DNA Transport across the Outer and Inner Membranes of Naturally Transformable *Vibrio cholerae* Is Spatially but Not Temporally Coupled

Patrick Seitz, Melanie Blokesch

Laboratory of Molecular Microbiology, Global Health Institute, School of Life Sciences, Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland

**ABSTRACT** The physiological state of natural competence for transformation allows certain bacteria to take up free DNA from the environment and to recombine such newly acquired DNA into their chromosomes. However, even though conserved components that are required to undergo natural transformation have been identified in several naturally competent bacteria, our knowledge of the underlying mechanisms of the DNA uptake process remains very limited. To better understand these mechanisms, we investigated the competence-mediated DNA transport in the naturally transformable pathogen *Vibrio cholerae*. Previously, we used a cell biology-based approach to experimentally address an existing hypothesis, which suggested the competence protein ComEA plays a role in the DNA uptake process across the outer membrane of Gram-negative bacteria. Here, we extended this knowledge by investigating the dynamics of DNA translocation across both membranes. More precisely, we indirectly visualized the transfer of the external DNA from outside the cell into the periplasm followed by the shuttling of the DNA into the cytoplasm. Based on these data, we conclude that for *V. cholerae*, the DNA translocation across the outer and inner membranes is spatially but not temporally coupled.

**IMPORTANCE** As a mode of horizontal gene transfer, natural competence for transformation has contributed substantially to the plasticity of genomes and to bacterial evolution. Natural competence is often a tightly regulated process and is induced by diverse environmental cues. This is in contrast to the mechanistic aspects of the DNA translocation event, which are most likely conserved among naturally transformable bacteria. However, the DNA uptake process is still not well understood. We therefore investigated how external DNA reaches the cytosol of the naturally transformable bacterium *V. cholerae*. More specifically, we provide evidence that the DNA translocation across the membranes is spatially but not temporally coupled. We hypothesize that this model also applies to other competent Gram-negative bacteria and that our study contributes to the general understanding of this important biological process.

---

**H**orizontal gene transfer (HGT) greatly contributes to the evolution of bacteria. As such, studying the regulatory networks and mechanistic aspects of HGT is of prime importance. One mode of HGT in bacteria is natural competence for transformation. Naturally competent bacteria are in a physiological state that allows them to take up free DNA from the environment and to incorporate this foreign DNA into their genome. Indeed, large pieces of DNA containing a series of genes can be transferred by transformation without the need for direct interaction with other microbes or the intercession of mobile genetic elements. This can quickly foster the spread of antibiotic resistance traits, adaptation of bacteria to new environmental niches, and emergence of new pathogens.

Although the regulation of natural competence differs widely between bacteria (reviewed in references 1–6), the core components of the DNA uptake machinery are often conserved, and the uptake process might be close to universal. However, major knowledge gaps exist with respect to the mechanistic aspects of the DNA uptake process, as also stated in a recent review by the groups of Patrice Polard and Jean-Pierre Claverys, who highlighted the fact that further investigations into this direction are needed (6). This is especially true for Gram-negative bacteria, where the transforming DNA needs to cross two membranes (and the periplasmic space) before entering the cytoplasm. The aim of our study was therefore to address the DNA uptake process in the naturally competent bacterium *Vibrio cholerae* and, more specifically, the transfer of transforming DNA (tDNA) from the periplasm into the cytoplasm.

Below, we describe a working model of how natural competence-driven DNA uptake might occur in Gram-negative bacteria. Variations of this model have existed for many years (see, for example, the model of the DNA uptake complex of *Neisseria gonorrhoeae* proposed by Fussenegger et al. in 1997 [7]). However, as is the case for many multiprotein complexes, all former and current DNA uptake models are derived from a combination of documented data combined with hypothetical assumptions, with
the latter still requiring experimental validations. The gene/protein nomenclature used throughout the text is based on the *V. cholerae* genome annotation (8) and on proteins of *V. cholerae* that show similarities to proteins of other naturally competent bacteria (9, 10). We note that several excellent review articles are available with an emphasis on other naturally competent bacteria and these reviews offer a more detailed description of the putative components of the DNA uptake machineries (e.g., references 5, 6, and 11–19 and references therein).

With respect to the DNA uptake process, it is believed that a core part of the uptake machinery is composed of a (pseudo)pilus with similarities to type IV pili (Tfp) (20–22). Tfp are assembled by the polymerization of the major pilin protein subunit and can be several micrometers in length. The opposite process, namely, the depolymerization of the pilin subunits, has been suggested to occur through pilus retraction (23, 24) driven by the ATPase PilT (25–27). We recently demonstrated that *V. cholerae* indeed produces a specific Tfp structure after competence induction (9).

For Gram-negative bacteria, it is assumed that the Tfp crosses the outer membrane through a secretin pore formed by the PilQ protein (28–31). With respect to the DNA uptake machineries, such Tfp-associated secretins might also serve as the entry point of the incoming tDNA into the periplasm. We have recently demonstrated that PilQ-independent DNA uptake, which still resulted in naturally transformed bacteria, can occur in *V. cholerae* even though such events are extremely rare (9). Thus, assuming that in the majority of cases the DNA enters the periplasm through the secretin, it is still unclear which protein-protein complex pulls the DNA through this pore. To date, most DNA uptake models suggest that the force for the DNA uptake process might come from repeating cycles of pilus extension and pilus retraction. Indeed, the mechanical characteristics of the DNA uptake process in *Bacillus subtilis* (32) were reminiscent of pilus retraction in *N. gonorrhoeae* (23, 33). Yet, it was recently speculated (17, 19) or experimentally addressed that the DNA-binding protein ComEA (34, 35) might also be involved in DNA transport across the outer membrane, even though the exact mode of action of ComEA is still elusive. The majority of naturally competent bacteria contain at least one copy of *comEA* (the homologs are *comE* and *comEI* in *Neisseria* spp. [36, 37] and *Haemophilus influenzae* [38], respectively). The four *comE* copies of *Neisseria gonorrhoeae*, which all encode the same ComE protein, were first identified in a seminal study by Chen and Gotschlich (37). The authors also further investigated this competence gene and demonstrated that a quadruple deletion strain that lacked all four copies of *comE* was severely impaired in DNA uptake and consequently also in natural transformation, despite a normal piliation and twitching motility phenotype. Moreover, they also suggested that ComE might be located in the periplasm (37). Indeed, Chen and Gotschlich used a signal peptide algorithm to predict the presence of a cleavable signal peptide, which was in accordance with the smaller size of the mature protein as observed by Western blot analysis of neisserial cell lysates (37). That purified ComE/ComEA binds DNA in vitro and in a sequence-unspecific manner was also demonstrated for other competent bacteria, including *B. subtilis* (35), *N. gonorrhoeae* (37), *Campylobacter jejuni* (39), *Pasteurella multocida* (40), and *V. cholerae* (41).

Based on these earlier data and supported by our recent study on ComEA in vivo (where we also experimentally confirmed its periplasmic localization within *V. cholerae*), we speculated that the protein might be compacting DNA (as demonstrated by heterologous expression of fluorescently tagged ComEA in *Escherichia coli*) and be pulling the tDNA into the periplasm of Gram-negative bacteria via a Brownian ratchet mechanism (41). Notably, while the present article was under review, Gangel et al. reported on the DNA translocation across the outer membrane of the Gram-negative bacterium *N. gonorrhoeae* and the involvement of ComE in this organism (42). This study nicely confirmed our earlier data in that it also established that tDNA is imported into the periplasm at random locations around the cell, that the incoming tDNA accumulated in the periplasm in a Tfp- and ComEA-dependent manner (visualized through the use of fluorescently labeled DNA), and that fluorescently tagged ComEA formed distinctive ComEA-tDNA clusters (41, 42). Notably, the authors even concluded that “ComE-dependent DNA-import into the periplasm can be considered a general mechanism for Gram-negative competent species” (42).

That the tDNA enters the cytoplasm of both Gram-positive and Gram-negative bacteria through the putative membrane channel protein ComEC (43) is widely accepted in the field. Next, and in accordance with what is known for Gram-positive bacteria, it is assumed that the incoming tDNA is single stranded and immediately decorated with single-stranded binding (Ssb) proteins and DprA to protect it from degradation and to promote loading of the RecA protein, which is required for homologous recombination with the chromosome (44, 45).

Here, we report on the translocation of tDNA across the inner membrane of naturally competent *V. cholerae* cells. We asked the following question (Fig. 1): does the tDNA enter the cytoplasm at the same position (Fig. 1B) where the translocation through the outer membrane occurred (Fig. 1A), or is the second translocation event spatially and temporally uncoupled from the first step (Fig. 1C)? To answer this question, we generated translational fusion constructs for ComEA and RecA, which were functional with respect to natural transformability. These fluorescently labeled protein markers allowed us to indirectly visualize the path of the tDNA, as both proteins bind to the tDNA in the course of natural transformation (Fig. 1). In summary, we provide evidence that the tDNA shuttling across the two membranes is spatially but not temporally coupled.

**RESULTS**

**Persistence of periplasmic ComEA-tDNA clusters differs significantly among competence-induced cells.** We have previously demonstrated that the periplasmic competence protein ComEA of *V. cholerae* is involved in DNA uptake across the outer membrane. More specifically, fluorescently tagged ComEA displayed high affinity for DNA binding in vivo and formed distinctive ComEA-tDNA clusters at a single site within the periplasm of competent cells (41). We therefore concluded that ComEA foci assemble upon translocation of the tDNA across the outer membrane, which most likely occurs at or in close proximity to the Tfp and the PilQ secretin (as no ComEA-tDNA clusters were detectable in the absence of PilQ or the major Tfp subunit PilA). Moreover, time-lapse microscopy movies suggested that the ComEA foci were resolved and that the even distribution pattern of ComEA was reconstituted over time (41). However, these downstream events were not further investigated.

To better understand what could initiate the resolution of the ComEA clusters, we now quantified the persistence of ComEA...
foci by measuring the time interval between the formation of the ComEA foci and the reconstitution of a uniform ComEA distribution pattern. Through time-lapse microscopy (40 min in total), we observed that the resolution of the ComEA foci was very heterogeneous, with some cells restoring the initial pattern within 2 min, whereas others took more than 20 min (Fig. 2A). Notably, we did not observe any difference between cells containing the putative inner membrane transporter protein (ComEC) and those that lacked it (Fig. 2B). Through the use of longer tDNA fragments, the persistence time of the ComEA foci was largely increased, with the majority of cells maintaining the ComEA-tDNA clusters for more than 20 min (e.g., 52% of cells) (Fig. 2C).

As the time distribution patterns for the resolution of the ComEA foci were indistinguishable between ComEC-positive and -negative cells (Fig. 2A and B), we reasoned that such resolution events might not or might only to a minor extent be caused by the transport of the tDNA across the inner membrane (A [shaded in gray]) and the translocation of DNA into the cytosol coincide at one large DNA uptake complex (B) or that the outer and inner membrane transports are spatially uncoupled (C). Previous studies on naturally competent Gram-positive bacteria indicated that the inner membrane protein ComEC translocates the DNA across the cytoplasmic membrane (43), most likely in conjunction with the competence protein ComF, after which the single-stranded DNA is decorated with the proteins Ssb, DprA, and RecA.

Establishment of fluorescent protein markers to visualize the transport of the tDNA across the inner membrane. Based on studies of other naturally competent bacteria, it is assumed that the transport of the tDNA across the inner membrane occurs through a putative channel formed by the ComEC protein (43). We have previously confirmed the importance of ComEC and ComF (a homolog of ComFC of B. subtilis [51]) in the DNA uptake process of V. cholerae (9, 41, 52). Thus, we reasoned that the
FIG 2 ComEA-mCherry focus persistence during DNA uptake. Competence-induced strains carrying chromosomal comEA-mCherry were exposed to 10.3-kb PCR fragments (A and B and D to F) or phage λ DNA (48.5 kb) (C) and imaged at 2-min intervals for 21 frames. n = 100 (B, C, D) or n = 200 (A, E, F) cells. Cells depicting early mCherry focus formation were analyzed for the duration of focus persistence. Frequencies (%) of the observed phenotypes are shown as bar plots. ComEA-mCherry focus persistence was quantified in a wild-type (WT) strain (A, C) or in strains lacking comEC (B), xds (D), dns (E), or xds and dns (F).

point of DNA translocation across the inner membrane could be visualized through the use of ComEC or ComF translational fusions with the green fluorescent protein (GFP). After generating such constructs and using them to replace the indigenous native alleles of comEC and comF, respectively, we first assessed their functionality by scoring the natural transformability of the resulting strains. Indeed, both fusion proteins were fully proficient to functionally score the natural transformability of the result-}

alleles of such constructs and using them to replace the indigenous native

sions with the green fluorescent protein (GFP). After generating

visualized through the use of ComEC or ComF translational fu-

point of DNA translocation across the inner membrane could be

visualized through the use of ComEC or ComF translational fu-

usions with the green fluorescent protein (GFP). After generating

such constructs and using them to replace the indigenous native

alleles of comEC and comF, respectively, we first assessed their func-

tionality by scoring the natural transformability of the resulting

strains. Indeed, both fusion proteins were fully proficient to

drive natural transformation (see Table S1 in the supplemental

material), whereas deletion strains lacking either comEC or comF

were nontransformable (9). Similar results were obtained for a

DprA-GFP fusion (see Table S1), which we also used to monitor

entry of tDNA into the cytoplasm (Fig. 1). Unfortunately, the

abundance of these three proteins in competence-induced cells

(without or with external tDNA) was too low for detection by

fluorescence microscopy. The low abundance of ComEC was in

accordance with early expression data, which were based on tran-

scription-PCR (qRT-PCR) (48). Therefore, we decided to

study the localization of another downstream-acting cytoplasmic

protein, RecA, instead (Fig. 1). In fact, RecA localization has been

previously studied in the noncompetent Gram-negative bac-

teria and their ability to be naturally transformed (Fig. 3). No-

tably, even though the growth of the RecA-GFP-expressing strain

was impaired to a certain extent, the strain maintained extremely

high levels of natural transformability (Fig. 3). Next, we studied

the localization of RecA-GFP in non-competence-induced

V. cholerae cells (Fig. 3C). Under those conditions, RecA-GFP

was either evenly dispersed within the cytoplasm or clustered into dis-

	inctive and strongly fluorescent foci (Fig. 3C). We quantified the

number of RecA-GFP foci per cell by using the MicrobeTracker

software (58), and the analysis revealed that 58.6, 36.8, and 4.6% of

cells contained none, one, or more than one fluorescence focus

per cell, respectively (Fig. 3D). Renzette and colleagues previously

showed that there were two types of RecA-GFP foci within E. coli

cells: 4′,6-diamidino-2-phenylindole (DAPI)-sensitive clusters

that contained DNA and DAPI-insensitive DNA-less foci (53). To

better understand the type of RecA-GFP foci that were observable

in V. cholerae, we tested whether their distribution within a popu-

lation would change upon DAPI staining, which turned out not to

be the case (Fig. 3D). Thus, the observed RecA-GFP clusters

could act as DNA-less storage structures or, alternatively, be the

result of nonfunctional protein aggregates. To verify whether the

latter was the case, we constructed a translational fusion be-

tween the gene encoding inclusion body-associated protein A (IbpA)

and mCherry. The small heat shock protein IbpA was originally

identified in E. coli during the high-level production of heterolo-

gous proteins (59). Subsequently, IbpA has been shown to faith-

fuley identify the localization of protein aggregates and inclusion
and RecA-GFP was only observed in antibiotic or heat stress, colocalization between IbpA-mCherry and material). Moreover, even after induction of IbpA-mCherry through protein aggregates (Fig. 4D; see Table S2 in the supplemental material), such foci did not attract the IbpA-mCherry fusion protein, indicating that the foci were not recognized as unfolded above) (Fig. 3), such foci did not attract the IbpA-mCherry fusion protein, indicating that the foci were not recognized as unfolded protein aggregates (Fig. 4D; see Table S2 in the supplemental material). Moreover, even after induction of IbpA-mCherry through antibiotic or heat stress, colocalization between IbpA-mCherry and RecA-GFP was only observed in <25% of the cells (Fig. 4D; see Table S2). We therefore concluded that the bright RecA-GFP foci depict DNA-less storage structures and not misfolded protein aggregates or inclusion bodies. Nonetheless, we cannot fully exclude that such bright and seemingly stable RecA-GFP foci, as also observed in E. coli (53), might be the result of the fluorescent tag, as recently addressed by Landgraf et al. (63). However, the dynamic and tDNA-dependent changes of the RecA-GFP localization as described below would not be expected in the latter case.

DNA transport across the outer and inner membrane is spatially but not temporally coupled. After validating the functionality of the RecA-GFP fusion and determining its subcellular distribution pattern in noncompetent bacteria, we aimed at using this construct to pinpoint the point of entry of the tDNA into the cytoplasm. The rationale behind this idea was that RecA would bind to ssDNA entering the cytoplasm, thereby changing its localization pattern to form protein clusters, which should be visible by fluorescence microscopy (Fig. 1). Indeed, earlier studies showed that RecA colocalized with the polar DNA uptake machinery of B. subtilis and that RecA filaments (threads) formed upon addition of tDNA, which seemingly emanated from the DNA uptake complex (54). Notably, the situation is different in V. cholerae as this bacterium does not contain polar competence machineries and the transport across the outer membrane does not strictly occur at the cell pole (9, 10, 41). Thus, to indirectly visualize the inner membrane transport process, we combined the recA-gfp and the comEA-mCherry alleles and imaged the resulting V. cholerae strain under competence-inducing conditions. In the absence of exogenous tDNA, competence-induced cells depicted a uniform distribution pattern of ComEA-mCherry, whereas the pattern of RecA-GFP distribution (see Fig. S1A and B in the supplemental material) was not significantly different from what was observed in competence-noninduced cells (Fig. 3C). However, when the competent cells were exposed to external tDNA, we frequently observed foci of ComEA-mCherry (see Fig. S1C to E) due to the uptake of tDNA into the periplasm. Notably, about half of the cells with ComEA-mCherry foci also contained clusters of RecA-GFP, and the two fluorescent signals colocalized often but not exclusively (see Fig. S1). We rarely observed foci from which RecA-GFP threads emanated, a finding that was in contrast to studies performed with B. subtilis (54, 64, 65). A hypothetical explanation for this difference could be that DNA transport across the inner membrane in V. cholerae proceeds in a 3′ to 5′ direction, in accordance with what has been demonstrated for H. influenzae (66), whereas the RecA protein polymerizes in the 5′ to 3′ direction (67). However, the absence of RecA filaments was not a concern for our bodies of E. coli and to assist chaperone-mediated protein disaggregation (60–62). Consequently, we replaced the ibpA gene of V. cholerae with an ibpA-mCherry translational fusion construct and studied its expression and localization under unstressed and stressed conditions (Fig. 4). Indeed, diverse stresses (exposure to either inhibitory concentrations of streptomycin [100 μg/ml] or a mild heat-shock at 40°C) induced the expression of IbpA-mCherry (Fig. 4A) and resulted in the formation of protein foci of IbpA-mCherry (Fig. 4B), as was the case for E. coli (60). Such an upregulation of IbpA-mCherry production upon external stress was equally observed in a V. cholerae strain that concomitantly produced RecA-GFP, indicating that the fusion protein per se does not induce ibpA-mCherry due to a putative aggregation effect (Fig. 4C). Importantly, whereas noncompetent V. cholerae strains often contained large and distinctive RecA-GFP foci (as described above) (Fig. 3), such foci did not attract the IbpA-mCherry fusion protein, indicating that the foci were not recognized as unfolded protein aggregates (Fig. 4D; see Table S2 in the supplemental material). Moreover, even after induction of IbpA-mCherry through antibiotic or heat stress, colocalization between IbpA-mCherry and RecA-GFP was only observed in <25% of the cells (Fig. 4D; see Table S2). We therefore concluded that the bright RecA-GFP foci depict DNA-less storage structures and not misfolded protein aggregates or inclusion bodies. Nonetheless, we cannot fully exclude that such bright and seemingly stable RecA-GFP foci, as also observed in E. coli (53), might be the result of the fluorescent tag, as recently addressed by Landgraf et al. (63). However, the dynamic and tDNA-dependent changes of the RecA-GFP localization as described below would not be expected in the latter case.

DNA transport across the outer and inner membrane is spatially but not temporally coupled. After validating the functionality of the RecA-GFP fusion and determining its subcellular distribution pattern in noncompetent bacteria, we aimed at using this construct to pinpoint the point of entry of the tDNA into the cytoplasm. The rationale behind this idea was that RecA would bind to ssDNA entering the cytoplasm, thereby changing its localization pattern to form protein clusters, which should be visible by fluorescence microscopy (Fig. 1). Indeed, earlier studies showed that RecA colocalized with the polar DNA uptake machinery of B. subtilis and that RecA filaments (threads) formed upon addition of tDNA, which seemingly emanated from the DNA uptake complex (54). Notably, the situation is different in V. cholerae as this bacterium does not contain polar competence machineries and the transport across the outer membrane does not strictly occur at the cell pole (9, 10, 41). Thus, to indirectly visualize the inner membrane transport process, we combined the recA-gfp and the comEA-mCherry alleles and imaged the resulting V. cholerae strain under competence-inducing conditions. In the absence of exogenous tDNA, competence-induced cells depicted a uniform distribution pattern of ComEA-mCherry, whereas the pattern of RecA-GFP distribution (see Fig. S1A and B in the supplemental material) was not significantly different from what was observed in competence-noninduced cells (Fig. 3C). However, when the competent cells were exposed to external tDNA, we frequently observed foci of ComEA-mCherry (see Fig. S1C to E) due to the uptake of tDNA into the periplasm. Notably, about half of the cells with ComEA-mCherry foci also contained clusters of RecA-GFP, and the two fluorescent signals colocalized often but not exclusively (see Fig. S1). We rarely observed foci from which RecA-GFP threads emanated, a finding that was in contrast to studies performed with B. subtilis (54, 64, 65). A hypothetical explanation for this difference could be that DNA transport across the inner membrane in V. cholerae proceeds in a 3′ to 5′ direction, in accordance with what has been demonstrated for H. influenzae (66), whereas the RecA protein polymerizes in the 5′ to 3′ direction (67). However, the absence of RecA filaments was not a concern for our
study, as RecA-GFP primarily served as a marker protein to indicate the entry of the ssDNA into the cytoplasm.

Although we frequently detected an association between RecA-GFP and ComEA-mCherry foci in these fluorescence microscopy snapshots (see Fig. S1 in the supplemental material), the results were indicative but not conclusive and certainly not proof that incoming single-stranded tDNA caused RecA-GFP focus formation, especially as RecA-GFP foci were also abundant in noncompetent cells (as discussed above). Thus, to be able to distinguish between already existing versus newly formed RecA-GFP foci, we performed time-lapse experiments (at 2-min intervals). The cells, which depicted a uniform distribution pattern of both fusion proteins, followed by the formation of fluorescent foci of ComEA-mCherry and RecA-GFP, were most informative in this context (Fig. 5A), although other events, including ComEA-mCherry foci, which are indicative of DNA uptake across the outer membrane, were also considered (Fig. 5B to D). Indeed, under the conditions where RecA-GFP foci developed de novo throughout the time-lapse experiment, we exclusively observed colocalization of the foci with the ComEA-mCherry signal (Fig. 5E, row a). Furthermore, RecA proteins accumulated with a certain delay to ComEA (~8 min in Fig. 5A), even though the duration of this delay was not characteristic and varied between ≤2 min and 18 min (the shortest and longest possible delays according to the time-lapse settings). In contrast, if RecA foci were already present at the beginning of the experiment, they only colocalized with the ComEA signal in roughly one out of two cases (Fig. 5E, rows c and d). In the majority of cells (~50%), RecA did not form any clusters. This finding suggests that the tDNA might have never reached the cytoplasm in those cells within the tested time frame of 20 min. As a control, we repeated the quantification of the RecA-GFP and ComEA-mCherry foci in a comEC-deficient strain in which tDNA should not reach the cytoplasm. In this case, the number of cells that formed new RecA-GFP foci throughout the time-lapse experiment was extremely low and without any preference toward colocalization with ComEA-mCherry (Fig. 5F).

Since we detected an increase in the duration of ComEA-mCherry focus persistence in the absence of Dns (Fig. 2), we wondered whether the nuclease could have an effect on the amount of tDNA that is imported into the cytosol and thus the de novo formation of RecA-GFP foci. Indeed, when we repeated the time-lapse experiment using a dns-negative strain, we observed a significant increase in de novo RecA-GFP focus formation (Fig. 5G) and an almost exclusive colocalization of these newly formed RecA

FIG 4 RecA-GFP accumulations are not part of misfolded protein aggregates. The native copy of ibpA on the chromosome of V. cholerae was replaced by an ibpA-mCherry translational fusion. (A) The protein level of IbpA-mCherry was verified by Western blotting using polyclonal anti-mCherry antibodies. Cell lysates of a wild-type (WT) strain as well as lysates of bacteria carrying ibpA-mCherry were probed. Lysates of bacteria producing IbpA-mCherry were either prepared from an unstressed control culture (ctrl) or after exposure to streptomycin (strep) or to a mild heat shock (40°C). (B) The production and distribution of IbpA-mCherry were tested in unstressed bacterial cultures (control) or after exposure of the bacteria to streptomycin (strep [100 µg/ml]) or to a mild heat shock (40°C). Cells were imaged in the red (mCherry) or phase-contrast (Ph) channel. (C) Lysates of WT cells and bacteria expressing ibpA-mCherry and recA-gfp were probed by Western blotting as in panel A. (D) Absence of colocalization between IbpA-mCherry and RecA-GFP. Representative fluorescence images (mCherry and GFP) and overlays (merge) are shown. Chromosomal DNA was stained with DAPI. The cells are outlined with dashed lines. Scale bars, 2 µm.
FIG 5  Time-lapse microscopy imaging of RecA-GFP ComEA-mCherry-expressing bacteria exposed to exogenous tDNA. Competence-induced *V. cholerae* strains expressing RecA-GFP and ComEA-mCherry were exposed to exogenous tDNA and imaged at 2-min intervals for a total of 20 min. Time points (min) are indicated above the images (A to D). Shown are examples of cells with distinctive ComEA-mCherry and RecA-GFP foci. Images taken in the fluorescence channels (mCherry, red; GFP, green) and overlays thereof (merge) are depicted. The cells are outlined with dashed lines. Observed phenotypes comprised cells that showed either de novo focus formation of mCherry and GFP (A), a preexisting mCherry focus (i.e., present at the beginning of the time-lapse experiment) and a newly formed GFP focus (B), a preexisting GFP focus and a newly formed mCherry focus (C), or preexisting foci of both constructs (D). (E) Quantification (Continued)
clusters with the ComEA-mCherry signal (~94.4%). Importantly, a deletion of dns in a comEC-deficient background did not have such an effect (Fig. 5H).

**DISCUSSION**

As one of three modes of horizontal gene transfer, natural transformation is an important contributor to bacterial evolution. Nonetheless, the mechanistic basis of the DNA uptake process is still poorly understood. Current DNA uptake models suggest that most naturally competent bacteria contain a (pseudo)Tfp as part of their DNA uptake machinery, which is required for DNA transport across the outer membrane of Gram-negative bacteria or through the thick cell wall of Gram-positive bacteria (11, 20–22). It has been proposed that the DNA shuttling might therefore be driven by repeated cycles of pilus extension and retraction. Subsequently, single-stranded DNA reaches the bacterial cytoplasm after passage through the putative membrane channel formed by ComEC (43). The proteins that are involved in the DNA translocation process across the cytoplasmic membrane and the DNA-protein interactions taking place in the cytosol are most likely conserved among Gram-positive and Gram-negative bacteria (6, 11, 68); however, the cellular localization of the inner membrane transport has so far only been experimentally investigated in *B. subtilis* (54, 56). Notably, in this organism the majority of the competence proteins are localized to the cell pole, which is indicative of the presence of a large multiprotein complex composed of cytosolic and membrane-bound proteins (54–57). However, a strict polar localization of the Tfp and/or competence proteins was not observed in the case of *S. pneumoniae* (47, 69) or for the Gram-negative bacterium *V. cholerae* (9, 10, 41). Indeed, there is still a major knowledge gap with respect to the cellular localization of the DNA uptake complexes among bacterial species other than *B. subtilis* and especially in Gram-negative bacteria, where the tDNA has to cross two membranes. Notably, the localization might have an impact on the assembly and the functionality of the DNA uptake machines and therefore deserves further attention.

In the present study, we took a cell biology-based approach to indirectly visualize the DNA translocation across the outer and inner membranes of *V. cholerae*. Through time-lapse microscopy to visualize the fluorescently tagged ComEA and RecA proteins, both of which showed almost full functionality with respect to natural transformation, we demonstrated that the passage of the tDNA over the outer membrane and its translocation into the cytosol occurred in close proximity to each other (Fig. 1C and 5). Interestingly, the timing between both passages was not constant, and a significant delay was observable between the two crossing events, indicating that the two steps are spatially but not temporally coupled. Such a spatial restriction was not predictable as it was previously suggested that DNA uptake across the two membranes of Gram-negative bacteria might involve two independent steps (19). Indeed, DNA uptake in Gram-negative bacteria was initially studied in *H. influenzae* and *Neisseria* spp., where it was demonstrated that DNA uptake into a DNase-resistant state (e.g., into the periplasm or the “transformasome” [70]) still occurred in the absence of the inner membrane channel ComEC (homologs Rec2 in *H. influenzae* and ComA in *Neisseria*) (38, 71, 72) indicating that the outer and inner membrane translocation events are uncoupled. Such a two-step mechanism (e.g., distinguishing the transport events across the outer membrane and the cytoplasmic membrane) should not be confused with other two-step mechanisms previously proposed for naturally competent bacteria, which differentiated between initial DNA binding followed by the DNA uptake event (as mentioned, for example, by Chen and Gotschlich [37]). Indeed, it is well documented for *Neisseria* that DNA binding can be dissociated from DNA uptake. Notably, *Neisseria* species (as well as *H. influenzae*) preferentially take up genus-specific DNA fragments, which are recognized by the presence of so-called DNA uptake sequences (DUS) or uptake signal sequences (USS) (references 73 and 74, and recently reviewed by Mell and Redfield [68]). Such DUS/USS elements are highly over-represented in these organisms. Interestingly, a minor pilin protein (ComP) of *Neisseria meningitides* has recently been shown to directly interact with DUS-containing DNA (75) thereby answering one of the many open questions in the field of natural transformation, namely, “which, if any, protein acts as a receptor for transforming DNA?” in *Neisseria* (13). However, comP mutants of *N. gonorrhoeae*, though impaired for natural transformation, were properly pilinated and exhibited normal Tfp function (36). We previously demonstrated that *V. cholerae* does not differentiate between species-specific and species-nonspecific DNA at the level of the DNA uptake process and therefore also with respect to the initial binding event (52). Furthermore, no obvious ComP homologues exist in *V. cholerae* (9), suggesting that the initial DNA binding step might be different in other Gram-negative bacteria than what was reported for *Neisseria* spp.

That DNA uptake across the two membranes might be a two-step event was also suggested based in single-cell experiments performed on *Helicobacter pylori* (76), *V. cholerae* (41), and more recently also on *N. gonorrhoeae* (42). It should be noted though that *H. pylori* is considered the “black sheep” (6) of transformation as it uses a type IV secretion system rather than a Tfp for the DNA translocation across the outer membrane (77) and does not contain a ComEA homolog (19). *H. pylori* is therefore not widely representative of naturally competent Gram-negative bacteria. In these cell biology-based studies, the authors used fluorescently labeled DNA (and fluorescently tagged marker proteins) to visualize the outer membrane transport. However, none of these studies addressed the inner membrane transport, most likely due to experimental limitation (e.g., loss of fluorescence of intercalating dyes in the periplasm and inability of covalently labeled DNA to cross the cytoplasmic membrane [41, 42, 76]). In the present study, we overcome these obstacles by visualizing the participating proteins instead of the DNA itself (Fig. 1). Unfortunately, neither fluorescently tagged ComEC nor ComF resulted in strong enough signals that would allow live-cell imaging and time-lapse microscopy. This was consistent with our earlier studies that indicated low expression of comEC and that this overexpression resulted in cell death (9, 48). Notably, we refrained from overexpressing any

*Figure Legend Continued*

of the observed phenotypes, as exemplified in panels A to D. The arrow indicates the change of focus occurrence from a dispersed signal (−) to a focal distribution pattern (+). ± indicates cases of persisting or disappearing foci. Events with *de novo* RecA-GFP focus formation are shaded in gray. n.a., not applicable. (F to H) Quantification of observed phenotypes as in panel E, but for strains lacking comEC (F), dns (G), or comEC and dns (H). Scale bar, 1 μm.
of the reporter constructs as a recent study on the type II secretion system of *V. cholerae* indicated that this could result in severe localization artifacts (78). Instead, we targeted the downstream-acting proteins, namely, DprA and RecA (Fig. 1), with the latter resulting in sufficiently bright signals to perform live-cell imaging.

Indeed, using a ComEA-mCherry RecA-GFP double-labeled strain of *V. cholerae*, we indirectly visualized the entry of the tDNA into the cytosol, which allowed us to conclude that the transport across the two membranes is spatially but not temporally coupled. Further studies are likely to indicate whether this also holds true for other naturally transformable Gram-negative bacteria such as *Neisseria*.

The DNA uptake mechanism might be different in Gram-positive bacteria. Indeed, Maier et al. used optical tweezers to investigate the DNA transport in *B. subtilis*. Such an experimental setting allowed the measurement of pulling forces exerted by the bacterium. Notably, no pulling of the tethered DNA occurred in a *B. subtilis* comEC mutant strain (32), even though DNA binding was readily observed in such mutants (32, 34). Based on these data, two hypothetical scenarios can be envisioned. (i) The transport of DNA in Gram-positive bacteria might be a one-step event where the pulling force is neither solely exerted by the pseudopilus, nor by ComEA, or a combination of both. Instead, it is suggested that the binding of cytoplasmic DNA-binding proteins (such as ComEA [79], Ssb, DprA, or RecA). (ii) The absence of ComEC might interfere with the full functionality of ComEA; thus, ComEA might still bind to the DNA, but the ComEC-dependent clustering, which we previously suggested as being a prerequisite for pulling the tDNA toward the bacterium (41), might be inhibited.

Based on the present study, we hypothesize yet another role for ComEA and propose that the periplasmic ComEA-tDNA complexes might serve as storage reservoir for transforming DNA. This idea is based on the fact that the ComEA-tDNA complexes were not quickly resolved in most *V. cholerae* cells within the population but could be maintained over time, especially in the absence of nucleases (Fig. 2). It should be noted though that our current experimental setup does not allow us to exclude that some of the periplasmic tDNA might never reach the cytoplasm. If that were the case, then the periplasmic DNA might serve as a food source once the bacteria leave the biofilm state on the intestinal surface (i.e., where competence is induced in *V. cholerae* [80]). Indeed, a switch from high cell density to low cell density would lead to an upregulation of the nuclease gene *dns* concomitantly with a discontinuation of *comEA* and *comEC* expression as these genes are regulated by quorum sensing (46, 48, 49, 52, 80, 81). That the nuclease Dns might foster rapid growth by providing nucleotides was suggested earlier by Blokesch and Schoolnik (46) and later experimentally confirmed by Seper et al. (82).

In line with the putative reservoir function of the periplasmic ComEA-tDNA clusters are recent findings concerning a cytoplasmic reservoir of ssDNA in naturally competent *S. pneumoniae* cells (83). Such ssDNA reservoirs were dependent on the second or alternative and competence-inducible single-stranded binding protein (SsbB) that is present in *B. subtilis* and *S. pneumoniae* but absent from many other bacteria, including *V. cholerae*. The SsbB protein of *S. pneumoniae* was produced at high levels upon competence induction to handle large quantities of incoming tDNA and to protect the ssDNA against degradation (83). Attaiech et al. speculated that such reservoirs would permit successive rounds of transformation, thereby contributing to the genetic plasticity of *S. pneumoniae* (83). We suggest that even though the two proteins SsbB and ComEA are not related to each other, they might fulfill a similar purpose in Gram-positive and Gram-negative bacteria, respectively, as they could form a complex with the tDNA, thereby creating a DNA reservoir that could transform the cells at a later time point (e.g., temporarily uncoupled from the initial DNA uptake event). Interestingly, such a periplasmic reservoir function was also proposed by Gangel and colleagues in a recent study that was published while the manuscript for the present article was under review (42). Moreover, in this elegant study the authors provided evidence that the periplasm of *N. gonorrhoeae* is saturated with tDNA within minutes and that ComE/ComEA quantitatively controls the carrying capacity of the periplasm (42). However, further experiments will enable a better understanding of the fate of the periplasmic ComEA-tDNA clusters over time.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The strains used throughout this study were derivatives of the *V. cholerae* El Tor strain A1552 (84) and are described in Table S3 in the supplemental material. Escherichia coli strain S17-1 λpir (85) served as the donor for bacterial mating with *V. cholerae*. Bacterial cells were propagated in Luria-Bertani (LB) medium under shaking conditions and at 30 or 37°C unless otherwise stated. Solid LB agar plates contained 1.5% (wt/vol) agar. Medium was supplemented with antibiotics at final concentrations of 50, 75, and 100 μg/ml for gentamicin, kanamycin, and ampicillin, respectively.

**V. cholerae strain construction.** Genes were deleted from the parental strain A1552 (84) using either a gene disruption method based on the counterselectable suicide plasmid pGPT704-Sac28 (86) or by natural transformation and FLP recombination, as recently described (TransFLP method [87–89]). Strains carrying genes coding for translational fusion proteins were generated by replacing the indigenous gene with a PCR-generated fusion construct using the TransFLP method (87–89). Standard molecular biology-based methods were used for DNA manipulations. For cloning purposes, the high-fidelity DNA polymerases Pwo (Roche) or KOD (Merck, Novagen) were used. Taq DNA polymerase (GoTaq; Promega) was used for colony PCRs. For the amplification of *V. cholerae* genes, the genomic DNA (gDNA) of strain A1552 served as the template. Modified DNA sequences were verified using Sanger sequencing (Microsynth, CH).

**Natural transformation assays.** Transformation assays were performed as described (48) using gDNA of the A1552- lacZ-kan strain (87) as donor tDNA. Each independent experiment was repeated at least three times, and the averages of all experiments ± standard deviations (SD) are indicated. Statistical analysis of transformation frequencies was conducted in R (90). Differences were considered significant when P values of Welch’s t tests on log-transformed data were below 0.05 or 0.01.

**Microscopy setup.** Microscopy images were obtained using a Zeiss Axio Imager M2 epifluorescence microscope. Details about the instrument, the configurations, and sample preparation and mounting, as well as sealing of the samples, are provided elsewhere (9, 41, 48). Bacterial colonies were imaged using a Leica DM IL LED Fluo microscope equipped with a HI PLAN 40×/0.10 objective and an ICC 50 HD camera module.

**Protein (co)localization experiments using fluorescence microscopy.** To determine the duration of ComEA-mCherry focus persistence.
the respective strains were grown aerobically in LB medium supplemented with 0.02% L-arabinose for 7 h at 30°C. Cells were washed once in phosphate-buffered saline (PBS) buffer before being mixed with a 10.3-kb PCR fragments or phage λ DNA (48.5 kb; Roche). Time-lapse microscopy was then performed by acquiring images at 2-min intervals for 40 min (corresponding to 21 frames). Only cells that generated new ComEA-mCherry foci within 4 to 20 min were further analyzed. The times (i.e., number of frames) for which these cells maintained the ComEA clusters were counted manually.

RecA-GFP foci in non-competence-induced cells were observed after growing the bacteria in plain LB medium for 7 h and at 30°C. At that time, the cells were either directly visualized or washed and incubated for 15 min in PBS containing 5 μg/ml DAPI prior to imaging. The distribution of the RecA-GFP foci was evaluated using the MicrobeTracker software (58).

For the detection of IbpA-containing protein aggregates, strains carrying chromosomal ibpA-mCherry or ibpA-mCherry and recA-gfp were grown in plain LB medium for 6 h at 30°C. At this time point, cultures were either incubated in the absence (control) or the presence of 100 μg/ml streptomycin for 1 h at 30°C or incubated for 1 h at 40°C as indicated in the figures. The staining of chromosomal DNA was performed through the addition of DAPI (final concentration, 5 μg/ml) to bacterial cultures for at least 5 min.

For test for any colocalization between ComEA-mCherry and RecA-GFP, strains were grown under competence-inducing conditions as described previously (9, 41). Quantification in random fields of view was performed manually. Counts from three independent experiments were tested for consistency and pooled for statistical analysis. For time-lapse imaging, 50 μl of bacterial suspension was mixed with 1 μg of phage λ DNA (48.5 kb) and immediately mounted on agarose pads. Image acquisition was started within 5 to 10 min after initial exposure of bacteria to DNA (e.g., time needed for mounting the bacteria, sealing the coverslips, and defining random fields of view in the microscope). Time-lapse series were recorded at 2-min intervals for 20 min (11 frames). As for the analysis of snapshots, results from three independent experiments were checked for consistency and pooled for statistical analysis.

**Analysis of microscopy images using MicrobeTracker.** The MicrobeTracker Suite (58) was used to determine the number of RecA-GFP foci per cell. The parameters of Algorithm 4 were altered slightly (splitThreshold set to 0.2 and split1 set to 1) to outline V. cholerae cells. Correct outlining was verified manually, and cells depicting severe morphological abnormalities were excluded. RecA-GFP foci were detected automatically in non-competence-induced cells using the spotFinderZ tool. Data from three biological replicates were checked for consistency and pooled for statistical analysis.

**Image processing and annotation.** Image processing and manual quantification were performed using ImageJ. Linear adjustments of the contrast and brightness of microscopy images were applied to whole images and only used to optimally display gray values within the observed range. Adobe Illustrator was used for image annotation. Cells were outlined by Bézier curves according to the size and shape of the bacteria observed in the phase-contrast images.

**Cell lysates, SDS-PAGE, and Western blot analysis.** For the preparation of cell lysates, bacteria expressing IbpA-mCherry were grown as described above. Cells were lysed in Laemmli buffer (adjusted for the optical density at 600 nm [OD₆₀₀] of the bacterial culture) and incubated at 98°C for 15 min. Proteins were separated through SDS-PAGE using 11% acrylamide gels (91, 92) and transferred at 4°C onto polyvinylidene difluoride (PVDF) Western blotting membranes (Roche). Membrane-bound proteins were exposed to a primary antibody directed against mCherry (Biovision 5993-100; 1:5,000 diluted) and a secondary goat anti-rabbit antibody conjugated to peroxidase (Sigma A9169; 1:20,000 diluted). Immunoblots were developed using the Western Lightning-ECL enhanced chemiluminescence substrate (PerkinElmer), and signals were detected on chemiluminescence films (Amersham Hyperfilm ECL; GE Healthcare).

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.orglookup/supp doi:10.1128/mBio.01409-14/-/DCSupplemental.

**REFERENCES**

1. Lorenz MG, Wackernagel W. 1994. Bacterial gene transfer by natural genetic transformation in the environment. Microbiol. Rev. 58:563–602.
2. Macfadyen LP. 2000. Regulation of competence development in Haemophilus influenzae. J. Theor. Biol. 207:349–359. http://dx.doi.org/10.1006/jtbi.2000.2179.
3. Cleverys JP, Prudhomme M, Martin B. 2006. Induction of competence regulons as a general response to stress in Gram-positive bacteria. Annu. Rev. Microbiol. 60:451–475. http://dx.doi.org/10.1146/ annurev.micro.60.080805.142139.
4. Kline KA, Sechman EV, Skaar EP, Seifert HS. 2003. Recombination, repair and replication in the pathogenic Neisseria: the 3 R’s of molecular genetics of two human-specific bacterial pathogens. Mol. Microbiol. 50:3–13. http://dx.doi.org/10.1046/j.1365-2958.2003.03679.x.
5. Seitz P, Blokesch M. 2013. Cues and regulatory pathways involved in natural competence and transformation in pathogenic and environmental Gram-negative bacteria. FEMS Microbiol. Rev. 37:336–363. http://dx.doi.org/10.1111/j.1574-6976.2012.00353.x.
6. Johnston C, Martin B, Fichant G, Polard P, Cleverys JP. 2014. Bacterial transformation: distribution, shared mechanisms and divergent control. Nat. Rev. Microbiol. 12:181–196. http://dx.doi.org/10.1038/nrmicro3199.
7. Fussenegger M, Rudel T, Barten R, Ryll R, Meyer TF. 1997. Transformation competence and type-4 pilus biogenesis in Neisseria gonorrhoeae—a review. Gene 192:125–134. http://dx.doi.org/10.1016/S0378-1119(97)00038-3.
8. Heidelberg JF, Eisen JA, Nelson WS, Clayton RA, Gwinn ML, Dodson RJ, Haft DH, Hickey EK, Peterson JD, Umayam L, Gill SR, Nelson KE, Read TD, Tettelin H, Richardson D, Ermolaeva MD, Vamathevan J, Bass S, Qin H, Dragoi I, Sellers P, McDonald L, Utterback T, Fleshmann RD, Nierman WC, White O, Salzberg SL, Smith HO, Colwell RR, Mekalanos JJ, Venter JC, Fraser CM. 2000. DNA sequence of both chromosomes of the cholera pathogen Vibrio cholerae. Nature 406:477–483. http://dx.doi.org/10.1038/35020000.
9. Seitz P, Blokesch M. 2013. DNA-uptake machinery of naturally competent Vibrio cholerae. Proc. Natl. Acad. Sci. U. S. A. 110:17987–17992. http://dx.doi.org/10.1073/pnas.1315647110.
10. Metzger LC, Blokesch M. 2014. Composition of the DNA-uptake complex of Vibrio cholerae. Mol. Genet. Genomics 287:1–14. http://dx.doi.org/10.1007/s00438-013-3424-7.
11. Chen I, Dubnau D. 2004. DNA uptake during bacterial transformation. Nat. Rev. Microbiol. 2:241–249. http://dx.doi.org/10.1038/nrmicro844.
12. Chen I, Christie PJ, Dubnau D. 2005. The ins and outs of DNA transfer in bacteria. Science 310:1456–1460. http://dx.doi.org/10.1126/ science.1114021.
13. Hamilton HL, Dillard JP. 2006. Natural transformation of Neisseria gonorrhoeae: from DNA donation to homologous recombination. Mol. Microbiol. 59:376–385. http://dx.doi.org/10.1111/j.1365-2958.2005.04964.x.
14. Johnsborg O, Eldholm V, Hävarstein LS. 2007. Natural genetic transformation: prevalence, mechanisms and function. Res. Microbiol. 158:767–778. http://dx.doi.org/10.1016/j.resmic.2007.09.004.
15. Claverys JP, Martin B, Polard P. 2009. The genetic transformation machinery: composition, localization, and mechanism. FEMS Microbiol. Rev. 33:643–656. http://dx.doi.org/10.1111/j.1574-6976.2009.00164.x.

16. Averhoff B. 2009. Shuffling genes around in hot environments: the unique DNA transporter of *Thermus thermophilus*. FEMS Microbiol. Rev. 33:257–262. http://dx.doi.org/10.1111/j.1574-6976.2008.00160.x.

17. Burton B, Dubnau D. 2010. Membrane-associated DNA transport machines. Cold Spring Harb. Perspect. Biol. 2:a004006. http://dx.doi.org/10.1101/cshperspect.a004006.

18. Allemann JF, Maier B. 2009. Bacterial translocation motors investigated by single molecule techniques. FEMS Microbiol. Rev. 33:359–610. http://dx.doi.org/10.1111/j.1574-6976.2009.00166.x.

19. Kröger NJ, Stingl K. 2011. Two steps away from novelty–principles of bacterial DNA uptake. Mol. Microbiol. 80:860–867. http://dx.doi.org/10.1111/j.1365-2958.2011.07647.x.

20. Stone BJ, Kwaik YA. 1999. Natural competence for DNA transformation by *Legionella pneumophila* and its association with expression of type IV pili. J. Bacteriol. 181:1395–1402.

21. Graupner S, Frey V, Hashemi R, Lorenz MG, Brandes G, Wackernagel L. 2009. Future of the DNA receptor for transformable *Legionella pneumophila* is dispensable for pilus biogenesis but essential for bacterial transformation. *FEMS Microbiol. Rev.* 33:570–580. http://dx.doi.org/10.1111/j.1574-6976.2008.00160.x.

22. Chen I, Gotschlich EC. 2001. ComE, a competence protein from *Neisseria meningitidis* required for pilus biogenesis with DNA-binding activity. J. Bacteriol. 183:3160–3168. http://dx.doi.org/10.1128/JB.183.11.3160-3168.2001.

23. Sinha S, Mell JC, Redfield RJ. 2012. Seventeen Sxy-dependent cyclic AMP receptor protein site-regulated genes are needed for natural transformation in *Haemophilus influenzae*. J. Bacteriol. 194:5245–5254. http://dx.doi.org/10.1128/JB.06671-12.

24. Bean B, Zhang Q. 2007. CG011c, a periplasmic single- and double-stranded DNA-binding protein, contributes to natural transformation in *Campylobacter jejuni*. J. Bacteriol. 189:7399–7407. http://dx.doi.org/10.1128/JB.01012-07.

25. Muller LM, Bossé JT, Nair SP, Ward JM, Rycroft AN, Robertson G, Langford PR, Henderson B. 2008. *Pasteurella multocida* ComE1 proteins combine the properties of fibronectin adhesins and DNA binding competence proteins. *PLoS One* 3:e3991. http://dx.doi.org/10.1371/journal.pone.0003991.

26. Seitz P, Peeseshi Modarres H, Borgeaud S, Bulushev RD, Steenbock IJ, Rycroft AN, Davidsen L. 2009. Averhoff B. 2010. Membrane-associated DNA transport machinery: composition, localization, and mechanism. FEMS Microbiol. Rev. 33:643–656. http://dx.doi.org/10.1111/j.1574-6976.2009.00164.x.

27. Collins RF, Davidsen L, Derrick JP, Ford RC, Tønjum T. 2009. A key presynaptic role in transformation for a widespread bacterial protein: DprA conveys incoming ssDNA to RecA. Cell 130:824–836. http://dx.doi.org/10.1016/j.cell.2007.07.038.

28. Bergé M, Mortier-Barrière I, Martin B, Claverys JP. 2003. Transformation of *Streptococcus pneumoniae* relies on DprA- and RecA-dependent protection of incoming DNA single strands. Mol. Microbiol. 50:527–536. http://dx.doi.org/10.1046/j.1365-2958.2003.03702.x.

29. Mortier-Barrière I, Delvaux M, Fauth G, Suckow G, Seitz P, Blokesch M. 2011. ComEA is essential for DNA uptake: membrane topology, oligomerization and formation of disulphide bonds. Mol. Microbiol. 85:881–896. http://dx.doi.org/10.1111/j.1365-2958.2004.04430.x.

30. Averhoff B. 2010. Membrane-associated DNA transport machinery: composition, localization, and mechanism. FEMS Microbiol. Rev. 33:643–656. http://dx.doi.org/10.1111/j.1574-6976.2009.00164.x.

31. Collins RF, Davidsen L, Derrick JP, Ford RC, Tønjum T. 2009. A key presynaptic role in transformation for a widespread bacterial protein: DprA conveys incoming ssDNA to RecA. Cell 130:824–836. http://dx.doi.org/10.1016/j.cell.2007.07.038.

32. Blokesch M, Schoolnik GK. 2008. The extracellular nuclease Dns and its role in natural transformation of *Vibrio cholerae*. J. Bacteriol. 190:7232–7240. http://dx.doi.org/10.1128/JB.01099-08.

33. Berge MJ, Kamgoue A, Martin B, Polard P, Camus N, Claverys JP. 2011. Midcell recruitment of the DNA uptake and virulence nuclease, EndA, for pneumococcal transformation. *PLoS Pathog.* 7:e1003596. http://dx.doi.org/10.1371/journal.ppat.1003596.

34. Lo Scurotto M, Blokesch M. 2012. The regulatory network of natural competence and transformation of *Vibrio cholerae*. *PLoS Genet.* 8:e1002778. http://dx.doi.org/10.1371/journal.pgen.1002778.

35. Lo Scurotto M, Blokesch M. 2013. A transcriptional regulator linking quorum sensing and chitin induction to render *Vibrio cholerae* naturally transformable. Nucleic Acids Res. 41:3644–3658. http://dx.doi.org/10.1093/nar/gkt401.

36. Blokesch M. 2012. A quorum sensing-mediated switch contributes to natural transformation of *Vibrio cholerae*. *PLoS Genet.* 8:e1003596. http://dx.doi.org/10.1371/journal.pgen.1003596.

37. Kidane D, Graumann PL. 2005. Intracellular protein and DNA dynamics in competent *Bacillus subtilis* cells. Cell 122:73–84. http://dx.doi.org/10.1016/j.cell.2005.04.036.

38. Suckow G, Seitz P, Blokesch M. 2011. Quorum sensing contributes to natural transformation of *Vibrio cholerae* in a species-specific manner. J. Bacteriol. 193:4914–4924. http://dx.doi.org/10.1128/JB.05396-11.

39. Romette N, Guimarães N, Nordman JT, Krieger M, Yeh SP, Long E, Centore R, Boosnambrot R, Sandler SJ. 2005. Localization of RecA in *Escherichia coli* K-12 using RecA-GFP. *MBio* 1175–1185. http://dx.doi.org/10.1111/j.1365-2958.2005.04755.x.
55. Hahn J, Maier B, Haiejma BJ, Sheetz M, Dubnau D. 2005. Transformation proteins and DNA uptake localize to the cell poles in *Bacillus subtilis*. Cell 122:59–71. http://dx.doi.org/10.1016/j.cell.2005.04.035.

56. Kramer N, Hahn J, Dubnau D. 2007. Multiple interactions among the competence proteins of *Bacillus subtilis*. Mol. Microbiol. 65:454–464. http://dx.doi.org/10.1111/j.1365-2958.2007.05799.x.

57. Kaufmann S, Shechter Laan M, Graumann PL. 2011. The three-layered DNA uptake machinery at the cell pole in competent *Bacillus subtilis* cells is a stable complex. J. Bacteriol. 193:1633–1642. http://dx.doi.org/10.1128/JB.01128-10.

58. Slusarenko O, Heintz J, Emonet T, Jacobs-Wagner C. 2011. High-throughput, subpixel precision analysis of bacterial morphogenesis and intracellular spatio-temporal dynamics. Mol. Microbiol. 86:612–627. http://dx.doi.org/10.1111/j.1365-2958.2011.07578.x.

59. Allen SP, Polazzi JO, Gierse JK, Easton AM. 2008. Asymmetric segregation of protein aggregates is associated with cellular aging and rejuvenation. Proc. Natl. Acad. Sci. U. S. A. 105:3067–3081. http://dx.doi.org/10.1073/pnas.0709311105.

60. Ratajczak E, Zietkiewicz S, Liberek K. 2006. *Escherichia coli* richia coli. Letesson JJ, De Bolle X.

61. Danner DB, Deich RA, Sisco KL, Smith HO. 2009. Evidence for different pathways during horizontal gene transfer in competent Bacillus subtilis cells. Proc. Natl. Acad. Sci. U. S. A. 106:3157–3162. http://dx.doi.org/10.1073/pnas.0909955107.

62. Hofreuter D, Odenbreit S, Haas R. 2001. Natural transformation competence in *Helicobacter pylori* is mediated by the basic components of a type IV secretion system. Mol. Microbiol. 41:379–391. http://dx.doi.org/10.1046/j.1365-2958.2001.02520.x.

63. Lybarger SR, Johnson TL, Griswold MD, Sikora AE, Sandkivist M. 2009. Docking and assembly of the type II secretion complex of *Vibrio cholerae*. J. Bacteriol. 191:3149–3161. http://dx.doi.org/10.1128/JB.01701-08.

64. Fronzes R. 2012. Segregating genetic variation within the thioester-binding domain of the thioesterase TraE allows identification of the trauma protein SsbB in pneumococcal transformation: maintenance of a reservoir of DNA uptake specificity. J. Bacteriol. 194:2631–2635. http://dx.doi.org/10.1128/JB.01293-13.

65. Antonova ES, Hammer BK. 2011. Quorum-sensing autoinducer molecules produced by members of a multispecies biofilm promote horizontal gene transfer to *Vibrio cholerae*. FEMS Microbiol. Lett. 322:68–76. http://dx.doi.org/10.1111/j.1574-6968.2011.02328.x.

66. Facius D, Meyer TF. 1993. A novel determinant (coma) essential for natural transformation competence in *Neisseria gonorrhoeae* and the effect of a comA deletion on pillin variation. Mol. Microbiol. 6:699–712. http://dx.doi.org/10.1111/j.1365-2958.1993.tb00492.x.

67. Barany F, Kahn ME, Smith HO. 2008. Docking and assembly of the type II secretion complex of *Vibrio cholerae*. J. Bacteriol. 190:1365–1376. http://dx.doi.org/10.1128/JB.00694-08.

68. Barany F, Kahn ME, Smith HO. 2008. Distinct activities of *Brucella abortus* competence proteins of *Bacillus subtilis* expression in *Escherichia coli*. J. Bacteriol. 174:4963–4974.

69. Lindner AB, Madden R, Demarez A, Stewart EJ, Taddei F. 2011. An eleven-base-pair DNA double strand break repair centers in live cells. J. Cell Biol. 193:3105–3116. http://dx.doi.org/10.1083/jcb.2011031106.

70. Seitz and Blokesch 2009. An eleven-base-pair nucleotide binding site of the *Bacillus subtilis* membrane protein CoMFA aboliishes the uptake of DNA during transformation. J. Bacteriol. 176:4641–4655.

71. Lichtman JA, Dubnau D. 2004. Mutation of the putative nucleotide binding site of the *Bacillus subtilis* competence proteins of *Bacillus subtilis* expression in *Escherichia coli*. J. Bacteriol. 190:1365–1376. http://dx.doi.org/10.1128/JB.00694-08.

72. Meibom KL, Blokesch M, Dolganov NA, Wu CY, Schoolnik GK. 2005. Chitin induces natural competence in *Vibrio cholerae*. Science 310:1824–1827. http://dx.doi.org/10.1126/science.1120096.

73. Antonova ES, Hammer BK. 2011. Quorum-sensing autoinducer molecules produced by members of a multispecies biofilm promote horizontal gene transfer to *Vibrio cholerae*. FEMS Microbiol. Lett. 322:68–76. http://dx.doi.org/10.1111/j.1574-6968.2011.02328.x.

74. Attaieh L, Olivier A, Mortier-Barrière I, Soulet AL, Granadel C, Martin B, Polard P, Claverys JP. 2011. Role of the single-stranded DNA-binding protein SsbB in pneumococcal transformation: maintenance of a reservoir for genetic plasticity. PLoS Genet. 7:e1002156. http://dx.doi.org/10.1371/journal.pgen.1002156.

75. Yildiz FH, Schoolnik GK. 1998. Role of rpoS in stress survival and virulence of *Vibrio cholerae*, J. Bacteriol. 180:773–784.

76. Simon R, Priefer U, Pühler A. 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram negative bacteria. Nat. Biotechnol. 1:784–791. http://dx.doi.org/10.1038/nbt183-784.

77. Meibom KL, Li XB, Nielsen AT, Wu CY, Roseman S, Schoolnik GK. 2004. The *Vibrio cholerae* chitin utilization program. Proc. Natl. Acad. Sci. U. S. A. 101:2524–2529. http://dx.doi.org/10.1073/pnas.0308707101.

78. Marvig RL, Blokesch M. 2010. Natural transformation of *Vibrio cholerae* as a tool—optimizing the procedure. BMC Microbiol. 10:155. http://dx.doi.org/10.1186/1471-2180-10-155.

79. De Souza Silva O, Blokesch M. 2010. Genetic manipulation of *Vibrio cholerae* by combining natural transformation with FLP recombination. PLoS Pathog 6:761–765. http://dx.doi.org/10.1371/journal.ppat.1000765.

80. Devaiah V, Sambroski J, Fritsch EF, Maniatis T. 1988. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.