Impaired competence in flagellar mutants of *Bacillus subtilis* is connected to the regulatory network governed by DegU

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Summary

The competent state is a developmentally distinct phase, in which bacteria are able to take up and integrate exogenous DNA into their genome. *Bacillus subtilis* is one of the naturally competent bacterial species and the domesticated laboratory strain 168 is easily transformable. In this study, we report a reduced transformation frequency of *B. subtilis* mutants lacking functional and structural flagellar components. This includes *hag*, the gene encoding the flagellin protein forming the filament of the flagellum. We confirm that the observed decrease of the transformation frequency is due to reduced expression of competence genes, particularly of the main competence regulator *comK*. The impaired competence is due to an increase in the phosphorylated form of the response regulator DegU, which is involved in regulation of both flagellar motility and competence. Altogether, our study identified a close link between motility and natural competence in *B. subtilis* suggesting that hindrance in motility has great impact on differentiation of this bacterium not restricted only to the transition towards sessile growth stage.

Originality-Significance statement

Understanding how versatile bacterial phenotypes influence each other is important for our basic understanding of microbial ecology. Our research highlights the novel intertwinement of bacterial differentiation and reveal how lack of single cell motility adjusts DNA exchange among bacterial strains.

42 **Introduction**

43 When facing stressful environmental conditions, bacteria can respond with a variety of post-
44 exponential modifications including secretion of degradative enzymes, sporulation, or genetic
45 competence. *Bacillus subtilis* is one of the bacterial species that are able to take up free DNA from
46 the environment and incorporate it into its own genome, a phenomenon referred to as natural
47 competence (Dubnau, 1991). To import extracellular DNA into *B. subtilis* cells, a pseudopilus formed
48 by proteins encoded by the *comG* operon facilitates binding to the receptor protein ComEA, which is
49 located in the bacterial cell membrane (Inamine and Dubnau, 1995; Chen *et al.*, 2005). As only single
50 stranded DNA is imported, the membrane-associated nuclease NucA catalyzes cleavage of the DNA
51 after successful binding (Provvedi *et al.*, 2001). Subsequent transport of the DNA through a
52 membrane channel formed by the protein ComEC is mediated by the ATPase ComFA that probably
53 requires the transmembrane proton motive force (Maier *et al.*, 2004).

54 To take up DNA, cells have to be in a developmental state, in which a specific set of genes and
55 regulators are expressed (Dubnau, 1991; Berka *et al.*, 2002). Regulation of the whole apparatus
56 required for competence development is complex. Briefly, entry into the competence state occurs in
57 a bistable manner during the early stationary phase, where a minority of cells produces high level of
58 the competence master regulator ComK above a certain threshold that is required to switch on
59 competence development, the so called 'K-state' (van Sinderen *et al.*, 1995; Maamar and Dubnau,
60 2005; Smits *et al.*, 2005; Dubnau and Losick, 2006). It was demonstrated that noise in the expression
61 of *comK* determines the competent subpopulation and allows a dynamic stress response regarding
62 competence development (Maamar *et al.*, 2007; Mugler *et al.*, 2016). Eventually, ComK activates the
63 expression of late competence operons encoding the DNA-binding and -uptake machinery as well as
64 genes, whose products are responsible for DNA integration (Berka *et al.*, 2002; Ogura *et al.*, 2002;
65 Hamoen *et al.*, 2003). Increase of the ComK level is linked to a quorum sensing-mediated
66 accumulation of the small ComS protein, which interferes with ComK degradation during the
67 exponential phase (Turgay *et al.*, 1998). ComK is able to bind the *comK* promoter, triggering its own
68 transcription, thus creating an auto-stimulatory loop (van Sinderen and Venema, 1994). This binding
69 is further stabilized by the non-phosphorylated form of the regulator DegU to increase the level of
70 ComK above the threshold sufficient for competence development (Hamoen *et al.*, 2000).

71 However, DegU is not only crucial for competence initiation, but also involved in the regulation of
72 many other processes including protease production, biofilm development, and, particularly,
73 flagellar motility (Murray *et al.*, 2009; Mukherjee and Kearns, 2014). The main components of the
74 hook and basal body of the flagellum are encoded by the large *fla/che* operon. Transcription of this
75 operon is activated by a complex formed by the regulator SwrA and phosphorylated DegU (DegU~P),

76 which binds to one of the *fla/che* promoters (Mordini *et al.*, 2013; Mukherjee and Kearns, 2014).
77 Amongst other genes, the operon contains the gene encoding the sigma factor σ^D that activates
78 transcription of motility genes outside the *fla/che* operon like the *hag* gene (encoding flagellin),
79 *motA* and *motB* (encoding flagellar stator proteins), as well as transcription of *lytF*, which is
80 necessary for separation of motile cells after cell division (Serizawa *et al.*, 2004; Chen *et al.*, 2009).
81 The level of σ^D and its position in the *fla*/*che* operon determines the cell fate, i.e. subpopulations of
82 motile single cells or non-motile chains (Cozy and Kearns, 2010). The function of DegU~P changes in
83 the absence of SwrA. In this case, DegU~P seems to inhibit motility via the same promoter of the
84 *fla*/*che* operon (Amati *et al.*, 2004). Additionally, DegU~P can activate the anti-sigma factor FlgM by
85 binding to its promoter region in the absence of SwrA (Hsueh *et al.*, 2011) allowing FlgM to
86 antagonize σ^D (Caramori *et al.*, 1996). Consequently, DegU~P indirectly suppresses transcription of
87 σ^D-dependent genes (Hsueh *et al.*, 2011). It was suggested that a completion of flagellum assembly
88 can be sensed by the DegSU two component system: FlgM, which is activated by DegU~P, causes
89 inhibition of σ^D-dependent genes, when the assembly of the flagellum is impeded (Cozy and Kearns,
90 2010; Hsueh *et al.*, 2011).
91 In addition to its role on modulating the expression of flagellum-related genes in *B. subtilis*, the
92 phosphorylation and therefore the activity of DegU, has been shown to be influenced by a
93 mechanical signal transmitted by the flagellum (Cairns *et al.*, 2013). Inhibition of flagellar rotation by
94 the flagellar clutch or by tethering the flagella results in an increased DegU~P level in the cell.
95 In this study, we report a correlation between motility function and competence development,
96 which in *B. subtilis* is connected by the multifunctional response regulator DegU. We show that
97 mutants lacking a functional flagellum such as Δ*hag*, Δ*motA*, and Δ*flgE* exhibited a reduced
98 transformation frequency. This was due to a decrease in competence gene expression, particularly
99 reduced levels of the competence master regulator ComK, which can be reverted by overexpressing
100 *comK* in the *hag* mutant. Finally, we suggest that the reduced transformation frequency was likely
101 due to an imbalance in the phosphorylation level of DegU.

102 **Results**

103 *Lack of active flagella impairs competence for DNA uptake in* B. subtilis.

104 While genetically modifying various *B. subtilis* strains, a striking difference in transformation
105 frequency was observed between the wild type and a non-motile mutant lacking the gene encoding
106 flagellin, *hag*. To explore this phenomenon, we tested the transformability of wild type (strain 168)
107 and *hag*-mutant in competence medium (see Experimental Procedures), where the *hag*-mutant
108 showed a more than 100-fold reduced transformation frequency relative to the wild type (Fig. 1A,
109 B): while the transformation frequency of the wild type ranged between $3{\cdot}10^{-5}$ and $5{\cdot}10^{-5}$, that of the
110 hag-mutant was reduced to values below $3{\cdot}10^{-7}$. Similarly, the undomesticated *B. subtilis* strains
111 DK1042 (transformable derivative of NCIB 3610) and PS216 showed reduced transformation
112 efficiency when the *hag* gene of these strains was disrupted (Fig. S1). To investigate whether this
113 difference in transformation frequency between the two strains resulted from a lower growth rate
114 of the *hag*-mutant, the growth behavior of wild type and *hag*-mutant grown in competence medium
115 was evaluated over time. As depicted in Fig. 1B, the *hag*-mutant showed a clear growth advantage
116 and reached a higher OD compared to the wild type (unpaired two-sample t-test with Welch
117 Correction: P = 0.001, n = 5), thus supporting our previous observations (Hölscher *et al.*, 2015).
118 Further, it was tested whether the addition of DNA at different time points would increase the
119 transformation frequency of the *hag*-mutant. However, the mutant showed a consistently low
120 transformation frequency over the course of several hours, indicating that a shifted timing of the
121 initiation of the competence state is unlikely to be the reason for the observed decrease in
122 transformation frequency (Fig. 1C). To test whether this phenomenon is restricted to the *hag*-
123 mutant or connected to the lack of an active motility apparatus in general, mutants lacking other
124 functional flagellum-related genes were investigated. The transformation frequencies of mutants
125 lacking the gene encoding one of the flagellar motor units, *motA*, and the gene encoding the hook
126 protein, *flgE*, were decreased in both cases compared to the wild type (Fig. 2; unpaired two-sample
127 t-test with Welch Correction: P = 0.01 for WT - Δ*motA*, P = 0.039 for WT - Δ*flgE*, n = 9 for both).
128 Although the wild type transformation frequency was slightly different, the transformation
129 frequencies of both Δ*motA* and Δ*flgE* were around 10-times lower than that of the wild type (Fig. 2).
130 In contrast, a *cheA*-mutant lacking the main chemotaxis sensor kinase showed a similar
131 transformation frequency than the wild type (Fig. S2; unpaired two-sample t-test with Welch
132 Correction: P = 0.232, n = 3), suggesting that the presence of an active flagellum, but not directed
133 motility *per se* is required for full competence development. In sum, these results demonstrate that
134 the observed impaired competence is linked to a loss of flagellar function.

135

136 *Lack of competence in flagellar mutants is due to the reduced expression of competence genes.*
137 To determine if the detected diminished transformation frequency of flagellar mutants was due to
138 altered competence gene expression, the fluorescent reporter P_{comG}-*gfp* was introduced into these
139 strains. This reporter allows the detection of cells expressing the *comG* operon-encoding genes
140 required for pseudopilus formation and DNA uptake. In addition, this reporter provides a proxy on
141 the activity of the ComK protein, the master regulator of competence. Qualitative microscopy
142 analyses of cultures harboring the reporter, and which were grown in competence medium for 5 h,
143 showed indeed a decreased number of fluorescent (i.e. *comG* expressing) cells in the *hag* mutant
144 compared to the wild type, whereas a control strain lacking *comK* showed no fluorescence (Fig. 3A).
145 For quantitative determination of competence gene expression within the population, flow
146 cytometric measurements were performed that revealed 24.7% of fluorescent cells in wild type
147 cultures (mean value), but only 4.6% of fluorescent cells for the *hag*-mutant (Fig. 3B, C; unpaired
148 two-sample t-test with Welch Correction: P = 0.004, n = 3), thus confirming the microscopy results.
149 Similarly, the *motA* and *flgE* mutants were analyzed microscopically as well as by using flow
150 cytometry. Both methods revealed fewer cells activated transcription of competence genes in these
151 mutants compared to the wild type (Fig. 4; unpaired two-sample t-test with Welch Correction: P =
152 0.017 for WT - Δ*motA*, P = $1.3 \cdot 10^{-9}$ for WT - Δ*flgE*, n = 3 for both; mean percentage of fluorescent
153 cells: 16.7% for wild type, 4.5% for Δ*motA*, 4.2% for Δ*flgE*). Flow cytometry measurements at
154 different time points during growth in competence medium confirmed a similarly reduced fraction of
155 competent cells in the *hag* mutant compared to the wild type strain (Fig. S3).
156
157 *Reduced competence in* hag *mutant can be rescued by overexpression of* comK.
158 The reduced competence gene expression in the tested flagellar mutants suggested a regulatory link
159 between flagellar motility and competence. To investigate if regulatory elements upstream of *comK*
160 were responsible for our observations and if a bypass of those could therefore rescue
161 transformation frequency in the flagellar mutant, we examined a strain with an additional copy of
162 *comK* under the control of a xylose-inducible promoter (P_{xyl}-*comK*). Indeed, in combination with P_{xyl}-
163 *comK*, the transformation level of the *hag* mutant increased back to a level that was statistically
164 indistinguishable from wild type levels (mean transformation frequency of $5.3 \cdot 10^{-6}$ for the wild type
165 and $8.5 \cdot 10^{-6}$ for Δ*hag* P_{xyl}-*comK*; Kruskal-Wallis test: P = 0.453, n = 9, Fig. 5A). Despite this observed
166 increase in the *hag* strain upon *comK* overexpression, the wild type strain, which contained an
167 inducible copy of *comK* showed a higher transformation frequency (Fig. 5A, Kruskal-Wallis test: P =
168 $3.4 \cdot 10^{-4}$ for WT – WT P_{xyl}-*comK*, P = $3.4 \cdot 10^{-4}$ for WT P_{xyl}-*comK* - Δ*hag* P_{xyl}-*comK*, n = 9 for both), which

169 was probably due to higher levels of *comK* transcription at the native locus as previously observed
170 (Hahn *et al*., 1996).
171
172 *Reduced competence in flagellar mutants is likely connected to unbalanced DegU phosphorylation.*
173 As the above results suggested that regulatory elements in response to impaired flagellar motility
174 are responsible for the decreased *comK* expression, we investigated DegU as a likely candidate
175 causing the reduced competence in flagellar mutants. As non-phosphorylated DegU was implicated
176 to be required for *comK* transcription (Dahl *et al.*, 1992; Hamoen *et al.*, 2000), two variants of *degU*
177 were tested: *degU*32, which harbors a mutation resulting in an extended half-life and thus higher
178 stability of the phosphorylated form of the DegU protein (DegU~P), and *degU*146, which is cannot be
179 phosphorylated (Dahl *et al.*, 1991; Dahl *et al.*, 1992; Kunst *et al.*, 1994). Both variants were tested in
180 wild type as well as the Δ*hag* background to observe differences in transformability compared to the
181 wild type strain. The results of this experiment indicated that the transformation frequency of the
182 *degU*32 strain was slightly decreased (Figure 5B), which is consistent with previous publications,
183 suggesting that non-phosphorylated DegU is required for priming *comK* transcription. The observed
184 difference, however, was only marginally significant in our experimental setup (Figure 5B; Kruskal-
185 Wallis test: P = 0.078, n = 6). Surprisingly, when combined with the Δ*hag* mutation, the
186 transformability of *degU*32 increased significantly far above wild type levels, despite presumably
187 possessing low levels of non-phosphorylated DegU to induce the ComK auto-stimulatory loop (Fig.
188 5B; Kruskal-Wallis test: P = 0.004, n = 6). Furthermore, we observed a tendency towards a reduced
189 albeit non-significant transformation frequency in the *degU*146 strain compared to the wild type
190 (Fig. 5B; Kruskal-Wallis test: P>0.05, n = 6). This result was similar to the one observed for *degU*32,
191 although no negative impact on transformability was expected in strain *degU*146 due to the
192 abolished phosphorylation of DegU. Interestingly, the *degU*146 strain combined with the Δ*hag*
193 mutation exhibited transformation frequencies at the same level than the wild type strain (Fig. 5B;
194 Kruskal-Wallis test: P>0.05, n = 6) that was significantly higher than the transformation frequency of
195 the single *degU*146 mutant (Fig. 5B; Kruskal-Wallis test, P = 0.007, n = 6). These results suggest that
196 altering the phosphorylation state of DegU in flagellar mutants can revert the negative impact on
197 competence, which was caused by a lack of motility.
198
199 *Increased viscosity enhances competence in* B. subtilis
200 A recent study showed that restricting the flagellar rotation by viscous medium results in induction
201 of flagellar gene transcription and activation of the DegSU two-component system in *Paenibacillus*
202 sp. NAIST15-1 (Kobayashi *et al.*, 2017). Accordingly, we tested whether an increased viscosity of the

203 medium changes the transformability in *B. subtilis*. Indeed, the average transformation frequency of
204 the wild type strain was three-fold higher in a medium of increased viscosity. The corresponding
205 statistical test, however, indicated only a trend towards a statistically significant difference (Fig. 6;
206 unpaired two-sample t-test: $P = 0.095$, $n = 4$).
207

208 **Discussion**

209 Many cellular processes in *B. subtilis* are tightly connected through their underlying regulatory
210 networks. Examples include motility and biofilm formation or biofilm formation and sporulation
211 (e.g. Vlamakis *et al.*, 2013; Marlow *et al.*, 2014; Hölscher *et al.*, 2015). Here, we report an additional
212 connection between flagellar motility and competence development. We could show that mutants
213 with impaired flagellar function have defects in competence development. Such mutants displayed a
214 considerably lower transformation frequency and expression of late competence genes, suggesting
215 that it is due to an altered expression of the competence master regulator gene *comK*. The rescue
216 experiment with an inducible *comK* confirmed that indeed competence could be rescued in the Δ*hag*
217 strain, since Δ*hag* P_{xyl}-*comK* exhibited a wild type transformation level.

218 In a recently published study, similar effects were observed even though different methods have
219 been used: Diethmaier and colleagues found that the expression of *comK* is lower in deletion
220 mutants of the *fla/che* operon, *hag*, and the second stator gene *motB* (Diethmaier *et al.*, 2017).
221 While in their study the expression of *comK* was primarily monitored using a *comK* promoter fusion,
222 our experiments predominantly assayed transformation frequency. Both studies, however, report a
223 negative effect of the deletion of flagellar components on competence development. The extent to
224 which wild type and mutant differ in competence development is in the same order of magnitude
225 between the studies: for example Diethmaier *et al*. observe a 10-fold reduced number of *comG*
226 expressing-cells in Δ*hag* (Diethmaier *et al.*, 2017), whereas our flow cytometry experiments showed
227 a slightly lower, 5-fold reduction. Additionally, by investigating the transformation frequency in a
228 *cheA* mutant, we could also show that the chemotactic response does not seem to have an influence
229 on competence development.

230 Investigating modified variants of the response regulator DegU, we found that the transformation
231 frequency of the *hag*-mutant could be restored to wild type level when the mutant carried a non-
232 phosphorylatable DegU variant (*degU*146). This result suggests that a high level of DegU~P in the
233 flagellar mutants was the reason for the decreased expression of the competence genes and *comK*,
234 which could be counteracted by introducing a non-phosphorylatable variant of DegU. By additionally
235 investigating a strain harboring a *degU-yfp* fusion, Diethmaier *et al*. also suggested an increased level
236 of DegU~P to be present in the *hag*-mutant (Diethmaier *et al.*, 2017), which is consistent with our
237 conclusions. In addition, the authors detected a reduced expression of *comK* in a strain with the
238 *degU*32 variant, which produces a form of DegU~P with higher stability (Diethmaier *et al.*, 2017).
239 Comparable results were obtained by Msadek *et al*., who found that high levels of DegU~P inhibit
240 competence (Msadek *et al.*, 1990). We observed a similar, although weak statistical trend towards a
241 reduced transformation frequency in *degU*32 strain. Miras and Dubnau (2016) have recently

242 highlighted that differences in the DegU phosphorylation pathway among diverse *B. subtilis* isolates
243 were likely responsible for variance in DNA transformation efficiency among certain domesticated
244 and undomesticated strains. Moreover, slight differences in competence induction levels could also
245 be affected by strain-specific characteristics. For example, *B. subtilis* 168 strains derived from
246 different laboratories can exhibit striking variations in biofilm robustness (Gallegos-Monterrosa *et*
247 *al.*, 2016). As suggested by Diethmaier and colleagues, the reduced transformation frequency in
248 *degU*32 might be caused by the high DegU~P levels of this strain. However, the *degU*32 strain
249 exhibits a non-motile phenotype and in the undomesticated strains DegU32 is not able to interact
250 with SwrA at the P_A promoter of the *fla/che* operon (*swrA* is inactive in domesticated strains),
251 leading to repression of P_A (*fla/che*) (Amati *et al.*, 2004; Mordini *et al.*, 2013). Due to low or no
252 expression of the basic flagellar genes, this phenotype could mimic the situation observed in the
253 flagellar mutants. In addition, we observed an increased transformation frequency when the *hag*
254 gene was deleted from the *degU*32 background. This is in contrast to the model assuming that
255 increased levels of phosphorylated DegU in the cells lowers competence. Therefore, it is possible
256 that yet unidentified factors are also involved in connecting motility and competence development
257 that might be independent of DegU~P. At this point however, we cannot provide a reasonable
258 explanation for the increased transformation frequency of Δ*hag degU*32.
259 Interestingly, induction of competence state has negative impact on motility in *B. subtilis*. ComK
260 negatively controls *hag* gene expression by stimulating the transcription of *comFA-C* operon and the
261 downstream located anti-sigmaD factor coding gene, *flgM* (Liu and Zuber, 1998). This feedback loop
262 presents another intriguing connection between these two cellular processes.
263 Diethmaier *et al*. proposed that increased DegU~P and lower *comK* expression in the flagellar
264 mutants and in a strain with straight flagella was caused by a lower viscous load (Diethmaier *et al.*,
265 2017). In line with this report, we also observed that higher viscosity in the medium resulted in an
266 increased transformation frequency. Nevertheless, a possible role of the DegSU two-component
267 system in sensing incomplete assembly of flagella and dysfunction as suggested previously (Hsueh *et*
268 *al.*, 2011; Cairns *et al.*, 2013) could also explain the increased DegU~P levels in the flagellar mutants.
269 Together, our results identify a connection between two major physiological processes, providing
270 another example of the complexity of intracellular regulatory networks and the vast amount of tasks
271 a single regulator can cover.
272
273

274 **Experimental Procedures**

275 *Strains and cultivating conditions*

276 The strains used in this study and their mutant derivatives are listed in Table S1. Mutants
277 constructed in this study were obtained by natural transformation of a *B. subtilis* receptor strain with
278 genomic DNA from a donor strain. Strain TB831 was created by transformation of strain 168 P_{xyl}-
279 *comK* with genomic DNA of strain GP902 (J. Stülke lab collection). To obtain strains TB926 and
280 TB925, genomic DNA of strain 168 P_{comG}-*gfp* was used to transform strain TB710 and TB689,
281 respectively. Strain TB928 was obtained by transforming strain 168 P_{xyl}-*comK* with genomic DNA of
282 GP901 (J. Stülke lab collection). To create strain TB935 and TB936, strain 168 was transformed with
283 genomic DNA obtained from strain QB4371 (Kunst *et al.*, 1994) and QB4458 (Dahl *et al.*, 1991),
284 respectively. Their derivatives harbouring also a mutation of *hag* (TB923 and TB924) were created by
285 transformation with genomic DNA, which was obtained from GP901. In-frame deletions of *motA*,
286 *flgE*, and *cheA* were created using plasmids pEC1, pDP306, and pDP338, respectively, as previously
287 described (Courtney *et al.*, 2012; Chan *et al.*, 2014; Calvo and Kearns, 2015). Strains were verified by
288 fluorescence microscopy (P_{comG}-*gfp* reporter), PCR (*hag* mutants), or sequencing (*degU* variants),
289 using the oligonucleotides listed in Table S2. For experiments with strains harboring the inducible
290 construct P_{xyl}-*comK*, 1% of xylose (final concentration) was added for induction (see van den Esker *et*
291 *al.*, 2017). To increase medium viscosity, 10% Ficoll400 (Carl Roth) was added to the medium before
292 culture inoculation and the mix was vortexed vigorously for ca 20 s.

293

294 *Transformation frequency assay*

295 To assess the transformation frequency of different strains, a modified version of the transformation
296 protocol from Konkol *et al*. (2013) was used. 1 ml of each culture grown in 3 ml Lysogeny broth (LB)
297 medium (LB-Lennox, Carl Roth; 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, and 5 g L^{-1} NaCl) for 16 h was
298 centrifuged for 2 min at 11,000 x g. The pellet was washed twice in de-ionized water and was re-
299 suspended in 100 µl de-ionized water. The re-suspended culture was diluted (1:80) in complete
300 competence medium (MC: 1,8 ml de-ionized water, 6.7 µl 1M $MgSO_4$, 50 µl 0.2% L-Tryptophan, 200
301 µl 10xMC; per 100 ml 10xMC: 14,036 g K_2HPO_4 [x3H_2O], 5,239 g KH_2PO_4, 20 g glucose, 10 ml 300 mM
302 tri-sodium citrate, 1 ml 83.97 mM ammonium iron (III) citrate, 1 g casein hydrolysate, 2 g potassium
303 glutamate [H_2O]) and incubated at 37°C, 225 rpm. For experiments with strains harboring P_{xyl}-*comK*,
304 10x MC with fructose instead of glucose was used. After 6 h incubation time, 5 µl DNA with an
305 antibiotic marker (PY79 *safA*::Tet gDNA, 60 ng/µl) was added to 500 µl culture. Any alteration in
306 incubation time before addition of the DNA is indicated in the results section. Each culture was
307 incubated for 30 min, then 500 µl fresh LB medium was added and the culture was incubated for

308 another 1 h under the conditions mentioned above. Serial dilutions of cultures supplemented with
309 DNA were prepared and plated on LB medium supplemented with 1.5 % agar to determine the
310 number of colony forming units (cfu). Additionally, 50 µl and 100 µl undiluted cultures
311 supplemented with gDNA as well as controls were plated on tetracycline (Tet) containing LB-agar
312 plates (10 µg ml^{-1} Tet) to determine the number of transformant colonies. The transformation
313 frequency was calculated by dividing the number of transformants per ml by cfu per ml.
314
315 *Growth curve experiments*
316 To examine growth properties, cultures were inoculated in LB medium from frozen glycerol stocks
317 and incubated for ca. 16 h at 37°C shaking at 225 rpm. Cultures were diluted 1:100 in 200 µl fresh
318 completed MC medium (see above) and the OD_{590nm} was recorded for 16 h using a TECAN Infinite
319 F200 PRO microplate reader. The cultures were incubated with orbital shaking with a duration of
320 800 s and an amplitude of 3 mm at 37°C and the OD_{590} was measured every 15 min.
321
322 *Fluorescence microscopy*
323 Strains were investigated using a confocal laser scanning microscope (LSM 780, Carl Zeiss) equipped
324 with an argon laser and a Plan-Apochromat/1.4 Oil DIC M27 63× objective. Cultures were grown
325 prior microscopy for 5 h (if not indicated otherwise) in competence medium under the same
326 conditions as described above (see section transformation frequency assay). Excitation of the
327 fluorescent reporter (GFP) was performed at 488nm and the emitted fluorescence was recorded at
328 493-598nm. For image visualization, Zen 2012 software (Carl Zeiss) was used, brightness and
329 contrast were adjusted equally in all images.
330
331 *Flow cytometry*
332 Flow cytometric measurements were performed using a Partec CyFlow® Space (Sysmex Partec
333 GmbH, Germany), which was equipped with a solid-state laser for excitation of green/yellow
334 fluorescent proteins at 488 nm. Single cells were detected in forward and sideward scatter channels
335 as well as in one fluorescent channel. A minimum of 40,000 cells were analyzed for the experiments.
336 To define the background fluorescence signal, non-labelled *B. subtilis* cultures were analyzed as
337 control. Cultures used for measurements were grown for 5 h (if not indicated otherwise) in
338 competence medium under the same conditions as described above (see section transformation
339 frequency assay). For evaluation of the data, the FlowJo® software (FlowJo LLC, Ashland, USA) was
340 used and a gate was set at 3 fluorescence units for all samples to isolate the fluorescent population
341 and determine the percentage of fluorescent cells.

342

343 *Statistics*

344 Statistical analyses were performed using OriginPro 2016 (V93E, OriginLab Northampton, USA).
345 Unpaired two-sample t-test with Welch Correction or a Kruskal-Wallis test was used to test for
346 significant differences.

347

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Conflict of interest. None declared

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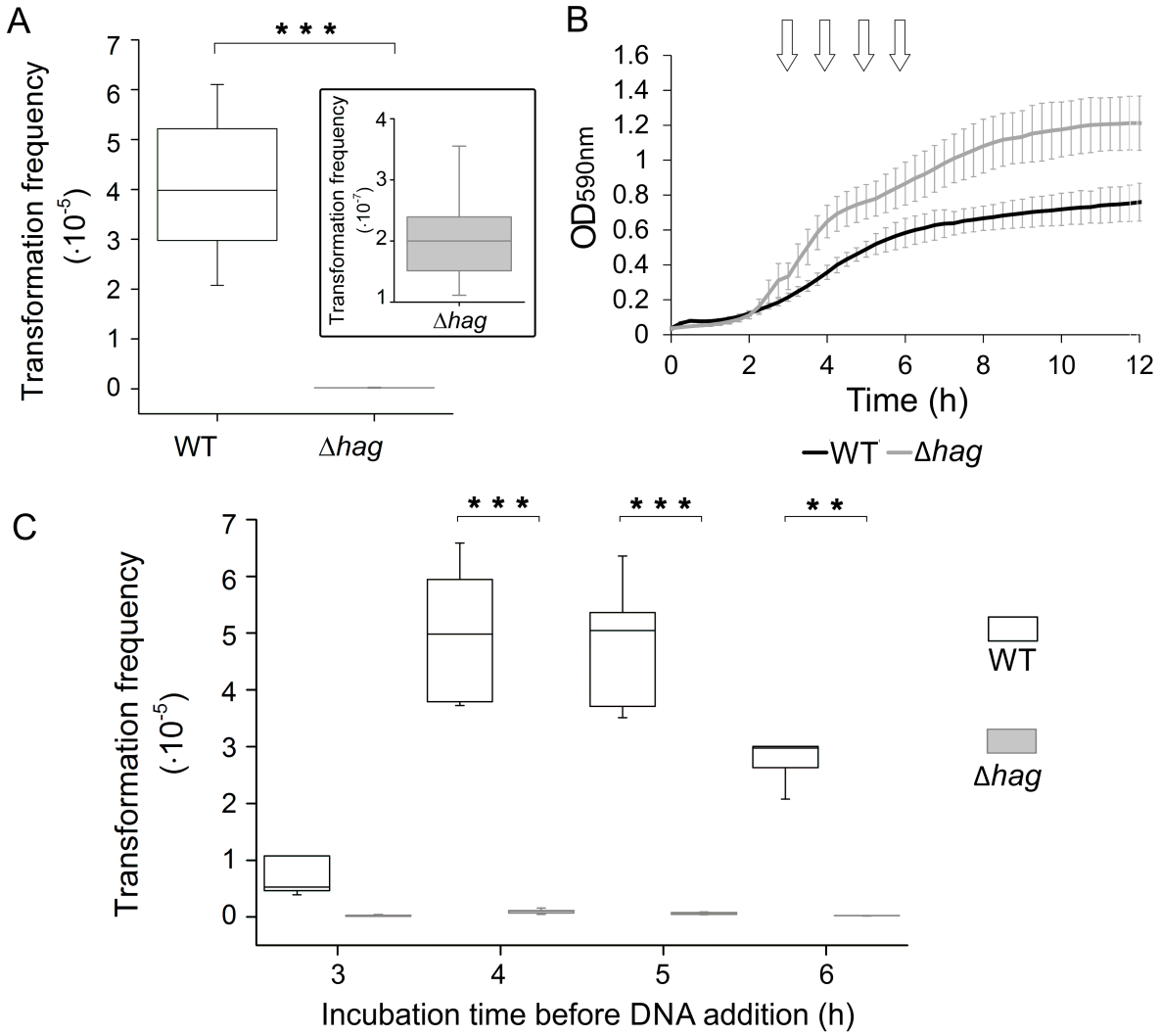
460

461 **Figures**

462



463

464 **Fig. 1.** Transformation frequency is reduced in a mutant lacking flagellin protein. (A) Transformation
465 frequency of *B. subtilis* wild type and *hag*-mutant after 6 h incubation in competence medium
466 (unpaired two-sample t-test with Welch Correction: $P = 3.1 \cdot 10^{-5}$, n = 9). The inset shows a zoom-in of
467 the *hag*-mutant data. (B) Growth dynamics of wild type and hag-mutant during 12 h incubation in
468 competence medium. Standard deviations for the measurements are depicted in light grey
469 (unpaired two-sample t-test with Welch Correction: P = 0.001, n = 5). Arrows indicate the time
470 points of DNA addition to investigate the transformation frequency over time, which is shown as
471 box-and-whisker plot in (C). The line in the boxes represents the median, the box indicates the 25th-
472 75th percentile. Asterisks indicate statistically significant differences between wild type and *hag*-
473 mutant (unpaired two-sample t-test with Welch Correction for WT - Δ*hag*:, P = 0.125 for 3 h, P < 0.01
474 for 4 h, 5 h, 6 h; n = 6).

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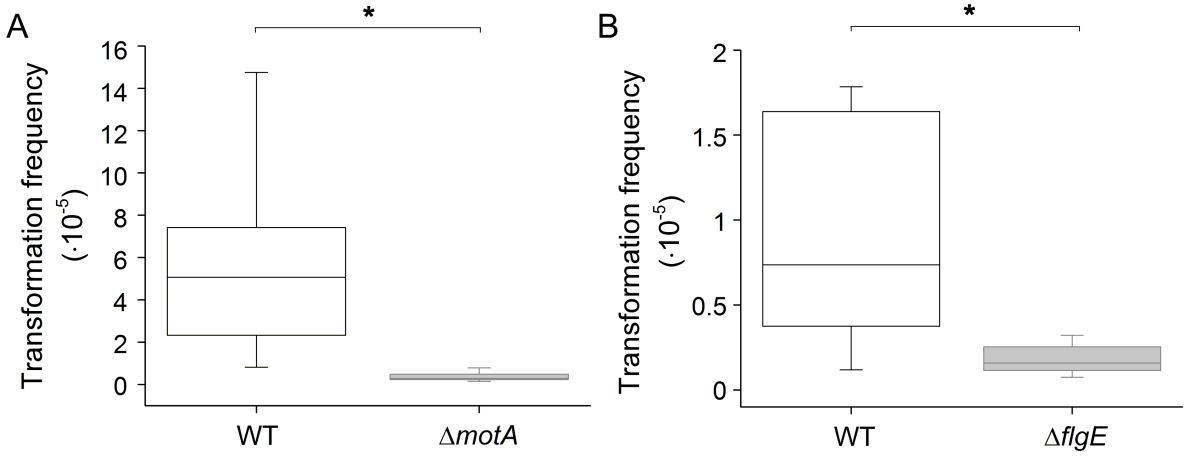
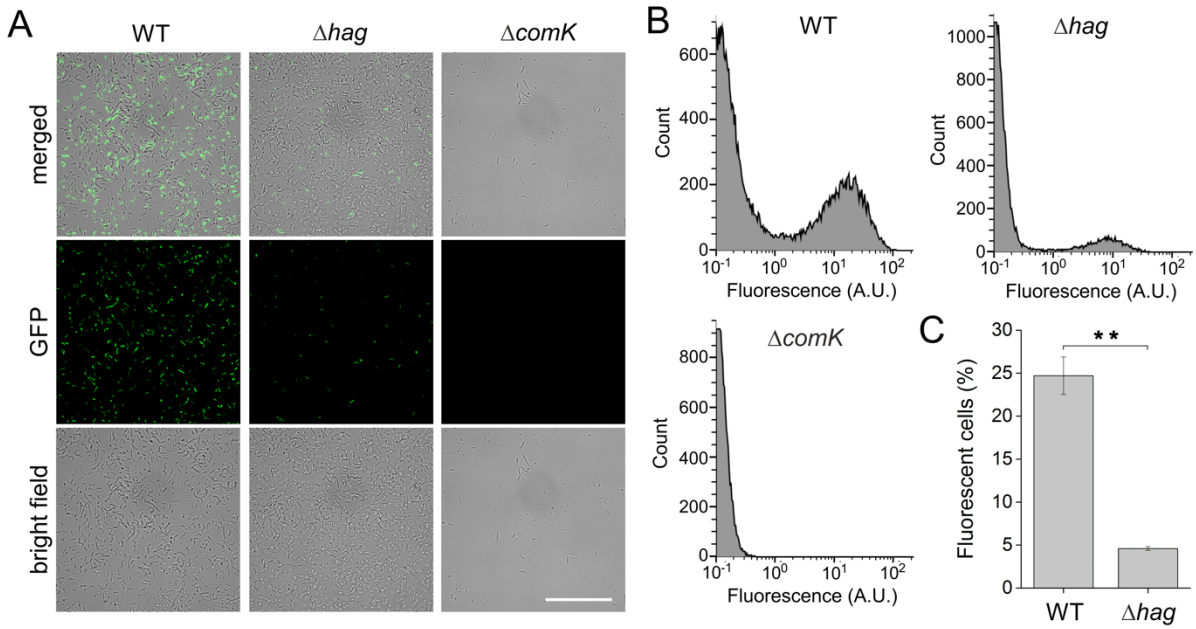


477

478 **Fig. 2.** Mutants impaired in flagellar function exhibit lower transformation frequencies. Deletion of
479 the gene encoding a flagellar stator (*motA*; A) or the gene encoding the hook protein (*flgE*; B) results
480 in significantly lower transformation frequency of the respective strain compared to the wild type
481 after incubation in competence medium for 6 h. The line in the boxes represents the median, the
482 box indicates 25th-75th percentile. Asterisks indicate statistically significant differences (unpaired
483 two-sample t-test with Welch Correction: P = 0.01 for Δ*motA*; P = 0.039 for Δ*flgE*; n = 9 for all).

484



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486

487 **Fig. 3.** Fewer cells of the *hag*-mutant express competence genes compared to the wild type. (A)
488 Representative microscopy images of strains harboring the P_{comG}-*gfp* reporter in wild type, Δ*hag* or
489 Δ*comK* genetic background. Images were recorded after incubation in competence medium for 5 h.
490 The scale bar represents 50 µm. (B) Histograms of flow cytometric measurements showing the cell
491 count and the fluorescence in arbitrary units for wild type, Δ*hag*, and Δ*comK* including background
492 fluorescence. Representative images are shown for each strain. (C) Percentage of fluorescent cells
493 determined from the data in (B) for wild type and *hag*-mutant by isolating the fluorescent
494 population with fluorescence intensities above 3 A.U. Asterisks indicate significant differences
495 (unpaired two-sample t-test with Welch Correction: P = 0.036, n = 3).

496

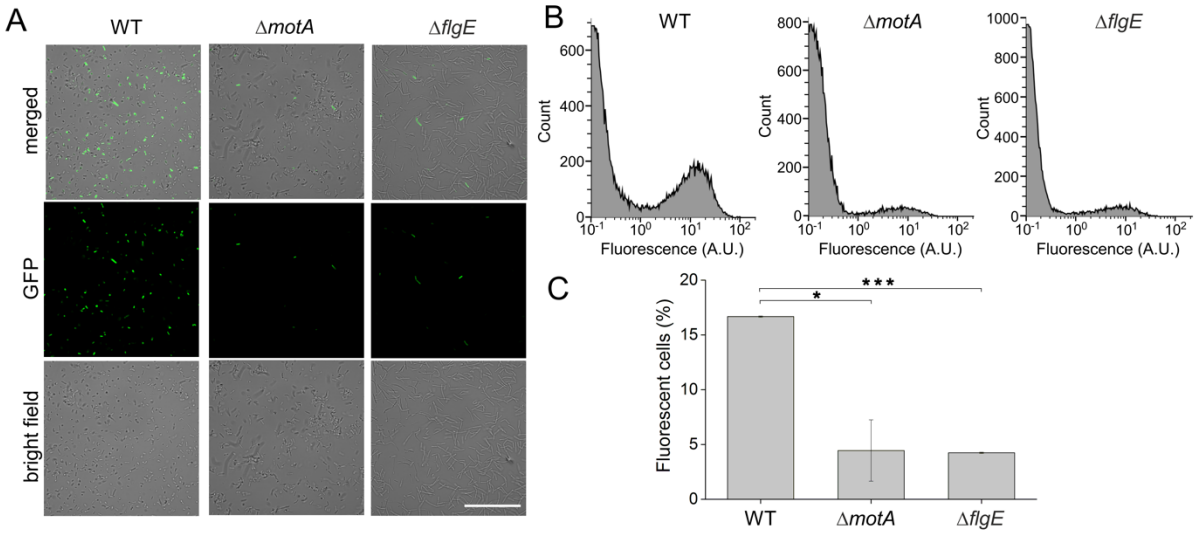
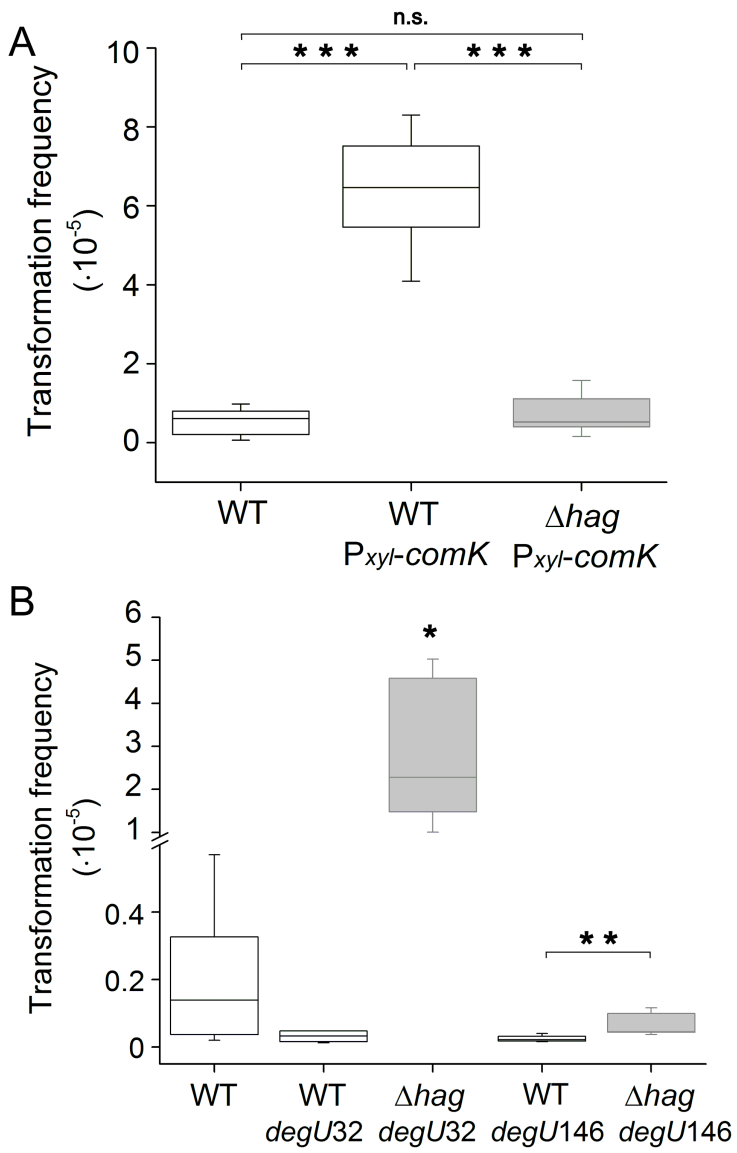


Fig. 4. Competence gene expression is reduced in mutants lacking a functional flagellum. (A) Representative microscopy images of strains harboring the P_{comG}-*gfp* reporter in wild type, Δ*motA*, or Δ*flgE* genetic background. Images were recorded after 5 h incubation in competence medium. The scale bar represents 50 µm. (B) Histograms of flow cytometric measurements showing the cell count and the fluorescence in arbitrary units for wild type, Δ*motA* or Δ*flgE*. Representative images are shown for each strain. (C) Percentage of fluorescent cells determined from the data in (B) by isolating the fluorescent population with fluorescence intensities above 3 A.U. showing a significant difference (asterisks) between wild type and Δ*motA* ($P = 0.017$) as well as wild type and Δ*flgE* ($P < 0.001$) with $n = 3$ for both (unpaired two-sample t-test with Welch Correction).

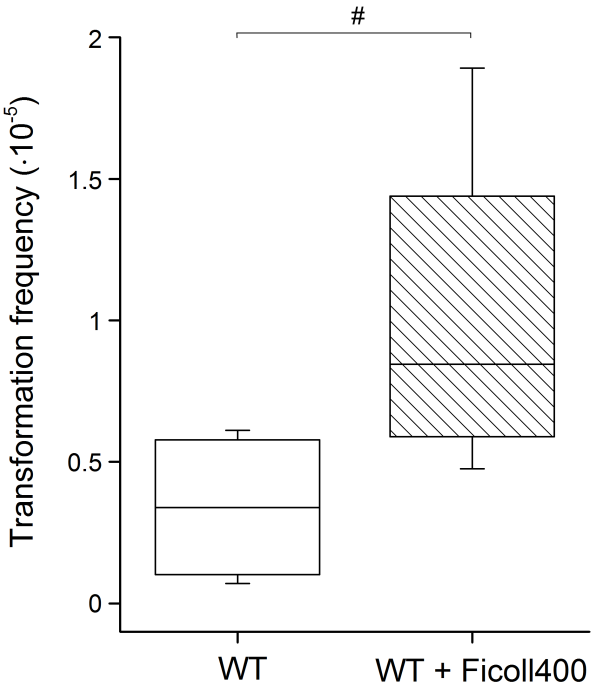


509

510

511 **Fig. 5.** Synthetically induced *comK* and *degU*146 increase competence of Δ*hag*. (A) Transformation
512 frequencies of wild type compared to strains harboring a xylose-inducible copy of *comK* (P_{xyl}-*comK*)
513 with wild type or Δ*hag* genetic background (Kruskal-Wallis test for WT - WT P_{xyl}-comK: $P = 3.4 \cdot 10^{-4}$;
514 for WT P_{xyl}-*comK* - Δ*hag* P_{xyl}-*comK*: $P = 3.4 \cdot 10^{-4}$, n = 9 for both). (B) Transformation frequencies of WT
515 compared to strains harboring either a phosphorylated DegU variant (*degU*32) or a non-
516 phosphorylatable DegU variant (*degU*146) in wild type or Δ*hag* background. Strain Δ*hag degU*32 is
517 significantly different from all other strains (Kruskal-Wallis test: $P < 0.05$ for all, n=6). The line in the
518 boxes represents the median, the box indicates 25th-75th percentile. Asterisks indicate statistically
519 significant differences (Kruskal-Wallis test: P = 0.007, n=6).

520



521

522

523 **Fig. 6.** Competence is improved in viscous medium. Transformation frequency of the wild type strain
524 grown in normal competence medium and in medium with increased viscosity. The line in the boxes
525 represents the median, the box indicates 25th-75th percentile, # indicates marginally significant
526 differences (unpaired two-sample t-test: P = 0.095, n = 4).

527