Rampant loss of social traits during domestication of a wild *Bacillus subtilis*

Hugo C. Barreto[1,2], Tiago N. Cordeiro[2], Adriano O. Henriques[2*], and Isabel Gordo[1*]

[1]Instituto Gulbenkian de Ciências, Oeiras, Portugal

[2]Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Oeiras, Portugal

*** Corresponding author**

E-mail: igordo@igc.gulbenkian.pt (I.G.), aoh@itqb.unl.pt (A.O.H.)

Abstract

Most well-studied bacteria have been domesticated to some extent. How fast can a wild strain diverge from its ancestral phenotypes under domestication to a novel laboratory environment is poorly known. Yet such information is key to understand rates of evolution, the time scale at which a wild strain can be propagated without loss of its natural adaptive traits and the reliability of experimental results across labs. Using experimental evolution, phenotypic assays and whole-genome sequencing, we show that within a week of propagation in a common laboratory environment, a wild strain of *Bacillus subtilis* acquires mutations that cause changes in a multitude of traits. A single adaptive mutational step, in the gene coding for the transcriptional regulator DegU, impairs a DegU-dependent positive autoregulatory loop and leads to loss of robust biofilm architecture, impaired swarming motility, reduced secretion of exoproteases and changes in the dynamics of sporulation across environments. Importantly, domestication also resulted in improved fitness when the bacteria face pressure from cells of the innate immune system. These results show that *degU* is a key target for mutations during domestication and also underscore the importance of performing careful and extremely short-term propagations of wild isolates to conserve the traits encoded in their original genomes.

Author Summary

Domestication is the process by which organisms are selected to live in specific conditions and an important phenomenon that shapes the evolution and variation in many animals and plants. In microbes, domestication is also a key driver of adaptation. It can be beneficial, when improving microbes abilities that are important for biotechnology, but also problematic, especially when studying microbe-host interactions and the microbe natural behavior. Using a wild isolate of *Bacillus subtilis*, we determined the speed and genetic basis of microbial domestication using experimental evolution. Within 1 week of growth in the common laboratory media, mutations in the pleiotropic transcriptional regulator, DegU, emerge and spread in the populations. These lead to loss of social traits, increased resistance to bacteriophages and macrophages. The data highlights the extreme caution that is needed when culturing natural microbial isolates and may help explain why some key microbial social traits and behaviors may differ between different laboratories, even when these study the same strains.

Introduction

Most bacteria grown in the laboratory face environmental conditions that are very distinct from those in their natural habitat. In nature, hardly anywhere a bacterium would find a niche with plentiful nutrients and optimal aeration. Therefore, when sampled from the diverse natural world and subsequently cultured in the lab, bacteria can adapt genetically to a truly novel environment. Such evolutionary domestication can result in increased fitness in the lab at the cost of loss of previous adaptations [1]. Some studies in *Escherichia coli*, *Bacillus subtilis*, *Caulobacter crescentus,* and *Saccharomyces cerevisiae* have shown that adaptation to laboratory environments occurs via diverse genotypic paths, but some evolutionary parallelism has also been observed [2–5]. The tempo and mode of evolution under domestication is still poorly known. Yet, it is important to quantify how many of the current model bacteria have taken undetermined domestication paths.

The model organism for spore-forming bacteria, *B. subtilis* strain 168, is known to have acquired mutations during its laboratory life, which lead to loss of traits likely to be important for its natural life cycle in the soil, root plants or the gastrointestinal tract of various organisms [6]. Robust biofilm formation is one of such traits. Using genetic engineering it was possible to restore biofilm phenotypes exhibited by the parental less domesticated strain NCIB 3610 [6]. The latter is commonly used as a model for biofilm development. A recent study, however, as shown that this strain carries two mutations, one in *rapP* and the other in *dtd* [7], which lead to impaired of biofilm formation [8,9], rendering it less than an ideal model for wild *B. subtilis* strains with high capacity to form biofilms.

The ability to produce endospores (spores for simplicity) is another important trait of the life cycle of *B. subtilis*. Spores are highly resistant to external stresses and largely

responsible for the widespread dissemination of *B. subtilis*. The production of spores is a response to extreme nutrient depletion and under laboratory conditions it is triggered at the onset of the stationary phase of growth [10]. A recently characterized wild isolate of *B. subtilis*, BSP1, starts the process of spore formation during growth, unlike its domesticated relatives, and reaches a higher spore titer [11]. This occurs as a result of the main activator of the sporulation process, Spo0A~P, reaching higher levels per cell and in a larger fraction of the population during exponential growth. Spo0A is activated by phosphorylation by means of a phosphorelay that integrates multiple environmental, cell cycle and nutritional cues [10,11]. The precocious and increased sporulation of BSP1 is due to the lack of two genes coding for Rap phosphatases, able to drain phosphoryl groups from the phosphorelay, through the dephosphorylation of Spo0F, a phosphorelay component [11].

The large number of traits that can potentially be lost during continuous growth of *B. subtilis* in classical laboratory environments and the lack of knowledge regarding its first steps of adaptation makes it imperative to understand when and how the domestication process occurs. Experimental evolution offers a powerful methodology to study the dynamics of the repeatability of evolutionary change under the same laboratory conditions and to study domestication [12]. When coupled with genome sequencing, it allows a real-time assessment of the speed and genetic basis of adaptation to novel environments, as well as the order at which adaptive mutations fix in evolving populations [13–16].

Here we have used experimental evolution to follow the evolutionary path taken by a wild *B. subtilis* during domestication to a common laboratory environment. We found that within one week (8 passages), mutations in DegU, coding for a global transcriptional regulator [17–19], spread and cause a rapid change in several social

traits of *B. subtilis*, likely to be important for its fitness in the wild [20]. Using gene-editing we show that one of the *degU* mutations causes attenuation of swarming motility, reduction of biofilm production and alteration of its architecture, increased resistance to bacteriophage SPP1 infection and reduced exoprotease secretion. Importantly, the initial process of domestication also resulted in a change of the sporulation dynamics across environments and a fitness improvement when the bacteria face pressure from the innate immune system. DegU thus emerges as a central mutational target during domestication. Overall, our results indicate that the propagation of wild isolates in the laboratory should be performed with extreme care as domestication can lead to rampant loss of traits that while important for the *B. subtilis* natural life cycle are largely dispensable under laboratory conditions.

Results

Emergence of a new adaptive colony morphology during *B. subtilis* domestication.

Five populations derived from a wild *B. subtilis* isolate (BSP1) hereinafter named Ancestral, were daily passaged for sixteen days, through dilution in a rich medium with agitation and aeration. Samples were frozen every two days so that evolutionary steps during short-term domestication could be followed through whole-genome sequencing. Daily plating revealed that, within the first week of the experiment, two new colony morphotypes emerged (Fig 1A). The ancestral type *a* dominated the initial populations, but a new type *b*, characterized by a flat colony morphotype, and a type *c*, an intermediate morphotype, reached appreciable frequencies rapidly. Type *b* was detected in all the populations, while type *c* was only observed in three out of five populations (Fig 1B). As type *b* achieved the highest frequency, reaching fixation after sixteen days in population 1 (Fig 1C), we conducted a detailed phenotypic and genetic characterization of its clones. The rapid spread of type *b*, as well as its repeated emergence, suggests that it carries a fitness advantage. To test this hypothesis, we selected a type *b* colony from population 1 at day eight, hereinafter named Evolved, and characterized its growth (S1A Fig). Indeed both the maximum growth rate and the carrying capacity of the Evolved were significantly higher than those of the Ancestral (S1B Fig). This shows that Evolved has increased fitness in the laboratory environment, and thus can outcompete Ancestral.

DegU as a main target for the adaptation to the laboratory environment.

To determine the genetic basis of the adaptive morphotype we performed whole-genome sequencing of the Ancestral and the Evolved clone. Only one mutation was observed, in the *degU* gene, coding for the response regulator DegU [21]. The non-

synonymous mutation is a T-to-G transition causing the substitution of isoleucine 186, in the helix-turn-helix motif (HTH), within the DNA-binding domain of DegU, by a methionine (I186M) [21] (Fig 2A). *degU* is part of the *degS-degU* operon, coding for a two-component system that controls social behaviors in *B. subtilis* [17,18,22,23]. This suggested that the I186M substitution caused an adaptive change capable of affecting several traits. The unphosphorylated form of DegU activates genes involved in the ability to uptake external DNA during competence development [24]. During growth, the intracellular concentration of the phosphorylated form of DegU (DegU~P), increases, leading to the progressive activation of genes required for swarming motility, biofilm formation and the secretion of extracellular enzymes [22,25]. Given the central importance of DegU in processes that could be costly in the laboratory environment we tested whether additional clones, that had evolved independently, also carried mutations in *degU*. Sanger sequencing revealed that all the five populations had mutations in *degU*: in addition to the mutation causing the I186M substitution (Evolved, in population 1; see above), the same mutation was identified in a clone from population 4; a mutation causing the substitution of histidine 200 by a tyrosine (H200Y) was identified in two populations (in population 1 and in population 5), and another, causing the replacement of valine 131 by an aspartate (V131D) was identified in one (population 2) (Figs 2A and 2B). Finally, one mutation caused the insertion of a TGA codon leading to premature translation arrest after codon D18 (Figs 2A and 2C; population 3). These populations exhibited distinct colony morphologies but all appeared less structured than the colonies formed by Ancestral (Fig 2C). The V131D substitution lies within the linker region that separates the receiver and DNA-binding domains of DegU, while H200Y (as for I186M; above) is located in the HTH motif (Fig 2B). All of the missense mutations affect amino acid residues that are conserved among DegU orthologs (S2A and S2B Figs) and are

thus likely to be functionally important. In particular, the I186M and H200Y substitutions are likely to affect DNA binding (Fig 2A; see also S1 Text for a discussion of the effect of the various substitutions). These results suggest a high degree of evolutionary parallelism at the gene level and implicate DegU as a main target for the domestication to the laboratory.

The I186M substitution in DegU is responsible for the colony phenotype of Evolved.

We proceeded with a detailed characterization of the Evolved from population 1 which carried a I186M substitution in DegU. To test whether this mutation caused the alteration in colony morphology, the wildtype *degU* allele (or $degU^{Anc}$, for Ancestral) and the allele coding for $DegU^{I186M}$ (found in population 1, or $degU^{Evo}$) were introduced ectopically at the non-essential *amyE* gene in a strain bearing a *degU* knockout constructed in the background of Ancestral (S3 Fig; see also S1 Text). The resulting strains, termed $degU^{Anc}$ and $degU^{Evo}$, had a colony morphology indistinguishable from Ancestral and Evolved / $degU^{Evo}$ respectively (Fig 3C). We infer that the $degU^{Evo}$ allele is responsible for the colony phenotype of Evolved.

***degU*^{I186M} causes adaptive loss of major social traits.**

Several social traits are regulated by the unphosphorylated form as well as by low, medium or high levels of DegU~P [18,19]. DegU functions as a “rheostat”, sensing environmental signals and allowing the expression of competence, social motility (or swarming), biofilm development and exoprotease production along a gradient in the cellular accumulation of Deg~P [18]. Competence development is positively regulated by the unphosphorylated form of DegU [24,26,27]. ComK is a regulatory protein required for competence development that drives transcription of the genes coding for the DNA uptake and integration machinery but also stimulates transcription of its own gene [28].

187 Unphosphorylated DegU functions as a priming protein in competence development by
188 binding to the *comK* promoter and facilitating ComK stimulation of *comK* transcription at
189 low ComK concentrations [24,28,29]. We found no differences in the development of
190 competence between Ancestral or $degU^{Anc}$ and Evolved or $degU^{Evo}$ (S4 Fig). This
191 suggests that the I186M substitution does not affect the function of the
192 unphosphorylated form of DegU in promoting competence development and thus that
193 $degU^{Anc}$ is not a loss-of-function allele. Low levels of DegU~P, however, activate
194 transcription of genes involved in social motility, or swarming [17,22,30,31]. Swarming
195 motility assays revealed that while the Ancestral and $degU^{Anc}$ have the ability to swarm,
196 the Evolved and $degU^{Evo}$ show poor swarming ability (Fig 3A). A widely used laboratory
197 strain, PY79, hereinafter termed Lab, as well as other laboratory strains, carry mutations
198 that prevent swarming motility [32–34]. In our assay, Lab as well as a *degU* insertional
199 mutant, are deficient in swarming motility (Fig 3A). Thus, the I186M substitution leads
200 to limited pleiotropic effects, *i.e.*, decreased swarming motility without affecting
201 competence.

202 The production of proteins responsible for biofilm formation is another trait under
203 the control of DegU~P [17,22,35–37]. To query whether biofilm architecture and
204 robustness were affected by the I186M substitution, we examined both the colony
205 architecture over time, the colony being a biofilm formed at the solid medium/air
206 interface, as well as the formation of biofilms at the liquid/air interface in liquid cultures.
207 Both Ancestral and $degU^{Anc}$ showed a complex colony architecture characterized by
208 many wrinkles after 24 h of incubation, and the complexity of the colony architecture
209 increased with time during the assay (Fig 3B). In contrast, Evolved and $degU^{Evo}$ formed
210 colonies that tended to be flatter and with fewer wrinkles (Figs 3B and 3C). As a control,
211 colonies formed by a *degU* insertional mutant show extremely low complexity and

compared to Ancestral, Lab also formed colonies with a simpler architecture (Fig 3C). These results are consistent with the importance of DegU for the formation of a biofilm at a solid medium/air interface [36,37]. Similarly, in liquid cultures, both Evolved and *degU*^{Evo} formed a biofilm at the liquid/air interface less robust than that of the Ancestral as determined by the quantification of the pellicle formed (Fig 4A and S5 Fig). Thus, *degU*^{Evo} strongly affects the ability of *B. subtilis* to form complex, robust biofilms.

Other processes important for *B. subitlis* social natural lifestyle that depend on high levels of DegU~P include the secretion of exoproteases and survival under bacteriophage predation [18,30,38,39]. We found decreased exoprotease secretion in Evolved and the *degU*^{Evo} strain, as compared to Ancestral and *degU*^{Anc} (Fig 4B). For reference, exoprotease secretion was severely impaired in both the *degU* insertional mutant and in Lab (Fig 4B). Lastly, when infected with the bacteriophage SPP1, both Evolved and *degU*^{Evo} showed increased resistance to phage infection, as compared to Ancestral and *degU*^{Anc} or Lab (Fig 4C). Overall, these results reinforce the view that the *degU*^{Evo} mutation is pleiotropic but not fully, as it affects the phenotypes regulated by DegU~P, including social mobility, biofilm formation, exoprotease production and resistance to phage infection, but does not impair the function of unphosphorylated DegU in priming competence.

Decreased transcription of DegU~P-target genes after domestication.

The phenotypic assays performed showed that the I186M substitution in DegU changed traits regulated by DegU~P in the first steps of domestication. The location of the I186M substitution in the HTH motif of DegU raised the possibility that the transcription of genes regulated by DegU~P could be impaired in Evolved in comparison with Ancestral. To test this, we constructed transcriptional fusions between the promoters for the *hag*, *bslA*, and *aprE* genes and the *gfp* gene. *hag* codes for flagellin,

the main component of the flagellum and is required for social motility; and the expression of *hag* indirectly requires low levels of DegU~P [17,40]. *bslA* codes for a self-assembling hydrophobin that forms a hydrophobic coat at the surface of biofilms; as shown in strain NC3610, a model for biofilm development, BslA is required for the formation of structurally complex colonies and biofilms [41–44]. Lastly, *aprE* codes for subtilisin, a major alkaline exoprotease [21,45]. Transcription of both the *bslA* and *aprE* genes is subject to a logic AND gate, in that it requires both derepression of both promoters under the control of Spo0A~P and in addition, DegU~P [17,21,41,46].

We then examined the transcription of these genes at the population (biofilms) and single-cell levels. In Ancestral, the P_{aprE}-, P_{bslA}- and P_{degU}-*gfp* fusions were expressed throughout the architecturally complex colonies, whereas expression of P_{hag}-*gfp* was expressed mostly at the colony edge (Fig 4D). Expression of P_{aprE}-, P_{degU}- and P_{hag}-*gfp* was also detected in Lab, but at lower levels, and expression of P_{bslA}-*gfp* was not detected, consistent with the simpler colony morphology (Fig 4D). Interestingly, in spite of the maintenance of a complex colony architecture, expression of P_{bslA}-, P_{degU}- and P_{hag}-*gfp* was detected at very low levels in Evolved, and expression of P_{aprE}-*gfp* was not detected (Fig 4D). Thus, $DegU^{I186M}$ strongly curtails expression of DegU target genes during biofilm development.

During planktonic growth, transcription of *hag* decreases markedly in the absence of DegU~P [22]. Moreover, expression of *hag* is heterogeneous, with free cells showing higher expression levels than chained cells [47]. Accordingly, in Ancestral, the free cells showed higher fluorescence intensity from the P_{hag}-*gfp* fusion than chained cells (Figs 5A and 5B). In contrast, Evolved showed a decrease in the GFP signal from P_{hag}-*gfp* both in free cells (~2.2 fold) and chains (~2.8 fold), relative to the Ancestral (Fig 5B). Although Lab does not exhibit swarming motility, as previously reported [47] it showed

increased expression of P_{hag}-*gfp* in both free cells and chains when compared with Ancestral, which exhibits swarming motility (Fig 5B). Expression of *bslA* is heterogeneous in the Ancestral, as also found for *hag* (Fig 5A; see also above), and was ~2.8 fold lower in free cells when compared to chained cells (Fig 5B). Strikingly, no heterogeneous expression of *bslA* was observed in the Evolved clone (Fig 5B). Moreover, the level of *bslA* expression was similar in free cells and chains, although *bslA* expression was ~1.5 fold lower in the free cells when compared to Ancestral (Fig 5B). In the Lab strain, which does not produce robust biofilms, transcription of *bslA* is markedly reduced (Fig 5B). DegU~P is a direct positive regulator of *aprE* and expression of *aprE* was shown to be bi-stable in a laboratory strain [46]. Consistent with this finding, low and high levels of *aprE* expression were detected for Lab, both in free cells and chains (Fig 5). In contrast, Ancestral did not express high levels of *aprE*, although it showed heterogeneity in *aprE* expression, both in free cells and chains (Fig 5). Lastly, Evolved showed greatly reduced expression of *aprE* (Fig 5). Thus, domestication is accompanied by a sharp decrease in the expression of *aprE*.

Overall, these results indicate that the I186M substitution in DegU strongly impairs the activity of the protein during biofilm development and planktonic growth.

Domestication impairs a *degU* positive auto-regulatory loop

In its high-level phosphorylated state, DegU~P activates transcription of *degU* itself [48,49] by binding to a site in the *degU* regulatory region [25,50]. Importantly, this positive auto-regulatory loop contributes to the heterogeneous expression of *degU* and the DegU~P-dependent genes [46]. Since Evolved shows reduced expression of DegU~P-target genes, we wanted to test whether $DegU^{I186M}$ also impaired expression of *degU*, reducing the levels of DegU. We found expression of P_{degU}-*gfp* to be heterogeneous in Ancestral, with chains showing a ~1.6 fold higher expression relative

to free cells in line with earlier results (Fig 5) [47]. In sharp contrast, no heterogeneous expression of *degU* was seen for the Evolved and Lab strains, in which expression levels, slightly reduced when compared to Ancestral, were similar between free cells and chains (Fig 5).

Together, these results suggest that I186M impairs the ability of DegU~P to activate transcription of *degU* itself. Impaired activation of the DegU auto-regulatory loop, in turn, reduces transcription of degU itself, explaining why the expression of genes directly or indirectly regulated by DegU~P in Evolved is strongly reduced.

Domestication leads to increased fitness in the presence of cells of the immune system.

B. subtilis has been isolated from the gastrointestinal tract of several animals including humans [51,52] and has been found to grow, sporulate and persist in the murine gut [53,54]. Thus, it can experience selective pressures inside a host. To determine if domestication could impair the ability of *B. subtilis* to withstand a hostile host environment, we measured the fitness of Evolved and Ancestral in the presence of cells of the innate immune system - macrophages. The Evolved strain showed a fitness advantage over the Ancestral in the presence of macrophages (Fig 6A). This result shows that the domestication to the laboratory environment coincidently leads to a fitness advantage for wild *B. subtilis* when facing cells of the host immune system.

Sporulation efficiency changes across environments.

High levels of DegU~P promote sporulation by increasing the levels of Spo0A~P, the master regulatory protein for entry into sporulation [23]. Since the I186M substitution reduced transcription of *degU*, we reasoned that changes in the frequency of sporulation could also have occurred during domestication. We first tested this phenotype in a host-related environment (RPMI medium) and found the sporulation efficiency of Ancestral

(and of $degU^{Anc}$) to be ~1.5-fold higher than that of Evolved (or $degU^{Evo}$) (Fig 6B). The levels of DegU, as assessed by immunoblot analysis with an anti-DegU antibody of established specificity [55], are slightly higher in RPMI for both Ancestral and $degU^{Anc}$ as compared to Evolved or $degU^{Evo}$ (Fig 6C).

We also tested the ability of our strains to sporulate in the environment where the domestication process occurred (LB medium). Most laboratory strains sporulate at very low levels in LB (about 10^4 spores/ml of culture as compared to 10^8 spores/ml in a medium such as DSM that support efficient sporulation) [11]. We found a small increase in the efficiency of sporulation for Evolved (and $degU^{Evo}$) relative to Ancestral (or $degU^{Anc}$) in LB (Fig 6D). In addition, all of the laboratory strains tested sporulated in LB at efficiencies lower than that of the Ancestral (or $degU^{Anc}$), although one strain, JH642, sporulated better than the other laboratory strains tested under these conditions (Fig 6D). This is consistent with the initial description of Ancestral, which enters sporulation during growth and reaches a higher titer of spores than any of the laboratory strains tested [11]. The steady-state levels of DegU in whole-cell lysates obtained 1 hour after the onset of stationary phase in LB revealed very similar levels of DegU for all the strains (Fig 6E).

These results show that the I186M mutation can affect the intracellular levels of DegU in a manner that depends on the environment and show that the influence of DegU on sporulation exhibits antagonistic pleiotropy.

Discussion

BSP1 is a gastro-intestinal isolate of *B. subtilis* in which sporulation initiates during growth. This happens because BSP1 lacks three *rap* genes, coding for phosphatases that normally drain phosphoryl groups from the phosphorelay, thus negatively regulating the activation of Spo0A. As such, more cells in the population have Spo0A active above a threshold level required to induce sporulation. Several other gastro-intestinal isolates of *B. subtilis*, including from the human gut, also lack combinations of the *rap* genes and show enhanced sporulation [11,54]. *B. subtilis* completes its entire life cycle in the gut [54], and it seems likely that sporulation is important for survival and/or propagation in the gut ecosystem, as shown for other spore-formers [56,57]. Sporulation is also important for the efficient dispersal of spore-formers through the environment and among hosts [58–60]. Sporulation is, however, a time and energetically costly process, requiring the differential expression of over 10% of the genome over a period of 7-8 hours [11,61]. Accordingly, the propagation of *B. subtilis* in the laboratory in the absence of selection for sporulation results in a reduction in the ability to sporulate [62,63]. *B. subtilis* has been used in laboratory conditions for more than fifty years, and has accumulated mutations likely to be adaptive in that environment and that, relative to wild strains, lead to the attenuation of phenotypic traits that include swarming motility [32,33], poly-γ-glutamate synthesis [35], production of antibiotics, the secretion of degradative enzymes [64] or the formation of robust biofilms [35,65,66]. These processes have either become neutral with respect to fitness, or selection favored its loss under laboratory conditions.

Here we traced the first steps of domestication of strain BSP1. Rapid changes in colony morphotypes were observed in parallel cultures, leading to complete fixation of a specific colonial morphotype, termed type *b*, in all the replicate cultures after two

weeks (Fig 1). The adaptive morphotype is characterized by a smooth and flat colony, lacking the complex architectural features of the original strain (Fig 2C). Similar colony morphology changes were previously observed during domestication of other *B. subtilis* strains [3,62,63,67,68]. Colonies are biofilms formed at the solid/air interface [1]. As such, these observations hinted at attenuation of an important social behavior. Studies in *Salmonella enterica*, *Saccharomyces cerevisiae*, *Bacillus licheniformis,* and *Aneurinibacillus migulanus* have also documented the appearance of smooth colonies within a short period of time [5,69–71]. This suggests that phenotypic parallelism across species is a broad pattern of adaptation to the laboratory environment.

At the genomic level we detected mutations in the coding region of the *degU* gene (Fig 2). DegU is the response regulator of the two-component system DegS-DegU and controls social traits such as biofilm formation, swarming motility and exoprotease secretion [17]. In its non-phosphorylated state, DegU is responsible for the development of competence while the rise in DegU~P levels sequentially activates swarming, biofilm formation and exoprotease secretion [22]. DegU belongs to the NarL/FixJ subfamily of DNA binding proteins [72]. We characterized in detail the effects of a mutation leading to the I186M substitution in DegU, as this substitution showed a fast rate of fixation during domestication (Fig 1). The I186M substitution occurs in the DNA-recognition helix of the DegU HTH motif, located in the C-terminal domain of the protein (Fig 2A). Modeling studies indicate that this substitution is likely to affect a contact of the HTH motif with bases in the major groove of DNA as also shown in the crystal structure of NarL, in which I186 is conserved, with DNA [73] (Fig 2 and S2 Fig; see also S1 Text). Moreover, alanine-scanning mutagenesis showed decreased transcription of the DegU-controlled genes *comG* (as a proxy for the activity of ComK, a direct target of DegU) and *aprE* in a strain producing DegU^{I186A}, and the substitution also affected the binding of

383 DegU to the *comK* and *aprE* target promoters [21]. I186 is also conserved in LuxR,
384 another NarL family member, and its replacement by Ala also resulted in reduced
385 binding to target DNA sequences [74]. One other substitution found in DegU in our study,
386 H200Y, is also likely to impair DNA binding, as suggested by the study of a single Ala
387 substitution in transcription and DNA binding to cognate sites in the promoters of the
388 DegU-responsive genes *comK* and *aprE* [21]. This residue, however, as suggested by
389 the structure of a NArL-DNA co-crystal, contacts the DNA phosphate backbone [73] (see
390 also S1 Text). Importantly, while the I186M substitution found in the domesticated clone,
391 Evolved, did not cause changes in the efficiency of transformation with exogenous DNA,
392 it impaired processes regulated by DegU~P as swarming motility, biofilm formation,
393 SPP1 bacteriophage sensitivity, and exoprotease secretion. In agreement with these
394 observations, transcription of genes regulated by DegU~P was reduced in the
395 domesticated clone and this phenotype, as well as the other phenotypes tested, was
396 due to the I186M substitution, as shown by complementation analysis (S3 Fig). The
397 transcription of *degU* itself was also reduced (Fig 5B); since our *degU* transcriptional
398 reporter fusion includes all promoters known to contribute to the expression of the gene
399 (S3 Fig), including the DegU~P-recognized P3 promoter, it follows that the I186M
400 substitution affects the auto-regulatory loop that controls the production and activity of
401 DegU [49]. Failure to activate the auto-regulatory loop most likely explains impaired
402 biofilm development, exoprotease production and SPP1 sensitivity, which require high
403 levels of DegU~P. It is important to note that the I186M substitution does not impair the
404 accumulation of DegU, indicating that it did not grossly alter the folding of the protein at
405 least under two conditions tested (Fig 6), as anticipated from the modeling studies.

406 The extensive propagation of *B. subtilis* in a nutrient-rich medium, *i.e.*, under
407 conditions of relaxed selection for sporulation, resulted in the emergence of a strain that

accumulated mutations in genes of biosynthetic pathways, sporulation competence, DNA repair and others [62,63]. Relative to the ancestral, the resulting strain showed different cell and colony morphologies, loss of sporulation and competence, but an overall increased fitness under laboratory conditions [62,63]. Interestingly, our selection, which was also performed in a rich medium, LB, did not result in loss of sporulation. Rather, the I186M substitution modulated the efficiency of sporulation across conditions, specifically, in a host-related condition (RPMI medium) and in LB (Fig 6). Importantly, the accumulation of DegU was only slightly higher in Ancestral (and $degU^{Anc}$) relative to Evolved when sporulation was tested in RPMI, where the sporulation efficiency of Ancestral (and $degU^{Anc}$) was also higher than that of Evolved. Since high levels of DegU~P control production of Spo0A~P [23], it is possible that I186M places DegU~P below a threshold required for the stimulation of sporulation through Spo0A. This is consistent with decreased transcription of *degU* by $DegU^{I186M}$ (Fig 5B). In contrast, Evolved sporulated slightly better than Ancestral under nutritional conditions (LB medium) that do not support efficient sporulation by most laboratory strains (Fig 6). Again, the accumulation of DegU differed only slightly between Evolved and Ancestral. We note, however, that the accumulation of DegU reflects both transcription/production of the protein and proteolysis; since DegU~P is a preferred substrate for the ClpXP protease, the steady-state levels of the protein will reflect the ratio of DegU/DegU~P levels [49]. The inability of some domesticated strains of *B. subtilis* to form robust biofilms results from the accumulation of mutations in four chromosomal genes (*sfp*, *epsC*, *swrA,* and *degQ*), in addition to the loss of plasmid-born gene, *rapP* [6]. In contrast, under our experimental conditions, the target for mutation during domestication is *degU*. It seems possible that our experimental conditions did not cause relaxed selection for sporulation; rather, our selection may have targeted the most costly

phenotypes under the test conditions, those directly controlled by DegU~P, including the formation of complex colonies and robust biofilms, at least in the context of the BSP1 genome. It is interestingly to note that a two-month culture of the Laboratory strain NCIB 3610, resulted in the emergence of strains with different levels of biofilm robustness, as shown by the colony architecture and the expression of genes required for matrix production [3]. These phenotypes were the result of mutations in the *sinR* gene, coding for a master regulator of biofilm development, and arose both on plates as well as in LB cultures [3]. One conclusion offered was that matrix overproduction can be neutral or advantageous in a rich medium [3]. The difference in the mutations obtained under our experimental conditions and the study of Leiman and co-workers is in line with the idea that adaptations to a new environment, depend both on the initial genome as well as the culture history of the strain [75].

Taken together, the results suggest that the I186M mutation impairs the ability of DegU to function as a transcription factor and that this feature confers an advantage to BSP1 when growing under laboratory conditions. Interestingly, *arcA* in *E. coli* and *rpoS* in *S. enterica* and *E. coli*, regulators of stationary phase processes, are also common targets of laboratory adaptation [69,76]. This strengthens the evidence for a general rule that the initial adaptations to a new environment involve changes in genes that act as regulatory hubs of networks that affect the stationary phase of growth.

The accumulation of mutations during adaptation to a laboratory environment over a relatively small number of passages also unraveled a signal of antagonistic pleiotropy: Evolved exhibited changes in traits in host-related environments, and interestingly, it showed increased fitness in the presence of macrophages. This provides support for the coincidental hypothesis that adaptations to new environments can lead to changes in complex interactions with hosts, including virulence.

Material and Methods

General methods. Luria-Bertani (LB) medium was used for the routine growth of *B. subtilis* and *Escherichia coli*. The *E. coli* strain DH5α was used as the host strain for the construction and maintenance of plasmids and was grown in the presence of 100 μg ml^{-1} ampicillin when carrying vectors or recombinant plasmids. When appropriate, *B. subtilis* strains were grown in the presence antibiotics, used at the following concentrations: 5 μg ml^{-1} chloramphenicol, 1 μg ml^{-1} erythromycin, 1 μg ml^{-1} neomycin for liquid cultures and 3 μg ml^{-1} neomycin on solid media.

Other Methods. The construction of all plasmids and strains is described in detail in the supplemental material. The *B. subtilis* strains used or constructed in this work are listed in S1 Table. Plasmids are listed in S2 Table and oligonucleotides in S3 Table. Modeling of the DegU structure is also described in the supplemental material.

Domestication experiments. Five independent populations, all derived from the ancestral wild isolate BSP1 [52], were grown for 16 days in LB with a 1:100 dilution into fresh medium every 24 h. This is a common media for growing *B. subtilis* in the laboratory environment and may introduce selective pressure against sporulation and biofilm formation. At the point of dilution, an aliquot from each culture was collected and kept frozen at -80ºC for subsequent analysis.

Whole-genome sequencing. The identify the mutations that emerged after 8 days of evolution we extracted DNA from the Evolved clone from population 1 and from the Ancestral. The DNA library construction and sequencing were carried out by the IGC genomics facility. Each sample was pair-end sequenced on an Illumina MiSeq Benchtop Sequencer. Standard procedures produced data sets of Illumina paired-end 250-bp read pairs. The mean coverage per sample was of 30 and 18, for the Evolved and Ancestral

482 respectively. Mutations were identified using the BRESEQ pipeline version 0.32.1 [77]
483 with default parameters and using the available BSP1 genome [78] as a reference
484 genome. Genome sequencing data have been deposited in the NCBI Read Archive,
485 http://www.ncbi.nlm.nih.gov/sra (accession no. XXXX). All predicted mutations were
486 manually inspected using IGV [79].

487 **Macrophages culture and infection assay.** The murine macrophage cell line RAW
488 264.7 was cultured in RPMI medium (Sigma), supplemented with 2 mM L-glutamine, 1
489 mM sodium pyruvate, 10 mM hepes, 50 µM 2-mercaptoethanol solution and 10 % heat-
490 inactivated Fetal Bovine Serum in an atmosphere of 5 % CO2. For the infection, *B.*
491 *subtilis* and the macrophages were grown separately in a 24-well tissue plate containing
492 fresh RPMI media as described above. At 24h of acclimatization, *B. subtilis* was diluted
493 1:100 into fresh RPMI. Macrophages were washed, re-suspended in fresh RPMI and
494 activated with 2 µg ml-1 CpG for another 24h [80]. Then, the macrophages were washed
495 to remove the remaining CpG, fresh RPMI media was added and *B. subtilis* added to a
496 1:8 MOI (multiplicity of infection; about 8 x 10^6 cells). At the indicated time points of
497 infection, the wells were scrapped and the contents centrifuged at 6000 g for 10 minutes
498 at room temperature. After centrifugation, the samples were serially diluted and plated
499 to determine the titer of total, viable, cells and heat-resistant spores.

500 **Sporulation assays.** Sporulation of *B. subtilis* was usually analyzed in LB and in
501 supplemented RPMI. When using LB, *B. subtilis* cultures were grown overnight, diluted
502 1:100 and incubated for 24 h at 37 ºC. At this time, dilutions of the cultures were plated
503 for total viable counts and treated for 20 min at 80ºC to determine the titer of heat-
504 resistant spores. For supplemented RPMI, the cultures were grown as described above
505 for 48h and plated for viable cells and spore counts as described above for LB. The

506 sporulation efficiency was defined as the ratio of heat-resistant spores relative to the
507 total viable cell count [11].

508 **SPP1 phage lysates and transduction.** SPP1 lysates were prepared as described by
509 Yasbin and Young [81]. Briefly, a dense culture of *B. subtilis* was infected with different
510 dilutions of SPP1 in a semisolid LB agar (LB containing 0.7% agar). The plate containing
511 near confluent phage plaques was washed with 4 ml of TBT, centrifuged at 5000 g for
512 10 min, treated with 12 µg ml^{-1} DNase and filtered through a 0.45 µm syringe filter. The
513 indicator strain PY79 was used for titration of the SPP1 lysates as described by São-
514 José et al [82]. SPP1 phage transduction was performed as described [33]. The recipient
515 strains were grown in LB until stationary phase after which 1 ml of the culture of the
516 recipient strain was mixed in a glass tube with 10 mM $CaCl_2$ and infected with an MOI
517 of 1 of the donor SPP1 lysate. The transduction mixture was then incubated at 37 ºC for
518 25 min with agitation, centrifuged at 5000 g for 10 min, washed with 2 ml of LB, and
519 centrifuged again at 5000 g for 10 min. The supernatant was discarded, the pellet was
520 resuspended in 100 µl of LB and plated onto LB plates fortified with 1.5 % agar with the
521 appropriate antibiotics and 10 mM of sodium citrate.

522 **Competence assay.** Development of competence was performed as described by
523 Baptista et al [39]. Briefly, *B. subtilis* cultures were grown overnight and diluted 1:100 in
524 GM1 at 37 ºC. Ninety minutes after the end of the exponential growth, the cultures were
525 diluted 1:10 in GM2 and incubated for 90 minutes at 37 ºC. At this point, a sample of the
526 cultures was serially diluted in LB and plated for determination of total colony forming
527 units (CFU) per milliliter. For transformation, DNA from strain AH7605 or W648 was
528 added to 500 µl of the culture samples, to a concentration of 5 µg ml^{-1}, the mixture
529 incubated for 30 min at 37 ºC and finally plated with the appropriate antibiotics. The

transformation efficiency is the ratio between the number of transformants and the total number of colonies.

Protease activity assay. Secreted proteases were observed essentially as described by Saran et al [83]. The strains were grown until they reached an absorbance of 0.8 at 600 nm. At this time the cultures were diluted to an absorbance of 0.01 at 600 nm. 10 µl of this dilution was spotted in a 2 % skimmed milk plate and incubated at 37 ºC for 48 h. Then, 6 ml of 10 % Tannic Acid was added for detection of the protease-positive strains. The diameter of the halos observed was measured, and the diameter of the colony was subtracted in order to obtain the real value of the halo.

Swarming and colony morphology assays. Swarming motility was examined according to the method described by Kearns and Losick [33]. For colony morphology, the *B. subtilis* cultures were grown overnight and 3 µl of the culture was spotted onto an MSgg [84] plate fortified with 1.5 % agar. The plates were incubated at 28 ºC or 37 ºC. The images were captured at the times indicated in the figures.

Biofilm quantification by crystal violet. The method used for estimating the solid-surface-associated biofilm formation with crystal violet was as described by Morikawa et al [85]. Briefly, an overnight culture was diluted to an absorbance of 0.03 at 600 nm and mixed 1:100 into 100 µl of MSgg in a 96-well plastic titer plate. The plate was incubated for 48 h at 25 ºC. Then, the culture was carefully removed from the wells. After washing two times with distilled water, 150 µl of 1 % crystal violet was added to the wells and incubated for 25 min at room temperature. The wells were washed again two times with distilled water and the crystal violet attached to the biofilm matrix was solubilized in 150 µl of DMSO and incubated for 10 minutes at room temperature. The removed culture was quantified by measuring its absorbance at 600 nm and the biofilm attached to the crystal violet was quantified measuring its absorbance at 570 nm.

555 **Biofilm fluorescence imaging.** For biofilm imaging, the *B. subtilis* cultures were grown
556 overnight and 3 µl of the culture was spotted onto an MSgg plate fortified with 1.5 %
557 agar and incubated for 96h at 28ºC. Images were acquired on a Zeiss Axio Zoom.V16
558 stereomicroscope equipped with a Zeiss Axiocam 503 mono CCD camera and
559 controlled with the Zeiss Zen 2.1 (blue edition) software, using the 1x 0.25 NA objective,
560 the fluorescence filter set GFP and the Bright Field optics.

561 **Fluorescence microscopy and image analysis.** Cultures were grown until one hour
562 after the end of the exponential phase. The cells were collected by centrifugation (1 min
563 at 2.400 x g, room temperature), and washed with 1 ml of phosphate-buffered saline
564 (PBS). Finally, the cells were resuspended in 100 µl of PBS and applied to microscopy
565 slides coated with a film of 1.7% agarose. Images were taken with standard phase
566 contrast and GFP filter, using a Leica DM 6000B microscope equipped with an
567 aniXon+EM camera (Andor Technologies) and driven by Metamorph software (Meta
568 Imaging series 7.7, Molecular Devices). For quantification of the GFP signal, 6x6 pixel
569 regions were defined in the desired cell and the average pixel intensity was calculated
570 and corrected by subtracting the average pixel intensity of the background, using
571 Metamorph software (Meta Imaging series 7.7, Molecular Devices).

572 **Immunoblot analysis.** Cultures were grown until one hour after the end of the
573 exponential phase and samples (10 ml) were withdrawn. The cells were collected by
574 centrifugation (5 min at 15300 x g, 4° C). The cells were resuspended in 1ml Lysis buffer
575 (50 mM NaH_2PO_4, 0.5 M NaCl, 10 mM Imidazole, pH 8.0) and whole-cell lysates
576 prepared using a French press cell (19,000 lb/in^2). Proteins in the lysates (10 µg) were
577 then separated on 15% SDS-PAGE gels and the gels subject to immunoblot analysis
578 using an anti-DegU antibody of established specificity at a 1:1000 dilution [86]. Gels run
579 in parallel were stained with Coomassie brilliant blue to be used as loading controls.

Acknowledgments

We thank Dr. Mitsuo Ogura for the gift of the anti-DegU antibody, Dr. Kazuo Kobayahis for the gift of strains WTF28 and W648, Dr. Jan-Willem Veening for the gift of strains 08G52, 08G57, and 08I09, and Dr. Carlos São José for the gift of the SPP1 lysate and advice on its use. We also thank Mónica Serrano for helpful discussions.

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Supporting information

S1 Fig. Evolved has better fitness than Ancestral. (A) Comparison of the growth curves of Ancestral (red line) and Evolved (blue line) in LB. (B) 95% family-wise confident intervals comparing Evolved with Ancestral for mu (slope), lambda (lag), A (max) and AUC (area under the curve), using ANOVA and Dunnett post-test. The analysis and the graphics were obtained from https://mdphan.shinyapps.io/GrowthCurvesAnalysis/.

S2 Fig. Alignment of DegU orthologs. (A) Sequence conservation profile of DegU homologs computed from a Hidden Markov Model multiple sequence alignment using Skylign [17]. The overall height indicates the conservation per position. The orange dashed line shows the position of D18, V131, I186, and H200. With the exception of V131, all other residues are in highly conserved regions; V131, however, is located in a conserved hydrophobic patch (expanded in panel B). (B) Alignment of the regions close to the observed substitutions in DegU. The panel was produced with ESPript 3.0 [18] following a Clustal Omega [19] alignment of the selected sequences. The red arrows indicate the residues which are the site of the V131D, I186M, and H200Y substitutions herein described. The boxes indicate blocks of high sequence identity; red indicates chemical similarity. The sequence of *E. faecium* LiaR, used for the homology modeling of the *B. subtilis* DegU protein, is indicated below the consensus. The positions of helices 8 (scaffolding helix) and 9 (DNA recognition helix) is also shown. The brown dots indicate residues important for binding of DegU to the *comK* and *aprE* promoters [13].

S3 Fig. Construction of strains bearing *degU^{Anc}* and *degU^{Evo}* at an ectopic site. The figure depicts the construction of strains HB13 and HB14. (A) left: genome organization of the native *degU* locus, at the left of *oriC,* as shown in the circle below

872 the genetic map, in Ancestral. Note the presence of the three promoters, P1 to P3, that
873 drive expression of *degU.* Right: strains HB11 and HB12 are BSP1 derivatives bearing
874 a *degU*::*em* insertion at the *degU* normal locus and an insertion of the *degU* region at
875 the non-essential *amyE* locus, to the right of *oriC*, as depicted. The region inserted at
876 *amyE* includes a *degS* in frame-deletion that removes nucleotides 75-912 of the coding
877 region, so that the strain has only one copy of the gene, at the normal locus. The *degU*
878 allele inserted at *amyE* codes for a form of the protein with the A10E substitution, in
879 strain HB11 (blue), or for the I186M substitution, in strain HB12 (red), as shown. Note
880 that in both strains, expression of *degU* from *amyE* can still occur from P1 to P3. (B)
881 The panel depicts the result of transforming HB11 or HB12 with plasmid pHB1. Using
882 HB11 as the recipient, a wild-type degU allele is restored, yielding HB13. Using HB12
883 as the recipient, the mutation leading to the A10E substitution is corrected, yielding
884 strain HB14, which expresses $degU^{Evo}$ ($degU^{I186M}$) from *amyE* (see text for details).

885 **S4 Fig. Competence is not affected in Evolved.** Transformation of Ancestral and
886 Evolved with genomic DNA from (A) AH7605 and (B) W648. The transformation
887 efficiency is expressed as the ratio between the number of transformants obtained and
888 the total number of colonies.

889 **S5 Fig. Pellicle formation is impaired in Evolved.** Representative images of pellicle
890 formation by Ancestral and Evolved after incubation in liquid MSgg medium at 28ºC for
891 the indicated time, in hours. The region boxed in red in the two sets of panels on the left,
892 for each time sample, are magnified on the right. Scale bar, 1 cm.

893 **S1 Text. Supporting information text.**

894 **S1 Table. Bacterial strains used in this study.**

895 **S2 Table. Plasmids used in this study.**

S3 Table. Oligonucleotide primers used in this study.

Figure captions

Fig. 1. Changes in colony morphology with domestication. (A) Representative image of the Ancestral colony morphology and of the three different types of colony morphology, *a*, *b* and *c*, observed at the eight and sixteen day of the domestication experiment in the five evolved populations; (B) Frequency of each morphotype in the five populations at day 8; (C) Frequency of morphotype *b* in population 1 along time. The scale bar represents 1 cm and applies to all panels.

Fig. 2. Domestication is accompanied by mutations in *degU.* (A) *degU* region of the *B. subtilis* chromosome (top) and domain organization of the DegU protein (bottom). The position of the various mutations found and the corresponding amino acid substitution is indicated. (B) Model of the full-length DegU protein *of B. subtilis* obtained by comparative modeling and using the crystal structure of the LiaR protein from *Enterococcus faecalis* as the template (PDB code: 5hev). The protein is thought to form a dimer and the two monomers are represented in blue and light brown, with the position of the receiver and DNA-binding domains indicated. The red arrows indicate the location of the single amino acid substitutions found in DegU. *a* and *b*, show a magnification of the regions encompassing the V131D (*a*) and I186M and H200Y (*b*) substitutions. In *b*, the region of the helix-turn-helix (HTH) motif is modeled with DNA, to highlight the likely involvement of residues I186 and H200 in DNA binding. The HTH motif was independently modeled using the crystal structure of the LiaR DNA-binding domain as the template (PDB code: 4wuh). (C) Representative images showing the complex biofilm morphology of Ancestral and clones representative of each population after 16 days of domestication. In red are indicated the mutation in DegU present in each clone. All strains were incubated in MSgg for 96h at 28ºC. Scale bar, 1 cm.

Fig. 3. *degU^{Evo}* is responsible for the alteration in swarming motility and colony architecture. (A) Swarming motility assay of Ancestral, Evolved, *degU^{Anc}* (HB13), *degU^{Evo}* (HB14), *ΔdegU* (HB4), and Lab (PY79). LB plates fortified with 0.7% of agar were inoculated incubated for 16h at 28ºC. Swarm expansion, resulting from bacterial growth, appears in white, whereas uncolonized agar appears in black. (B) Representative images showing the complex colony architecture development along with the indicated time points of the Ancestral and Evolved. The strains were grown in MSgg medium at 28ºC. (C) Representative images showing the complex colony architecture of the indicated strains on MSgg agar plates incubated for 96h at 28ºC. Scale bars, 1 cm.

Fig. 4. *degU^{Evo}* is responsible for the alteration in biofilm complexity, exoprotease secretion, phage resistance and the pattern of gene expression during biofilm formation. (A) Quantification by the crystal violet method of the biofilms formed by the indicated strains in MSgg broth incubated at 25ºC for the indicated time points. For the statistics the Mann-Whitney test was used. For ****, $p<0.0001$. (B) Dimension of the halos produced by the indicated strains in LB fortified with 1.5 % agar and supplemented with 2% of skimmed milk incubated at 37ºC for 48h. For the statistics an unpaired t-test with Welch's correction was used. For ****, $p<0.0001$, for ***, $p=0.0066$. The error bar represents the standard deviation. Scale bar, 10 mm (C) Efficiency of Plating (EOP) shown in white numbers for the Ancestral, Evolved, *degU^{Anc}* (HB13), *degU^{Evo}* (HB14), *ΔdegU* (HB4) using as a reference the indicator strain Lab (PY79), which is phage sensitive. The yellow arrows indicate SPP1 phage plaques. Note that Ancestral is sensitive to SPP1 but the plaque size is reduced when compared with the Lab strain, while the Evolved is resistant. Scale bars, 1 cm. (D) Representative images of the

945 expression of transcriptional fusions between the *aprE*, *bslA*, *hag* and *degU* promoter
946 regions and *gfp* after 96h of incubation in MSgg at 28ºC. Scale bar, 1 cm.

947 **Fig. 5. *degU*^{Evo} alters the pattern of gene expression at the single-cell level.** (A)
948 Representative images of the expression of *hag*-, *bslA*-, *aprE*- and *degU-gfp*
949 transcriptional fusions one hour after the onset of stationary phase in LB. The cultures
950 were grown with agitation at 37ºC. Scale bar, 1 µm. (B) Relative frequency of expression
951 of transcriptional fusions of the indicated promoters to *gfp* in the same conditions as
952 above.

953 **Fig. 6. degU^{Evo} increases survival in the presence of macrophages and changes**
954 **sporulation efficiency in an environment-dependent manner.** (A) Macrophages
955 were infected with Ancestral and Evolved and colony-forming units of both the
956 intracellular and extracellular bacteria obtained by plating the at the indicated time
957 points. The error bar represents the standard deviation of 9 replicates. (B) Comparison
958 of the sporulation efficiency in RPMI medium between Ancestral, Evolved, *degU*^{Anc}
959 (HB13) and *degU*^{Evo} (HB14). The efficiency of sporulation was calculated as the ratio
960 between the heat resistant spore counts and total (viable) cells. The dashed line
961 indicates the average sporulation efficiency for the Ancestral in LB. For the statistics an
962 unpaired t-test with Welch's correction was used. For ****, p<0.0001. (C) Accumulation
963 of DegU in Ancestral, Evolved, *degU*^{Anc} (HB13), *degU*^{Evo} (HB14) and the *degU*
964 insertional mutant (HB4) in RPMI. (D) Comparison of the sporulation efficiency and
965 variance in LB between Ancestral, Evolved, *degU*^{Anc} (HB13), *degU*^{Evo} (HB14), Lab and
966 three other commonly used laboratory strains (MB24, JH642 and 168). For the statistics
967 an unpaired t-test with Welch's correction was used. For *, p=0.0113. (E) The levels of
968 DegU are similar between Ancestral and Evolved in LB. Accumulation of DegU in
969 Ancestral, Evolved, *degU*^{Anc} (HB13), *degU*^{Evo} (HB14), and the *degU* insertional mutant

(HB4). In (C) and (D), the cells were collected 1 hour after the onset of stationary phase in RPMI (C) or LB (D) and whole-cell lysates prepared. Proteins (20 µg) in whole-cell lysates were resolved by SDS-PAGE and subject to immunoblot analysis with an anti-DegU antibody. The arrow shows the position of DegU; the red arrows indicate slightly higher levels of DegU. The panel below the immunoblot shows part of a Coomassie-stained gel, run in parallel, as a loading control. The position of molecular weight markers (in kDa) is shown on the left side of the panels.

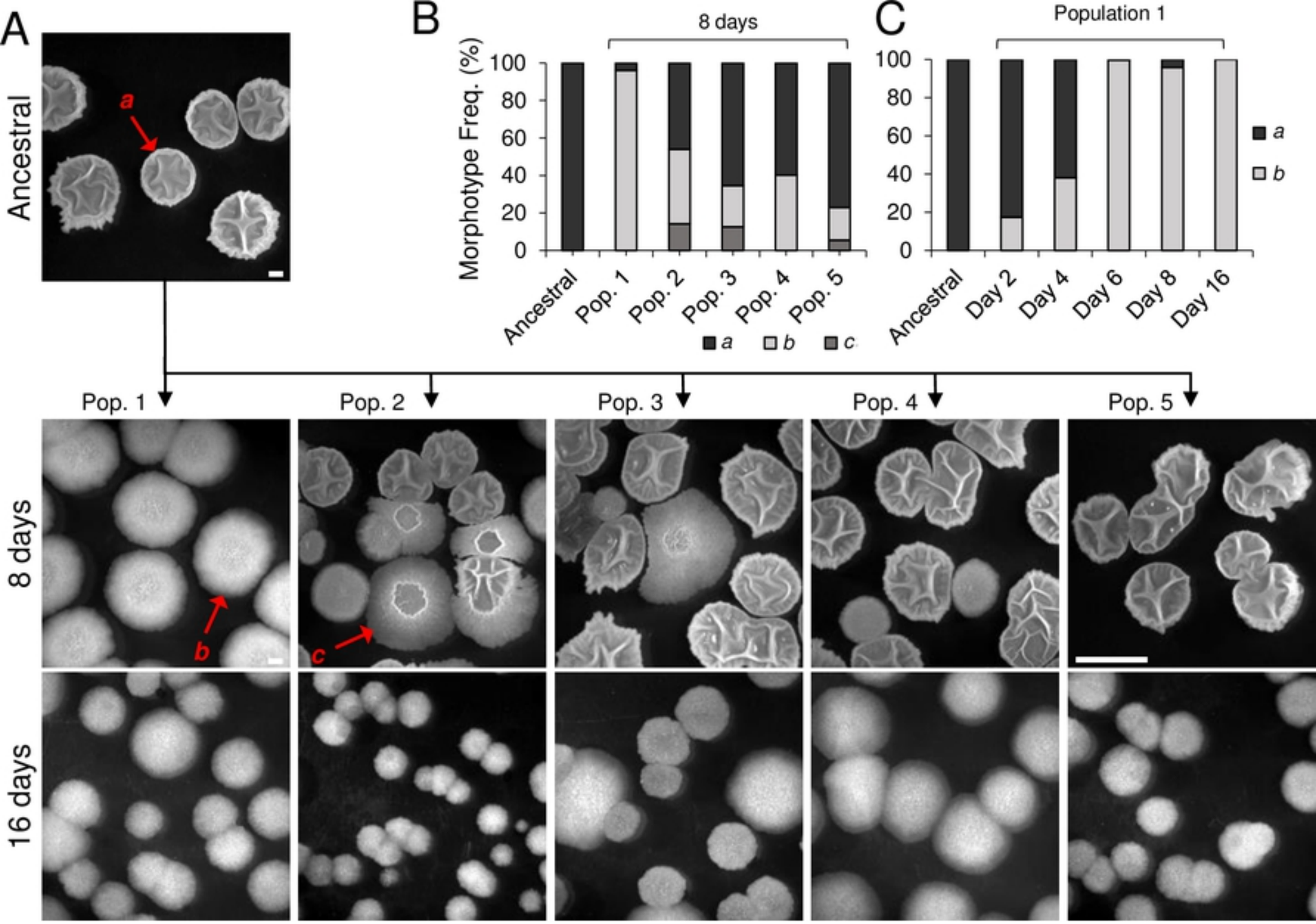

A
Ancestral
a
B
8 days
Morphotype Freq. (%)
100
80
60
40
20
0
Ancestral
Pop. 1
Pop. 2
Pop. 3
Pop. 4
Pop. 5
a
b
c
C
Population 1
Ancestral
Day 2
Day 4
Day 6
Day 8
Day 16
a
b
Pop. 1
Pop. 2
Pop. 3
Pop. 4
Pop. 5
8 days
16 days
b
c

Figure 1

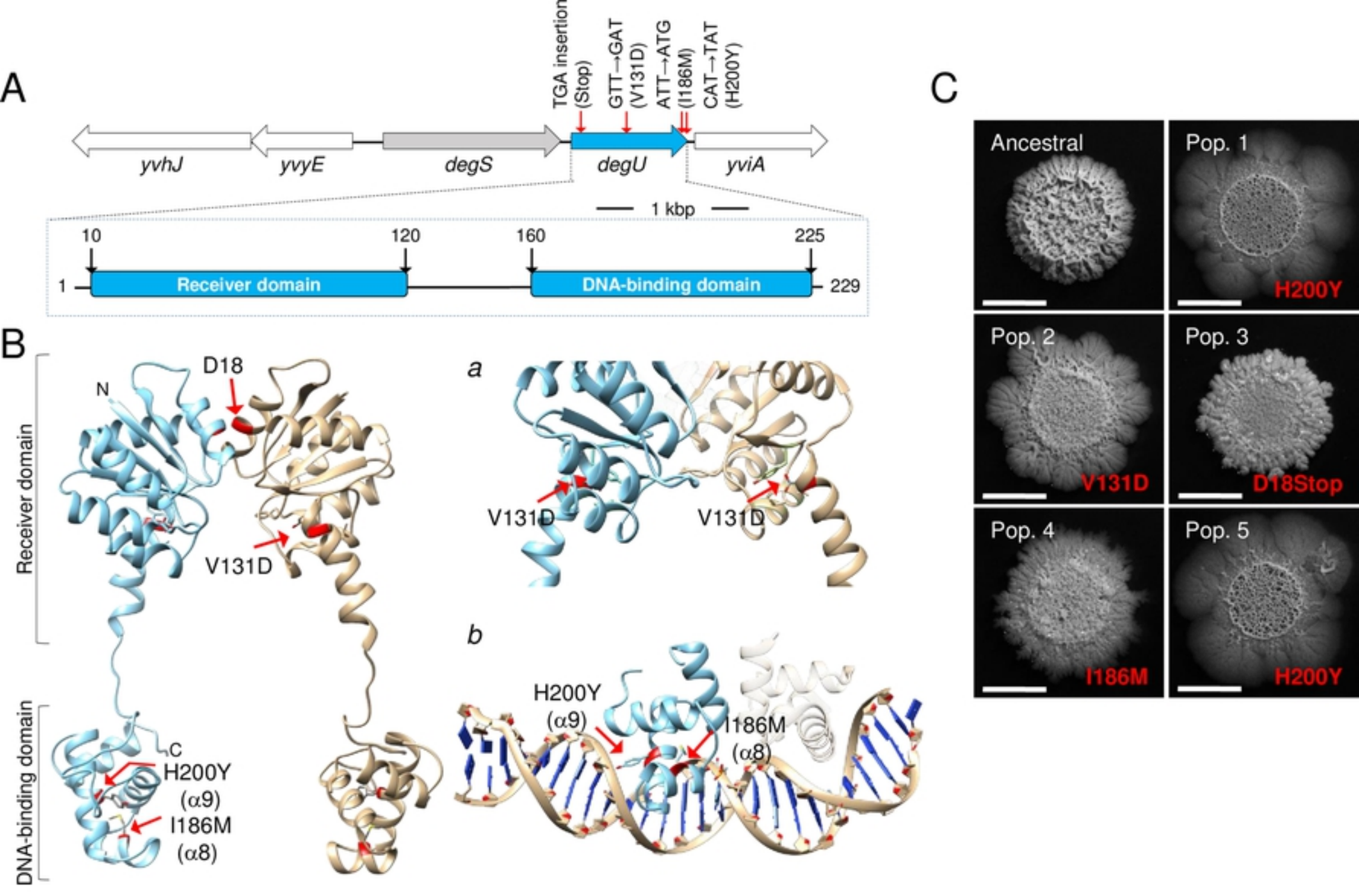
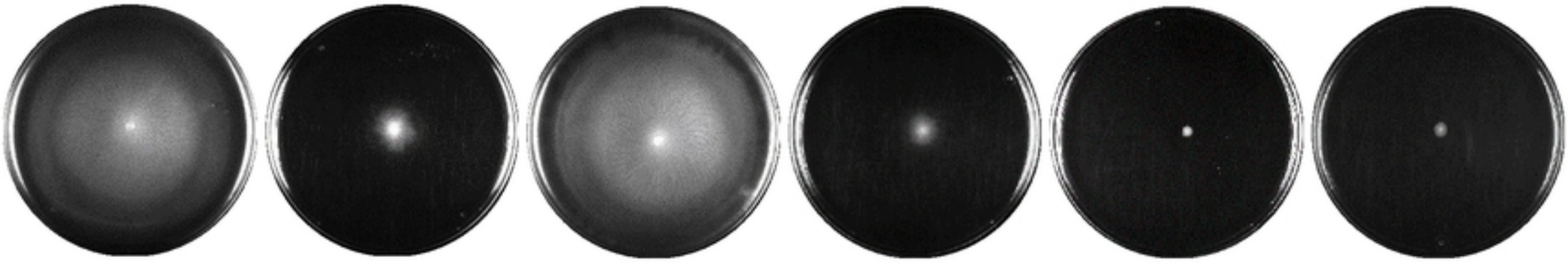
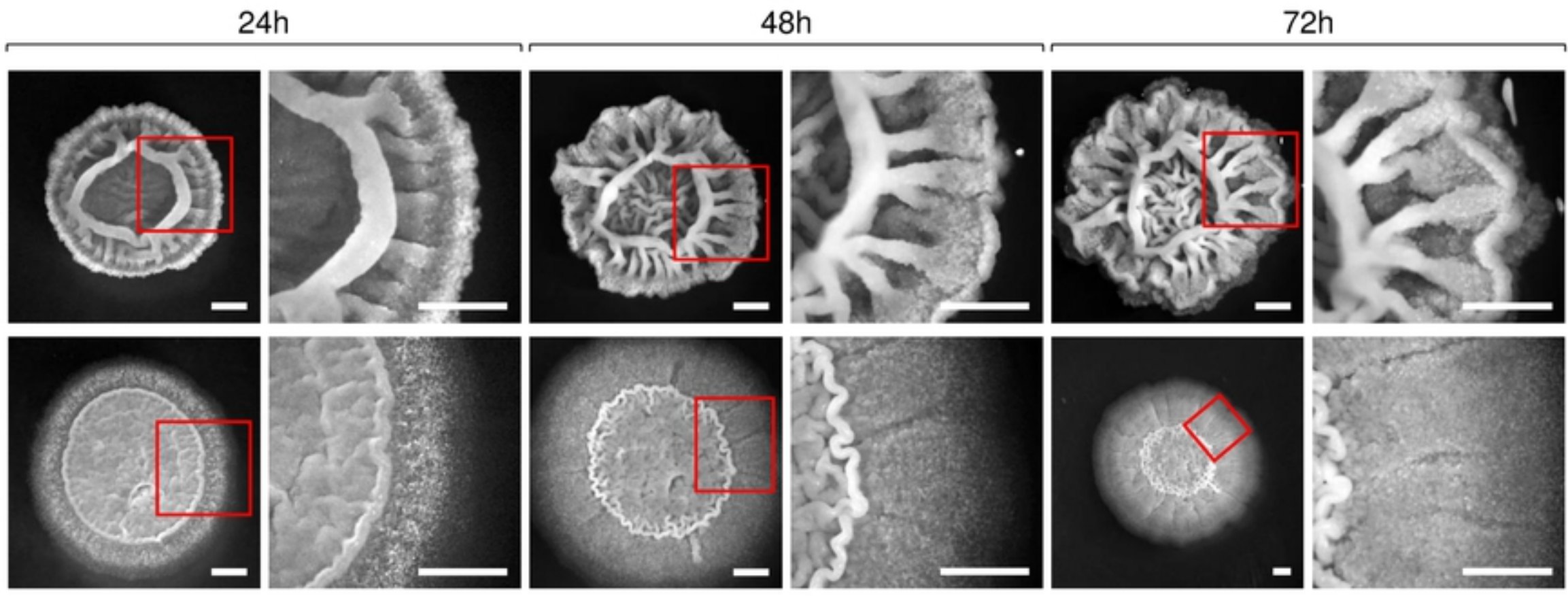

A
TGA insertion (Stop)
GTT→GAT (V131D)
ATT→ATG (I186M)
CAT→TAT (H200Y)
yvhJ
yvyE
degS
degU
yviA
1 kbp
10
120
160
225
1
Receiver domain
DNA-binding domain
229
B
Receiver domain
DNA-binding domain
N
D18
V131D
C
H200Y
(α9)
I186M
(α8)
a
V131D
V131D
b
H200Y
(α9)
I186M
(α8)
C
Ancestral
Pop. 1
H200Y
Pop. 2
V131D
Pop. 3
D18Stop
Pop. 4
I186M
Pop. 5
H200Y

Figure 2

A


Ancestral
Evolved
degU^Anc
degU^Evo
ΔdegU
Lab

B


24h
48h
72h
Ancestral
Evolved

C

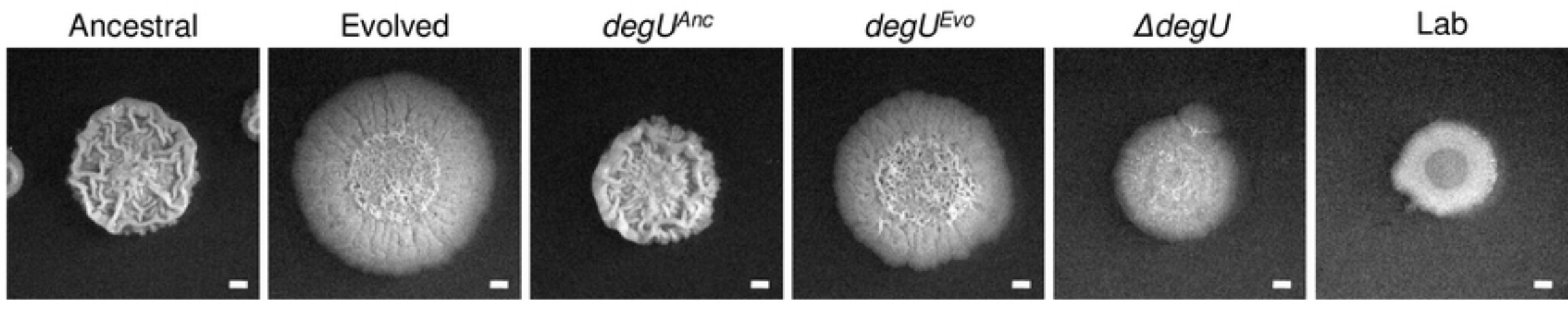

Ancestral
Evolved
degU^Anc
degU^Evo
ΔdegU
Lab

Figure 3

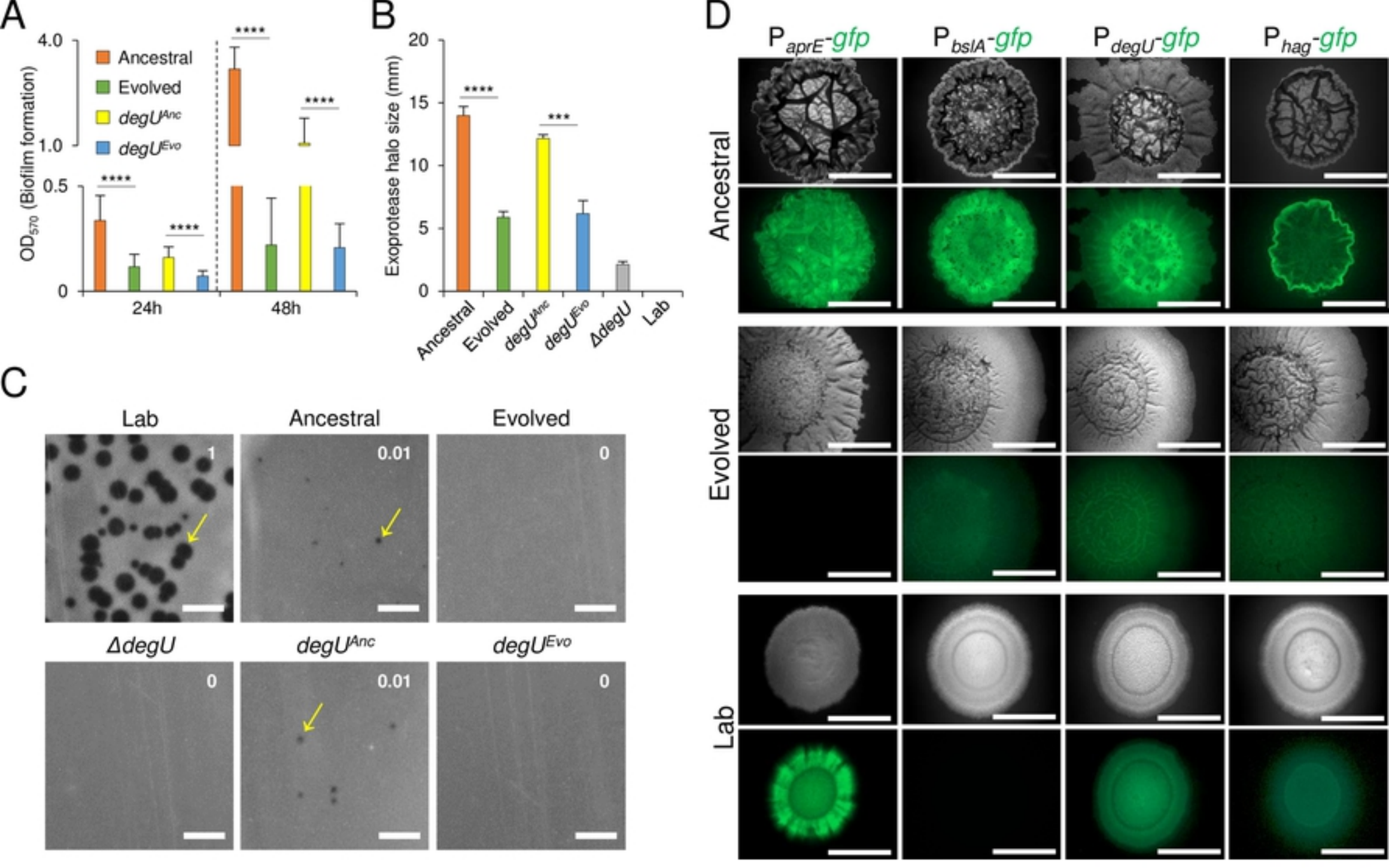

A
4.0
1.0
0.5
0
OD570 (Biofilm formation)
Ancestral
Evolved
degUAnc
degUEvo
****
****
****
24h
48h
B
20
15
10
5
0
Exoprotease halo size (mm)
****
***
Ancestral
Evolved
degUAnc
degUEvo
ΔdegU
Lab
C
Lab
Ancestral
Evolved
1
0.01
0
ΔdegU
degUAnc
degUEvo
0
0.01
0
D
PaprE-gfp
PbslA-gfp
PdegU-gfp
Phag-gfp
Ancestral
Evolved
Lab

Figure 4

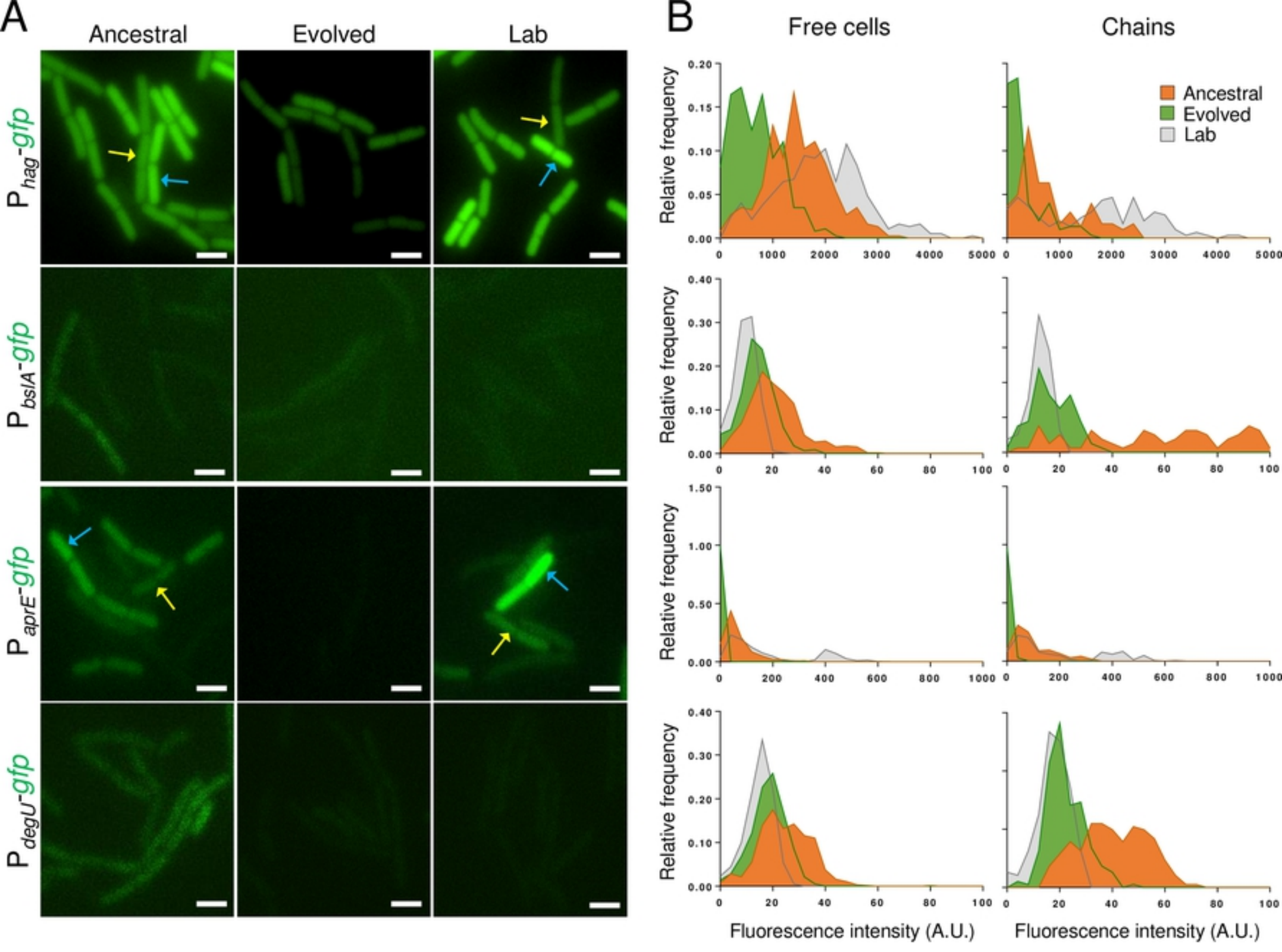

A
Ancestral
Evolved
Lab
P hag -gfp
P bslA -gfp
P aprE -gfp
P degU -gfp
B
Free cells
Chains
Ancestral
Evolved
Lab
Relative frequency
Fluorescence intensity (A.U.)
Fluorescence intensity (A.U.)

Figure 5

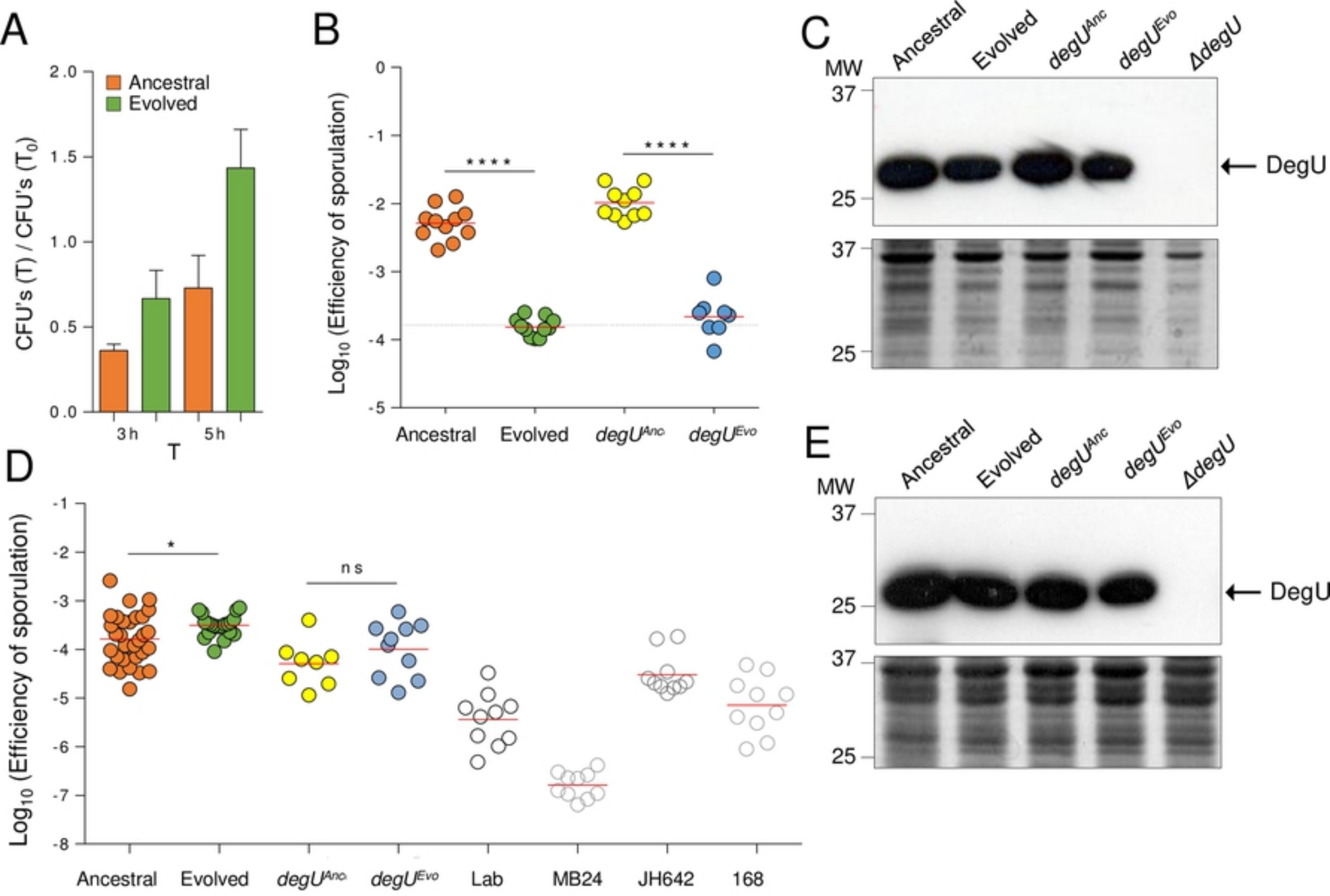

A
Ancestral
Evolved
CFU's (T) / CFU's (T0)
2.0
1.5
1.0
0.5
0.0
3 h
5 h
T
B
Log10 (Efficiency of sporulation)
0
-1
-2
-3
-4
-5
****
****
Ancestral
Evolved
degUAnc
degUEvo
C
MW
Ancestral
Evolved
degUAnc
degUEvo
ΔdegU
37
25
37
25
DegU
D
Log10 (Efficiency of sporulation)
-1
-2
-3
-4
-5
-6
-7
-8
*
n s
Ancestral
Evolved
degUAnc
degUEvo
Lab
MB24
JH642
168
E
MW
Ancestral
Evolved
degUAnc
degUEvo
ΔdegU
37
25
37
25
DegU

Figure 6