Mechanisms of transforming DNA uptake to the periplasm of *Bacillus subtilis*

Jeanette Hahn^a, Micaela DeSantis^a, David Dubnau^{a#}

^aPublic Health Research Institute and Department of Microbiology, Biochemistry and Molecular Genetics, New Jersey Medical School, Rutgers University

Running Head: Uptake of DNA to the periplasm during transformation

Jeanette Hahn: Public Health Research Institute, New Jersey Medical School, Rutgers University, hahnje@njms.rutgers.edu

Micaela De Santis: Public Health Research Institute, New Jersey Medical School, Rutgers University, md1203@njms.rutgers.edu

David Dubnau: Public Health Research Institute and Department of Microbiology, Biochemistry and Molecular Genetics, New Jersey Medical School, Rutgers University, dubnauda@njms.rutgers.edu

Address correspondence to David Dubnau, dubnauda@njms.rutgers.edu

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34 **ABSTRACT**

35 We demonstrate here that the acquisition of DNAase resistance by transforming
 36 DNA, often assumed to indicate transport to the cytoplasm, actually reflects
 37 uptake to the periplasm, requiring a re-evaluation of conclusions about the roles
 38 of several proteins in transformation. The new evidence suggests that the
 39 transformation pilus is needed for DNA binding to the cell surface near the cell
 40 poles and for the initiation of uptake. The cellular distribution of the membrane-
 41 anchored ComEA of *B. subtilis* does not noticeably change during DNA uptake as
 42 does the unanchored ComEA of *Vibrio* and *Neisseria*. Instead, our evidence
 43 suggests that ComEA stabilizes the attachment of transforming DNA at localized
 44 regions in the periplasm and then mediates uptake, probably by a Brownian
 45 ratchet mechanism. Following that, the DNA is transferred to periplasmic portions
 46 of the channel protein ComEC, which plays a previously unsuspected role in
 47 uptake to the periplasm. We show that the transformation endonuclease NucA
 48 also facilitates uptake to the periplasm and that the previously demonstrated role
 49 of ComFA in the acquisition of DNAase resistance actually derives from the
 50 instability of ComGA when ComFA is deleted. These results prompt a new
 51 understanding of the early stages of DNA uptake for transformation.

52

53 **IMPORTANCE** Transformation is a widely distributed mechanism of bacterial
 54 horizontal gene transfer that plays a role in the spread of antibiotic resistance and
 55 virulence genes and more generally in evolution. Although transformation was
 56 discovered nearly a century ago and most, if not all of the proteins required have
 57 been identified in several bacterial species, much remains poorly understood
 58 about the molecular mechanism of DNA uptake. This study uses epifluorescence
 59 microscopy to investigate the passage of labeled DNA into the compartment
 60 between the cell wall and the cell membrane of *Bacillus subtilis*, a necessary early
 61 step in transformation. The roles of individual proteins in this process are
 62 identified, and their modes of action are clarified.

63

64 INTRODUCTION

65 Transformation, the ability to internalize high molecular weight
66 environmental DNA, is widespread among bacteria and is a major mechanism of
67 horizontal gene transfer (reviewed in (1-3)). Several proteins that mediate
68 transformation are widely conserved, suggesting that the core mechanism is of
69 ancient origin. Although most of these proteins were identified more than three
70 decades ago in a genetic screen (4, 5), much remains to be learned of the
71 mechanisms that mediate the transfer of DNA across the multiple layers that
72 comprise the bacterial surface barrier.

73 In the Gram-negative bacteria *Vibrio cholerae* and *Neisseria gonorrhoeae*, a
74 type 4 pilus (t4 pilus) snares transforming DNA (tDNA) and retracts, threading
75 the tDNA through a pore in the outer membrane formed by a ring of secretin
76 subunits and thus into the periplasm (6-9). There, the tDNA encounters the DNA-
77 binding protein ComEA, a periplasmic protein that serves as a Brownian ratchet
78 (10), preventing back diffusion of the tDNA which consequently is believed to
79 accumulate in the periplasm before crossing the inner cell membrane (7-9, 11,
80 12). In these bacteria, ComEA diffuses to the site of transforming tDNA uptake
81 where it concentrates dramatically as it binds to inward-diffusing tDNA.

82 Gram-positive bacteria lack an outer membrane. Instead, *Bacillus subtilis*, the
83 subject of the present study, is surrounded by a formidable cell wall, roughly 30-
84 35 nm in thickness, enclosing a 20-40 nm thick periplasm, defined as the
85 compartment between the wall and the cell membrane (13-15). This
86 compartment is probably gel-like, containing proteins, small molecules and
87 membrane-anchored lipoteichoic acid (14, 16), all of which may restrict diffusion
88 by molecular crowding. The wall of *B. subtilis* is a complex structure, including
89 peptidoglycan, proteins and wall teichoic acid (14). These differences in the outer
90 layers of Gram-positive and Gram-negative bacteria suggest consequent
91 differences in their mechanisms of DNA uptake to the periplasm. In fact, wall
92 teichoic acid, which is exclusive to the Gram positives, has been proposed to play
93 a role in DNA binding to competent cells (17). Also suggestive of such differences
94 is that *B. subtilis* ComEA is an integral membrane protein with its C-terminal DNA

95 binding motifs exposed to the periplasm (18, 19) as opposed to the unanchored
96 ComEA proteins of *V. cholerae* and *N. gonorrhoeae* that can diffuse within the
97 periplasm.

98 Transformable Gram-positive bacteria, like their Gram-negative counterparts,
99 encode t4pilus-like proteins that form filamentous structures. In *Streptococcus*
100 *pneumoniae*, these proteins assemble fibers that can extend several micrometers
101 into the extracellular environment and can bind DNA (20-22). No such extended
102 structure has been described in *B. subtilis*, which instead seems to form a shorter
103 “pseudopilus” that is probably long enough to traverse the wall (23). This
104 transformation pseudopilus (tpilus) is encoded by the 7 genes of the *comG* operon
105 and also requires *comC* and *bdbDC* for its construction (23-28). ComC is a
106 membrane peptidase that processes ComGC, the major pre-pilin subunit as well
107 as several minor pre-pilins, while BdbD and BdbC are thiol-disulfide
108 oxidoreductases that introduce intramolecular and intermolecular disulfide
109 bonds into ComGC and ComEC, the latter a component of the transformation
110 permease (29). *comGA*, the first gene of the *comG* operon, encodes a traffic ATPase
111 that is required for tpilus assembly (30). By analogy with *V. cholerae* (6) it is likely
112 that the Gram-positive tpili retract to bring DNA into the periplasmic
113 compartment. Indeed, a recent study shows convincingly that the pili of *S.*
114 *pneumoniae* extend and rapidly retract (22), although no such evidence has been
115 reported for *B. subtilis*.

116 In all transformable bacteria, transport across the cell membrane is
117 accomplished with the participation of ComEC, which is believed to form a
118 channel for the passage of DNA. In *B. subtilis* and *S. pneumoniae*, transport also
119 requires the membrane associated ComFA ATPase and ComFC, its binding partner
120 (31-35). During transport, one strand of the transforming tDNA is degraded while
121 the transforming strand enters the cytosol (36), where it associates with DNA
122 binding proteins and recombines with homologous DNA sequences to yield a
123 transformant.

124 In both Gram-negative and Gram-positive bacteria, tDNA becomes resistant to
125 added DNAase I (hereafter DNAase) during an early step in transformation. In the

Gram-negatives, DNAase-resistance signals that the tDNA has breached the outer membrane. In Gram-positive bacteria the proper interpretation of DNAase resistance is less clear and has been equated with “uptake” or “internalization” (37-39) without precise definition, although often assumed to indicate transport to the cytoplasm. The latter assumption was reasonably suggested by the observations that ComFA and ComEC, both required for the acquisition of DNAase resistance, are almost certainly needed for transport to the cytoplasm, based on their membrane localizations and molecular properties.

This study elucidates the process of tDNA uptake into the periplasm of *B. subtilis*. We show here that DNAase resistance actually reflects entry of tDNA to the periplasm, a step that we refer to as “uptake” to distinguish it from “transport” across the cell membrane. With this understanding, we have visualized the process of uptake using fluorescently labeled tDNA, enabling a re-evaluation of the roles of several transformation proteins. Based on our results we present an updated understanding of the early steps in transformation of *B. subtilis*, which may be applicable to other transformable firmicutes.

142

143 RESULTS

Experimental design. To visualize uptake, bacteriophage lambda DNA (48.5 kbp) was labeled using the Label-IT Rhodamine TM reagent (Mirus Bio) that covalently couples the fluorophore predominantly to the N7-position of guanine through a flexible linker, without disrupting Watson-Crick base pairing. To minimize perturbation of the rhodamine-DNA (rDNA) structure we used a labeling density that modifies fewer than 1% of the bases.

In *B. subtilis*, competence for transformation is expressed in only 10-20% of the cells. To identify competent cells, the promoter of *comG* was fused at the *amyE* locus to either yellow fluorescent protein (YFP) or cyan fluorescent protein (CFP), which delineate the cytosolic compartments of the competent cells. These isogenic strains behaved identically in our assays. For each experiment, aliquots of YFP- and CFP-expressing competent cells were incubated separately with rDNA, fixed with paraformaldehyde to stop transformation and then combined

157 and visualized for epifluorescence and phase contrast microscopy. Depending on
158 the experiment, the YFP- and CFP-expressing samples were derived from wild-
159 type and mutant strains or from samples incubated with and without DNAase
160 after transformation. This permitted direct comparisons without differences due
161 to image processing and the vagaries of agarose pads.

162 **DNAase as a tool for transformation studies.** Studies in *B. subtilis* and *S.*
163 *pneumoniae* have used 50-200 µg/ml of DNAase under varying conditions to
164 investigate transformation (4, 19, 34, 37, 38, 40). Because we wished to study
165 uptake, we sought to standardize a DNAase treatment that would remove
166 attached rDNA from the surface of the cells, without degrading rDNA in the
167 unidentified protected compartment. Preliminary experiments comparing
168 treatment with DNAase concentrations from 10 to 100 µg/ml after 15 minutes of
169 incubation with rDNA, when the acquisition of DNAase resistance has reached
170 about half its maximum level (37), showed no differences in the intensities of the
171 rhodamine signals after DNAase treatment, comparing the lowest and highest
172 amounts of DNAase. If DNAase significantly penetrated the protected
173 compartment, we would have expected to see a dose-dependent decrease in the
174 residual rDNA signal. We therefore settled on a 3-minute incubation with 100
175 µg/ml of DNAase at 37°C as an appropriate method for our experiments.

176 Wild-type cultures expressing YFP or CFP were each incubated with 0.2 µg/ml
177 rDNA, close to a saturation concentration for transformation. The CFP samples
178 were treated with DNAase and were then mixed with untreated YFP cells from the
179 same time points and visualized for fluorescence in the YFP, CFP and rhodamine
180 channels as well as by phase contrast after 2- and 30-minutes incubation with
181 rDNA. In the 2-minute sample (Figure 1A), few competent cells and very few of
182 the non-competent cells were associated with rDNA and after DNAase treatment
183 virtually no cells retained a significant rhodamine signal. After 30-minutes
184 incubation (Figure 1B), most of the competent cells showed an rDNA signal
185 without DNAase treatment while the non-competent cells were rarely (<1%)
186 associated with rDNA, showing that association is biologically relevant. After

187 DNAase treatment, many of the cells from the 30-minute sample were still
188 associated with rDNA (Figure 1B). The data presented to this point, are consistent
189 with a process in which DNA first binds specifically to the surface of competent
190 cells in a DNAase sensitive fashion and later becomes DNAase resistant by
191 entering a protected compartment. DNAase resistance is largely complete at 30
192 minutes, consistent with previous observations using radiolabeled tDNA (37),
193 suggesting that the uptake of rDNA is not markedly impeded by its covalent
194 adducts.

195 **rDNA enters the periplasm where it becomes resistant to added DNAase.**

196 It is likely that rDNA cannot cross the cell membrane because of its bulky adducts
197 (rhodamine plus a positively charged linker) and therefore accumulates in the
198 periplasm. Although we have found that rDNA with a labeling density similar to
199 that used in this study is reduced in its ability to transform, two considerations
200 make it impossible to interpret this reduced transforming capacity. First, low
201 density random labeling will leave variable stretches of label-free DNA that may
202 be capable of transforming. On the other hand, transformation could be blocked
203 by the adducts at the levels of recombination, gene expression or replication.

204 To investigate the location of DNAase-resistant rDNA further, we generated
205 3D-deconvolved, volume-reconstructed images of transformed CFP- and YFP-
206 labeled competent cells with and without DNAase treatment (Figure 1C and D).
207 The images show representative cells, viewed from 4 directions, related by 90°
208 rotations around the long axis of the cell. The three images in Figure 1C show a
209 typical cell not treated with DNAase and imaged after 2 minutes of incubation with
210 rDNA, a time when very little tDNA is protected from DNAase. The projected views
211 show a blob of rDNA, exhibiting limited contact with the surface of the YFP
212 volume. λ DNA molecules in solution are coils with an average radius of gyration
213 equal to about 500 nm (41). Figure 1C is consistent with binding of such an rDNA
214 coil to the cell surface, involving a limited portion of the rDNA surface. In cells
215 imaged after 30 minutes of incubation, whether DNAase-treated or not, the rDNA
216 is more closely juxtaposed to the surface (Figure 1D). In Figure 1D some of the
217 rhodamine signal is stretched across the surface, and comparison of the rotated

views shows rDNA that appears to wrap around the cell volume, consistent with its location within the confined space of the periplasm and its extension across the outer face of the cell membrane. Figure 1E shows a single optical slice through three cells, imaged after DNAase treatment following 30-minutes incubation with rDNA, confirming that the rDNA signal is localized near the edges of the cells, consistent with a periplasmic location and with the failure of rDNA to cross the membrane. Figure 1C-E thus suggests a process in which rDNA initially associates with the wall surface by localized contact and then enters the periplasmic compartment where it becomes DNAase-resistant, while some of it then extends across the cell membrane surface.

ComFA stabilizes ComGA. Before studying the roles of individual proteins in rDNA uptake, it was important to determine the impact of each deletion mutation on the stabilities of other proteins needed for transformation. Figure S1 shows Western blots in which we have examined $\Delta comFA$, $\Delta comGA$, $\Delta nucA$, $\Delta comC$, $\Delta comEA$ and $comEC518$ loss-of-function mutant extracts using antisera raised against ComGA, ComFA, NucA, ComEA and ComEC. All the mutations are deletions except that $comEC518$ is a Tn917 insertion, which exhibits no transformability and is located near the start of the $comEC$ coding sequence after residue V50. All of the proteins are present at close to normal levels in the mutants, with the important exception of ComGA, which is destabilized in the absence of ComFA (Figure S1B), as reported previously (42) and suggesting that ComGA and ComFA are binding partners. However, ComGA is present at the normal level in an inactivating K152E mutant of $comFA$ (33, 35). K152 lies in the Walker A motif of ComFA, demonstrating that the ATPase activity of ComFA is not needed for ComGA stability. In fact, ComGA interacts with both ComFA and ComFC (43). Thus, any uptake-associated $\Delta comFA$ phenotype may be ascribed to an indirect effect on ComGA

Testing mutants for rDNA binding and uptake. We next examined mixtures of YFP- and CFP-labeled wild-type and mutant strains. To analyze the raw images, we utilized several tools. First, we counted the percent of competent cells for mutant and wild-type strains that were associated with detectable rDNA signals.

249 Second, when required, we recorded deconvolved images and performed volume
250 reconstructions. These separate types of measurement were helpful because not
251 all the competent cells were associated with rDNA, even in the wild-type and
252 because in theory a given mutant might not have 100% penetrance, so that a few
253 cells might exhibit relatively normal binding or uptake while others are totally
254 deficient. It is important to recognize that the images recorded without DNAase
255 treatment reflect rDNA binding plus uptake, whereas after treatment they reflect
256 uptake to the periplasm.

257 **ComGA and ComC.** The $\Delta comGA$ and $\Delta comC$ mutants exhibited no detectable
258 association with rDNA even without DNAase treatment (Figure S2). Because
259 ComGA is needed to assemble tpili and ComC is a peptidase that processes the
260 major and minor pilins for assembly (23), these results support the hypothesis
261 that tDNA binding requires tpili, most likely because the DNA binds directly to
262 these organelles, as it does in *S. pneumoniae* (20) and in *V. cholerae* (6).

263 **ComFA.** In the $\Delta comFA$ strain, there is a significant decrease in the percent of
264 cells that were associated with rDNA after 30-minutes, either with or without
265 DNAase treatment (Table 1). Despite this, many $\Delta comFA$ cells did bind and take
266 up rDNA. In the absence of DNAase treatment the signal intensities of the wild-
267 type and mutant cells were similar (Figure S3, panel A) but with treatment the
268 signals in the mutant were markedly weaker (Figure S3, panel B), in agreement
269 with results obtained using radiolabeled tDNA (32, 34). If the decrease in signal
270 intensity in the $\Delta comFA$ strain is due to a deficiency in ComGA (Figure S1, panel
271 B), we would expect the *comFA* K152E mutant to behave like the wild-type.
272 Indeed, no significant effect of the K152E mutant was observed on the percent of
273 cells showing rDNA fluorescence either with or without DNAase treatment (Table
274 1) and the images in Figure 2A and 2B show that there are no obvious effects of
275 the mutation on either the total or DNAase resistant signal intensities. We
276 conclude that the effect of $\Delta comFA$ on the acquisition of DNAase resistance and on
277 rDNA binding to the cells is largely due to ComGA deficiency. Despite the non-
278 requirement of ComFA for uptake, the rDNA signal in the K152E mutant cells is
279 more localized after DNAase treatment than in the wild-type cells, suggesting an

280 unexpected ATP-dependent requirement for ComFA in the spreading of the rDNA
281 in the periplasm (Figure 2C).

282 **ComEA.** In a $\Delta comEA$ strain the percent of cells with associated rDNA signals
283 was reduced about 7-fold in the absence of DNAase treatment (Figure 3A and
284 Table 1) and the frequency of cells with DNAase resistant rDNA was reduced 57-
285 fold (Figure 3B and Table 1), consistent with results obtained with *B. subtilis* and
286 *S. pneumoniae* using radiolabeled DNA (19, 37, 38). In the $\Delta comEA$ cells that did
287 exhibit binding, the rDNA remained on the cell surface (Figure 3C), like the wild-
288 type image at 2-minutes (Figure 1A). Thus, ComEA assists in binding and is
289 required for uptake. Most likely, the initial binding of tDNA to the tpilus is labile
290 and must be stabilized by contact with periplasmic ComEA, a known DNA-binding
291 protein. Indeed, reversible attachment of DNA to the surface of *B. subtilis*
292 competent cells has been reported (44). The nearly total DNAase susceptibility of
293 rDNA in the mutant demonstrates that ComEA is also needed for uptake to the
294 periplasm.

295 **NucA.** A $\Delta nucA$ strain exhibits decreased rates of transformation, tDNA
296 binding and the acquisition of DNAase resistance (37). To explore this phenotype
297 with rDNA, we used samples incubated with rDNA for 15- rather than 30-minutes
298 to maximize the differences between the mutant and wild-type strains, because
299 the final yield of transformants in the mutant is only reduced about 2-fold (37).
300 The percent of $\Delta nucA$ cells associated with rDNA (Table 1) was reduced about 2-
301 fold and the rDNA signal intensities were clearly reduced after DNAase treatment
302 (Figure S4). To determine if the uptake deficiency of the $\Delta nucA$ strain was due to
303 the nuclease activity of NucA, we inserted a D98A active site mutation at the native
304 *nucA* locus. The design of this mutation was based on analysis of NucB (45), which
305 exhibits 58% identity with NucA. D98A corresponds in position to residue D87A
306 in NucB. This mutant exhibits a transformation deficiency similar to that of the
307 $\Delta nucA$ strain. The data in Table 1 show that the D98A strain has a similar
308 phenotype to the knockout of *nucA*, although the effect on the frequency of cells
309 associated with rDNA is not as great, perhaps due to residual nuclease activity.
310 Figure 4 shows that the intensities of the rDNA signals in this mutant were

311 decreased following DNAase treatment, confirming that the nuclease activity of
312 NucA is needed for uptake to the periplasm and excluding a polar effect of *nucA*
313 inactivation on the downstream *nin* gene (46). Volume reconstructions after
314 DNAase treatment for the Δ *nucA* (Figure S5 A) and D98A (Figure S5 B) mutants
315 show that the rDNA is more localized than in the wild-type. similar to the
316 phenotypes of the *comFA* K152E and *comEC518* mutants (see below). Based on
317 the earlier assumption that DNAase resistance corresponds to entry to the
318 cytoplasm, it was proposed that NucA produced ends for transport through the
319 membrane channel (37). Although this may be true, the present results show that
320 the nuclease activity also plays a role in uptake to the periplasm.

321 **ComEC.** As noted above, the use of radiolabeled rDNA showed that the
322 elimination of ComEC did not affect the total association of tDNA with competent
323 cells but markedly reduced the label after DNAase treatment (4, 37, 38). The use
324 of rDNA should therefore show a previously unexpected role for ComEC in uptake.
325 To test this, we used the *comEC518* mutation, which has a transposon insertion
326 near the N-terminus of the *comEC* coding sequence (47). After 30 minutes, the
327 fraction of competent cells showing DNAase-resistant rDNA was moderately
328 decreased in the *comEC518* mutant (31%) compared with 67% for the wild-type
329 (Table 1), probably a significant difference ($p=0.01$). In contrast, there is little if
330 any effect on the frequency of rDNA attachment before DNAase treatment. This
331 trend is confirmed by the images contained in Figure 5. Panel A shows that in the
332 absence of DNAase treatment, the intensities of the rDNA signals are comparable
333 in wild-type and mutant cells. However, Figure 5B shows that the rDNA signal in
334 those DNAase-treated *comEC518* cells that exhibit uptake is markedly lower than
335 in the wild-type, in agreement with measurements made using radiolabeled tDNA
336 (4, 19, 34, 37). Interestingly, the volume reconstructed images in Figure 5C reveal
337 a marked difference between the DNAase-resistant rDNA signals in wild-type and
338 *comEC* cells. In the wild-type cells, as noted above (Figure 1B), some of the rDNA
339 is extended across the volume surface. But in the *comEC518* cells, the DNAase
340 resistant rDNA is more localized. Thus, although binding to the cell surface is

essentially normal in the *comEC* mutant, uptake is strongly affected and the reduced amount of rDNA that manages to enter the periplasm is confined locally.

rDNA closely associates with YFP-ComEA during uptake. In the Gram-negative systems, periplasmic ComEA is initially distributed uniformly within the periplasm and then dramatically forms foci, accumulating at the sites of DNA uptake (8, 9). To examine the interactions of rDNA with ComEA *in vivo*, we constructed a YFP-ComEA mutant in which the N-terminal YFP moiety was in the cytosol and the DNA binding portion of ComEA was appropriately located in the periplasm. The *yfp-comEA* construct was placed at the *comE* locus, expressed normally under competence control. The resulting strain, with *yfp-comEA* as the only source of *comEA* in the cell, was normally transformable, although the rate of appearance of DNAase resistant transformants was decreased about 2-fold. As reported previously using immunofluorescence with native protein (48) and by Kaufenstein et al (49) with a YFP-ComEA construct similar to ours, ComEA forms large foci at several locations around the membrane. Uniquely among the transformation proteins there is no obvious preference of these foci for polar or sub-polar locations. The foci are not caused by the uptake of DNA from occasional lysed cells, because the addition of DNAase (100 µg/ml) during 90 minutes of growth prior to sampling had no effect on this distribution.

After the addition of rDNA, the distribution of YFP-ComEA foci was not obviously different in the cells with and without bound rDNA. Figure 6A shows deconvolved and volume reconstructed images of four representative cells from a single field that exhibit significant rDNA attachment after 1 minute incubation with rDNA. In these images, the rhodamine signal is almost always localized near the poles as reported previously using a different DNA-labeling protocol (48). The rDNA in Figure 6A, is consistently near a blob of YFP-ComEA in all the cells shown except in panel Aa, which may show a cell with initial attachment to a tpilus that is not located near an accumulation of ComEA. In the remaining cells of panel A, the rDNA and YFP-ComEA appear to be connected by faint regions of YFP signal intensity. After 10 minutes of incubation the images are strikingly different (Figure 6B). At this time, the rDNA is often intimately associated with foci of YFP-

372 ComEA and is sometimes located away from the cell poles (Figure 6B, cells c and
373 d). At this time, in this same experiment, much of the rDNA intensity is resistant
374 to DNAase. These images are consistent with a scenario in which tDNA initially
375 attaches to tpili at the poles, and is then pulled into the periplasm, where it binds
376 to locally mobile molecules of ComEA. The Graumann laboratory has reported
377 that the diffusive behavior of YFP-ComEA in the membrane is similar to that of
378 tDNA in the periplasm, suggesting that these molecules are associated (49),
379 consistent with this conclusion. After the capture event, which results in stabilized
380 binding but no major redistribution of ComEA, the tDNA is plausibly pulled into
381 the periplasm by a Brownian ratchet mechanism involving ComEA and ComEC,
382 where it is stretched across clusters of ComEA molecules.

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384 DISCUSSION

385 The new appreciation of the meaning of DNAase resistance allows us to
386 interpret data acquired in this study and to reinterpret data from the literature. It
387 had been generally assumed that the acquisition of resistance was due to
388 transport of tDNA across the cell membrane, but our present results show that
389 resistance is actually imparted by the cell wall barrier. DNAase, has a calculated
390 molecular weight of 29 kDa and it has been estimated from *in vitro* studies that
391 globular proteins of more than about 25 kDa cannot diffuse through the cell wall
392 of *B. subtilis* (50). The wall preparations used to produce this estimate were
393 treated to remove associated proteins and the intact wall is likely to be even
394 denser and thus more restrictive to diffusion.

395 The conceptual partition of transformation proteins into three exclusive
396 categories based on their requirements for binding, uptake and transport, does
397 not fit with the new data. Thus, ComEA is needed for both tight binding and
398 uptake. ComGA appears to be needed for both initial binding and for uptake
399 because the $\Delta comFA$ mutation, which lowers the amount of ComGA, decreases the
400 frequency of rDNA binding (Table 1) and is deficient in the amount of uptake in
401 individual cells (Figure S3), while the *comFA* K152E mutation does not reduce

uptake. NucA, ComEC and ComFA help to distribute tDNA within the periplasm and the latter two proteins are certainly also needed for transport to the cytoplasm, while NucA helps with binding as well as uptake. This complexity is consistent with the participation of the proteins in a nanomachine, in which they interact to guide tDNA through the wall, periplasm and membrane. Further support for this notion is provided by the observations that ComFA stabilizes ComGA (Fig, S1) and that ComFC binds to ComFA (31). Co-immunoprecipitation experiments in *B. subtilis* have shown that ComGA associates with ComFA, ComFC, DprA, SsbB, RecA and ComEC (43), all proteins that participate in transformation, providing support for the notion of a nanomachine.

The results for $\Delta comGA$ and $\Delta comC$ conform to previous data for *B. subtilis* and *S. pneumoniae* obtained using radiolabeled DNA (24, 44, 51) showing that in the absence of these proteins there is no detectable binding of tDNA to the mutant cells. This reinforces the belief that the tpilus is the initial site of tDNA binding and is consistent with the localization of both ComGA and the initial sites of DNA binding near the poles (48). tpilus retraction has now been observed in both *V. cholerae* and *S. pneumoniae* (6, 22), and is very likely to also occur in *B. subtilis*, although both of these Gram-positive organisms lack retraction ATPases. Based on a number of recent studies it is tempting to conclude that the *B. subtilis* pilus retracts by spontaneous disassembly into the membrane without an external energy source (6, 52-54). However, a dual function ATPase has been described that drives both assembly and retraction of a t4 pilus (55), and this remains a possibility for ComGA. In fact, the major effect of $\Delta comFA$ on uptake compared to binding (Table 1, Figure S3), could be explained by the decreased amount of ComGA (Figure S1),

In the absence of ComEA, tDNA binding is reduced, and uptake is eliminated. There is experimental evidence for an initially transient form of binding (44), suggesting that attachment of tDNA to the tpilus is reversible. ComEA is too small to extend from the membrane through the wall to aid in the initial binding, and attachment of tDNA to the cell is likely to be stabilized by contact between the tDNA and ComEA in the periplasm. During the uptake of tDNA, no dramatic

relocation of the membrane-anchored YFP-ComEA comparable to the reorganization reported for *Vibrio* and *Neisseria* (8, 9, 12) was observed. We propose that inwardly diffusing DNA segments are first captured by fixed or locally mobile ComEA molecules. Further uptake and the spreading of rDNA within the periplasm then results from the inward diffusion of additional segments of DNA, which are trapped by more distant ComEA molecules, in an ongoing Brownian ratchet process. The dependence of uptake on NucA and ComEC and the restricted distribution of DNAase resistant rDNA in the *comEC518*, *nucA* and *comFA* K152E mutants suggests that a more complex mechanism takes place than in the Gram-negatives, where uptake depends only on the tpilus and ComEA. This complexity may be characteristic of bacteria with membrane-anchored, relatively immobile ComEA.

A role for ComEC in uptake to the periplasm was unexpected. The *B. subtilis* ComEC contains two conserved periplasmic domains that are obvious candidates for involvement in uptake (29, 56, 57): the N-terminal N-loop or OB-domain (PFAM13567) and the C-terminal metallo- β -lactamase domain (PFAM00753) (57). Between the two is the competence domain (PFAM03772) that contains several transmembrane helices and must contribute to forming the transport channel. The restricted localization of rDNA in *comEC* and *comFA* mutants suggest that tDNA is transferred to ComEC in a process that depends on ComFA. The absence of ComEC might then arrest uptake by confining the rDNA to locally available ComEA. Transfer to ComEC would also position tDNA for entry to the channel and for degradation of the non-transforming strand. A recent finding in *Helicobacter pylori* (58) is consistent with these ideas. This organism encodes neither tpili nor ComEA but uses a type 4 secretion system to bring tDNA into the periplasm and ComH, a periplasmic DNA binding protein that interacts with tDNA in the periplasm. Suggestively, ComH, which does not resemble ComEA but plays an analogous role, interacts directly with the OB-domain of ComEC (58). It has been proposed that the β -lactamase domain contains the nuclease activity that degrades the non-transforming strand of tDNA (57). If degradation does take

place in the periplasm, ComEC may provide an entropic boost to uptake by converting tDNA to single strands and free nucleotides.

The role of NucA in uptake is also a novel finding. NucA is anchored in the membrane with its catalytic site protruding into the periplasm. Its nuclease activity assists in uptake (Table 1, Figure 4) implying that either a nick on one strand of tDNA or a double strand cut facilitate uptake. Indeed, a double strand cut is likely, as suggested by studies of the NucA paralog, NucB (57). Transformation with circular DNA has shown NucA-dependent conversion to the full length linear form shortly after binding (37). This result reinforces the idea that tDNA crosses the wall as a folded dsDNA molecule. Folding DNA, which has a persistence length of about 50 nm (~147 bp), requires local deformation of the DNA structure, presumably caused by binding to the tpilus. After release from the tpilus, strain in the folded tDNA would impel the tDNA to straighten locally and pull back through the wall. A double strand break induced by NucA would relieve the strain and accelerate the ratchet. An additional explanation for the role of NucA, which is not mutually exclusive with this one, is suggested by the finding that the ComEA protein of *V. cholerae* prefers binding to a DNA end (8). If this is also true of the *B. subtilis* protein, NucA might not only relieve the strain in folded tDNA but would also facilitate the initial binding of ComEA to the incoming DNA. These ideas are consistent with our finding that both binding and uptake are reduced in the *nucA* mutants (Table 1, Figure 4). The termini produced by NucA would also be available to enter the ComEC channel (37).

The results presented in this study allow us to propose an updated model for transformation in *B. subtilis*. First, tDNA binds reversibly to the tpilus, which retracts, bringing a folded loop of DNA into the periplasm. The *S. pneumoniae* tpilus is 6 nm thick and retraction would likely leave a hole in the wall of the same size. A folded DNA molecule would have thickness of 4 nm, permitting the tpilus to bring the DNA into the periplasm with its hydration shell intact, and allowing subsequent inward diffusion of the DNA. NucA then cuts the tDNA as it is released from the tpilus, relieving strain introduced by tDNA folding and possibly facilitating binding to ComEA at a terminus. This binding stabilizes the association

of tDNA with the cell and as successive segments of DNA diffuse into the periplasm through the cell wall, they are captured by additional ComEA molecules and prevented from diffusing outward. As the ComEA binding sites near the locus of entry become occupied, tDNA spreads in a process dependent on ComFA and ComEC and is captured by ComEC for transport to the cytoplasm, accomplished by ComEC and the ComFA/ComFC complex, which comprise the transformation permease.

The use of labeled DNA that cannot cross the membrane may obscure what is actually a seamless process of uptake and transport. In other words, transformation may not be a two-step process in which tDNA accumulates in the periplasm. Another open question concerns the extent to which our revised understanding of DNAase resistance applies to other Gram-positive transformable bacteria, such as *S. pneumoniae*. Despite the shared requirements for ComEA, ComEC and t4 pilus proteins, there are likely to be important mechanistic differences between the uptake mechanisms of these two model Gram-positive bacteria. In *S. pneumoniae*, degradation of the non-transforming strand is accomplished by EndA (59, 60) and degradation takes place in the absence of ComEC (38), implying that the latter is not needed for uptake to the periplasm. In contrast, *B. subtilis* does not encode EndA and when *comEC* is inactivated, degradation ceases (37), consistent with the role of ComEC in uptake and perhaps with its proposed action as a nuclease. Finally, in *B. subtilis* the tpilus is probably short while in *S. pneumoniae* it extends from the cell surface (20, 23). Many outstanding questions remain, most importantly, how these various processes are accomplished on the molecular level.

Materials and Methods

Strains and growth conditions. All mutant strains (Table S1) of *B. subtilis* were constructed in IS75 (his leu met) and are derivatives of the domesticated strain 168. All the strains expressed YFP or CFP controlled by the *comG* promoter, inserted ectopically in *amyE*. *B. subtilis* was grown to competence for

all experiments by the two-step method (61) except that unfrozen cultures were used and cultures were started from plates grown overnight instead of from spore suspensions.

Transformation for microscopy. Competent cultures of P_G-CFP and P_G-YFP expressing mutant and wild-type strains were mixed 1:1 and the mixture was incubated with 0.2 µg/ml of rhodamine-labeled DNA for the indicated times, in final volumes of 300-500 µl. Samples were taken and either immediately fixed by the addition of 3.2% paraformaldehyde and incubation at room temperature for 30 minutes or were first incubated for 3 minutes with 100 µg/ml DNAase I at 37°C before fixation. In the experiment shown in Figure 1, where DNAase-treated and untreated samples were combined for visualization, the cells were centrifuged briefly and resuspended in the same medium used to achieve competence, but containing no Mg and EDTA (10 mM) to stop the nuclease activity before mixing and fixation. Samples were then washed and imaged.

Preparation of rDNA. Bacteriophage λ DNA (New England Biolabs) was diluted to 10 µg/ml and incubated for 60 minutes with a MIRUS Label-IT rhodamine TM reagent (Mirus Bio) according to the manufacturer's recommendations. The DNA was then processed through a spin column to remove excess reagent.

Point mutation of *nucA*. A fragment of DNA carrying the *nucA* sequence with a D98A mutation and *Bam*H1 ends, was synthesized by Biomatik (Canada) and cloned into the *Bam*H1 site of pMiniMAD2, a gift from Dan Kearns (Indiana University). After transformation with selection for erythromycin (5 µg/ml), the markerless chromosomal mutant was isolated as described by Patrick and Kearns (62).

YFP-ComEA construction. Three fragments were produced by PCR and assembled using NEBuilder HiFi into pUC18CM, a derivative of pUC18 with a chloramphenicol resistance cassette for selection in *B. subtilis*. Fragment #1 carried 1500 base pairs upstream from the *comEA* coding sequence including the promoter, the ribosomal binding site and start codon. To produce this fragment primers 1 and 2 were used (Table S2). Fragment #2 carried the *yfp* open reading

555 frame with the stop codon omitted, produced using primers 3 and 4. Fragment
556 #3 consisted of the *comEA* open reading frame without its start codon, produced
557 using primers 5 and 6. After assembly, the clone was verified by sequencing and
558 transformed into the native locus via a single crossover event. The duplicated
559 native *comEA* open reading frame was then deleted by transformation using
560 DNA from strain BD8739 (*comEA::ery trpC2*) and the final construction was
561 again verified by sequencing.

562 **Microscopy.** For microscopy 1 µl samples of transformed cells were placed on
563 thin agarose pads before visualization. All images were acquired with a 100x Plan
564 Appo immersion objective, NA=1.40 on a Nikon Ti microscope, equipped with LED
565 excitation sources and an Orca Flash 4.0 camera (Hamamatsu). Nikon Elements
566 was used for data acquisition and image analysis. Z-stack images were recorded
567 with 200 nm steps and processed for 3D deconvolution with the Landweber
568 algorithm in Nikon Elements. Volume reconstructions were assembled with alpha
569 blending. Images were exported to Adobe Photoshop and Microsoft PowerPoint
570 for final processing.

571 **Western blotting.** Western blotting was carried out using standard methods
572 with semi-dry blotting and the nitrocellulose blots were developed using ECL (GE
573 Healthcare). The images were recorded with a BioRad ChemiDoc MP imager. In
574 most gels, for a loading control, the top of the membrane was cut off after blotting
575 and developed separately with anti-EFG antiserum, a kind gift from Jonathan
576 Dworkin (Columbia University Medical school).

577 **Statistical analysis.** Proportions of mutant and wild-type cells that were
578 associated with rDNA were compared using a two-tailed z-test.

579 **Data availability statement.** The data that support the findings of this study
580 are available from the corresponding author, [author initials], upon reasonable
581 request.

582

583 SUPPLEMENTAL MATERIAL

584 Table S1 Strains

585 Table S2 Primers

586

587 Figure S1. Western blots for transformation proteins in mutant strains.

588 Figure S2. DNA binding in $\Delta comGA$ and $\Delta comC$ strains.

589 Figure S3. Binding and uptake in the $\Delta comFA$ mutant.

590 Figure S4. Binding and uptake in the $\Delta nucA$ mutant.

591 Figure S5. Volume reconstructions of the $\Delta nucA$ and $nucA$ D98A mutant strains.

592

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 600

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Table 1

Binding and uptake of rDNA by transformation mutants^a

Mutant	No DNAase treatment				With DNAase treatment			
	With signal	N	Percent With signal	p-value ^b	With signal	N	Percent With signal	p-value ^b
<i>ΔcomEA</i>	12	99	12.1		2	133	1.5	
Wild-type	82	99	82.8	<0.00001	92	108	85.2	<0.00001
<i>ΔnucA</i>	39	137	28.5		13	126	10.3	
Wild-type	88	138	63.8	<0.00001	70	123	56.9	<0.00001
<i>nucA D98A</i>	45	114	39.5		21	126	16.7	
Wild-type	70	124	56.5	<0.009	47	162	29.0	0.015
<i>ΔcomFA</i>	45	110	40.9		32	104	30.8	
Wild-type	86	110	78.2	<0.00001	80	103	77.7	<0.00001
<i>comFA K152</i>	82	136	60.3		91	163	55.8	
Wild-type	65	118	55.1	0.40	68	103	66.0	0.10
<i>comEC518</i>	66	107	61.7		47	150	31.3	
Wild-type	112	147	76.2	0.01	91	136	66.9	<0.00001

796

797 ^aData for each mutant was obtained in a separate experiment in mixed
798 suspension with a wild-type control.

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800 ^bDetermined with a two-tailed z test.

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808 **FIGURE LEGENDS**

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810 Figure 1. Binding of tDNA and acquisition of DNAase resistance. Competent cells
811 expressing CFP (BD5810) or YFP (BD6011) from the *comG* promoter were
812 incubated separately with rDNA and samples were taken after (A) 2- and (B) 30-
813 minutes of incubation. The CFP samples were treated with DNAase and then
814 combined with YFP samples from the same time points. In panels A and B, images
815 are presented with superimposed rhodamine, CFP, YFP and phase contrast
816 channels as well as one showing only the rhodamine channel for clarity. Cells
817 associated with rDNA are indicated by arrows. The circled cell in panel A was
818 imaged by 3D deconvolution and volume reconstructed and views produced by
819 successive 90° rotations are shown in panel C. In panel D volume reconstructed
820 images are shown for the single cyan (with DNAase) and yellow (no DNAase) cells,
821 circled in panel B. Panel E contains a 3D-deconvolved optical slice from the center
822 of a Z-stack, showing of a group of cells after DNAase treatment.

823

824 Figure 2. Binding and uptake in the *comFA* K152A mutant. Wild-type (YFP) and
825 mutant (CFP) cells were combined before 30 minutes incubation with rDNA.
826 Panels A and B show results for *comFA* K152E without and with DNAase
827 treatment respectively. In each panel the top and bottom rows show 9 each of
828 wild-type and mutant cells respectively. All the cells were taken from a single
829 representative microscope field and show all or nearly all of the rDNA-associated
830 cells in that field. In the cropped images of a given panel, rDNA signals were
831 enhanced identically but rDNA signal intensities cannot be compared between
832 panels A and B. Panel C shows volume reconstructed images of several
833 representative wild type (top row) and mutant (bottom row) cells. In each case a
834 single view is shown except for the adjacent images joined by brackets, which
835 show two views of the same cell related by 180° rotations.

836

837 Figure 3. Binding and uptake in the $\Delta comEA$ mutant. Wild-type (YFP) and $\Delta comEA$
838 cells were combined before incubation for 30-minutes with rDNA. Panel A shows

the cells without DNAase treatment and B shows cells with treatment. In the top images the rhodamine, CFP and YFP channels were merged and the bottom images in A and B show only the rhodamine channel. The arrows indicate the position of all the rhodamine signals. Panel C shows one aspect from a 3D deconvolution of the CFP- and YFP-expressing cells circled in Panel A.

844

Figure 4. Binding and uptake in the *nucA* D98A mutant. Wild-type (YFP) and D98A (CFP) mutant cells were made competent, combined, and after 15 minutes incubation with rDNA were imaged without (panel A) and with DNAase (panel B). In each of these panels the top and bottom rows show nearly all the wild-type and mutant cells from a single field, respectively. The rhodamine signal was enhanced before cropping the cells and the intensities of the rhodamine signal can be directly compared. The YFP and CFP images were separately adjusted in each image so as not to obscure the rDNA signal. The images in these two panels were ordered by apparent rDNA signal strengths decreasing from left to right, to facilitate comparisons of the mutant and wild-type cells. The boxed image in panel B shows both a YFP- and a CFP-expressing cell.

856

Figure 5. Binding and uptake in the *comEC518* mutant. Wild-type (YFP) and $\Delta comEC518$ (CFP) cells were combined before 30-minutes incubation with rDNA. The images were processed as described in the legend to Figure 4. Panel A shows the cells without DNAase treatment and B shows cells with treatment. The single image to the right of panel B shows two YFP cells and one CFP cell. The images in these two panels were ordered by apparent rDNA signal strengths decreasing from left to right, to facilitate comparisons of the mutant and wild-type cells. Panel C shows selected volume reconstructed images of wild-type and *comEC518* cells selected from the same field, representing almost all the cells in the field. Only one view is shown of each cell. Image intensities cannot be compared either within panel C or with the images in panels A and B.

868

869 Figure 6. Association of rDNA with YFP-ComEA without DNAase treatment. In
 870 each group of four images (a-d in panel A and a-h in panel B), the top and bottom
 871 pairs show views of a single cell, related by 180° rotations. The left-hand images
 872 in each group show the superimposed YFP, rhodamine and CFP channels, where
 873 CFP delineates the cytoplasmic volume. The right-hand images show only the YFP
 874 and rhodamine channels, for clarity. Panel A shows images collected after 1
 875 minute incubation of the cells with rDNA and Panel B after 10 minutes.
 876

A

B

C

D

E

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B

A

B

C

A

B

Table S1

Strains

Strain ^a	Genotype	Source
BD5810	<i>amyE::P_G-CFP (spc)</i>	(48)
BD6011	<i>amyE::P_G-YFP (spc)</i>	(48)
BD8739	<i>trpC2 ΔcomEA (ery)</i> ^b	
BD8800	<i>amyE::Pr_G-CFP (spc) ΔcomGA(ery)</i> ^b	This work
BD8802	<i>amyE::Pr_G-CFP (spc) ΔcomEA(ery)</i> ^b	This work
BD8805	<i>amyE::P_G-CFP (spc) ΔnucA(ery)</i> ^b	This work
BD8883	<i>amyE::P_G-CFP (spc) ΔcomC (ery)</i> ^b	This work
BD8884	<i>amyE::P_G-CFP (spc) comFA K152E</i>	(33)
BD8911	<i>amyE::P_G-CFP (spc) ΔcomFA (ery)</i> ^b	This work
BD8949	<i>amyE::P_G-CFP (spc) yfp-comEA ΔcomEA (ery)</i> ^b	This work
BD8951	<i>amyE::P_G-CFP (spc) nucAD98A</i>	This work
BD8954	<i>amyE::P_G-CFP (spc) comEC-518</i>	(29)

^aAll of the listed strains except BD8739 were derivatives of IS75 *his leu met*.

^bThese deletion mutations, constructed by Koo et al (63), were obtained from the Bacillus Genetic Stock Center

Table S2

Primers

Primer number	Sequence (5'->3')
1	AGAGGATCCCCGGGTACCGAGCTCGAATTCAAAGGTATCGAGTAGCCAG
2	CTCCTTTACTCATATTCATCGTGCATGTTCC
3	ATGCACGATGAATATGAGTAAAGGAGAAGAAC
4	TCAACCAATTCATTTTGTATAGTTCATCCATGC
5	GGAAACAGCTATGACCATGATTACGAATTCACCTTTACTGTAATGGAAG
6	ACTATACAAATAAATGAATTGGTTGAATCAGC

Figure S1. Western blots for transformation proteins in mutant strains. All of the mutants were deletions except for *comEC518* which is a transposon insertion near the beginning of the coding sequence. In all cases the relevant knockout mutant was used as a negative control for the antiserum. There is a consistent cross-reacting band at the position of the ComEC signal. For most of the gels a loading control is shown using anti-elongation factor G (EFG) antiserum, a kind gift from J. Dworkin. In panel D, the cross-reacting band just below the ComEA signal serves as a loading control. In panel E, a cross-reacting band is visible in the *comEC518* lanes at the position of ComEC (29).

Figure S2. DNA binding in $\Delta comGA$ and $\Delta comC$ strains. $\Delta comGA$ (A) and $\Delta comC$ (B) strains, expressing CFP, were combined with transformed wild-type bacteria expressing YFP. Transformation was for 30 minutes without DNAase treatment. The arrows indicate all the cells with detectable rDNA signals. The CFP labeled $\Delta comGA$ cells are slightly filamented (Hahn J, Tanner AW, Carabetta VJ, Cristea IM, Dubnau D. 2015. Mol. Microbiol 97:454-71).

Figure S3. Binding and uptake in the $\Delta comFA$ mutant. Wild-type (YFP) and mutant (CFP) cells were combined before 30-minutes incubation with rDNA. Panels A and B show results for $\Delta comFA$ without and with DNAase treatment respectively. In each panel the top and bottom rows show 9 each of wild-type and mutant cells respectively. All the cells were taken from a single representative microscope field and show all or nearly all of the rDNA-associated cells in that field. In the cropped images of a given panel, rDNA signals were enhanced identically but rDNA signal intensities cannot be compared between panels A and B.

Figure S4. Binding and uptake in the $\Delta nucA$ mutant. Wild-type (YFP) and mutant (CFP) cells were combined before 15-minutes incubation with rDNA and imaged without (panel A) and with DNAase (panel B). In each of these panels the top and bottom rows show nearly all the wild-type and mutant cells from a single field, respectively. The

45 rhodamine signal was enhanced before cropping the cells and the intensities of the
 46 rhodamine signal can be directly compared. The YFP and CFP images were separately
 47 adjusted in each image so as not to obscure the rDNA signal. The images in these two
 48 panels were ordered by apparent rDNA signal strengths decreasing from left to right,
 49 to facilitate comparisons of the mutant and wild-type cells. The boxed images both a
 50 YFP- and a CFP-expressing cell.

51

52 Figure S5. Volume reconstructions of the $\Delta nucA$ (panel A) and *nucA* D98A (panel B)
 53 mutant strains. Wild-type (YFP-expressing) and mutant (CFP-expressing) cells are
 54 shown in each panel for comparison.

55

56

57

58

A

B

A

B

A

B