1 **Impaired competence in flagellar mutants of *Bacillus subtilis* is connected to the regulatory**

2 **network governed by DegU**

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19 **Running title:** Hindered competence by lack of motility

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22 **Summary**

23 The competent state is a developmentally distinct phase, in which bacteria are able to take up and

24 integrate exogenous DNA into their genome. *Bacillus subtilis* is one of the naturally competent

25 bacterial species and the domesticated laboratory strain 168 is easily transformable. In this study,

26 we report a reduced transformation frequency of *B. subtilis* mutants lacking functional and

27 structural flagellar components. This includes *hag*, the gene encoding the flagellin protein forming

28 the filament of the flagellum. We confirm that the observed decrease of the transformation

29 frequency is due to reduced expression of competence genes, particularly of the main competence

30 regulator *comK*. The impaired competence is due to an increase in the phosphorylated form of the

31 response regulator DegU, which is involved in regulation of both flagellar motility and competence.

32 Altogether, our study identified a close link between motility and natural competence in *B. subtilis*

33 suggesting that hindrance in motility has great impact on differentiation of this bacterium not

34 restricted only to the transition towards sessile growth stage.

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36 **Originality-Significance statement**

37 Understanding how versatile bacterial phenotypes influence each other is important for our basic

38 understanding of microbial ecology. Our research highlights the novel intertwinement of bacterial

39 differentiation and reveal how lack of single cell motility adjusts DNA exchange among bacterial

40 strains.

41

Introduction

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

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

However, DegU is not only crucial for competence initiation, but also involved in the regulation of many other processes including protease production, biofilm development, and, particularly, flagellar motility (Murray *et al.*, 2009; Mukherjee and Kearns, 2014). The main components of the hook and basal body of the flagellum are encoded by the large *fla/che* operon. Transcription of this 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 = $1.3\cdot10^{-9}$ 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 $5.3\cdot10^{-6}$ for the wild type

165   and $8.5\cdot10^{-6}$ 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    $3.4\cdot10^{-4}$ for WT – WT P*xyl*-*comK*, P = $3.4\cdot10^{-4}$ 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

Discussion

208

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

Experimental Procedures

Strains and cultivating conditions

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

Transformation frequency assay

To assess the transformation frequency of different strains, a modified version of the transformation protocol from Konkol *et al*. (2013) was used. 1 ml of each culture grown in 3 ml Lysogeny broth (LB) 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 centrifuged for 2 min at 11,000 x g. The pellet was washed twice in de-ionized water and was re-suspended in 100 µl de-ionized water. The re-suspended culture was diluted (1:80) in complete competence medium (MC: 1,8 ml de-ionized water, 6.7 µl 1M MgSO$_4$, 50 µl 0.2% L-Tryptophan, 200 µl 10xMC; per 100 ml 10xMC: 14,036 g K$_2$HPO$_4$ [x3H$_2$O], 5,239 g KH$_2$PO$_4$, 20 g glucose, 10 ml 300 mM tri-sodium citrate, 1 ml 83.97 mM ammonium iron (III) citrate, 1 g casein hydrolysate, 2 g potassium glutamate [H$_2$O]) and incubated at 37°C, 225 rpm. For experiments with strains harboring P$_{xyl}$-*comK*, 10x MC with fructose instead of glucose was used. After 6 h incubation time, 5 µl DNA with an antibiotic marker (PY79 *safA*::Tet gDNA, 60 ng/µl) was added to 500 µl culture. Any alteration in incubation time before addition of the DNA is indicated in the results section. Each culture was incubated for 30 min, then 500 µl fresh LB medium was added and the culture was incubated for

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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.

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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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359

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460

461   **Figures**

462
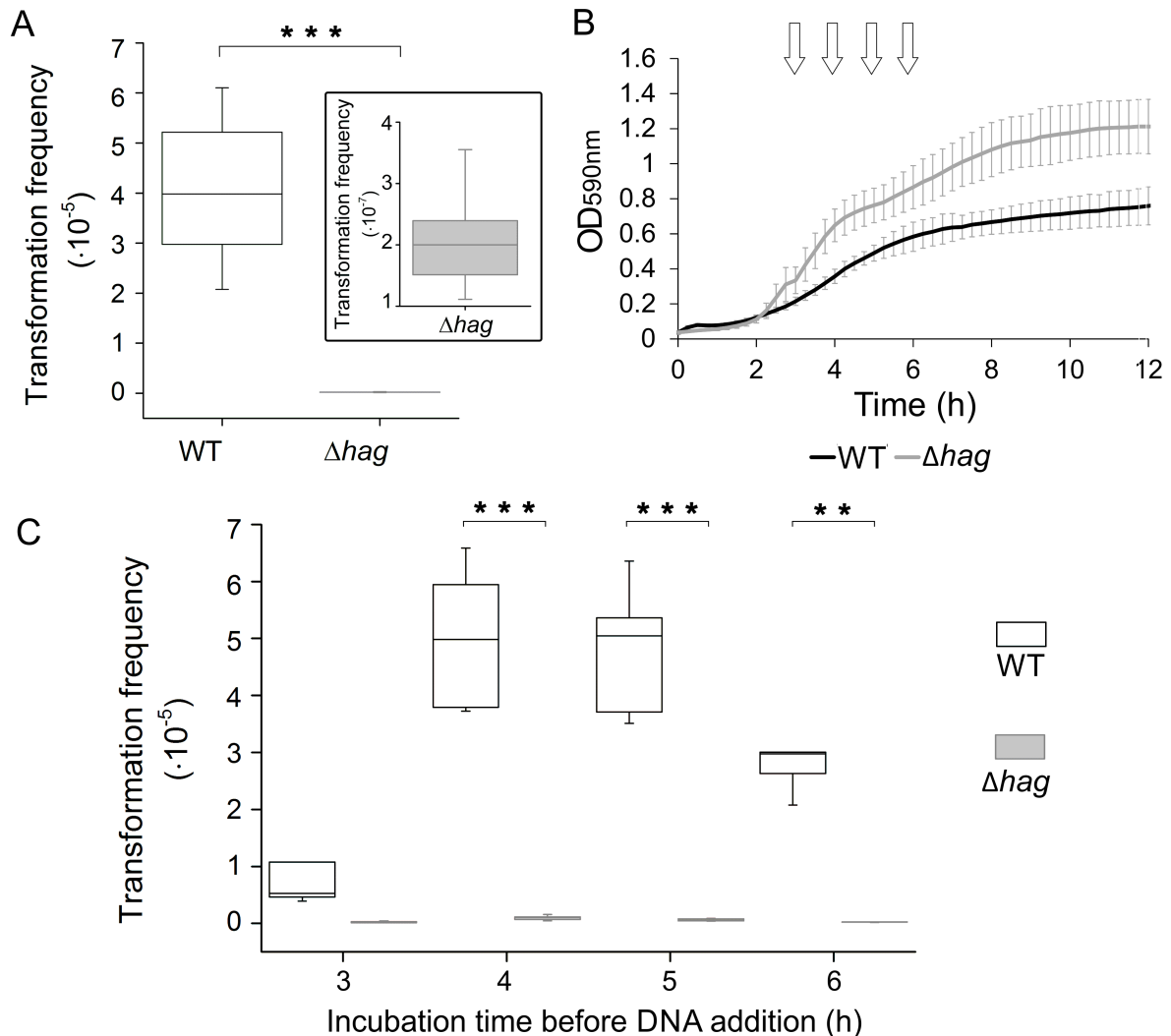
463

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).

475

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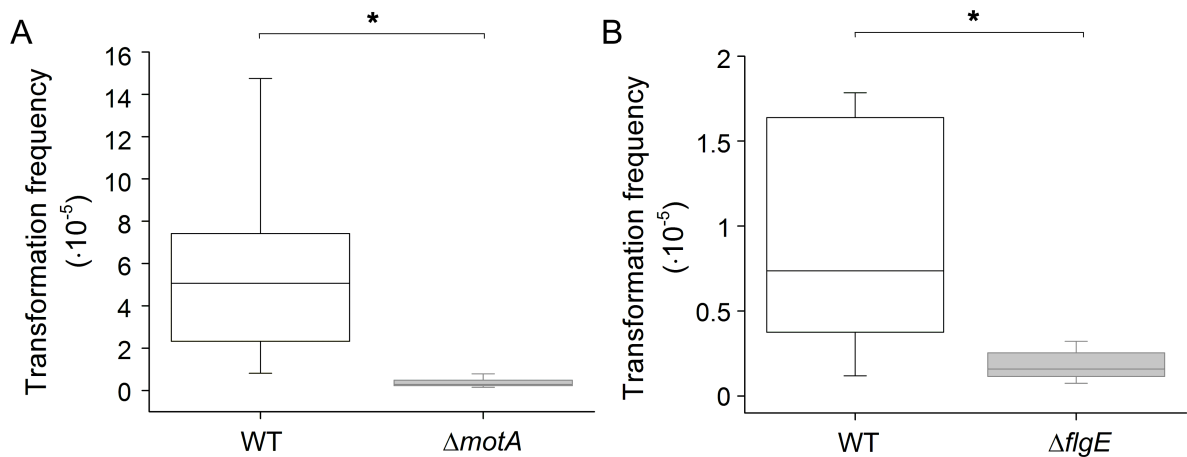
477

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
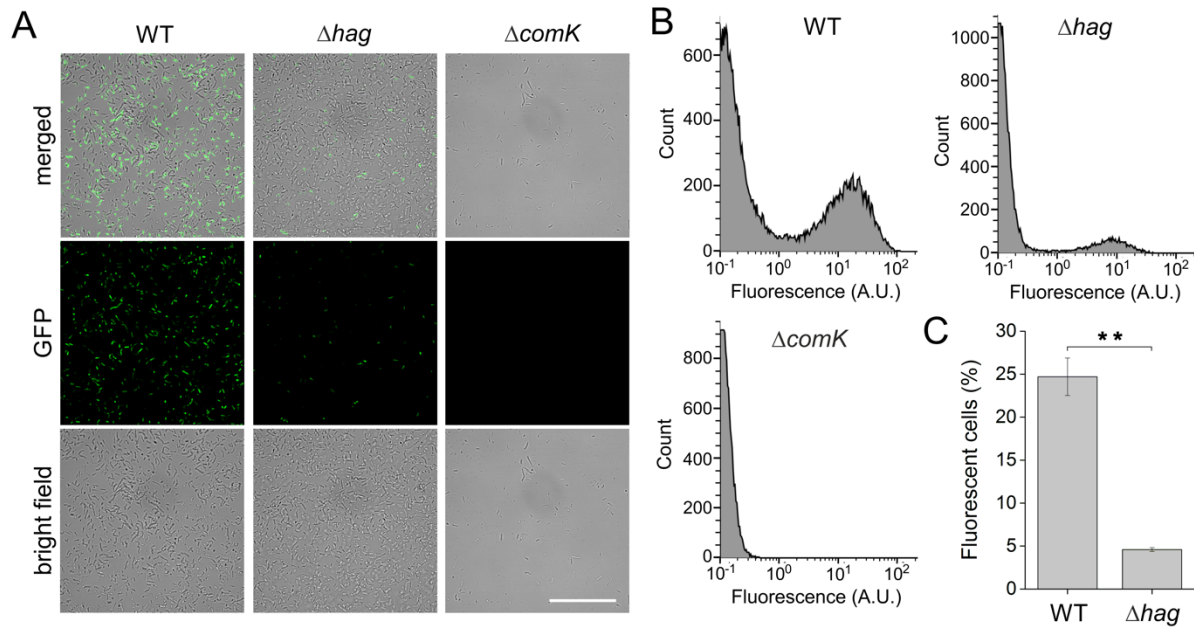
Fig. 3. Fewer cells of the *hag*-mutant express competence genes compared to the wild type. (A) Representative microscopy images of strains harboring the P*comG*-*gfp* reporter in wild type, Δ*hag* or Δ*comK* genetic background. Images were recorded after incubation in competence medium for 5 h. The 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*hag*, and Δ*comK* including background fluorescence. Representative images are shown for each strain. (C) Percentage of fluorescent cells determined from the data in (B) for wild type and *hag*-mutant by isolating the fluorescent population with fluorescence intensities above 3 A.U. Asterisks indicate significant differences (unpaired two-sample t-test with Welch Correction: P = 0.036, n = 3).
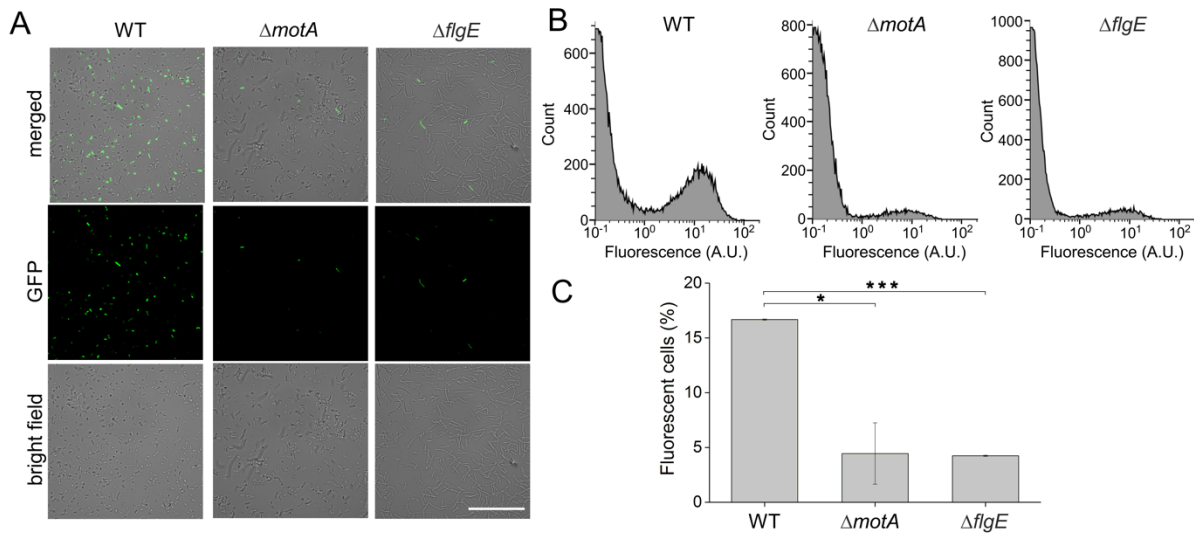
Fig. 4. Competence gene expression is reduced in mutants lacking a functional flagellum. (A) Representative microscopy images of strains harboring the P$_{comG}$-*gfp* reporter in wild type, Δ*motA*, or Δ*flgE* genetic background. Images were recorded after 5 h incubation in competence medium. The scale 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).
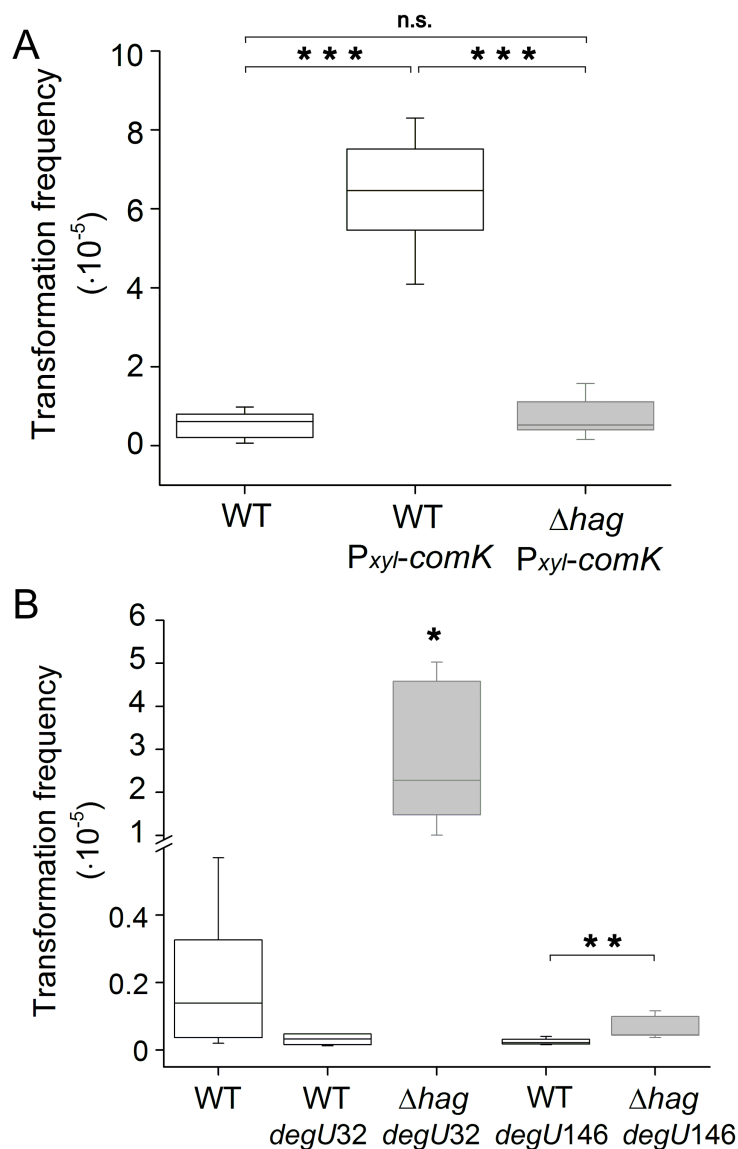
21

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

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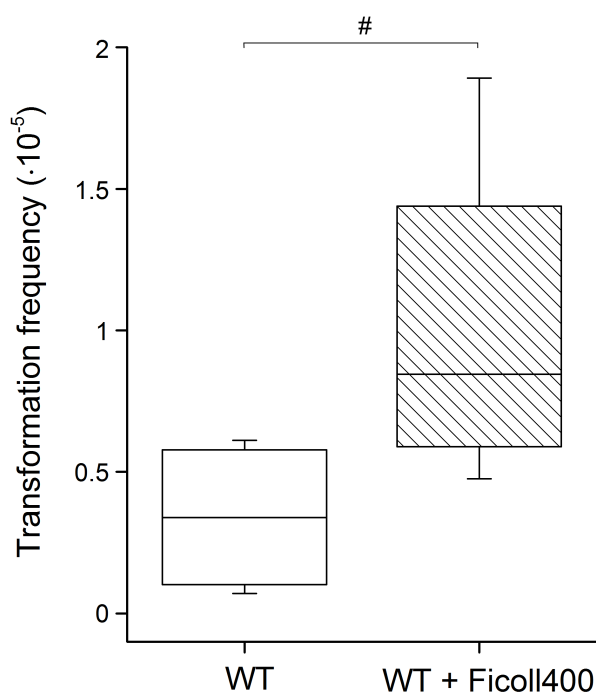
Fig. 6. Competence is improved in viscous medium. Transformation frequency of the wild type strain grown in normal competence medium and in medium with increased viscosity. The line in the boxes represents the median, the box indicates 25th-75th percentile, # indicates marginally significant differences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