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1 **SwrA as global modulator of the two-component system DegS/U in *B. subtilis***

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5 Keywords: SwrA, DegU, DegS, DegQ, competence, subtilisin, γ-PGA

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7 ABSTRACT

8 The two-component system DegS/U of *Bacillus subtilis* controls more than one hundred genes involved
9 in several different cellular behaviours. Since the consensus sequence recognized by the response regulator
10 DegU has not been clearly defined yet, mutations in either component have been crucial in the
11 identification of the cellular targets of this regulatory system. Over the years, the *degU32*^{Hy} mutant allele,
12 that was supposed to mimic the activated regulator, has been commonly used to define the impact of this
13 TCS on its regulated genes in domestic strains.

14 SwrA encodes a small protein essential for swarming motility and for poly-γ-glutamate biosynthesis
15 and is only present in wild strains. Previous work indicated that SwrA is partnering with DegU~P in exerting
16 its role on both phenotypes.

17 In this work, inserting a *degS200*^{Hy} mutation in *swrA*$^{+}$ and *swrA*$^{-}$ isogenic strains we demonstrate that
18 SwrA modulates the action of DegU~P on two new phenotypes, subtilisin expression and competence for
19 DNA uptake, with a remarkable effect on transformation. These effects cannot not be appreciated with the
20 DegU32^{Hy} mutant as it does not mirror the wild-type DegU protein in its ability to interact with SwrA.

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22 INTRODUCTION

23 Two-component systems (TCS) are signal transduction modules common in bacteria and archaea,
24 composed by a sensor histidine kinase and a cognate response regulator. Sensor kinases auto-
25 phosphorylate themselves on a histidine residue in response to specific environmental signals and then
26 transfer the phosphate group to a specific aspartic acid residue of the regulator inducing a structural
27 rearrangement that enables it to modify its DNA binding properties and regulate gene expression.
28 Moreover, sensor kinases can often quench spurious signals by dephosphorylating their cognate regulators
29 (1). The DegS/U TCS, composed by the cytoplasmic DegS kinase and the DegU transcription factor, is
30 involved in the regulation of several important physiological pathways of *Bacillus subtilis*, among which
31 flagella-mediated motility, degradative enzyme synthesis, genetic competence, and sporulation (2). The
32 extremely wide impact of this TCS has been evidenced through several transcriptional profiling experiments
33 (3-5). A particular class of mutations in either DegS or DegU leads to the hyperproduction of several
34 degradative enzymes, including the *aprE*-encoded protease subtilisin, and has therefore been named "Hy"
35 (6-8). Besides promoting the synthesis of several degradative enzymes, the pleiotropic Hy mutations also
36 cause the so called Hy phenotype which includes loss of DNA competence, absence of flagella, sporulation
37 in the presence of glucose and elongated cell morphology (6, 9, 10). A Hy phenotype is also observed when
38 two small proteins, DegQ and DegR, are overexpressed. They are both involved in the DegS/U signalling
39 pathway: DegQ stimulates the transfer of the phosphate moiety from DegS to DegU (5), while DegR
40 stabilizes DegU~P by preventing DegS-mediated DegU~P dephosphorylation (11). The overexpression of
41 DegQ naturally occurs in wild *B. subtilis* strains, thanks to a nucleotide change in the -10 box of its promoter
42 that leads to 10-fold increase in transcription with respect to domestic strains (12, 13); however, the
43 *degQ*^{Hy} mutation present in undomesticated strains only generates a mild phenotype, as these strains,
44 differently from *degS/U*^{Hy} mutants, do not copiously produce γ-PGA (see below).

45 Among the number of originally isolated Hy mutants (6), subsequent studies have heavily relayed
46 on *degU32*^{Hy}, a particular *degU* allele which carries an A-to-T transversion at nucleotide 35 of the *degU*
47 ORF, leading to a His to Leu amino acid change at position 12 (14). In early studies, all DegU32^{Hy} phenotypes
48 matched those obtained with the DegS200^{Hy} mutant, in which the Gly to Glu mutation at position 218 of
49 DegS impairs the phosphatase activity of the sensor kinase, thus leading to the accumulation of DegU~P
50 (15). From the perfectly overlapping phenotypes of the two mutants, DegU32^{Hy} has been since considered
51 as a constitutively active proxy of DegU~P, without any further structural characterization. Only recently
52 such interpretation has been challenged, thanks to the introduction of a new player, SwrA (16).

53 SwrA is also a small protein, 117 aa, which has been discovered thanks to its fundamental role in
54 swarming motility (17). Its existence remained long undisclosed due to the fact that domestic *B. subtilis*
55 strains only encode a non-functional 13 aa truncated peptide because of the insertion of an extra adenine

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56 in a poly-A tract in *swrA* ORF causing a frameshift mutation (17-19). This type of mutations can easily flip
57 back to the wild-type form (wt) and vice versa with the frequency of a phase-variation event (17). It is thus
58 frequent to obtain mixed *swrA*$^{+/-}$ populations even in laboratory strains upon prolonged incubations. When
59 functional, SwrA stimulates flagella production through its activity at the $P_{fla/che}$ promoter, thereby
60 promoting *sigD* transcription which also permits efficient cell septation (16, 19-21). Although initially
61 confined to motility regulation, the role of SwrA was also shown to be essential for the expression of the
62 otherwise-silent *pgs* operon, encoding the enzymes required for the synthesis of the biopolymer poly-γ-
63 glutamic acid (γ-PGA) (22). Interestingly, γ-PGA production not only strictly depends on SwrA but also on a
64 Hy mutation in either DegQ (23) or DegU/S (24). This was the first evidence of a strict connection between
65 SwrA and the DegS/U TCS.

66 Further genetic evidence of the link between SwrA and the DegS/U was gained by studies on the
67 *fla/che* operon, in which the direct interaction between DegU~P and SwrA was demonstrated genetically
68 and biochemically. Genetically, it was observed that while *degU32*^{Hy} and *degS200*^{Hy} mutants completely
69 suppress $P_{fla/che}$ expression in laboratory strain lacking SwrA, leading to the classically non-flagellated Hy-
70 phenotype (6, 10), the restoration of a functional *swrA* allele leads to hyperflagellation in *degS200*^{Hy}
71 mutants as well as in *degS/U*^{wt} backgrounds, although not in *degUS32*^{Hy} mutants (16). Biochemically, it was
72 shown that, in electro mobility shift assays, the DegU~P-bound *fla/che* promoter is super-shifted in the
73 presence of SwrA while DegU32^{Hy} is not; moreover, DegU32^{Hy} does not require phosphorylation for DNA
74 binding (16). The physical interaction between DegU and SwrA was also evidenced in other studies (25).
75 Ultimately, the impact of SwrA on motility is to remarkably turn the $P_{fla/che}$ repressive effect of DegU~P,
76 naturally produced or induced by a *degS200*^{Hy} mutation, into a transcriptional boost, thus allowing
77 swarming motility (16, 21). This dramatic overturn of DegU~P impact on the motility operon could not be
78 appreciated in domestic strains, because of the absence of SwrA and in wild strains if the *degU32*^{Hy} allele is
79 used as a proxy of DegU~P (26).

80 Although the above data suggest that SwrA does not interact with DegU32^{Hy}, this is not always true.
81 Indeed, γ-PGA production is induced by SwrA in the presence of either *degU32*^{Hy} or *degS200*^{Hy} (24).
82 However, a deep characterization of the differential impacts of the two Hy alleles on P_{pgs} has yet to be
83 conducted.

84 In competence, Hy mutants have been shown to have a negative impact on the overall process in
85 laboratory strains. However, *degS/U* null mutants were also shown to be non-competent, suggesting the
86 requirement of this TCS in the pathway (9). The current model is the following: DegU~P has a negative
87 impact on *comS,* while unphosphorylated DegU is required, possibly because it mediates the binding of
88 ComK to its own promoter (27). More recently, the *degQ*^{Hy} allele was shown to negatively affect *comS* and
89 *comK* expression in both domestic and wild strains (28).

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90 In this work we demonstrate that SwrA heavily impacts not only motility and γ-PGA production but
91 also other DegS/U regulated behaviours; SwrA positively modulates DegS/U activity in competence for
92 transformation and reduces *aprE* transcription. Moreover, we characterized the differential influence of
93 *degU32*^{Hy} and *degS200*^{Hy} mutations on *pgs* transcription. Finally, we once more demonstrate that the
94 *degU32*^{Hy} allele encodes a constitutively active mutant protein whose activity dramatically differs from the
95 phosphorylated DegU~P protein. Our results suggest that, as it happened in the past for motility, the use of
96 this mutant may lead to misleading interpretations of the real physiological role of DegS/U TCS in *B. subtilis*
97 physiology.

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RESULTS

SwrA and motility in undomesticated strains. Our previous work showed that SwrA acts by subverting the impact of DegU~P on the *fla/che* promoter, transforming its action into a positive boost on flagellar gene expression. The functional interaction SwrA-DegU~P only occurs with the wild-type phosphorylated form of the response regulator, while the DegU32^{Hy} mutant protein does not effectively interface with SwrA at this promoter (16). To generalize this effect also to undomesticated strains, either *degU32*^{Hy} or *degS200*^{Hy} mutation was introduced in the transformable *comI*^{Q12L} mutant of the undomesticated NCIB3610 (29). The introduction of the DegU32^{Hy} mutation caused a complete loss of motility, as already shown by Stanley-Wall and collaborators (26). Conversely, the *degS200*^{Hy} derivative of the undomesticated strain proficiently swam and swarmed, paralleling the results obtained in domestic strain (Fig. S1). The only difference with domestic strains is the presence of a well-defined “lump” of γ-PGA that can be observed in the central part of the *degS*^{Hy} plates in Fig. S1. This characteristic is due to the abundant production of the polymer in DegS200^{Hy} mutants, which is much higher than in the *degU32*^{Hy} background. Interestingly, γ-PGA production was never visible in *degS/U*^{wt} undomesticated strains, although they naturally contain the *degQ*^{Hy} mutation. This finding suggests that the *degQ*^{Hy} mutation, which does not impact on the protein structure of DegU, is less effective than the *degS200*^{Hy} mutation in generating DegU~P.

Therefore, we concluded that the powerful overturn of the DegU~P action on motility genes is a general phenomenon occurring not only in laboratory strains, but also in wild, undomesticated strains, even when the phosphorylation of DegU is maximal.

SwrA and competence for DNA uptake. The voluminous literature data reporting the negative effect of Hy mutations on competence were acquired in domestic *B. subtilis* strains which lack the SwrA protein. To establish whether SwrA has a general role as regulatory factor for DegS/U activity in competence, genetic transformation was analyzed in isogenic mutants differing for the status of the *swrA* allele as well as for the source of DegU~P: either the intact phosphoprotein obtained in the presence of a *degS200*^{Hy} or the mutant DegU32^{Hy} protein.

Transformation efficiency was assessed in PB5370 and PB5249, respectively the *swrA*$^{-}$ and *swrA*$^{+}$ versions of the commonly used JH642 domestic strain (30), which do not contain any selectable marker. The *swrA*$^{-}$ and *swrA*$^{+}$ isogenic strains did not show any significant difference in transformation efficiency (Fig. 1), but for a slightly better performance of the *swrA*$^{+}$ strain, PB5249, which was thus taken as reference strain for determining the efficiency of the others. Both strains were transformed with the Hy mutation in either *degU* or *degS* and the resulting Hy mutants were transformed with a selectable genomic DNA. As shown in Fig. 1, consistently with literature data, in *swrA*$^{-}$ strains transformation efficiency was abolished by both *degU*^{Hy} and *degS*^{Hy} mutations (efficiency 0.7% and 2.7%, respectively). Even in the presence of a functional *swrA* allele the *degU*^{Hy} strain did not substantially modify competence, i.e., the

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133 *swrA*$^+$ *degU32*^{Hy} strain remained non-transformable (0.7% efficiency). However, the presence of SwrA in the
134 *degS*^{Hy} strain was sufficient to restore competence to 36% of efficiency (Fig. 1).

135 Taken together, these data indicate that if SwrA is functional, competence is reduced but no longer
136 abolished by phosphorylation of DegU. Thus, SwrA is able to modulate the activity of DegU~P, partially
137 suppressing its negative effect. This positive action is possible only in the presence of a *degS*^{Hy} mutation,
138 i.e., in the presence of a phosphorylated wild-type DegU protein.

139 **SwrA and subtilisin expression.** The restoration of competence in a *degS*^{Hy} strain prompted us to extend
140 our investigation to *aprE* expression, which is known to be induced by the presence of a Hy mutation in
141 either *degS* or *degU* (31). As already described, *swrA*$^+$ revertants often arise in the *swrA*$^-$ population upon
142 long incubations and might generate confusing results. To avoid the development of such revertants, a
143 *swrA* null mutant was created together with an isogenic *swrA*$^+$ strain. To verify whether SwrA has a role also
144 in subtilisin production, the P_{aprE}-GFP reporter, developed by Veening et al. (32), was inserted in *swrA*$^+$ and
145 Δ*swrA* JH642-derived strains. Analyses were carried out by imaging flow cytometry, which not only allows
146 to quantify the average single cell fluorescence, but also to dissect the *aprE*-ON and -OFF populations due
147 to heterogeneity in *aprE* expression (32, 33). As the expression of the reporter was not detected in these
148 conditions (data not shown), a *degU32*^{Hy} or *degS200*^{Hy} allele was introduced in each strain. The analyses of
149 the P_{aprE}-GFP *degS*^{Hy} or *degU*^{Hy} in both *swrA*$^+$ and Δ*swrA* were focused on the transition phase (T_0), and 5
150 and 15 h later (T_5 and T_{15}). As shown in Fig. 2A, in DegU^{Hy} strains, the percentage of *aprE*-ON cells did not
151 substantially vary in dependence of the presence of SwrA both at T_0 and at later time points. Conversely, in
152 DegS^{Hy} strains the percentage of *aprE*-ON cells was highly affected by SwrA. The presence of SwrA led to a
153 substantial decrease in the number of ON cells, particularly at T_0 (-70%). Moreover, the percentage of ON
154 cells was similar between DegU32^{Hy} or DegS200^{Hy} mutants in Δ*swrA* strains, but it was significantly reduced
155 in the *swrA*$^+$ *degS200*^{Hy} background.

156 Also, the expression level of P_{aprE}, i.e., the single cell fluorescent intensity, did not vary in the
157 presence or absence of SwrA in DegU^{Hy} strains; however, in DegS^{Hy} mutants the presence of SwrA
158 significantly decreased fluorescence intensity (Fig. 2B). Also in this case, there are no appreciable
159 differences in GFP levels among *ΔswrA* strains, while in the *swrA*$^+$ background the *degS200*^{Hy} allele is not as
160 efficient as *degU*^{Hy} in driving *aprE* expression. A gallery of images of *aprE* ON and OFF cells acquired during
161 flow cytometry are provided in Fig. S2.

162 These data allow to conclude that SwrA modulates the activity of DegU~P also at the *aprE*
163 promoter. SwrA reduces the efficacy of DegU~P on subtilisin expression. Analogously to what observed in
164 competence, the SwrA-mediated effect only occurs in the presence of a *degS*^{Hy} mutation, i.e., in the
165 presence of a wild-type phosphorylated DegU protein.

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167 **DegS^{Hy} and DegU^{Hy} mutants in *pgs* expression.** The activation of the *pgs* operon expression is known to
168 depend on the co-presence of at least a *degS*/U^{Hy} allele and SwrA. However, so far, most of the data have
169 been collected using DegU^{Hy} mutants, while scant information is given on γ-PGA production in DegS^{Hy}
170 strains (24). To fill this gap, a P_{pgs}-sfGFP reporter construct was devised and inserted *in locus* in the *swrA*$^{+}$
171 laboratory strain. Since no fluorescence was detected in this strain (data not shown), it was further
172 transformed with either *degS*^{Hy} or *degU*^{Hy} alleles. The Hy strains were grown under vigorous shaking in a
173 glutamate-rich medium that supports γ-PGA production, with periodic sampling over a 48-h prolonged
174 incubation. At relevant time-points, P_{pgs} expression was quantified by imaging flow cytometry. In the
175 DegU^{Hy} mutant, P_{pgs} appeared to be homogeneously active from the beginning of the analysis (2-h post
176 inoculum, data not shown), with intensity reaching a peak at T_{-2} (8-h post inoculum). This early peak of
177 maximal intensity was followed by a monomodal decline over the next 40 h (Fig. 3A), with the majority of
178 the population already OFF after T_{18}. Conversely, in the DegS^{Hy} strain, P_{pgs} activation showed a 2-h delay:
179 cells started displaying fluorescence at T_0 (10-h post inoculum), with a gradual increase over time. Intensity
180 reached a peak at T_{14} which was followed by a slower decline of the GFP signal, which remained however
181 appreciable, in most of the cell population, up to the end of the experiment (T_{38}) (Fig. 3B).

182 From these data it emerges that the impact of SwrA on *pgs* transcription is dramatic for both Hy
183 mutants: no transcription is observed in *swrA*$^{-}$ backgrounds (22-24), and data not shown). However, there is
184 a remarkable difference in the expression profile using the two partner proteins; upon interaction with
185 SwrA, the constitutively active mutant protein DegU32^{Hy} immediately exerts its pressure on the *pgs*
186 promoter but the effect is rapidly relieved. In the *degS*^{Hy} strain, a delay in *pgs* activation is observed, most
187 likely due to the requirement of the physiological trigger of the signalling pathway. However, once
188 activated, the SwrA-DegU~P stimulus on P_{pgs} is sustained up to 24 h (T_{14}), although the intensity of the
189 fluorescent signal is reduced with respect to what observed in cells containing DegU^{Hy}. These data are in
190 line with our experimental evidence that DegS^{Hy} strains produce a much higher amount of γ-PGA (data not
191 shown) and indicate that, although an interaction between SwrA and DegU^{Hy} occurs, the effect on
192 transcription is considerably different from that obtained when SwrA interacts with DegU~P.

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194 DISCUSSION

195 This work extends the array of DegS/U regulated phenotypes in which SwrA plays a pivotal role.
196 The data have been summarized in Table 2. Considering the phenotypes thus far analyzed, SwrA emerges
197 as key modulator of DegS/U on all the promoters tested so far, P_{aprE}, P_{pgs} (Figs. 2 & 3) and $P_{fla/che,}$ (16) (Fig. 4).
198 Notably, SwrA also mitigates the negative effect of DegU~P on genetic competence (Fig. 1) and makes
199 *degS*^{Hy} *swrA*$^{+}$ strains easily transformable.

200 The results shown in this work do not confute literature data obtained in *swrA*$^{-}$ domestic *B. subtilis*
201 strains (168, JH642, and others) (18). The non-transformability of *degU*^{Hy} as well as *degS*^{Hy} *swrA*$^{-}$ strains is
202 indeed validated in our experimental settings (Fig. 1). Rather, a piece of literature data appears to support
203 our results. In 1991, Hahn and Dubnau, analyzing the impact of *degU32*^{Hy} and *degS200*^{Hy} alleles on P_{srfA}
204 expression, could not interpret the fact that, differently from DegU^{Hy}, DegS^{Hy} did not repress *srfA*
205 transcription (34). It is tempting to imagine that a high percentage of *swrA*$^{+}$ revertant cells arose in the
206 DegS^{Hy} strain used in the experiment, due to the high frequency of phase variation events (10^{-4}) (17), and in
207 those revertants SwrA was able to mitigate -or supress- the negative effect of DegU~P, turning it into a less
208 negative -or positive- signal.

209 Presently, the main target of DegU~P in competence has not been clearly identified, because of the
210 coexistence of at least two possible target genes: P_{comK} (8, 27, 28) and P_{srfA} (28, 34). A negative effect of the
211 *degQ*^{Hy} allele has been evidenced on both promoters, in domestic and undomesticated strains (*swrA*$^{-}$ and
212 *swrA*$^{+}$, respectively) (28). Since the *degQ*^{Hy} mutation increases DegU~P levels (5) without impacting on the
213 DegU structure, its interaction with SwrA is preserved. The way in which the effects of SwrA and DegQ are
214 balanced needs to be further analyzed in well-defined genetic backgrounds.

215 A second fundamental result that emerges from this work is that the DegU32^{Hy} mutant protein
216 does not behave as the phosphorylated wild-type DegU protein. A proxy for DegU~P is represented by the
217 *degS200*^{Hy} mutation, which produces high levels of DegU~P without directly modifying the structure of the
218 transcriptional activator DegU. Moreover, from the lack of activation of the *pgs* promoter in
219 undomesticated strains, which are naturally *swrA*$^{+}$ *degQ*^{Hy} (data not shown), it can be hypothesized that the
220 level of DegU phosphorylation attained in *degQ*^{Hy} cells is lower compared to that gained with the *degS200*^{Hy}
221 mutation.

222 From P_{pgs} analyzes it can be hypothesized that DegU32^{Hy} is able to bind directly to DNA, even
223 before activation of the signalling pathway that would lead to its phosphorylation, i.e., in the non-
224 phosphorylated form (see Fig. 3A). This notion is not novel: Stanely-Wall and collaborators showed that γ-
225 PGA production in a *degU32*^{Hy} background also occurs in a *degS* null mutant (35). Also *in vitro*, Mordini et
226 al. (2013) showed that DegU32^{Hy} binds to DNA independently from the presence of its cognate kinase.
227 Moreover, the interaction of SwrA (physical or genetical) with the mutant DegU32^{Hy} protein is

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228 compromised. Either it does not occur at all, as it appears by the lack of differences between the
229 phenotypes of *degU32*^{Hy} *swrA*$^{+}$ and *swrA*$^{-}$ strains in competence, *aprE* expression and motility (Figs. 1 & 2
230 and ref. 16), or it markedly differs from the interaction with DegU~P produced by *degS200*^{Hy}, as it appears
231 from the differential activation profile of P_{pgs} in the two mutants. In any case, the physiological role of
232 DegU~P in *B. subtilis* should be approached using a DegS200^{Hy} mutant. This also suggests that our current
233 view of the impact of the DegS/U on *B. subtilis* physiology gained through the use of *degU32*^{Hy} mutants
234 might require some revamping, as it happened for motility.

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235 MATERIALS AND METHODS

236 Strain construction

237 All strains used in this study are listed in Table 1.

238 PB5630, corresponding to strain DK1042 obtained by D. Kearns and co-workers (29) by introducing the
239 *comI*^{Q12L} mutation in the resident plasmid of the undomesticated NCIB3610, was transformed with
240 pLoxSpec/degSU(Hy) and pLoxSpec/degS200 (24). PB5814 and PB5815, respectively, were obtained after
241 selection for spectinomycin resistance (60 µg/ml).

242 The clean deletion of the *swrA* gene was obtained by transforming PB5249 with pCCΔswrA, a non-
243 replicative plasmid that, completely removing the *swrA* ORF, inserts a kanamycin resistance gene upstream
244 of the *swrA* promoter to control the expression of the downstream *minJ* gene. pCCΔswrA plasmid was
245 obtained through the following steps: a PCR fragment comprising the region upstream the *swrA* gene,
246 containing all the regulatory elements, was amplified from PB5249 genomic DNA with primers UPPromA/E
247 (*Eco*RI)5'-ccgaattctttgtgcttaaagagattatggatc-3' and CC_A_rev (*Xho*I) 5'-
248 aacgctcgagttgtgaacccccattttctttatacagataagcac-3'; the initial part of the following ORF, *minJ*, was amplified
249 from the same source with primers CC_B_for (*Xho*I) 5'-accgctcgaggtgtctgttcaatggggaattgaactgttaaaaagc-3'
250 and CC_C_rev (*Sma*I) 5'-tcccccggggtttgccagctgctgtccgatcg-3'. The two products were digested with *Xho*I
251 (restriction sites underlined) and ligated. The 934 bp resulting product was inserted between the *Eco*RI and
252 *Sma*I sites of the pJM114-derived pCC1 (21). The plasmid pCCΔswrA, verified by multiple restriction
253 digestion and by sequencing of the relevant portions.

254 The plasmid pCCΔswrA was used to transform PB5249. PB5606 was obtained by selecting one clone for
255 kanamycin (2 µg/ml) resistance; deletion of the coding sequence of *swrA* and the integrity of its promoter
256 and *minJ* were verified by PCR and DNA sequencing.

257 The P_{aprE}-*gfp* strains were obtained by *in-locus* integration of the pGFP-aprE plasmid (a generous gift from
258 Prof. J.W. Veening, ref. 32) into the chromosome of *swrA*$^{+}$ and *ΔswrA* isogenic strains, respectively PB5393
259 (21) and PB5606 (described above), both carrying a kanamycin resistance gene upstream of the *swrA*
260 promoter region. The resulting strains were named PB5717 and PB5719, respectively (Table 1). *degU32*(Hy)
261 and *degS200*(Hy) alleles, were introduced in PB5717 and PB5719 by transformation with
262 pLoxSpec/degSUHy) and pLoxSpec/degS200 (24) and selection for spectinomycin resistance (60 µg/ml). In
263 the derived strains, PB5720, PB5722, PB5723 and PB5725 (Table 1), the single copy insertion of each
264 construct was assessed.

265 The P_{pgs}-SF*gfp* strains were obtained using a modified pMutin vector (pMATywsC). The construction of the
266 plasmid occurred in multiple steps. First, in the pMutin-GFP vector (ECE149, obtained from the Bacillus
267 genetic stock centre, http://www.bgsc.org/) the *gfp* gene was substituted by Gibson assembly with a super

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268 folder version of the GFP (SFgfp) amplified from pECE323 plasmid (Bacillus genetic stock centre) with
269 primers RXeGFPda321- 5'-ggctgcactagtgctcgaattcattatttataaagttcgtccataccgtg-3' and FXeGFPda321- 5'-
270 tcggccggaaggagatatacatatgtcaaaaggagaagaactttttacag-3' to give pMutinsfGFP. The 5' portion of *ywsC*
271 together with the Phyperspank promoter were inserted in the resulting pMutinsfGFP through a tripartite
272 Gibson assembly. The Phyperspank promoter was amplified from plasmid Phyp.R0.sfGFP(sp).LacI_operon
273 (36) using primers PHypFor 5'-agcttccaagaaagatatccctcggatacccttactctgttg-3' and

274 PHypRev 5'-ggctataatgagtaaccacatgtttgtcctccttattagttaatc-3'; the 5' portion of *ywsC* (647 bp) was amplified
275 from PB5249 chromosomal DNA using primers ywsCFor 5'-
276 taactaataaggaggacaaacatgtggttactcattatagcctgtg-3' and ywsCRev 5'-
277 gtaaaaagttcttctccttttgacagagaagcgttatcagggaatac-3'. In the plasmid obtained, pPhsywsCsfGFP, the spoVG
278 RBS and initial codons were translationally fused to the sfGFP using the partially overlapping oligos
279 oligoFORSpoVG 5'-
280 ccctgataacgcttctctggaattcccgggatccccagcttgttgatacactaatgcttttatatagggaaaaggtggtgaactactatgTCAAAAGGAG
281 - 3' and oligoREVSpoVG 5'-
282 CTCCTTTTGAcatagtagttcaccaccttttccctatataaaagcattagtgtatcaacaagctggggatcccgggaattccagagaagcgttatcaggg
283 - 3', derived from the pJM116 vector (37) The final construct was verified by sequencing and saved as
284 pMATywsC. This plasmid was used to transform PB5249 (*swrA*$^{+}$) and PB5370 (*swrA*$^{-}$), using erythromycin
285 resistance (5 μg/ml) for selection, resulting in PB5741 and PB5742 strains, respectively. *degU32*(Hy) and
286 *degS200*(Hy) alleles were introduced in PB5741 by transformation with pLoxSpec/degSU(Hy) and
287 pLoxSpec/degS200 (24) by spectinomycin resistance (60 μg/ml) selection, giving rise to PB5743 and
288 PB5745, respectively. The single copy insertion of each construct was assessed.

289 Competence evaluation

290 Cells were inoculated in LM (LB supplemented with $MgSO_4$, 9μM; tryptophan, 50 μg/mL; phenylalanine, 50
291 μg/mL) at OD_{600}=0.2 and grown at 37°C shaking. At OD_{600}=1, cells were diluted 1:20 in MD (K_2HPO_4, 9.8
292 mg/ml; KH_2PO_4, 5.52 mg/ml; $Na_3Citrate{\cdot}5H_2O$, 0.92 mg/ml; glucose, 20 mg/ml; tryptophan, 50 μg/ml;
293 phenylalanine, 50 μg/ml; ferric ammonium citrate, 11 μg/ml; K-aspartate, 2.5 mg/ml; $MgSO_4$, 0.36 mg/ml)
294 and grown at 37°C until stationary phase (T_0). About 200 ng of chloramphenicol (Cm)-selectable *B. subtilis*
295 chromosomal DNA was added to 0.5 ml cells which were further incubated for 1.5 h at 37°C with shaking.
296 Transformants were isolated on 5 mg/ml chloramphenicol on several selective plates. Resistant colonies
297 were counted and related to cell density at T_0 to calculate the transformation efficiency, taking into account
298 each dilution step before plating. Data shown in Fig. 1 represent the average of three independent
299 experiments.

300 Gene expression evaluation by flow cytometry

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301 For the analysis of P_{aprE} activity, cells were inoculated in Shaeffer's sporulation medium (38) at 0.2 OD_{600} and
302 grown at 37°C under continuous shaking for 20 h. Aliquots were collected every 60' for OD_{600} readings; at
303 the transition point (5h), 5 and 15 h later, 10% glycerol (final concentration) was added to culture aliquots
304 for storage at -20°C.

305 For the analysis of P_{pgs} activity, cells were inoculated in E-medium (39) at OD_{600}=0.1 and grown at 37°C
306 under continuous shaking for 48h. Aliquots were collected at 2-h intervals for OD_{600} readings and direct
307 cytofluorimetric analyses. Before analyses, fresh and/or frozen samples were centrifuged for 5 minutes at
308 14000 xg; cell pellets were re-suspended in D-PBS for flow cytometry (Gibco).

309 Samples were acquired on an Amnis® ImageStream®X Mk II Imaging Flow Cytometer using the INSPIRE
310 software with the following set up: Channel 02 (GFP fluorescence), Channel 06 (scattering channel); the
311 Brightfield was visualized on Channel 01 and on Channel 05, depending on the GFP expression level, to
312 avoid interference from Channel 02. The 488 nm laser was used at either 50 mW or 100 mW power,
313 according to the GFP expression level, in order to avoid sensor saturation. The flow rate was set to low
314 speed/high sensitivity and images were taken at 60X magnification. For each sample at least 10000 events
315 were acquired.

316 All data were analyzed using the IDEAS software (version 6.2). In-focus events were gated in a histogram
317 displaying the Gradient RMS_M01_Ch01 on the x-axis. A plot of the Area versus Aspect Ratio Intensity in
318 the Brightfield channel was used to exclude doublets from the analysis. A plot of the Area versus Intensity
319 in the Scattering channel was used to exclude events characterized by high scatter such as beads. To avoid
320 any bias due to cell size in evaluating fluorescence intensity, the GFP level of each cell was calculated
321 through the Median Pixel feature on the fluorescence channel. The threshold value to distinguish the ON
322 population was set at the maximum autofluorescence of a non-fluorescent population used as negative
323 control (OFF). Data presented in Figs. 2 and 3 represent the average of three independent experiments.

324

325 ACKNOWLEDGEMENTS

326 This research was supported by the Italian Ministry of Education, University and Research (MIUR):
327 Dipartimenti di Eccellenza Program (2018–2022) - Dept. of Biology and Biotechnology "L. Spallanzani",
328 University of Pavia. Authors are grateful to Prof. J. W. Veening for the P_{aprE} plasmid. The contribution of
329 Erlinda Rama, Matteo Cavaletti, Jessica Bollini, Laura Nucci is acknowledged. We also thank Dr A. Azzalin,
330 from the departmental imaging facility, and Dr A. Serra, from Luminex Corporation, for support with the
331 Amnis ImageStream data acquisition and analyses.

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332 Table 1

Strain	**Relevant genotype**	**Source or reference**
PB5630	*comI*^{Q12L}	BGSC # 3A38
PB 5814	*comI*^{Q12L} *degU32*(Hy); Sp	This study
PB 5815	*comI*^{Q12L} *degS200*(Hy); Sp	This study
PB5249	*trpC2 pheA1 swrA*$^{+}$	(Calvio *et al.*, 2008)
PB5370	*trpC2 pheA1 swrA*$^{-}$	(Calvio *et al.*, 2008)
PB5606	*trpC2 pheA1 ΔswrA*; Km	This study
PB5383	*trpC2 pheA1 swrA*$^{+}$ *degU32*(Hy); Sp	(Osera *et al.*, 2009)
PB5384	*trpC2 pheA1 swrA*$^{-}$ *degU32*(Hy); Sp	(Osera *et al.*, 2009)
PB5390	*trpC2 pheA1 swrA*$^{+}$ *degS200*(Hy); Sp	(Osera *et al.*, 2009)
PB5391	*trpC2 pheA1 swrA*$^{-}$ *degS200*(Hy); Sp	(Osera *et al.*, 2009)
PB5717	*trpC2 pheA1 swrA*$^{+}$ P_{aprE}-*gfp*; Km, Cm	(This study)
PB5719	*trpC2 pheA1 ΔswrA* P_{aprE}-*gfp*; Km, Cm	(This study)
PB5720	*trpC2 pheA1 swrA*$^{+}$ P_{aprE}-*gfp degU32*(Hy); Km, Cm, Sp	(This study)
PB5722	*trpC2 pheA1 ΔswrA* P_{aprE}-*gfp degU32*(Hy); Km, Cm, Sp	(This study)
PB5723	*trpC2 pheA1 swrA*$^{+}$ P_{aprE}-*gfp degS200*(Hy); Km, Cm, Sp	(This study)
PB5725	*trpC2 pheA1 ΔswrA* P_{aprE}-*gfp degS200*(Hy); Km, Cm, Sp	(This study)
PB5741	*trpC2 pheA1 swrA*$^{+}$ *ywsC::RBSspoVG:sfGFP_Phyperspank::ywsC*; Em	(This study)
PB5742	*trpC2 pheA1 swrA*$^{-}$ *ywsC::RBSspoVG:sfGFP_Phyperspank::ywsC*; Em	(This study)
PB5743	*trpC2 pheA1 swrA*$^{+}$ *ywsC::RBSspoVG:sfGFP_Phyperspank::ywsC degU32*(Hy); Em, Sp	(This study)
PB5745	*trpC2 pheA1 swrA*$^{+}$ *ywsC::RBSspoVG:sfGFP_Phyperspank::ywsC degS200*(Hy); Em, Sp	(This study)

333

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334 Table 2

335

		Effect of DegU~P [*degS*(Hy)]		
Target	***degS/U*^{wt}*swr*A^{-}**	***swr*A^{-}**	***swrA*$^{+}$**	
$P_{fla/che}$	motile	NEGATIVE (non-motile)	POSITIVE (hyperflagellation)	Mordini et al, 2013
P_{aprE}	no expression	POSITIVE (production)	PARTIALLY NEGATIVE (-38%)	Fig. 2
P_{pgs}	no expression	NONE	POSITIVE (mucoid colonies)	Fig. 3
Competence	competent	NEGATIVE (non-competent)	PARTIALLY POSITIVE (+37%)	Fig. 1

336

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427 FIGURE LEGENDS

428 **Fig. 1. Transformation efficiency.** Competence was evaluated in laboratory strains derived from JH642
429 (*swrA*$^{-}$ *degQ*^{wt} *trpC1 pheA2*), differing for the status of the *degS/U* and *swrA* alleles (listed in Table 1), by
430 counting resistant colonies obtained by transformation with selectable genomic DNA. Each experiment is
431 the average of at least three independent replicates; error bars account for the standard error of the mean.
432 The genotype of each strain is given on the x-axis. In each experiment, 100 % efficiency was assumed for
433 the *swrA*$^{+}$ strain (PB5249).

434

435 **Fig. 2. Expression of P_{aprE}-GFP.** Domestic strains, differing for the status of the *degS/U*^{Hy} alleles and for the
436 presence of a functional *swrA* gene, were analysed by imaging flow cytometry to evaluate the percentage
437 of GFP-ON/OFF cells and the peak of fluorescence intensity. Cultures were sampled at the transition point
438 (T_0), 5 and 15 hours later (T_5 and T_{15}), as indicated below each graph, where the *swrA* status is also
439 indicated. A, C: data collected for the *degU*32^{Hy} strains; B, D: data collected for *degS*200^{Hy} strains. The upper
440 panels, A and B, represent the percentage of cells expressing the reporter gene (ON-population). The lower
441 panels, C and D, show the peak of intensity of the ON-population. Values represent the average of at least
442 three independent replicates; error bars account for the standard error of the mean.

443

444 **Fig. 3. Expression profile of P_{pgs}-GFP.** Strains (A) *swrA*$^{+}$ *degU*^{Hy} and (B) *swrA*$^{+}$*degS*^{Hy} were grown in E-
445 medium and cells were collected for imaging flow cytometry at different time points. The colour of the
446 plots refers to the collection time (in h relative to the transition point), which is indicated in each graph. A
447 dashed line marks the ON threshold. The intensity values, represented in logarithmic scale on the x-axis,
448 refer to the median pixel intensity of each single event.

449

450 **Fig. 4. SwrA effects on DegS/U regulated genes.** The schematic representations above summarizes the
451 data collected on *aprE*, *pgs* and *fla/che* transcription on the global effects exerted DegU~P alone (left) and
452 with SwrA (right). The size of the curved arrows in front of each gene is indicative of the efficiency of
453 transcription. Symbols are described in the box at the bottom.

454

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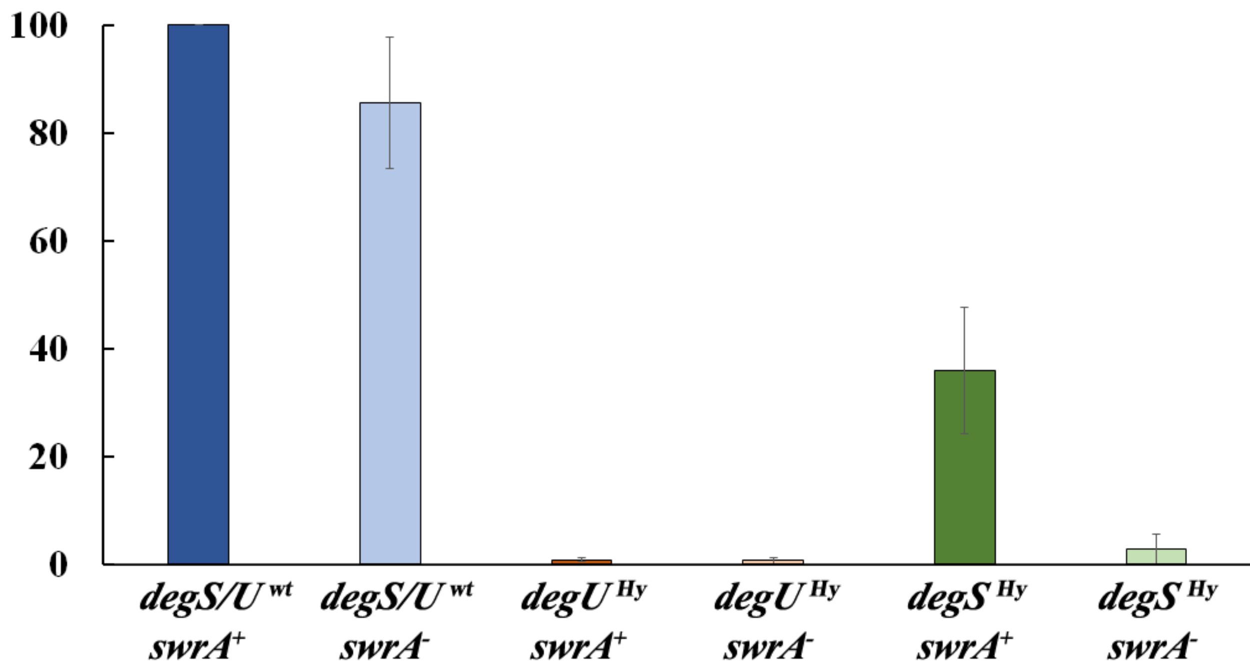
455

FIGURE 1



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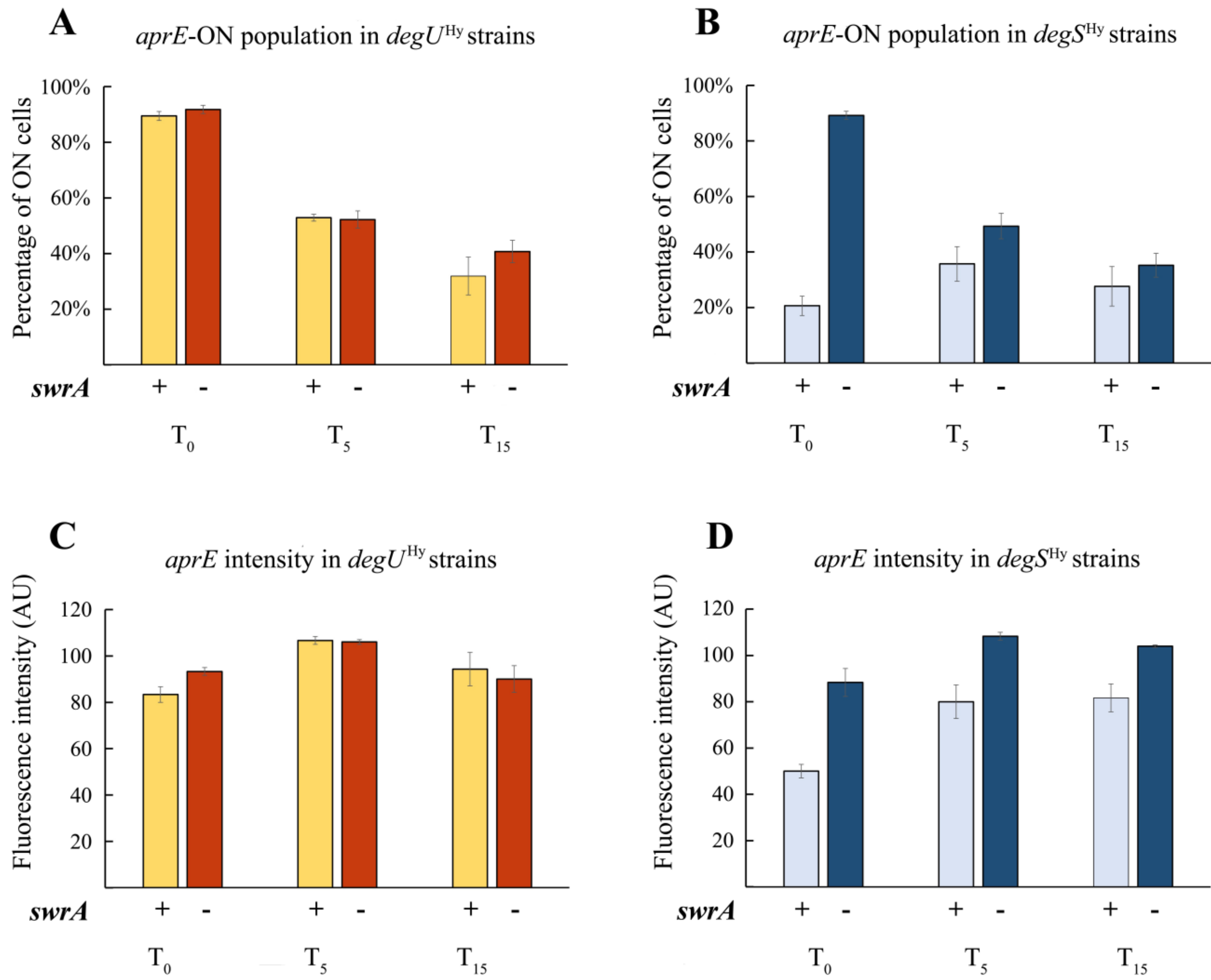
458

FIGURE 2



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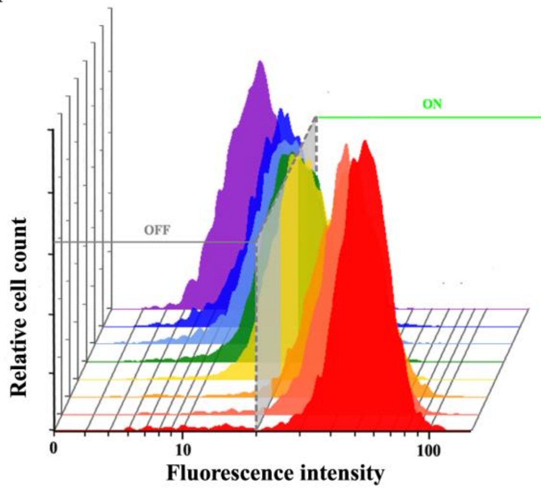
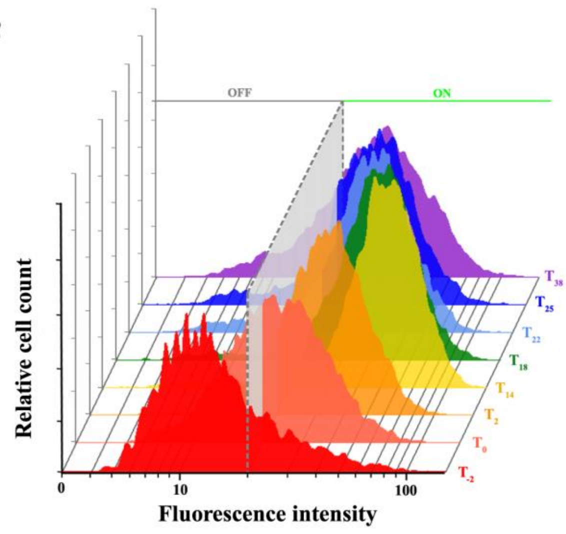
461

FIGURE 3





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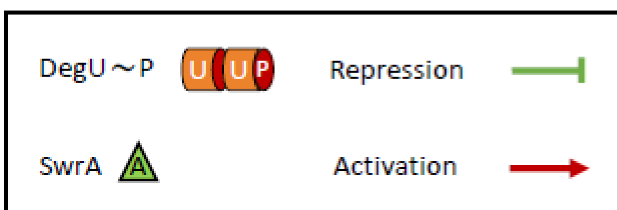
464

FIGURE 4

aprE *aprE*

pgsB *pgsB*

fla/che *fla/che*



465

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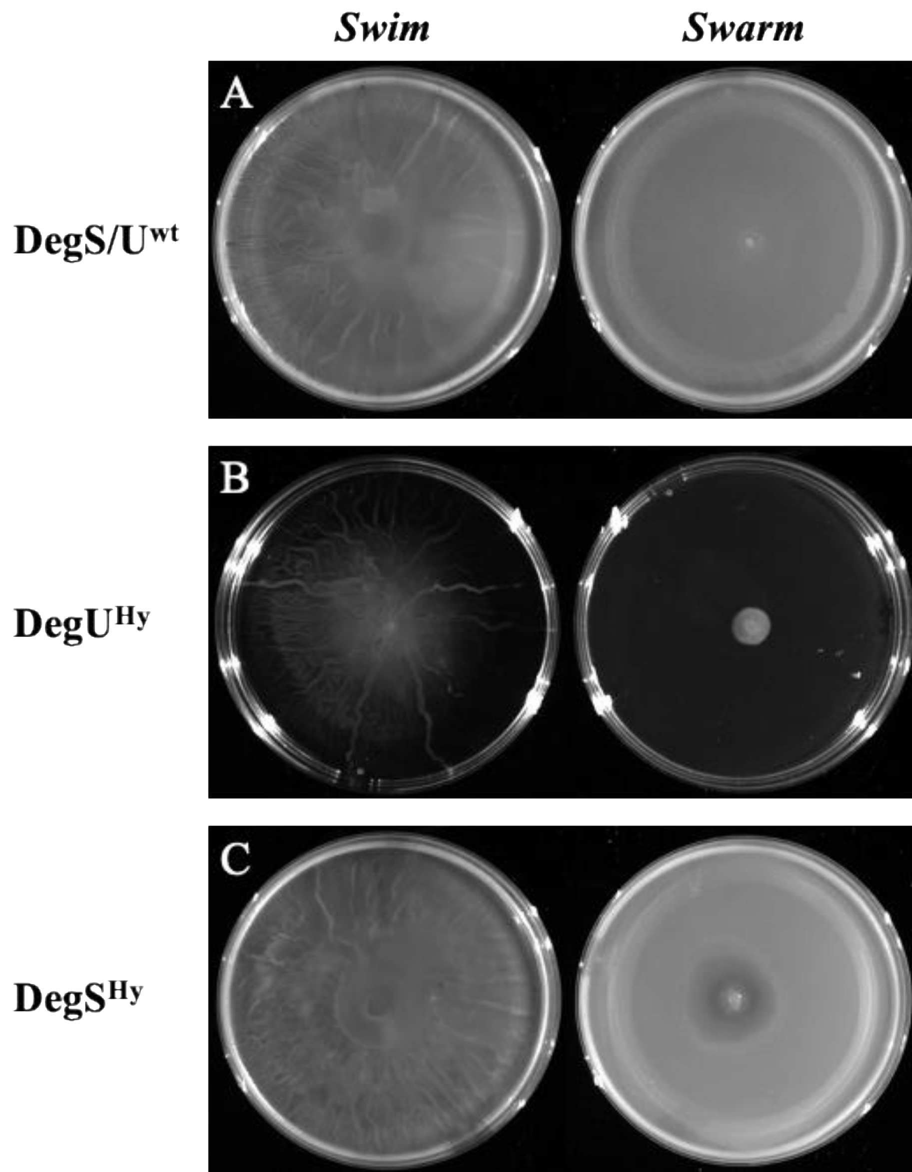
466 # SUPPLEMENTARY MATERIALS

467

468



469–489

490 **Fig. S1. Motility of undomesticated DegU/S^{Hy} strains.** Swimming (on the left of each panel) and swarming
491 (on the right) motility performances of undomesticated strains (*swrA*$^{+}$). A): PB5630 *degS/U*^{wt}; B): PB5814
492 *degU*^{Hy}; C) PB5815 *degS*^{Hy}. The genotype of each strain is also indicated on the right. Strains are listed in
493 Table 1.

494 Motility plates were prepared as described in Mordini *et al.*, 2013, except for the addition of surfactin, that
495 was omitted in the swarming plates.

496

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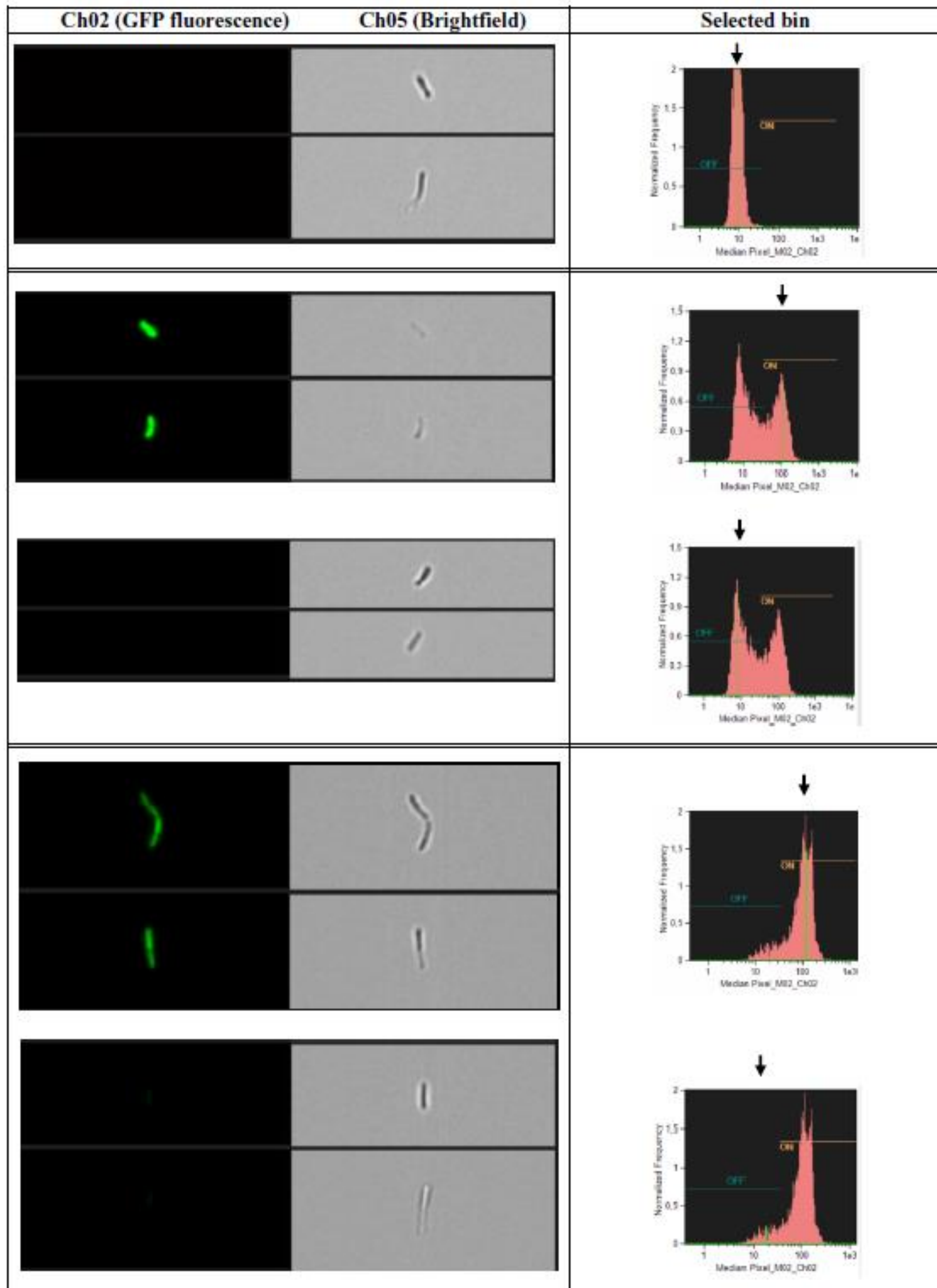

497

498 **Fig. S2. Cell images collected during Flow Cytometry.** Representative images of P_{aprE}-GFP
499 containing cells collected from the different intensity bins which are pointed by an arrow on the
500 graphs in the right panels. On the left, identical images as acquired in the fluorescence channel
501 (Ch02) and in the brightfield channel (Ch05).