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

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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.

Horizontal 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 *comE1* 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
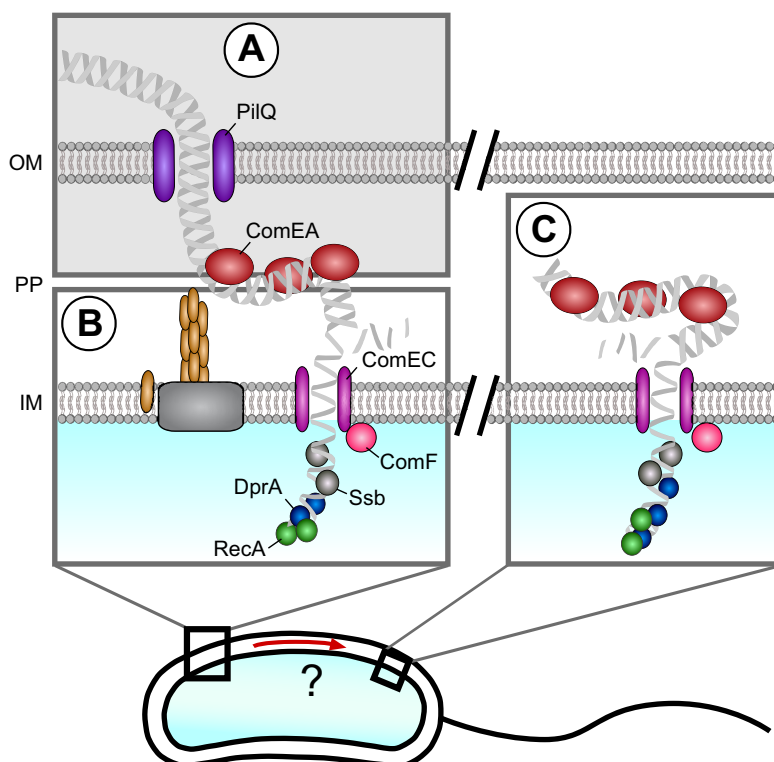
FIG 1 Putative positions of the DNA translocation events across the two membranes. The aim of this study was to assess the relative position of the DNA translocation across the inner membrane (IM) compared to the DNA passage across the outer membrane (OM). PP, periplasm. Two hypotheses were addressed in this study, namely, that the ComEA-assisted DNA uptake across the outer 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.

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 but could be due to another event that leads to the resolution of the complex. One option for such an effect would be the cleavage of one or both strands of the tDNA by nucleases. In this context, we have previously demonstrated that *V. cholerae* cells that lack the nucleases Dns and Xds are more transformable (46) and accumulated larger amounts of tDNA within the periplasm than nuclease-proficient strains (41). However, tDNA did not accumulate in these strains when ComEA was concomitantly absent, in which case the DNA uptake process seemed completely abolished. Thus, we suggested

that ComEA's major task was to pull tDNA into the periplasm rather than protecting the DNA against nucleases (41) or to modulate the nuclease activity, as has been suggested for *Streptococcus pneumoniae* (47). Here, we asked the question of whether these nucleases play a role in the resolution of the ComEA clusters. To test this question, we repeated the time-lapse microscopy experiments described above in *V. cholerae* strains that lacked either one or both nuclease genes (*dns* and *xds*). The absence of *xds* did not have an effect on ComEA focus persistence (Fig. 2D), which is in agreement with earlier transformation data (41, 46). In contrast, the ComEA clusters were maintained for at least 20 min in the vast majority of *dns*- and *dns xds*-negative cells (Fig. 2E and F). We therefore speculate that even though the expression of *dns* is downregulated at the time of competence induction in a quorum-sensing-dependent manner (as shown both on the transcriptional and cell-associated protein levels [46, 48–50]), the residual Dns protein is sufficient to degrade part of the periplasmic tDNA, thereby freeing ComEA from the complex.

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
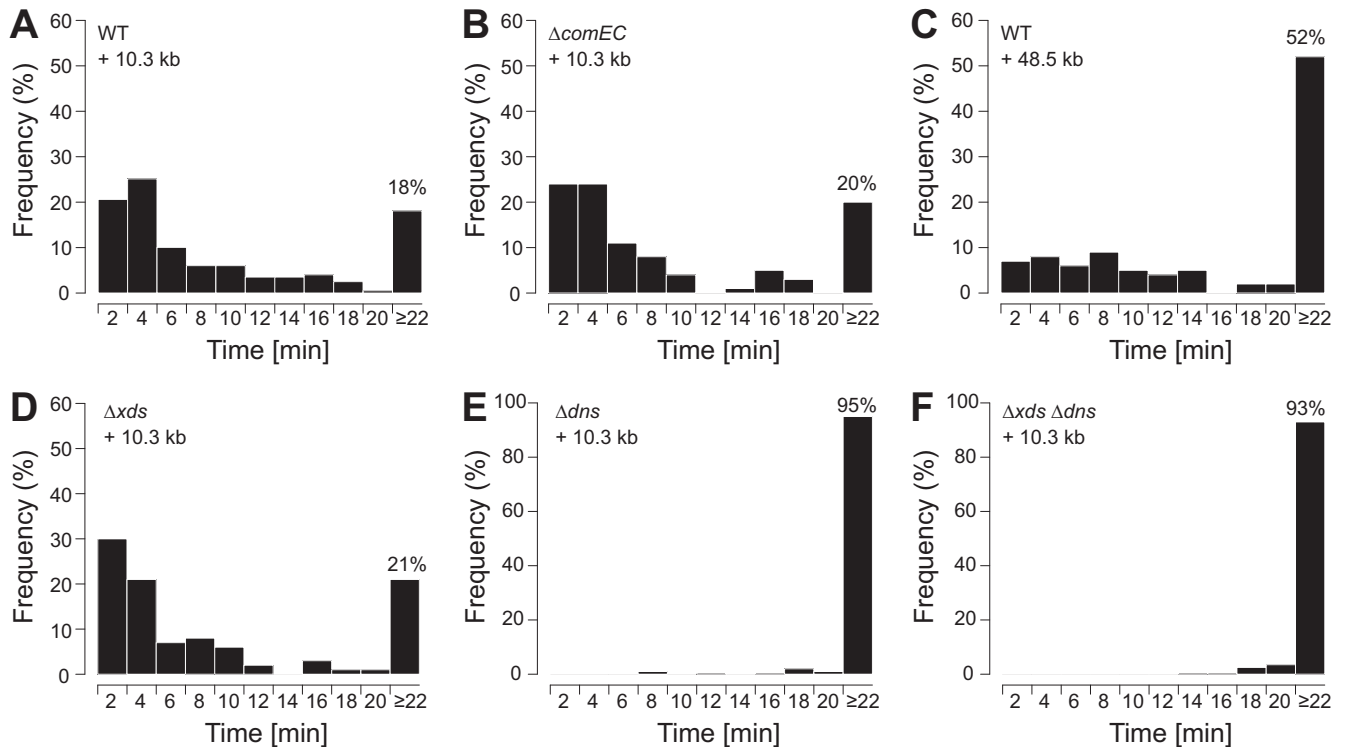
FIG 2 ComEA-mCherry focus persistence during DNA uptake. Competence-induced strains carrying chromosomal *comEA-mCherry* were exposed to 10.3-kb PCR fragments (A and B and D to F) or phage λ DNA (48.5 kb) (C) and imaged at 2-min intervals for 21 frames. $n = 100$ (B, C, D) or $n = 200$ (A, E, F) cells. 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 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 transcriptional reporters and on quantification by quantitative reverse transcription-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 bacterium *E. coli* (53) and in the Gram-positive competent bacterium *B. subtilis*, where it colocalized with other competence proteins of the DNA uptake machinery at a single cell pole of the bacterium (54–57). Moreover, a direct interaction between DprA and RecA has been demonstrated using a yeast two-hybrid system and pulldown assays (45).

After replacing the chromosomal copy of *recA* with the *recA-*

gfp allele in *V. cholerae*, we analyzed the growth behavior of the bacteria and their ability to be naturally transformed (Fig. 3). Notably, 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 distinctive 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 population 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 between 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 heterologous proteins (59). Subsequently, IbpA has been shown to faithfully identify the localization of protein aggregates and inclusion
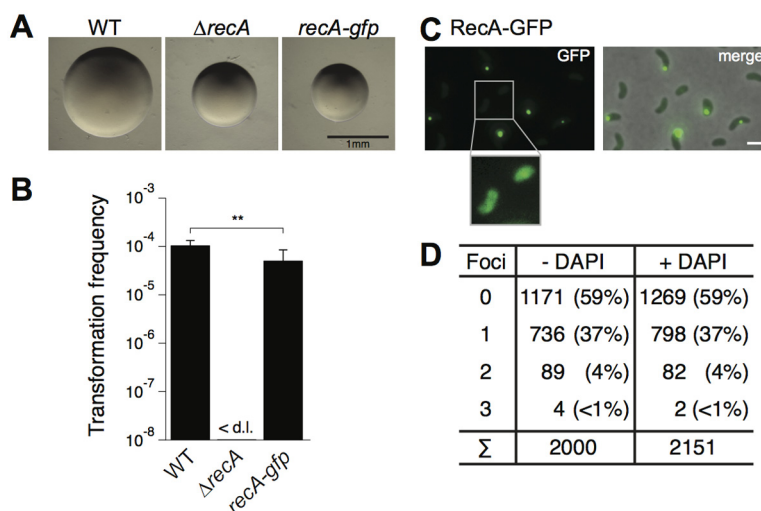
FIG 3 Functionality of chromosomally encoded RecA-GFP and localization of the protein. The native copy of *recA* on the chromosome of *V. cholerae* was replaced by a translational reporter fusion construct (*recA-gfp*). The functionality and localization of RecA-GFP were tested in live cells. (A) Colony morphology and size of a wild-type (WT) strain, Δ*recA* strain, and *recA-gfp* strain. (B) Functionality of RecA-GFP in chitin-independent natural transformation assays. All strains contain the Tn*tfoX* transposon for competence induction. <d.l., below the detection limit. Shown are average transformation frequencies of at least three independent experiments ± SD. Statistical significance was tested using Welch's t test on log-transformed values. **, $P < 0.01$. (C) Subcellular localization of RecA-GFP. Depicted is a representative fluorescence image of the green channel (GFP) and an overlay with the phase-contrast channel (merge). The inset was enhanced in contrast and brightness to visualize two cells that show homogenous RecA-GFP fluorescence. Scale bar, 2 μm. (D) Quantification of the number of GFP foci per cell in untreated (−DAPI) and DAPI-stained (+DAPI) cells.

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
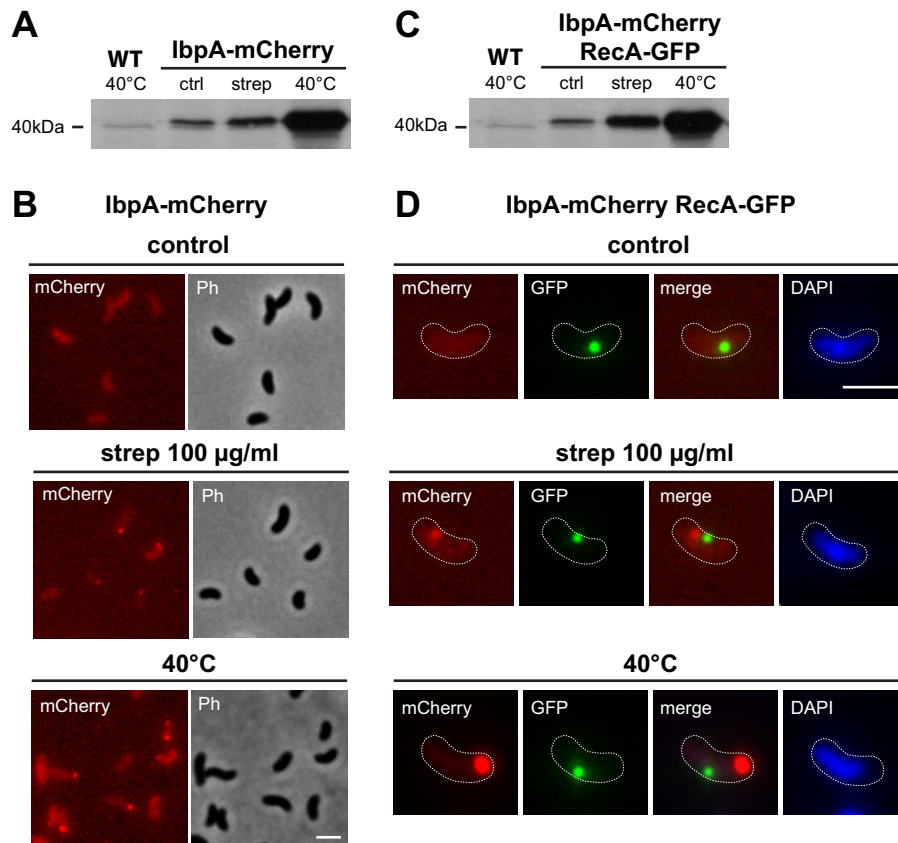
FIG 4 RecA-GFP accumulations are not part of misfolded protein aggregates. The native copy of *ibpA* on the chromosome of *V. cholerae* was replaced by an *ibpA-mCherry* translational fusion. (A) The protein level of IbpA-mCherry was verified by Western blotting using polyclonal anti-mCherry antibodies. 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.

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
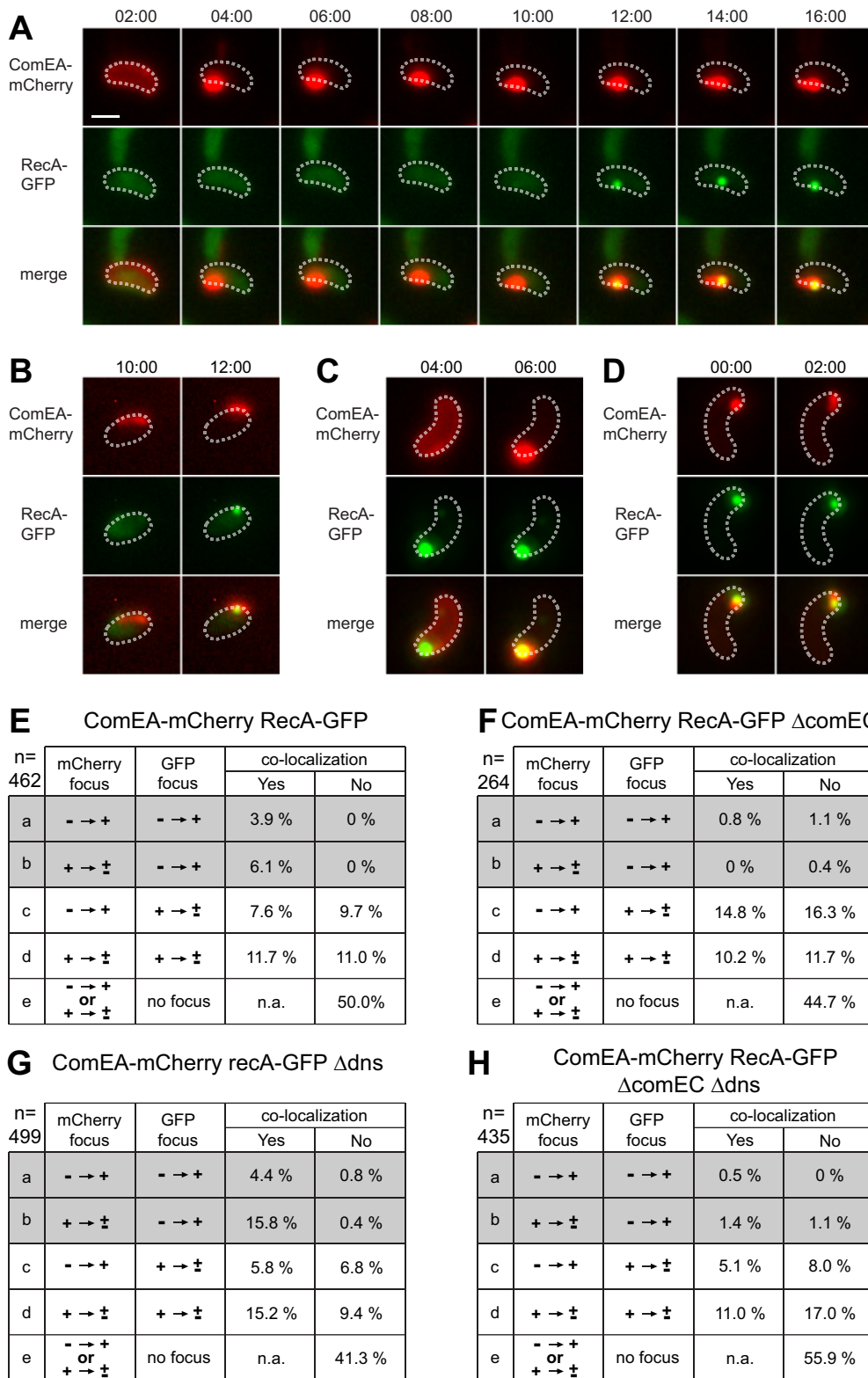
FIG 5 Time-lapse microscopy imaging of RecA-GFP ComEA-mCherry-expressing bacteria exposed to exogenous tDNA. Competence-induced *V. cholerae* strains expressing RecA-GFP and ComEA-mCherry were exposed to exogenous tDNA and imaged at 2-min intervals for a total of 20 min. Time points (min) are indicated 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 overrepresented 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 piliated 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 overcame 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 its 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, by ComEA, or a combination of both the pilus and ComEA but in addition requires the binding of cytoplasmic DNA-binding proteins (such as ComFA [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 required ComEA 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 chitin 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., temporally 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 S17-1λpir (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. For chitin-independent competence induction in Tn*tfoX*-carrying strains, the medium was supplemented with 0.02% L-(+)-arabinose as described previously (48). Thiosulfate-citrate-bile salts-sucrose (TCBS) agar plates were prepared as instructed by the manufacturer (Fluka) and used to counterselect *E. coli* after bacterial conjugation. For sucrose-based *sacB* counterselection, NaCl-free LB-medium containing 6% (wt/vol) sucrose was used. The medium was supplemented with antibiotics at final concentrations of 50, 75, and 100 μg/ml for gentamicin, kanamycin, and ampicillin, respectively, if required. The ampicillin concentration was lowered to 50 μg/ml for competence-induced *V. cholerae* strains.

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 pGP704-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 instrumentation, 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 4×/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.

To 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 [OD$_{600}$] 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

ACKNOWLEDGMENTS

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