at an OD₆₀₀ of 1.0 ($\pm$ 0.05) were supplemented with 2 μ g of purified gDNA and further incubated statically for 2 h at 30°C. The bacteria were then harvested by centrifugation and subjected to the DNase I treatment. Whole cells, adjusted for their newly measured OD₆₀₀ values, served as the template for the duplex PCR.

RESULTS

Uptake of non-species-specific DNA by naturally competent *V. cholerae* cells. As mentioned in the introduction, some Gram-negative bacteria preferentially take up DUS-containing DNA derived from their own species (23). As DUS have not been identified for *V. cholerae*, we tested experimentally whether non-species-specific DNA can be taken up by this organism. We first established a duplex PCR assay to investigate DNA uptake in *V. cholerae* (a detailed description appears in Materials and Methods). Briefly, we designed primers that specifically anneal to donor genomic DNA (gDNA) derived from either *V. cholerae*, *E. coli*, or *B. subtilis*. We also constructed oligonucleotides that exclusively prime the genome of the acceptor *V. cholerae* strains (Fig. 2A). The discriminatory power of both primer sets was proven in a duplex PCR setup as depicted in Fig. 2B; no cross-reactivity between acceptor strain-specific and donor strain-specific primer pairs was observed.

DNA uptake was induced in *V. cholerae* by growth on chitin surfaces and based on the simplified model for DNA uptake

FIG. 2. Uptake of non-species-specific DNA by naturally competent *V. cholerae* cells. (A) Scheme of acceptor and donor strain-specific gDNA regions. All *V. cholerae* acceptor strains used in this experiment were lacZ positive and therefore semiquantitatively detectable with lacZ-specific primers (LacZ-missing-fw/LacZ-missing-bw, indicated by #1 and #2 here and in Table 2). The donor gDNA of the *V. cholerae* strain A1552-LacZ-Kan (37) lacks this part of the lacZ gene due to replacement with a kanamycin resistance cassette (*aph*; primers #3 and #4). A chromosomal region specific to *E. coli* BL21(DE3) gDNA encodes the T7 DNA-directed RNA polymerase (CP001509 [29]; primers #5 and #6); for *B. subtilis* 168, the *spoEIII* gene served as a species-specific genome template (primers #7 and #8). The expected PCR fragment sizes are indicated (not to scale). (B) Control experiment for validation of primers in the duplex PCR. Duplex PCR with the oligonucleotide combinations #1/#2 (acceptor strain specific; lanes 1 to 6) and donor gDNA-specific primer pairs #3/#4 (lanes 1 and 2), #5/#6 (lanes 3 and 4), and #7/#8 (lanes 5 and 6) was performed. The different templates were 30 ng gDNA of the *V. cholerae* acceptor strain A1552 (acceptor strain; lanes 1, 3, and 5) and donor gDNA derived from the *V. cholerae* strain A1552-LacZ-Kan (lane 2), *E. coli* strain BL21(DE3) (lane 4), or *B. subtilis* strain 168 (lane 6). (C) Detection of DNase I-resistant donor gDNA after induction of natural competence on chitin surfaces. *V. cholerae* strains A1552 (wild type, lane 1), A1552ΔpilQ (lane 2), A1552ΔcomEC (lanes 3, 6, and 7), A1552ΔdprA (lane 4), and A1552ΔrecA (lane 5) were induced for natural competence by growth on chitin flakes. After 24 h from the provision of donor gDNA of strains, namely, *V. cholerae* A1552-LacZ-Kan (lanes 1 to 5) (*V.c.*), *E. coli* BL21(DE3) (lane 6) (*E.c.*), and *B. subtilis* 168 (lane 7) (*B.s.*), the cells were analyzed for uptake of donor DNA by whole-cell duplex PCR. The asterisk indicates the quantification of acceptor strains using the primer pair #1 and #2 (1:10 diluted), as indicated for panel A. Both images depict the same area, but the upper image has been uniformly enhanced for contrast and brightness. Primer combinations are indicated above the figure. (D) Semiquantitative DNA uptake assay following artificial competence induction in liquid medium. Chitin- and surface-independent DNA uptake was performed in liquid medium by artificial overexpression of *tfoX*. This method allowed for the quantification of bacterial cells by optical density measurements. *V. cholerae* strains tested were A1552Δdns/pBAD-*tfoX*-stop (lanes 1, 5, and 9), A1552ΔdnsΔpilQ/pBAD-*tfoX*-stop (lanes 2, 6, and 10), and A1552ΔdnsΔcomEC/pBAD-*tfoX*-stop (lanes 3, 7, and 11). As a negative control, strain A1552ΔdnsΔcomEC/pBAD-*tfoX*-stop was tested in the absence of the *tfoX* inducer (lanes 4, 8, and 12). Donor gDNAs were derived from *V. cholerae* A1552-LacZ-Kan (lanes 1 to 4), *E. coli* BL21(DE3) (lanes 5 to 8), and *B. subtilis* 168 (lanes 9 to 12). Primer combinations are indicated above the figure. Shown are the results of one representative experiment of at least three independent replicates. L, ladder (left, 1 = kb ladder; right, 100 = bp ladder [Invitrogen]).

described in the introduction. We tested several different mutant strains in this assay (Fig. 2C): wild-type strain A1552 (lane 1), A1552ΔpilQ (lane 2), A1552ΔcomEC (lane 3), A1552ΔdprA (lane 4), and A1552ΔrecA (lane 5). As the amounts of bacteria on the chitin flakes might differ between samples, we normalized the PCRs according to the intensity of the acceptor strain-specific DNA band (lower band in Fig. 2C). In accordance with

our model, we detected donor gDNA in the wild-type strain (lane 1) and the strain lacking ComEC (annotated inner membrane transporter). Whereas the PCR fragment derived from the wild-type sample (lane 1) could be the result of the three different locations of the template (donor gDNA), namely, in the periplasm, in the cytoplasm, or recombined into the chromosome (transformants; around 1×10^{-4}), a *comEC*-negative

strain (lane 3) is nontransformable, as it is blocked for periplasm-to-cytoplasm transport of the incoming DNA. Thus, the donor gDNA exclusively accumulates in the periplasmic space. In contrast, no donor DNA was taken up in a strain devoid of the outer membrane secretin PilQ (lane 2). This also represents an important control for whole-cell duplex PCR, confirming that the DNase treatment removed all traces of extracellular donor gDNA. We detected minimal amounts of donor gDNA in the two strains lacking DprA and RecA, respectively (Fig. 2C, upper image [after enhancement of brightness and contrast]). Based on the knowledge of other naturally competent bacteria and from our simplified model, the donor gDNA is not trapped in the periplasm in these strains and thus can enter the cytoplasm. However, as the incoming ssDNA is not protected from degradation and/or is unable to homologously recombine into the chromosome, it is prone to quick degradation in this compartment. Indeed, both strains were nontransformable based on the lack of detection of transformants on selective agar plates. Therefore, we hypothesize that the faint donor gDNA-specific PCR band shown in Fig. 2C is derived from leftover gDNA that has not yet been transported from the periplasmic space into the cytoplasm. We also demonstrated that the DprA strain is not impaired in the first step of the DNA uptake process, namely, the transport across the outer membrane, as a *dprA comEC* double mutant resulted in the same intense donor gDNA-specific PCR band as observed for the *comEC* single mutant (data not shown).

After establishing this assay, we were able to test for non-species-specific DNA uptake. We used donor gDNA from a representative sampling of both Gram-negative (*E. coli*) and Gram-positive (*B. subtilis*) bacteria (Fig. 2C, lanes 6 and 7). In both cases, the donor gDNA successfully accumulated in the periplasm of a *comEC*-deficient acceptor strain, proving that the DNA uptake machinery of *V. cholerae* does not differentiate between species-specific and non-species-specific DNA.

To obtain more-quantitative results, we switched from the chitin surface experimental setup to artificial *tfoX* overexpression in chitin- and surface-independent natural-transformation conditions (Fig. 2D). Furthermore, we deleted the extracellular nuclease gene *dns* in these strains to exclude uneven donor-gDNA degradation outside the cells. Apart from the *dns* deletion, the genotypes of the *V. cholerae* strains were unchanged (Fig. 2D, lanes 1, 5, and 9), deleted in *pilQ* (lanes 2, 6, and 10), or devoid of the *comEC* gene (lanes 3, 4, 7, 8, 11, and 12). For the last strain, two cultures were tested: one culture contained the *tfoX* inducer arabinose (Fig. 2D, lanes 3, 7, and 11), and one control culture was not induced for competence (lanes 4, 8, and 12). As illustrated in Fig. 2D, no difference was observed with respect to the uptake process between species-specific (lanes 1 to 4) and non-species-specific (lanes 5 to 12) DNA in this semiquantitative assay. Furthermore, no DNA uptake occurred in the absence of the outer membrane secretin PilQ or in the absence of artificial *tfoX* induction (Fig. 2D, even lanes). These data indicate that DNA uptake by *V. cholerae* requires the induction of the competence genes, including *pilQ*, and that the DNA uptake process itself does not depend on species-specific donor DNA.

Decrease in the transformability of *V. cholerae* strains with defects in autoinducer synthesis. As we did not observe any preference at the level of DNA uptake, we wondered how else

species specificity, which would greatly contribute to DNA repair, could still occur in this scenario. One possibility that we considered was the coupling of natural competence/transformation with quorum sensing. As mentioned in the introduction, *V. cholerae* can differentiate between species-specific autoinducers such as CAI-1 and those autoinducers that are more universally used by all kinds of bacteria (e.g., AI-2) (43). We also knew from previous studies that natural competence and transformation of *V. cholerae* are indeed dependent on the major regulator of quorum sensing, HapR (6, 38). However, in these studies, the upstream signaling cascade was mostly disregarded. To further understand the link between natural competence/transformation and quorum sensing and to distinguish between the roles of quorum systems 1 and 2, we created and investigated several knockout strains of *V. cholerae* (see Table 1 and Fig. 1 for an explanation): A1552Δ*cqsA*, a strain unable to produce CAI-1; A1552Δ*luxS*, a strain unable to produce AI-2; and A1552Δ*cqsA*Δ*luxS*, a strain defective in the production of both autoinducers. We subjected all of these strains and the respective controls (wild type and A1552Δ*hapR*) to transformation assays on chitin flakes (37) and scored their transformation frequency (Fig. 3A). While there was no significant difference between the wild-type strain and a strain defective in AI-2 synthesis (Fig. 3A, lane 3), there was a highly significant drop in transformation frequency in the strain lacking the ability to produce CAI-1 (Fig. 3A, lane 2). This effect was even more pronounced in the double-knockout strain, with which we did not detect any transformants in more than 40 experiments (Fig. 3A, lane 4). This is in agreement with the transformation-negative phenotype of strains missing functional HapR (Fig. 3A, lane 5) (38). Such strains are unable to respond to autoinducers and are therefore considered “locked” in a low-cell-density state.

To verify that the decrease in transformability can be directly linked to the lack of *cqsA* in strains A1552Δ*cqsA* and A1552Δ*cqsA*Δ*luxS*, we generated two *cqsA*-carrying plasmids: pBR-[own]-*cqsA* and pBR-[Tet+own]-*cqsA* (Table 1). With these constructs, we tested the complementability of the *cqsA* deletion strain. As shown in Fig. 3B, we could fully restore chitin-induced natural transformation by providing *cqsA* in *trans* (lanes 3 and 4). The two plasmids differ from each other in the promoter region upstream of *cqsA*; the expression of *cqsA* from the plasmid pBR-[own]-*cqsA* is driven solely by the endogenous promoter of *cqsA*, and expression from the plasmid pBR-[Tet+own]-*cqsA* is driven by the combination of the endogenous promoter of *cqsA* preceded by the constitutively active *tet* promoter present in plasmid pBR322 (8). As *cqsA* expression is also regulated at the posttranscriptional level in *V. cholerae* (34), it is not surprising that complementation by both plasmids resulted in comparable transformation frequencies. These results suggest that the CAI-1 synthase CqsA plays a major role in natural competence/transformation of *V. cholerae*.

Regulation of natural competence and transformation by quorum-sensing system 1 occurs through the canonical quorum-sensing cascade. To check whether impaired natural transformability in strains lacking the CAI-1 synthase CqsA is due to the canonical quorum-sensing circuit or whether CAI-1 might contribute to natural transformation in a manner independent of CqsS, LuxO, and HapR (Fig. 1), we performed

FIG. 3. Effect of quorum-sensing systems 1 and 2 on the natural transformability of *V. cholerae*. (A) Transformation frequencies of *V. cholerae* strains with defects in the quorum-sensing circuit. Strains tested were *V. cholerae* strain A1552 (wild type; lane 1), A1552Δ*cqsA* (CAI-1⁻ AI-2⁺; lane 2), A1552Δ*luxS* (CAI-1⁺ AI-2⁻; lane 3), A1552Δ*cqsA*Δ*luxS* (CAI-1⁻ AI-2⁻; lane 4), and A1552Δ*hapR* (lane 5). Natural-transformation frequencies are indicated on the y axis. < d.l., below the detection limit. The detection limit varied between 3.2×10^{-8} and 1.2×10^{-6} (44 independent experiments) and 4.0×10^{-8} and 8.7×10^{-7} (11 independent experiments) for A1552Δ*cqsA*Δ*luxS* and A1552Δ*hapR*, respectively. Data are averages of results from at least 11 independent experiments. #, for strain A1552Δ*cqsA*, the transformation frequency was below the detection limit in 12 out of 23 experiments. To allow the calculation of the average, the transformation frequency was set to the detection limit for these strains. Thus, the indicated average slightly overestimates the residual transformability of this strain. A statistically significant difference from the transformation frequency of the wild-type strain (lane 1) is indicated (**, $P < 0.01$, as determined by Student's *t* test of log-transformed data). (B) Complementation of the *cqsA* deletion in trans. Transformation frequencies were scored for *V. cholerae* wild-type strain A1552 (lane 1) or its *cqsA*-minus derivative (A1552Δ*cqsA*; lanes 2 to 4). Complete restoration of natural transformability was possible by providing *cqsA* in trans preceded solely by its endogenous promoter (A1552Δ*cqsA*/pBR-[own]-*cqsA*; lane 3) or in combination with the constitutive *tet* promoter provided by the plasmid backbone (A1552Δ*cqsA*/pBR-[Tet+own]-*cqsA*; lane 4). The vector control is shown in lane 2 (A1552Δ*cqsA*/pBR322). Averages of results from four independent experiments are shown. The detection limit was between 1.8×10^{-7} and 5.8×10^{-7} for strain A1552Δ*cqsA*/pBR322.

epistasis analyses (Fig. 4). First, we successfully restored natural transformation to wild-type levels in the Δ*cqsA* mutant by concomitantly deleting the *luxO* gene in this strain (Fig. 4A), proving that LuxO also acts downstream of CqsA/CAI-1 in the case of quorum-sensing-dependent natural transformation. Strains lacking LuxO mimic a higher cell density state as the posttranscriptional repression of HapR production is abol-

ished (33). Such strains are naturally transformable already at low cell densities (6).

Our second aim was to analyze whether the effect on genes downstream of the major regulator of quorum sensing, HapR, in the autoinducer synthase-deficient strains was comparable to that in strains lacking HapR. We knew from previous studies that the repression of the extracellular nuclease gene *dns* by HapR is essential for natural transformation to occur. The defect in the natural transformability of *hapR* mutant strains, including the first sequenced strain of *V. cholerae* N16961 (25), could be overcome by codeleting *dns* in this genetic background (Fig. 4B, lane 8). Accordingly, we deleted *dns* in the strains negative for *cqsA* and *cqsA luxS*. In both cases, natural transformability was significantly restored (Fig. 4B, lanes 6 and 7). However, as with the *hapR dns* double mutant (Fig. 4B, lane 8), the frequency of natural transformation did not reach that of the *dns* single mutant (Fig. 4B, lane 5). This again suggested a secondary role for HapR in the regulation of natural transformation, which is not essential for natural transformation *per se* but is required for the high numbers of transformants observed in nuclease-deficient strains (Fig. 4B, lane 5). As suggested before (6), we believe that this is due to an activation of *comEA* expression by HapR (38; Lo Scrudato and Blokesch, unpublished). Deletion of *comEA* abolished the transformability of all tested strains (Fig. 4B, lanes 9 to 11). These data provide convincing evidence that the signaling cascade from the synthesis of CAI-1 toward the regulation of natural competence follows the regular flow of information via phosphorylated and dephosphorylated LuxO and HapR (Fig. 1).

Cholera autoinducer-1 is of major importance for natural transformation. As described above, deletion of the CAI-1 synthase gene *cqsA* significantly decreased the transformation efficiency in *V. cholerae*. Although we were able to complement this defect by expressing *cqsA* in trans (Fig. 3B), we were also curious to see whether this is an effect of intracellular CAI-1 or Ea-CAI-1 (enamino-CAI-1) (57) production and detection or, more likely, a result of autoinducer secretion and detection (e.g., “cross-feeding”). We cultured *V. cholerae* strains in mixed communities on the same chitin surface and tested natural transformability (Table 3). The autoinducer-sensing strain in our experiments, which lacks both autoinducer synthases (A1552Δ*cqsA*Δ*luxS*“*str*”), was not by itself naturally transformable. The autoinducer-producing strains used in this assay all lacked *comEA*, rendering them nontransformable (Fig. 4B and data not shown). However, coculturing the autoinducer-sensing strain with either of the two autoinducer-producing strains, A1552Δ*comEA* or A1552Δ*comEA*Δ*luxS*, fully restored natural transformation (Table 3). This was not the case if the sensing strain was cocultured with strains A1552Δ*comEA*Δ*cqsA* and A1552Δ*comEA*Δ*luxS*Δ*cqsA*, which produce only AI-2 and no autoinducer at all, respectively (Table 3). This highlights the necessity of CAI-1 in this autoinducer cross-feeding experiment.

Synthetic CAI-1 can overcome a *cqsA* deletion in *V. cholerae*. After Higgins et al. solved the structure of CAI-1, they suggested that it could be a valuable molecule in the treatment of cholera patients (27). The authors of this study were able to chemically synthesize CAI-1 (synthetic CAI-1) and demonstrated its activity with a transcriptional reporter strain of *V.*

FIG. 4. Epistasis analysis indicates that the transformation-negative phenotype of a *cqsA*-minus strain occurs via the canonical quorum-sensing cascade. (A) Complete restoration of natural transformability through the deletion of *luxO* in a Δ *cqsA* background. Natural-transformation frequencies, as scored on chitin flakes, are indicated on the y axis. Strains tested were A1552 (lane 1), A1552 Δ luxO (lane 2), A1552 Δ cqsA (lane 3), and A1552 Δ cqsA Δ luxO (lane 4). (B) Partial restoration of natural transformability through elimination of the extracellular nuclease Dns (*dns*⁻). Strains tested for natural transformability were A1552 (lane 1), A1552 Δ cqsA (lane 2), A1552 Δ cqsA Δ luxS (lane 3), A1552 Δ hapR (lane 4), A1552 Δ dns (lane 5), A1552 Δ cqsA Δ dns (lane 6), A1552 Δ cqsA Δ luxS Δ dns (lane 7), A1552 Δ hapR Δ dns (lane 8), A1552 Δ comEA Δ dns (lane 9), A1552 Δ cqsA Δ luxS Δ dns Δ comEA (lane 10), and A1552 Δ hapR Δ dns Δ comEA (lane 11). #, for strain A1552 Δ cqsA, the transformation frequency was below the detection limit in 3 out of 4 experiments (A) and 4 out of 8 experiments (B). To allow calculation of the average, the transformation frequency was set to the detection limit for these strains. Thus, the indicated average slightly overestimates the residual transformability of this strain. The averages of results from at least three independent experiments are shown. < d.l., below the detection limit, which varied between 4.4×10^{-8} and 8.3×10^{-6} for all indicated strains. Statistically significant differences from the transformation frequency of the wild-type strain (lane 1) are indicated in both panels (*, $P < 0.05$; **, $P < 0.01$) determined by Student's *t* test of log-transformed data.

cholerae, which produces bioluminescence upon exposure to CAI-1 (27). To test the effect of synthetic CAI-1 on chitin-induced natural transformation, we synthesized CAI-1 according to the published protocol (27) (EPFL synthetic platform). The syn-

thetic CAI-1 molecule was initially tested for functionality using two methods. First, we measured the release of HA/protease secretion over time according to the protocol provided by Benitez et al. (3). HA/protease production is activated at high cell density by the quorum-sensing regulator HapR. Indeed, we

TABLE 3. Cholera autoinducer-1 is of major importance for natural transformation

Autoinducer-producing strain (autoinducer produced) ^a	Autoinducer-sensing strain (autoinducer produced)	Transformation frequency ^b (±SD)
A1552“str” (C ⁺ , A ⁺)	None	1.4 × 10 ⁻⁴ (±1.3 × 10 ⁻⁴)
None	A1552ΔcqsAΔluxS“str” (-)	<d.l.
A1552ΔcomEA (C ⁺ , A ⁺)	None	<d.l.
A1552ΔcomEA (C ⁺ , A ⁺)	A1552ΔcqsAΔluxS“str” (-)	1.8 × 10 ⁻⁴ (±1.3 × 10 ⁻⁴)
A1552ΔcomEAΔcqsA (A ⁺)	A1552ΔcqsAΔluxS“str” (-)	<d.l.
A1552ΔcomEAΔluxS (C ⁺)	A1552ΔcqsAΔluxS“str” (-)	1.8 × 10 ⁻⁴ (±8.2 × 10 ⁻⁵)
A1552ΔcomEAΔcqsAΔluxS (-)	A1552ΔcqsAΔluxS“str” (-)	<d.l.

^a C, CAI-1; A, AI-2; -, none.
^b Transformation frequencies were calculated as the number of transformants divided by the number of colony-forming streptomycin-resistant autoinducer-sensing cells (indicated as “str”). <d.l., below the detection limit, which varied between 1.8×10^{-8} and 5.3×10^{-7} . Averages of results from four independent experiments are shown.

TABLE 4. Chemically synthesized CAI-1 restores natural transformation of autoinducer-deficient *V. cholerae* strains

<i>V. cholerae</i> strain	Synthetic CAI-1	Transformation frequency ($\pm$ SD) ^a
A1552		1.1×10^{-4} ($\pm 6.6 \times 10^{-5}$)
A1552 Δ cqsA Δ luxS		<d.l.
A1552 Δ cqsA Δ luxS	5 μ M	1.2×10^{-5} ($\pm 1.2 \times 10^{-5}$)

^a <d.l., below the detection limit. The range of the detection limit was from 4.9×10^{-8} to 1.2×10^{-7} in the four independent experiments.

were able to fully and partly restore HA/protease secretion in strains A1552 Δ cqsA and A1552 Δ cqsA Δ luxS, respectively, by adding synthetic CAI-1 (data not shown). Second, we tested HapR-mediated biofilm repression using a crystal violet staining assay (slightly modified from reference 56). Whereas strains A1552 Δ cqsA and A1552 Δ cqsA Δ luxS had a semirugose appearance on plates and displayed an increased ability to form biofilms, the latter phenotype could almost completely revert to wild-type levels upon supplementation with synthetic CAI-1 (data not shown). With this knowledge of HapR-mediated activation and repression in hand, we then tested for chitin-induced natural transformation using crab shell fragments as chitin surfaces (38). As illustrated in Table 4 and in accordance with the data described above, a strain unable to produce its own autoinducers (A1552 Δ cqsA Δ luxS) is nontransformable in this experimental setup. However, by providing synthetic CAI-1 at a final concentration of 5 μ M, a concentration known to fully induce reporter expression in *V. cholerae* (27), we were able to rescue natural transformability (Table 4). The difference in transformation frequency between wild-type *V. cholerae* and strain A1552 Δ cqsA Δ luxS supplemented with synthetic CAI-1 was not statistically significant (P value = 0.065). However, we still believe that this lower natural transformability is real and due to the incomplete repression of *dns* by the HapR regulator. A similar effect was observed for incomplete HapR-mediated repression of the virulence gene *tcpA* upon treatment with 10 μ M synthetic CAI-1 (27).

CAI-1-producing *E. coli* strains restore natural transformation in autoinducer synthase-deficient *V. cholerae* strains. Because the use of *cqsA*-carrying/CAI-1-producing *E. coli* strains as probiotics has recently been suggested (15, 16), we investigated the effect of such strains on the natural transformability of *V. cholerae*. *E. coli* is unable to grow on chitin surfaces and cannot feed on chitin as a sole carbon source. The addition of alternative carbon sources, such as phosphotransferase system

(PTS)-transported sugars, would allow *E. coli* to grow but would concomitantly lead to catabolite repression and inhibition of natural transformation in *V. cholerae* (M. Blokesch, submitted for publication). We therefore adjusted the experimental setup accordingly with chitin- and surface-independent natural transformation. This method requires artificial overexpression of the gene encoding the major regulator of natural competence/transformation, *tfoX*, and can be performed in rich medium, such as LB. The induction of *tfoX* leads to an upregulation of natural competence genes (38) and to the subsequent uptake of exogenous DNA (Fig. 2D).

We grew the wild type and the A1552 Δ cqsA Δ luxS mutant strain of *V. cholerae*, both harboring the plasmid pBAD-*tfoX*-stop, as cocultures with *E. coli* strain DH5 α . The DH5 α strain contained either an empty vector or the plasmid pBR-[Tet+own]-*cqsA* (Table 1; proof of functionality in *V. cholerae* is shown in Fig. 3B). Arabinose was added as an artificial inducer of *tfoX*, as indicated in Table 5. In the absence of *tfoX* induction, no transformants of either of the two *V. cholerae* strains (wild-type or A1552 Δ cqsA Δ luxS) were detectable. Upon *tfoX* induction, only the wild-type strain became naturally transformable in the presence of the *E. coli* vector control. However, when the cocultured *E. coli* strain expressed *cqsA* from a plasmid, natural transformability was restored in *V. cholerae* strain A1552 Δ cqsA Δ luxS (Table 5). This proves that *cqsA*-carrying *E. coli* strains can cross-feed CAI-1 or its amino/enamino derivative (44, 57) and lead to natural transformation of *V. cholerae*.

DISCUSSION

Certain naturally competent bacteria, such as *N. gonorrhoeae*, were thought to be able to take up only certain kinds of DNA. This uptake mechanism is based on the recognition of specific DNA uptake sequences (DUS) on the bacterial surface by currently unidentified receptor proteins that are coupled to the DNA uptake machinery. All *Neisseria* species use the same DUS (23), making this a species- and genus-specific DNA uptake system. However, a recent study has slightly relaxed this notion, as the requirement for a DUS varied significantly between different strains (17). Repetitive *V. cholerae* DUS have not been described. With our established DNA uptake assay, we observed similar levels of uptake of donor DNA derived either from another Gram-negative bacterium, *E. coli*, or from an unrelated and Gram-positive bacterium, *B. subtilis*. Thus, we conclude that *V.*

TABLE 5. Coculturing of autoinducer-deficient *V. cholerae* strains with *cqsA*-carrying *E. coli* strains restores natural transformation

<i>V. cholerae</i> strain	TfoX induction ^a	<i>E. coli</i> strain	Transformation frequency ($\pm$ SD) ^b
A1552/pBAD- <i>tfoX</i> -stop	—	DH5 α /pBR322	<d.l.
A1552/pBAD- <i>tfoX</i> -stop	—	DH5 α /pBR-[Tet+own]- <i>cqsA</i>	<d.l.
A1552/pBAD- <i>tfoX</i> -stop	+	DH5 α /pBR322	8.3×10^{-6} ($\pm 4.5 \times 10^{-5}$)
A1552/pBAD- <i>tfoX</i> -stop	+	DH5 α /pBR-[Tet+own]- <i>cqsA</i>	1.2×10^{-5} ($\pm 6.0 \times 10^{-5}$)
A1552 Δ cqsA Δ luxS/pBAD- <i>tfoX</i> -stop	—	DH5 α /pBR322	<d.l.
A1552 Δ cqsA Δ luxS/pBAD- <i>tfoX</i> -stop	—	DH5 α /pBR-[Tet+own]- <i>cqsA</i>	<d.l.
A1552 Δ cqsA Δ luxS/pBAD- <i>tfoX</i> -stop	+	DH5 α /pBR322	<d.l.
A1552 Δ cqsA Δ luxS/pBAD- <i>tfoX</i> -stop	+	DH5 α /pBR-[Tet+own]- <i>cqsA</i>	5.6×10^{-6} ($\pm 4.5 \times 10^{-5}$)

^a *tfoX* was induced by the addition of 0.02% arabinose.

^b <d.l., below the detection limit, which ranged from 7.0×10^{-9} to 8.2×10^{-8} (based solely on the number of *V. cholerae* CFU). Averages of results from four independent experiments are shown.

cholerae does not distinguish between species-specific or non-species-specific DNA at the level of DNA uptake. However, we hypothesize that *V. cholerae* enhances the probability that it will take up species-specific DNA by coupling the regulation of natural transformation to quorum sensing.

QS in *V. cholerae* is accomplished mainly by the production and sensing of two distinct autoinducers, CAI-1 and AI-2. Whereas AI-2 is a universal autoinducer produced by a large variety of bacteria (59), CqsA/CqsS system 1, including CAI-1, is conserved only in certain *Vibrio* species and is therefore suggested to be used for communication between *Vibrios* (intraspecies/intragenic) (26, 27, 40). For example, the close relative of *V. cholerae*, *V. harveyi*, uses CqsA to produce Vh-CAI-1, a (Z)-3-aminoundec-2-en-4-one (44). The detection of CAI-1 and its derivatives by CqsS is genus or even species specific; *V. cholerae*'s CqsS preferentially but not exclusively recognizes the 10-carbon-tailed CAI-1 molecule, whereas *V. harveyi*'s CqsS responds only to the 8-carbon tail of Vh-CAI-1 (44). Our data indicate that natural transformation is strongly enhanced by the species/genus-specific autoinducer CAI-1. This could be an alternative mechanism, separate from DUS-driven DNA uptake, to ensure the uptake of DNA derived from siblings or close relatives. Such a mechanism resembles the competence pheromones/competence-stimulating peptides produced by Gram-positive bacteria. For example, the extracellular accumulation of the ComX pheromone triggers the onset of the competence program in *B. subtilis* (36). One hypothesis is that QS system 1 coevolved with natural transformability in *Vibrios*. It might be worth noting that after the initial discovery of natural competence in *V. cholerae* (38), several other *Vibrio* species turned out to also be naturally transformable; these include, among others, *V. fischeri*, *V. vulnificus*, and *V. parahaemolyticus* (11, 22, 47).

QS is involved in the downregulation of the extracellular nuclease Dns and thus significantly contributes to the natural transformation of *V. cholerae* (6). We tested whether the absence of CqsA and LuxS had a negative effect on the repression of *dns*, which is normally observed at high cell density. This was indeed the case, as the transformation-negative phenotype in the absence of autoinducers (Fig. 4B, lane 3) could be partly overcome by deleting *dns* in the same genetic background (Fig. 4B, lane 7). However, the nuclease-minus strains that are devoid of autoinducer synthases were not hypertransformable, as was the nuclease-minus but *cqsA*⁺ *luxS*⁺ strain (Fig. 4B, lane 5). The lowered transformability of *dns*-negative strains was even more pronounced in the complete absence of HapR (Fig. 4B, lane 8). This indicates that HapR also positively contributes to natural competence and transformation. We suggest that HapR enhances *comEA* transcription based on the following facts: (i) *comEA* is upregulated upon growth of *V. cholerae* on chitohexaose (38; M. Blokesch and G. K. Schoolnik, unpublished data); (ii) this chitin-dependent upregulation is not observed in "natural" *hapR* mutant strains, such as N16961 (39); and (iii) comparison of a chitin-induced wild-type strain to an in-frame *hapR* deletion strain indicated a significant difference in *comEA* expression (38). However, this decreased *comEA* expression in the absence of HapR is still sufficient to allow transformation to occur in nuclease-negative *V. cholerae* strains (Fig. 4B, lane 8). We hypothesize that the decrease in transformation frequencies in the tested nuclease-minus strains (Fig. 4B, lanes 5 to 8) might be a reflection of the

amount of HapR proteins in the cell and their ability to enhance *comEA* transcription. More specifically, if we compare strains A1552Δ*dns*, A1552Δ*cqsA*Δ*dns*, A1552Δ*cqsA*Δ*luxS*Δ*dns*, and A1552Δ*hapR*Δ*dns* at high cell density, we expect the amount of HapR proteins in the cell to decrease from very high (A1552Δ*dns*; Fig. 4B, lane 5) via intermediate levels of HapR (A1552Δ*cqsA*Δ*dns* and A1552Δ*cqsA*Δ*luxS*Δ*dns*; lanes 5 and 6) toward its total absence in strain A1552Δ*hapR*Δ*dns* (lane 8). This is in good agreement with what was shown for *V. harveyi* at the protein level (55). Those authors performed Western blot analysis to test the amount of LuxR protein, the HapR homolog in this organism, in a strain deficient in autoinducer synthesis. The level of LuxR protein synthesized within the cells upon external provision of both autoinducers (HAI-1, a *V. harveyi* species-specific autoinducer, and AI-2) was quantified and set at 100%. The relative percentages of LuxR protein were then determined to be 26, 6.8, and 1.6% by provision of solely HAI-1, solely AI-2, and no autoinducer, respectively (55). The level of bioluminescence in this bacterium, which reports LuxR abundance in the cell, followed the same pattern (55). We therefore believe that our data are in good agreement with that study and support the notion that different combinations of autoinducers produce a gradient of HapR protein concentration, which results in differential expression of target genes (43). Deletion of *comEA*, on the other hand, completely abolished transformability in all genetic backgrounds (Fig. 4B, lanes 9 to 11). Further studies to understand the complete complexity of this regulatory circuit are forthcoming.

If CAI-1 indeed contributes positively to natural competence and transformation, this might be a striking finding given the above-described research and the suggested use of CAI-1 as a therapeutic for the treatment of cholera (27). We synthesized CAI-1 and tested the direct effect of CAI-1 on natural transformation. As indicated in Table 4, an autoinducer-deficient strain was nontransformable under standard experimental conditions, but transformation was restored upon the addition of synthetic CAI-1. This provides evidence that the supplemented CAI-1 is sensed, the signal is transduced, and the QS pathway then leads to natural competence and the transformability of the bacterium (Table 4). Furthermore, we showed that CAI-1-producing *E. coli* strains restored natural transformation in autoinducer-negative *V. cholerae* strains (Table 5). Therefore, the safety of using CAI-1 as a therapeutic treatment (27) or probiotic treatment (with CAI-1-producing *E. coli* strains) for individuals at risk of ingesting *V. cholerae* (15, 16) should be reassessed.

ACKNOWLEDGMENTS

We thank Olga de Souza Silva for technical assistance and Onya Opota and Bruno Lemaitre for *B. subtilis* strain 168. We also thank Ronan Euzen from the EPFL synthetic platform for the synthesis of CAI-1. M.B. acknowledges her former mentor at Stanford for his generosity in sharing strains designed by M.B. while in his lab.

This work was supported by Swiss National Science Foundation (SNSF) grant 31003A_127029.

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