Chance and pleiotropy dominate genetic diversity in complex bacterial environments

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Abstract

How does environmental complexity affect the evolution of single genes? Here, we measured the effects of a set of mutants of *Bacillus subtilis* glutamate dehydrogenase across 19 different environments – from homogenous single cell populations in liquid media to heterogeneous biofilms, plant roots and soil communities. The effects of individual gene mutations on organismal fitness were highly reproducible in liquid cultures. Strikingly, however, 84% of the tested alleles showed opposing fitness effects under different carbon and nitrogen sources (antagonistic pleiotropy). In biofilms and soil samples, different alleles dominated in parallel replica experiments. Accordingly, we found that in these heterogeneous bacterial communities the fate of mutations was dictated by a combination of selection and drift. The latter was driven by programmed prophage excisions that occurred along biofilm development. Overall, per individual condition, by the combined action of selection, pleiotropy and chance, a wide range of glutamate dehydrogenase mutations persisted and sometimes fixated. However, across longer periods and multiple environments nearly all this diversity would be lost – indeed, considering all environments and conditions we have tested, wild-type is the fittest allele.

The function of most genes may be essential in some conditions, but only marginally contributing, or even redundant, in other conditions [1–4]. The effects of mutations on organismal fitness are therefore environment-dependent, giving rise to complex, pleiotropic genotype-by-environment interactions [5,6]. Moreover, bacterial populations often do not comprise single cells, but rather have a structure as in biofilms. Under this complexity: changing environments and heterogeneous bacterial communities, the fate of mutations could also be dictated by population bottlenecks (drift) or rapid takeover of beneficial mutations in other genes (selective sweeps) [7–9].Consequently, the frequency of a given gene allele may change dramatically (from perishing to fixation) with no relation to its molecular function [10,11].

We aimed at an experimental setup would examine how complex bacterial growth states and environments might shape protein evolution. Previous systematic mappings were based on a direct linkage between protein stability and function and organismal survival, thus enabling measurements of effects of mutations at the protein level [5,12–15]. However, how mutations in a single gene-protein affect organismal fitness under varying environments and conditions is largely unexplored [16]. We thus chose as our model *Bacillus subtilis* NCIB 3610, a non-domesticated strain capable of growing in diverse aquatic and terrestrial environments [17]. We explored the effects of mutations in different conditions: in dispersed cells in liquid, but also in biofilms where phenotypic and genetic variability prevails [18]. We also mapped the effects of mutations during spore formation and germination [19] and in more complex and close to natural environments including soil, rhizosphere and plant roots.

A catabolic glutamate dehydrogenase (GDH) was our model protein. This enzyme is essential when amino acids such as proline serve as sole carbon-nitrogen sources [20]. However, in the presence of ammonia and glycolytic sugars, GDH activity is redundant as glutamate must be synthesized rather than catabolized. GDHs therefore respond to changes in carbon-nitrogen sources, and as regulators of glutamate homeostasis, are also associated with biofilm development [21,22]. *B. subtilis* has two catabolic GDHs, RocG and GudB. The latter is constitutively expressed, and is regulated via association of its hexameric form [23]. GudB has also regulatory roles [24,25] via interactions with the transcriptional activator of glutamate synthase [25] and with an essential transcription termination factor, NusA, that also modulates the stringent response [26–28]. We explored mutations in the oligomeric interface of GudB, aiming at multilateral effects on GudB's enzymatic and regulatory functions.

Altogether, these choices of organism and enzyme allowed us to readily examine and quantify the fate of GudB alleles in a range of different growth conditions and environments, also mimicking natural habitats where strong evolutionary forces act [11].

Experimental setup and data processing

We anticipated that the effects of the explored mutations would be complex and condition-dependent. We thus opted for high rather than broad coverage and mapped 10 positions within a single ~150 base pairs segment that resides at GudB's oligomeric interface while choosing highly conserved (D58) as well as highly diverged positions (M48, or S61; **Table S1**). Positions were diversified using NNS (whereby N represents any of the 4 bases, and S, G or C). The resulting

GudB library contained in total 320 single mutant alleles (including wild-type), whose genomes differ, in principle, by a single mutation: 200 different amino acid alleles and 10 stop-codons. The library therefore included synonymous alleles whereby the same amino acid was encoded by 2 or 3 different codons. This allele library was incorporated into the chromosome of *B. subtilis* NCIB 3610 under *gudB*'s original promoter and terminator.

This starting population (the initial mix, hereafter) was used to inoculate cultures grown in an array of different conditions. We tested 7 different growth states where the population complexity varies from single cells to community: liquid, pellicles (air-liquid biofilms), spores, germinated spores, biofilms grown on agar including on carbon-nitrogen gradients, and soil colonization. Up to 5 different carbon-nitrogen sources were used that, at least as far as the phenotypes of the GudB knockout indicate, inflict different levels of selection on GudB: Glutamate *plus* ammonia (GA), where ΔGudB has no growth effect; glutamate *plus* glycerol (GG), arginine (A), and arginine *plus* proline (PA), where ΔGudB exhibits a slight growth defect, and proline (P), where ΔGudB exhibits the strongest growth defect (**Fig. S1**). Thus, in total, we tested 19 conditions. At each condition, three to five biological replicas were performed by inoculating from the same initial mix. The cultures were grown in parallel, and individually analyzed. Illumina sequencing was applied to determine the frequency of each of the *gudB* alleles in the initial mix and after growth. Following filtering (see Methods), we obtained data for 244 up to 269 individual alleles per experiment (**Data S1** & **Fig. S2)**.

The ratio between an allele's frequency at the end of growth and in the initial mix was derived, and this ratio is referred to as the frequency coefficient

(FC; **Data S2**). Given the experimental error in determining FC values, values between 0.8 and 1.2 were classified as 'neutral', FC ≤0.8 assigned a mutation as 'deleterious', and FC >1.2 as 'beneficial'. Mutations with FC ≤0.1 were classified as 'highly deleterious', and similarly, FC ≥10 as 'highly beneficial' (see Methods). Note that the number of generations in liquid growth (~50 generations) and spores (a dormant non-replicative form of *B. subtilis*), for example, differs fundamentally. Moreover, in pellicles and biofilms, the number of generations cannot be readily determined –different cell types having different growth rates [29]. So, while we could not calculate selection coefficients (*s*), one should keep in mind that an FC value of 0.8 in the spores is in effect equivalent to extinction across 50 generations in liquid ($0.8^{50} = 10^{-5}$).

Irreproducibility – selection *versus* drift

Our first observations indicate two contrasting scenarios. In liquid cultures, for example, we observed highly reproducible FC values in biological replicas (**Fig. 1a**). Given the small sample numbers (3 replicas, 5 in few cases like the rhizosphere), the observed variance may underestimate the actual variance. However, the repetitively low variance levels in a range of different liquid conditions, and in other replica measurements in liquid [30], supports high reproducibility. In biofilms, however, despite the fact that we did not bottleneck any population upon inoculum, the correlation between replicas was very low **(Fig. 1b**). The reproducibility between biological replicas indicates selection, thus indicating that that protein and organismal fitness are tightly coupled. In biofilms however, the lack of reproducibility indicates the dominance of drift, i.e., random sampling of GudB alleles.

To quantify the contribution of selection versus drift in different conditions, we used two criteria. Firstly, we compared the variability in FC values between replicas by calculating the standard deviation (SD) per allele (using the logarithm of the FC values; see Methods). The average SD value for all alleles in each experiment ($\overline{SD}$) is given for 7 general growth states (**Fig. 1c; Fig. S3a** & **Table S2**). As can be seen, in liquid, pellicles and spores, the $\overline{SD}$ values between biological replicas were low (< 0.06). In biofilms and bulk soil, however, the $\overline{SD}$ values were > 0.25 indicating low reproducibility.

Secondly, if drift dictates the fate of GudB alleles, codons of the same amino acid would exhibit very different FC values. The deviations between synonymous codons of the same amino acid alleles were calculated, averaged for all alleles in the same experiment, and then for all replica experiments per condition ($\overline{SD}$syn, in log values; **Fig. 1d; Fig. S3b** & **Table S3**). Note that the $\overline{SD}$syn criterion holds within individual replica experiments and is thus independent of the comparison of $\overline{SD}$ between biological replicas. Nonetheless, these criteria are clearly correlated (**Fig. 1c** & **d**). Overall, it appears that in liquid, pellicles and spores, the FC values report the outcome of selection acting on GudB alleles at the amino acid level as expected (in few alleles, selection also acted reproducibly at the codon level, **Fig. S4**). In contrast, in biofilms and bulk soil we consistently observed higher $\overline{SD}$ and $\overline{SD}$syn values. In some biofilm experiments, the $\overline{SD}$syn values exceeded 3 (*i.e.*, > 10^3 SD values on a liner scale). Thus, in effect, a single codon had taken over.

Given that some conditions were selection-dominated and others are subject to chance, we divided our analysis in two. Firstly, we analyzed selection

dominated conditions (liquid, pellicles and spores) to examine whether and how the fate of GudB mutations change under different environments. Secondly, conditions where drift prevailed (germination, biofilms and soil colonization) were analyzed to reveal the relative contributions of selection *versus* chance and the molecular mechanisms of drift.

Pleiotropy - fitness-effects of mutations are condition-dependent

While the FC values, and hence the fitness effects of mutations, were reproducible under many conditions, their distribution varied widely between conditions, including between carbon-nitrogen sources (**Fig. S5**). This indicates pleiotropy – individual GudB alleles have different fitness effects in different conditions. To quantify the level of pleiotropy, we compared the FC values of the same GudB mutation across the 9 individual selection-dominated conditions. Because the number of generations differs from one condition to another, we focused on shift from beneficial to deleterious, and vice versa (sign, or antagonistic pleiotropy) as the sign of the FC values of mutations indicates their fitness sign irrespective of generation numbers. Representative dot plots comparing the FC values across 3 different liquid conditions are shown (**Fig. 2a**). These indicate that pleiotropy is common, even when comparing liquid cultures with overlapping carbon-nitrogen sources. In particular, a significant number of GudB mutations show antagonistic pleiotropy (dashed squares, **Fig. 2a**). Indeed, the Pearson correlation values for the 36 possible pair-wise comparisons of 9 conditions were below 0.7 (**Fig. 2b**). Across all selection-dominated conditions, up to 84% of alleles showed antagonistic pleiotropy in one or more of the 36 pair wise comparisons, and 70% of alleles showed mild or strong antagonistic

pleiotropy. These pleiotropic effects are far beyond experimental noise, as indicated by comparison to a control sample (**Fig. 2c**).

Overall, the dominance of pleiotropy meant that across all protein – organismal fitness coupled conditions, 86% of the alleles were beneficial in at least one condition. However, not a single mutation was beneficial across all conditions. Further, if a mutation were to be considered deleterious if it was purged in at least one condition, then 98% of the tested GudB mutations were deleterious. To our knowledge, the degree of pleiotropy in protein mutations across multiple environments has not been measured so far. The exceedingly high degree of pleiotropy we found may relate to GudB's multiple roles, as an enzyme and regulator, and also to the chosen mutated positions (oligomer interface), but it may well be a general characteristic of proteins with key physiological roles. In some samples, only two alleles were present at >1% frequency, one being wild-type (**Fig. 3** & **Data S1**). The near-fixation of relatively few alleles could indicate very strong selection acting on GudB. However, the high $\overline{SD}$ and exceedingly high $\overline{SD}$syn values suggest fixation by chance (**Fig. 1c** & **d**). What is then the nature of these few GudB 'winners', are they merely lucky?

Combined action of selection and drift in heterogeneous environments

While drift dominated in biofilms and soil colonization, curiously, wild type GudB was enriched in up to 85% of these experiments suggesting that selection does play a role (**Fig. 3**). To assess the action of selection, we compared the three biofilm areas. There appears a systematic trend, whereby enriched alleles in the edge are more likely to arise from alleles that persisted or even enriched in the center (**Fig. 4a**). Similarly, although gradient biofilms were clearly dominated by

211 drift, 75% of the enriched alleles were neutral or beneficial under liquid growth
212 with proline, a condition under which GudB experiences the strongest selection
213 (**Fig. 4b**). This suggested that GudB is under selection at the early stages of
214 biofilm development. Accordingly, we found that in biofilm centers, the FC values
215 are less skewed and more reproducible (**Fig. S5**), and also, the center $\overline{SD}$syn
216 values are half than in the edge or wrinkles (**Table S3** & **Fig. S3b**). The $\overline{SD}$syn
217 values are obviously higher in the biofilms' center compared to liquid cultures,
218 but the trend suggests that at the onset of the biofilm's development, selection
219 acts on GudB (**Table S3** & **Fig. S3b**).

220 Similarly, we tested for signatures of selection in soil colonization. As in
221 biofilms, there is a statistically significant trend, whereby enriched alleles in the
222 root are more likely to arise from alleles that were enriched in the soil (**Fig. 4c**).
223 Further, 19 amino acid substitutions were enriched in at least 10 out of the 15
224 sequenced populations, suggesting reproducibility. Of these, in two amino acid
225 alleles, both synonymous codons enriched (D59A and D59V; $\overline{SD}$syn = 0.42 and
226 0.35; **Table S4**) thus indicating selection. Selection during soil colonization is
227 also manifested in the variation between biological replicas ($\overline{SD}$ values) of alleles
228 that were enriched in root populations being on average 20 % smaller than those
229 that were not (**Fig. 4d**). Finally, stop codons were purged in all biofilms and soil
230 populations, indicating, as expected, that GudB's activity is required for *B.*
231 *subtilis'* survival under these conditions [22,23].

232 Altogether, as expected [11], in biofilms, and particularly in soil colonization,
233 both drift and selection determine the fate of GudB alleles. The drivers of drift in
234 biofilms were further unraveled as described in the next section.

235

Drift in biofilms is driven by programmed prophage excisions

Mutagenic rates in biofilms are high and mutations with a selective advantage rapidly take over (genetic sweeps) [31]. Growth in biofilms is also spatially defined, giving rise to segregated lineages whereby an entire segment of the biofilm's edge stems from a single cell in which a beneficial mutation had first emerged [11]. GudB mutations that happen to be in these 'founder' cells might therefore fixate along these lineages. To examine this hypothesis, we sequenced samples for which enough genomic DNA was available (6 ordinary and 12 gradient biofilms, and for comparison, 2 Initial Mixes, 6 liquid and 4 pellicle samples). A range of single nucleotide polymorphisms (SNPs) in various loci was identified across these samples (**Data S3**). We focused, however, on identifying genomic mutations that were not, or scarcely observed in the Initial Mix and/or in liquid samples and were thus emerged and enriched in the biofilms.

Foremost, we observed two large genome deletions that occurred in all biofilms with a frequency approaching 100% (**Figs. 5a** & **5b**). These deletions correspond to the excision of two mobile genetic elements, or prophages, *skin* and SP-β [32–34]. Excision of *skin* generates a functional protein: sigK - a sporulation-specific transcription factor essential for cell differentiation in *B. subtilis* [35]. The excision of SP-β generates a functional CapD – an enzyme mediating production of poly-γ-glutamate, an essential component in capsule formation and biofilm development [36,37]. Nearly all biofilm cells carried one of these variations, and most cells carried both (**Fig. 5a & b**; **Table S5** & **Data S3**). Given their dominance [38], these structural variations are likely to be the primary cause of genetic sweeps and of GudB's drift (**Fig. 5c**). These prophage excisions

are also likely to occur in the soil, but the DNA recovered from these samples was insufficient to allow genome sequencing.

Exclusively in biofilms, we also detected 59 enriched SNPs in a conserved region of 16S rRNA (**Table S5** & **Data S3**). However, *B. subtilis* has ten 16S rRNA gene copies. Since these are essentially identical, we could not determine which of these 10 paralogues carried mutations. However, per population, 98% of the 16S rRNA mutations occurred in the same Illumina read suggesting that one paralogue was highly mutated while others remained intact (**Fig. S6**). At this stage, the mechanism of inactivation by multiple proximal 16S mutations, and how inactivation affects biofilm development, remains unclear. Large differences in expression levels of 16S rRNA genes were identified *in P. aeruginosa* biofilms [39], and ribosomal heterogeneity has been linked to biofilm development in *B. subtilis* [40]. However, to our knowledge, mutations in the 16S rRNA genes have not been reported in biofilms.

Overall, the 16S rRNA SNPs, and the structural variations in particular, seem to have a key role in biofilm development in *B. subtilis*. Accordingly, most of these genetic variations were reproducible between replica experiments (**Table S5**) suggesting that they arose during biofilm growth, and then enriched due to their adaptive potential [11]. Thus, although selection dictated the fate of GudB mutations in the early stages of biofilm development, once biofilm promoting mutations appeared and rapidly took over, they drove the fixation of any GudB that happened to be present in the mutated cell.

Concluding remarks

Pleiotropy of mutations is assumed but not at the magnitude unraveled here. Environmental changes, including minute ones like addition of arginine to a proline medium, completely revert the effect of up to 84% of the tested GudB mutations. Pleiotropy severely restricts protein sequence space –if all tested conditions are considered, only 2% of the tested GudB mutations are neutral in the 9 reproducible conditions -when selection strongly acts on GudB. This suggests that wild-type GudB's sequence is in fact unique in being shaped under multiple constrains and environments, as also indicated by its dominance in many conditions. Together, pleiotropy and drift dictate the evolution of short-term polymorphism (micro-evolution), but also the evolution of protein sequences along long evolutionary times and across species (macro-evolution). The correlation between the effects of mutations in laboratory mappings under one specific condition and the natural sequence diversity is therefore limited [12]. Merging of data from multiple reproducible conditions does not seem to improve correlation, also when applying a number of machine learning techniques (stochastic gradient descent classifier, support vector machines, or random forest classifier; **Fig. S7**).

Thus, along short evolutionary periods, proteins experience variable and opposing selection pressures. Additionally, drift may lead to rapid fixation of alleles that are marginally fit or even deleterious. The effects of drift have been extensively studied initiated by Kimura's neutral theory [10]. Our results quantify its effects in bacterial populations and the potential effect of drift in combination with selection across different environments. For example, nearly 80% of the tested mutations survived or enriched sporulation, and a single spore can start a whole new population. However, once the environment changes, such alleles will

309 be rapidly lost unless compensated by other mutations. Indeed, along macro-

310 evolutionary time scales, epistasis dominates gene and genome sequences [41].

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Authors Contribution

L.N.G. and D.S.T. designed experiments and wrote the manuscript. L.N.G., D.D. and D.S.T. analysed the data. L.N.G. performed all experiments, except selection in soil colonization that was performed in collaboration with E.K. and A.A. D.D. and A.E. wrote the scripts used for data analysis and visualization. E.P. applied machine learning classification.

454 **Fig. 1. Selection *versus* chance-dominated conditions. (A)** Dot-plot indicating
455 reproducible measurements of frequency coefficients of individual mutations
456 (FC values) in three parallel replica liquid cultures with proline as carbon-
457 nitrogen source. $\overline{SD}$ is the average standard deviation between biological
458 replicas. The S.D. values were calculated per each amino acid allele based on
459 logFC values and averaged for all alleles in a given condition. **(B)** The same
460 analysis of three parallel biofilms with arginine as carbon-nitrogen source
461 indicates low reproducibility. **(C)** The $\overline{SD}$ values categorized by the 7 general
462 growth states tested here. Each point represents the $\overline{SD}$ value between replicas
463 of the same experiment (the distributions of SD values per each condition are
464 shown in **Fig. S3a**). **(D)** $\overline{SD}$syn represents the standard deviation between the
465 logFC values of synonymous codons. The standard deviations per allele were
466 averaged for all synonymous alleles in the same replica experiment, and then
467 averaged across the 3 replica experiments in a given condition (the distributions
468 of SD*syn* values per experiment are shown in **Fig. S3b**).
469

Fig. 2. The pleiotropic effects of alleles across different conditions. (**A**) A dot-blot correlation of FC values of individual alleles in three different liquid carbon-nitrogen sources. The red squares encompass alleles that show sign pleiotropy – i.e., a change from beneficial to deleterious, or vice versa. (**B**) Pairwise correlation of the FC values in all 9 different reproducible conditions (average FC values of all alleles and replicas). Colors indicate the Pearson correlation values (-1, negative correlation; 0, no correlation; 1, positive correlation). The strongest anti-correlation was found with arginine as carbon-nitrogen source (black square). **(C)** The distribution of alleles by their level of sign pleiotropy. From pale to dark): (*i*) Weak sign pleiotropy (changes between deleterious and beneficial); (*ii*) Mild sign pleiotropy (changes from highly deleterious to beneficial, or from highly beneficial to deleterious); and (*iii*) Strong sign pleiotropy (changes from highly deleterious to highly beneficial, or vice versa). The fraction of alleles showing mild or strong sign pleiotropy is shown above the bars. The control dataset comprises 4 completely independent growth experiments in liquid proline, each inoculated from a different initial mix and grown on separate occasions (**Fig. S8**). Nonetheless, none of the alleles in this control set exhibited strong pleiotropy.

Fig. 3. Genetic sweeps in biofilms and soil. Photographs of: **(A)** normal biofilms; **(B)** gradient biofilms; **(C)** a scheme of soil colonization (shown biofilms with proline as carbon-nitrogen source). **(D)** The distribution of frequency of individual alleles for different growth states with proline as carbon-nitrogen source. Bar widths represent allele frequency from raw read counts (Rf values**; Data S1**). Magenta corresponds to wild type GudB. **(E)** Alleles with Rf ≥ 1% were identified and their number and sum of frequencies are shown (averages and standard deviations for all experiments in a given condition). Blue designates selection-dominated conditions and red drift-dominated ones, as in **Fig. 1**.

499 **Fig. 4. The combined action of selection and chance in biofilms (red) and**
500 **soil colonization (green). (A)** Alleles that enriched in the edge of the biofilms
501 are more likely to arise from alleles that were neutral or enriched in the center.
502 The distribution of categorized FC values from all biofilm centers (grey)
503 compared to the distribution of FC values of center alleles that were enriched in
504 the edge (red). **(B)** The distribution of categorized FC values of all alleles in all
505 liquid conditions (grey) compared to the distribution of FC values of liquid
506 alleles that were enriched in the edge of biofilms (red). **(C)** Alleles enriched in
507 the root are more likely to arise from alleles that were enriched in the bulk soil.
508 The distribution of categorized allele FC values in all soil samples (grey)
509 compared to the distribution of FC values of soil alleles that were enriched in the
510 root (green). T-tests were computed per each FC category. One, two or three
511 asterisks indicate a p-value < 0.05, <0.001, or < 0.005, respectively. **(D)** The
512 distribution of SD values (variability between replica experiments, as in **Fig. 1c**)
513 of alleles enriched in one or more root populations compared to alleles that were
514 never enriched in the roots.
515

Fig. 5. Programmed genomic excisions drive GudB's drift in biofilms. Schematic representation of *B. subtilis* genomic organization before and after the excision of the prophage mobile elements SP-β (**A**) and *skin* (**B**) and their position in the genome. (**C**) These excisions were absent in the initial mix yet dominated biofilms and went to near fixation in the edge of gradient biofilms (for frequencies in individual experiments see **Table S5**). Excision of the mobile elements occurred in two different genomic locations within the same experiment. The values were summed and averaged according to the general condition shown. The details of the excisions (location and frequency) per experiment are shown in **Data S3**.

Materials and Methods

Strains

B. subtilis NCIB 3610 DS7187 (gently gifted by Dr. Daniel B. Kearns [42]) that lacks the ComI peptide and has high competence capacity similar to domesticated *B. subtilis* strains was recruited to this study. *Bacillus subtilis* NCIB 3610 *gudB::tet* strain [23] genomic DNA was transformed into *B. subtilis* NCIB 3610 DS7187. *B. subtilis* NCIB 3610 *ΔcomI gudB::tet* was thus isolated, and was phenotypically and genetically tested.

GudB allele library construction

We performed site directed mutagenesis in 10 codons (amino acids: M46, L48, K52, D58, D59, S61, K63, T66, Y68, S75) of the *gudB* gene cloned in the pDG_GudB plasmid [23]. The codons were mutated to NNS (N = all bases & S = C or G) whereby the 20 standard amino acids and 1 stop codon is encoded. The codon mutagenesis was done in one step PCR protocol and independently for each position. Thus, we created 10 libraries, each containing 20 different amino acid alleles (non-synonymous, missense mutations), 1 stop-codon (nonsense), and 11 synonymous alleles (alternative codons encoding the same amino acid). All mutagenic PCRs were performed with Kapa HiFi HotStart Ready Mix (Kapa Biosystems) following manufacturers conditions (**Table S6** shows the sequence of all primers). The 10 PCR products were purified and used to transform the *E. coli* T10 strain (Thermo Fisher Scientific). Clones were pulled together after an overnight growth on LB + Ampicillin (100 µg/ml) agar plates at 37°C. At this stage, 4 to 6 clones per library were isolated and analyzed by sequencing. Total plasmid DNA from these library transformations was extracted and also analyzed by sequencing. Each of the 10 libraries contained, after transformation, at least 10^5 clones, corresponding to ≥ 1000-fold coverage per allele. Approximately 10 µg of plasmid DNA, from each library, was linearized (XhoI, New England Biolabs, following manufactures conditions), purified, and used to transform the *B. subtilis* NCIB 3610 *gudB::tet ΔcomI* strain. Transformations were performed as described [23]. After transformation, overnight growth on in + Spectinomycin (100µg/ml) + Glucose (0.5 mg/ml) agar plates was used as selection. The resulting cells were pulled together and kept at -20°C in 50%

glycerol. In total, 10 *B. subtilis* libraries were constructed in parallel and each contained, after transformation, at least 10^4 clones (≥100-fold coverage per allele). Genomic DNA extraction of each library was performed (GenElute - Sigma). The integrity of the mutagenic process was verified by sanger sequencing the *amyE::gudB* locus indicating that mutations were observed only in the diversified codon.

Selection and growth conditions

10 ml of LB with Glucose (0.5%), ammonium sulfate (0.5%) and spectinomycin (100 µg/ml) cultures were inoculated with 1 ml of each library stock. The cultures were grown overnight at 37°C with shaking. 500 µl of the overnight culture was used to inoculate 3 ml of LB plus glucose (0.5%) and ammonium sulfate (0.5%). The cultures were incubated at 37°C with shaking and once the $O.D._{600}$ reached 0.8 they were mixed equally and used as the starting population (Initial Mix). A fraction of the cells at this stage were harvested by centrifugation and stored for genomic DNA purification. In total, three different initial mixes were used for the experiments described here. Initial Mix #1 was used to inoculate most liquid conditions (4 carbon-nitrogen sources), pellicles and gradient biofilms. Initial Mix #2 was used to inoculate 1 liquid condition, spores, germination and biofilms, and Initial Mix #3 was used to inoculate bulk soil (**Data S1**). Detailed selection conditions are listed below:

For selection under liquid serial passages 100 ul of the Initial Mix was used to inoculate 10 ml cultures of MS medium [23] with glucose (0.5%) plus ammonium sulfate (0.5%), glutamate (0.5%) plus glycerol (0.5%), proline (0.5%), arginine (0.5%) or proline (0.25%) plus arginine (0.25%). The cultures were incubated at 37°C with shaking until $O.D._{600}$ reached 1 – 1.5, after which 100 µl was used to inoculate 10 mL of fresh medium. The serial passages were done every 24 hours when proline (0.5%), arginine (0.5%) or proline (0.25%) plus arginine (0.25%) where used as carbon-nitrogen sources, and every 12 hours when glucose (0.5%) plus ammonium sulfate (0.5%), or Glutamate (0.5%) plus glycerol (0.5%), were applied. In total, all liquid passages were maintained for approximately 50 generations.

For selection in pellicles, 100 ml of media (b), (c) and (d) were inoculated with 100 µl of the initial mix cells. The culture was incubated at 30°C without shaking, for 5 days.

For selection of spores and germinated spores, three ml of the Initial Mix was used to inoculate 25 ml of Difco Sporulation Medium (DSM) in 250 ml flasks and incubated at 37°C with 150 rpm shaking until O.D.600 reached 0.4. This culture was used to inoculate 250 ml of fresh DSM in 1L flasks. The cultures were incubated 48h at 37°C with 150 rpm shaking. Cells were subsequently harvested by centrifugation and stored at 4°C over night. After, cells were re-suspended with 200 ml of cold deionized sterile water (dW) and incubated for 30 min at 4°C. Cells were harvested and re-suspended with 200 ml of cold distilled water (dW) and incubated overnight at 4°C with slow orbital agitation, to kill all planktonic of vegetative cells. The culture was harvested, re-suspended in 30 ml of dW and heated to 80°C for 20 min. Finally, spores were harvested, re-suspended in 10 ml of dW, and stored at -20°C. To germinate these spores, they were diluted 1000 times in phosphate-buffered saline solution and 100 µl of this suspension was used to inoculate LB plus glucose (0.5%) agar plates (10 plates). Approximately 10,000 colonies were obtained and pulled together.

For selection in biofilms, MS agar (1.5%) plates supplemented with different carbon-nitrogen sources were prepared. For gradient biofilms, gradient agar plates were prepared. First, square plates (12x12 cm) with MS agar (1.5%) medium were poured. After the agar solidified, an area of 2x14 cm was removed from the top of the plate. In this area, a solution of either Proline 5%, Arginine 5%, monosodium glutamate 5% or Glycerol 5% in 1.5% agar was poured into the removed section. For the glutamate plus glycerol gradient biofilm, two opposite areas of the agar plate were removed. Into one, a solution of monosodium glutamate (5%) in 1.5% agar was poured, and into the other, glycerol (5%) plus 1.5% agar solution (see **Fig. S9a** for a graphic representation of the agar plates preparation). All gradient agar plates were incubated for 24 h at room temperature before use. We also calibrated the place in the gradient plate where we inoculated the cells such that we observed growth after 1 night incubation at 30°C (**Fig. S9b**). For growth in biofilms and gradient biofilms, 5 µl of the Initial Mix were used as inoculum. Plates were incubated for 4 days at

30°C and 2 more days at room temperature. The colony was then dissected in 3 areas (center, wrinkle and edge) for normal biofilms, and in 2 areas (center and upper) for gradient biofilms (illustrated in **Fig. S9c-g**). After selection in all the above-mentioned conditions the biomass was harvested and storage at -20°C. All growth experiments were performed in triplicate by inoculating with the same Initial Mix.

For selection in soil and plant roots, the Initial Mix was generated as above-mentioned except that the process was scaled up (instead of 3 ml, 10 ml of culture was prepared per library). In total, 200 ml of the Initial Mix ($O.D._{600}$ = 0.8) was applied. This LB culture was washed three times (by means of centrifugation and re-suspension) with 100 ml half strength Hoagland solution [43]. Since Hoagland's solution is not isotonic, the washes resulted in death of about a third of the *B. subtilis* cells. Thus, handling the samples at this stage was performed as fast as possible. After the final wash, the cells were re-suspended in half strength Hoagland solution to a final $O.D._{600}$ of 0.1. Natural soil was collected at the Ha-Masrek Reserve, Israel (31.793 N, 35.042 E), sifted through 2 mm sieve and autoclaved three times for 30 min at 121°C. A total of five pots (size 10 x 8 x 5 cm) with autoclaved natural soil were drenched with the Initial Mix suspended in half strength Hoagland Solution [43]. These potted soils drenched with bacterial suspensions were used to plant tomato seedlings grown first in sterile conditions. Seeds of tomato (*Solanum lycopersicum L.*; cv. Micro-Tom) were surface-sterilized with 70% ethanol for 5 minutes and, 10 minutes with 3% bleach with 0.01% Tween 20. Surface-sterile seeds were germinated on sterile filter paper (Whatman, catalog # 1001-085) saturated with half strength Hoagland Solution for 7 days (23°C and 16 hours photoperiod). Six tomato seedlings were transferred to each pot and grown for one month (21°C, 16h light, 8h dark) with drenching with half strength Hoagland twice a week. Plants were subsequently harvested from the five pots. Roots and rhizosphere samples were collected for each replica experiment consisting a pool of six roots. First, the plants were carefully removed from the soil. Roots were then cut out from the plants and vortexed in 20 ml of washing solution (0.85% NaCl) for 30 s. This step was repeated one more time with a fresh washing solution. The combined root washing solutions (40 ml) was centrifuged for 30 min at 3000 rpm and the

658 resulted pelleted samples corresponding to the rhizosphere were frozen in liquid
659 nitrogen and stored at -80°C. The washed roots were blotted in filter paper and
660 stored at −80°C until further use. Finally, bulk soil without roots was also stored
661 at −80°C.
662
663 **Genomic DNA extraction**
664 All samples, including pellicle, spores, biofilm and gradient biofilm samples, were
665 defrosted and re-suspended in 10 ml of dW. The samples were sonicated at 40%
666 power, VibraCell, Sonics, for 10 min at 60 s intervals. Cells debris was harvested
667 by centrifugation (13,000 g for 20 min). Genomic DNA from all samples was
668 extracted using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich)
669 generally following the manufacturer's instructions, with the exception of the
670 soil, rhizosphere soil and plant roots samples. For these samples, the PowerSoil
671 DNA Isolation kit of Mo Bio was used, following its manufacturer's instructions.
672
673 **Illumina sample preparations**
674 The mutagenized *gudB* fragment (from amino acids 45 to 81) was amplified
675 using the primers GudB_In_For (5'-
676 CTCTTTCCCTACACGACGCTCTTCCGATCTnnnnnnCCCGAAGAGGTATACGAATTGT
677 TAAAAGAG), and GudB_In_Rev (5'-
678 CTGGAGTTCAGACGTGTGCTCTTCCGATCTCGCCTTTCGTTGGACCGAC). To the
679 GudB_In_For primer, 6 N's were added to increase the sequence variability
680 between amplicons. PCRs were performed with the KapaHiFi HotStart Ready Mix
681 (Kapa Biosystems) using approximately 100 ng of genomic DNA as template and
682 following manufacturer's instructions. Using 10 µl of the PCR as template, a
683 second PCR was performed to add the Illumina adaptor sequence, using primers
684 GudB_Out_For (5'-
685 AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGC) and
686 GudB_Out_Rev (5'-
687 CAAGCAGAAGACGGCATACGAGATTGTTATACGTGACTGGAGTTCAGACGTGTGC).
688 The Illumina index (underlined) was changed in the GudB_Out_Rev primer to
689 different Illumina indexes. Each condition was differently barcoded. All PCRs
690 were purified using the Agencourt AMPure XP (Beckman Coulter). The

concentration of PCR products was verified using Qu-bit assay (Life Technologies).

Analysis of the Illumina reads

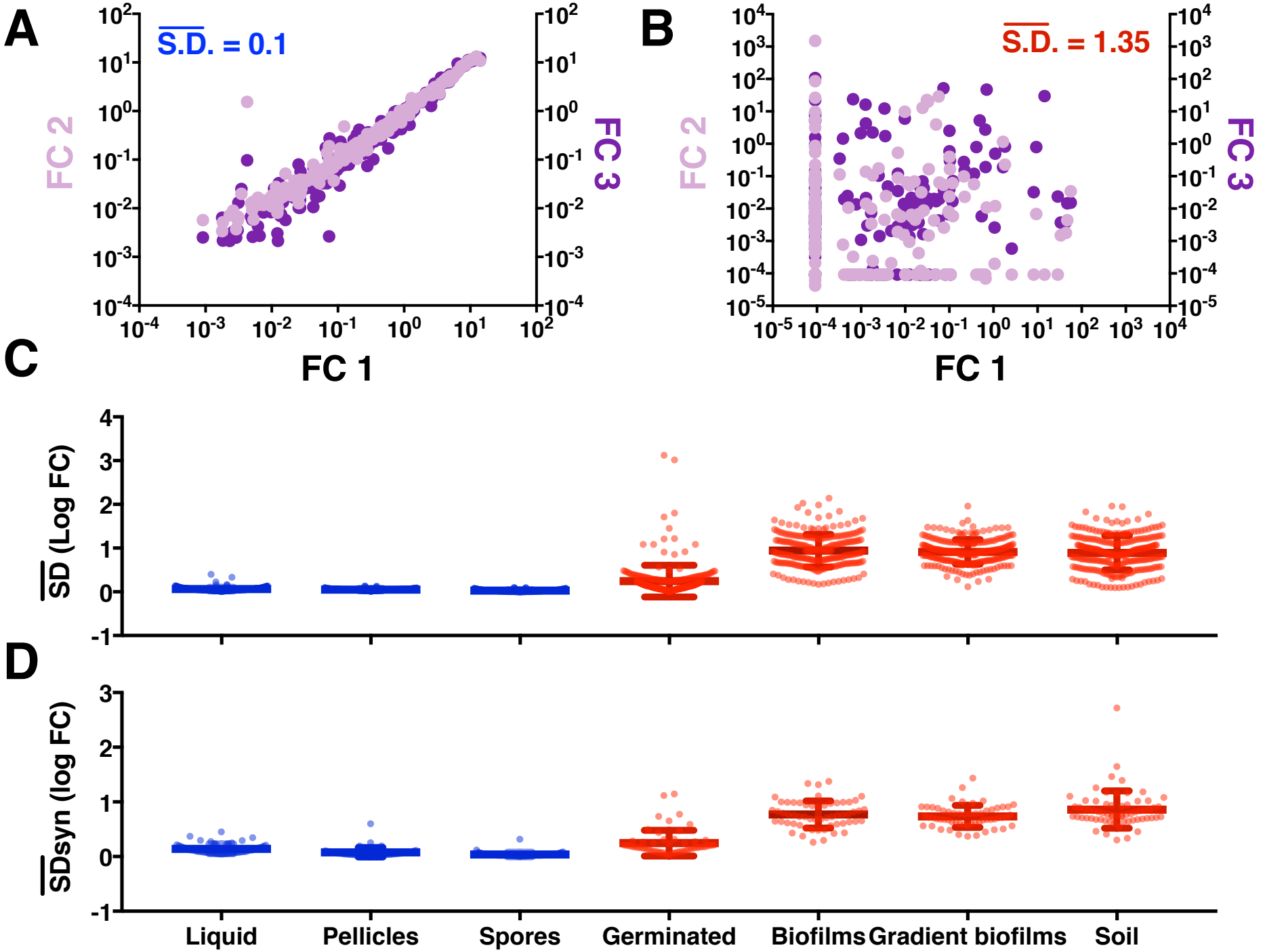
DNA samples were run using the Illumina NextSeq 150-bp paired-end kit. The FASTQ sequence files were obtained for each run and customized using MatLab 8.0 and Python 3.6 scripts designed to count the number of each individual allele in each sequenced sample. We filtered the reads to exclude any reads that have mutations outside the mutagenized codons. All codons encoding for the wild-type amino acid were summed in one and assigned as WT. All other codons were counted independently. The unprocessed read counts are shown in **Data S1**. Further filtering excluded alleles with < 100 counts in the Initial mix to avoid statistical uncertainty with respect to FC values. In total, we obtained data for up to 269 individual alleles per condition out of the originally introduced 320 alleles. Per condition, a minimum of 380,000 reads was obtained. Thus, in average, we obtained 1500 reads per allele.

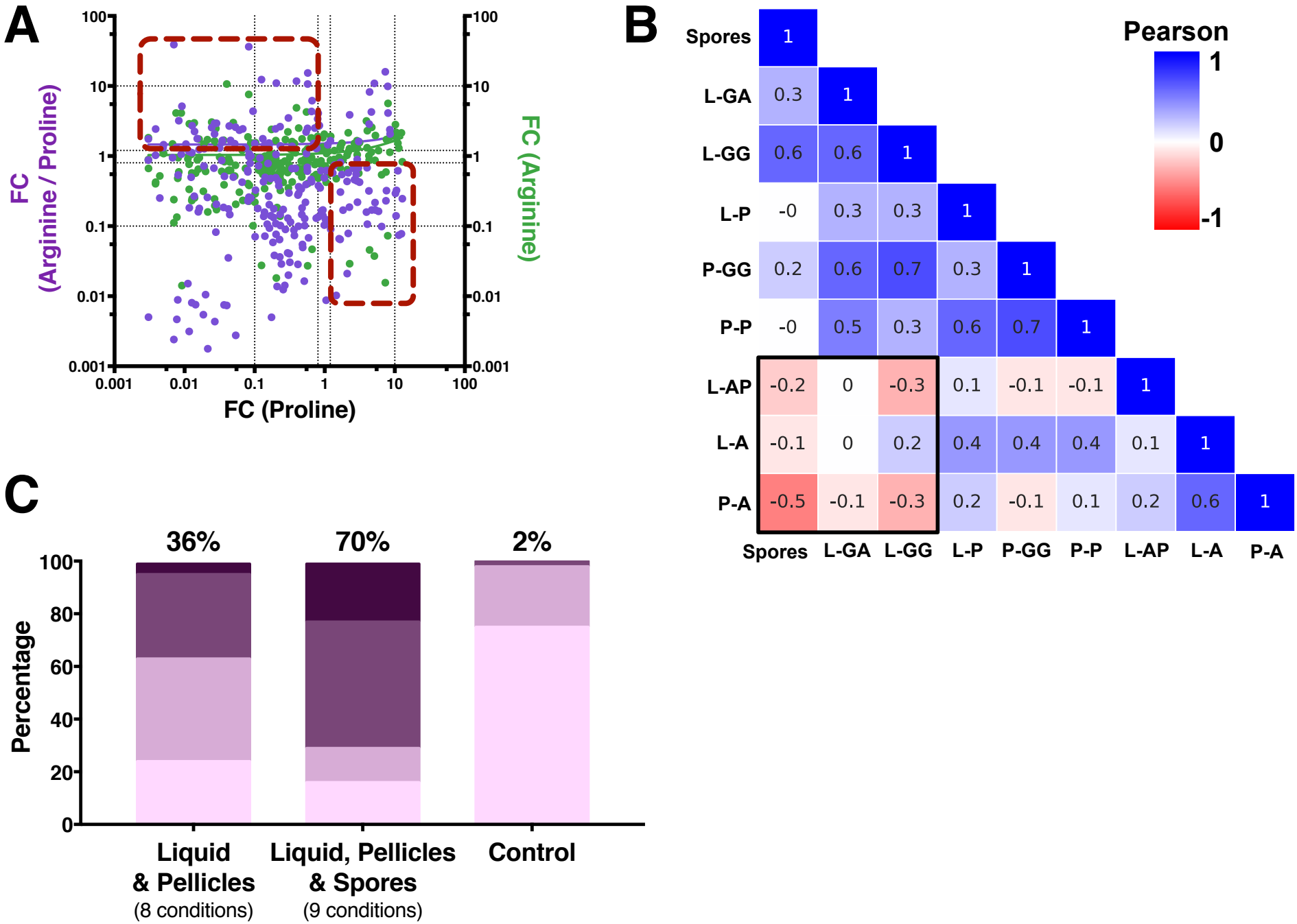
Data Analysis

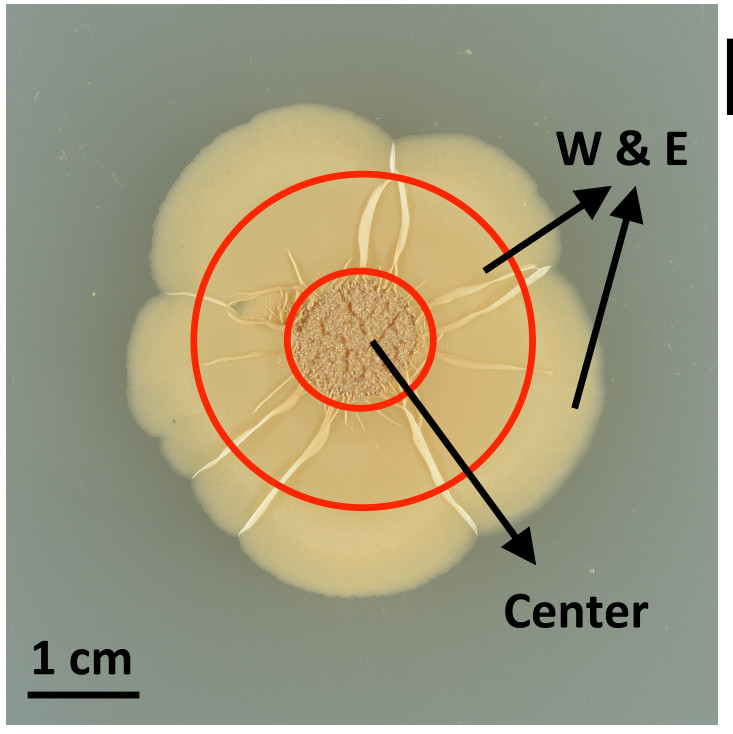
The frequency of each allele (f_i) was calculated as the ratio between the number of reads for allele i divided by the total number of reads. The allele frequency coefficient (FC_i) was subsequently calculated as the ratio of after selection (f_i) divided by the frequency of the same allele in the initial mix (**Fig. S2** & **Data S2**). Normalization by the number of wild-type reads rather than by the total number of reads gave essentially identical FC values for the majority of samples. However, in the few samples where wild-type frequency was significantly reduced after selection, normalization by wild-type reads resulted in high noise and large biases including large changes in sign (higher sign pleiotropy). FC values relate to fitness logarithmically, and thus logFC values were compared. To this end, all FC's equal to zero had to be changed, and we opted for a tenth of the minimum FC value found amongst all experiments. For the liquid, pellicles, biofilms, spores and germinated spores experiments (**Data S2, sheet 1**) the zeros were changed to 4.2×10^{-6}. For the bulk soil experiments (**Data S2, sheet 2**) zeros were changed to 1.14×10^{-5}. The logarithm of all FC values was calculated and was also used to derive mean FC values. The logFC values were

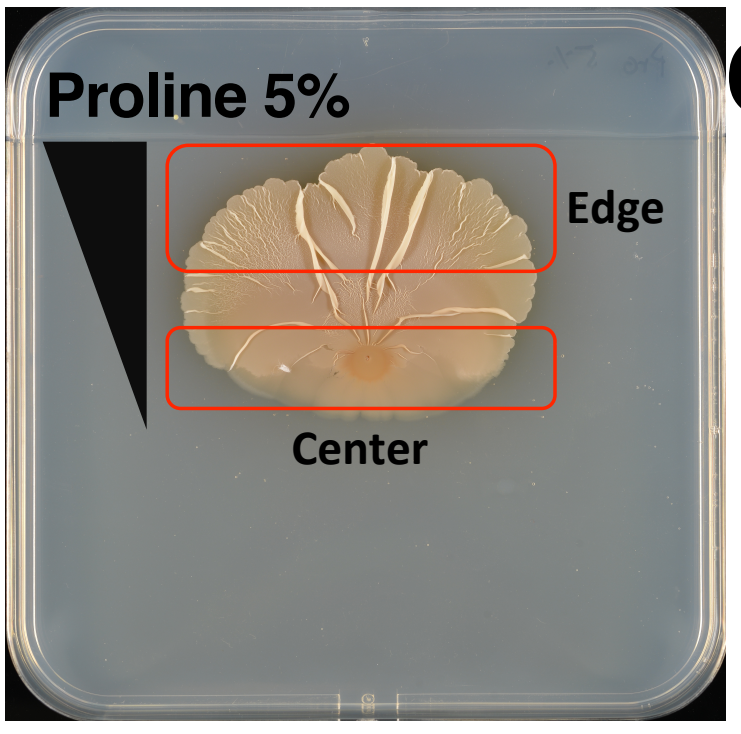
724 then used to calculate: (i) the standard deviation for all alleles across conditions
725 ($\overline{SD}$; the standard deviation between logFC values observed per each allele in
726 replica experiments were averaged for all alleles measured in a given condition);
727 (ii) the standard deviation between synonymous codons within the same replica
728 experiment (deviations between logFC values of synonymous codons of the same
729 amino acid allele were calculated, averaged for all alleles in the same experiment,
730 and then for all replica experiments per condition).
731
732 **Defining the limits of neutrality**
733 From all conditions tested here, only in glucose plus ammonia the GudB
734 knockout had no growth effect (**Fig. S1**). Hence, this condition is largely neutral,
735 and the variation observed in FC values would primarily be the outcome of noise.
736 The standard deviation between 3 biological replicas was calculated per allele,
737 and these values spanned over the range of 0.002 to 0.199. We rounded this
738 number to 0.2. Thus, by the strictest measure, FC values between 0.8 and 1.2
739 were classified as 'neutral'. Accordingly, FC ≤0.8 unambiguously assigned a
740 mutation as 'deleterious', and FC >1.2 as 'beneficial'.
741
742 **Genome sequencing**
743 We sequenced the genomic DNA of all biofilm populations for which we had ≥ 1
744 µg of DNA after extraction (6 normal and 12 gradient biofilm). For comparison,
745 we also sequenced Initial mix populations 1 and 2, 6 Liquid and 4 pellicle
746 populations. The Illumina HiSeq2500 platform was used, with 2x125 base pairs
747 read length. We obtained a total of 300 million reads. The reads were assembled
748 using as reference the *B. subtilis* NCIB 3610 genome (NCBI Accession number:
749 CP020102). Overall, 95% of all reads were successfully mapped to the reference
750 genome with minimal coverage of x300 for all samples analyzed. The Breseq
751 program was used to identify genomic variants, including single nucleotide
752 polymorphisms (SNPs) and insertion-deletion polymorphisms (INDELs) [44] (**Data**
753 **S3** & **Table S5**).
754
755 **Comparison of FC values and to GudB's natural sequence variability**

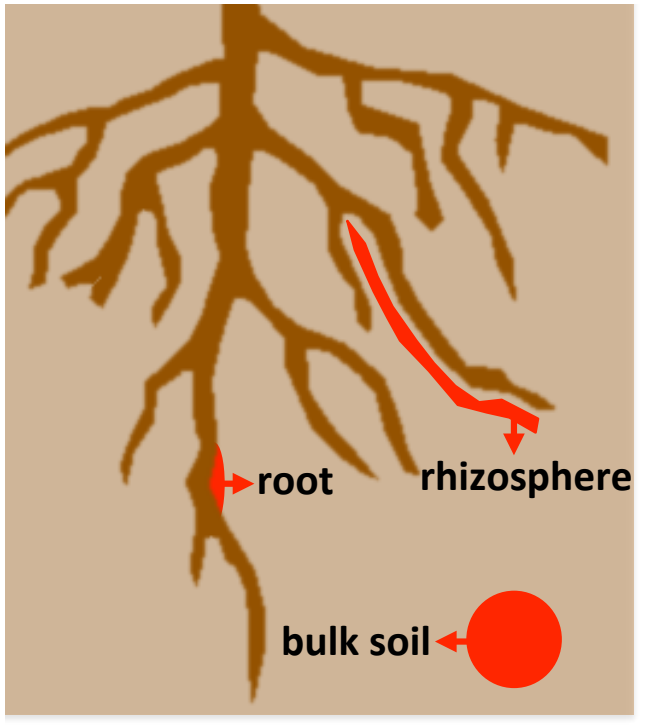
We examined whether the FC values for individual mutations, in individual conditions, might predict whether or not a certain sequence exchange is observed, or not, amongst the sequences of extant GDHs. To this end, we constructed a number of different support vector machines (SVM) classification models with a variety of kernels (such as linear, Gaussian, polynomial etc.). The feature vector of each GudB allele was composed from the normalized FC values from specific condition. The values from replica experiments of the highly reproducible liquid conditions were averaged prior to training. Based on the multiple sequence alignment containing 1013 GDH sequences, we divided the GudB mutations in our dataset into 3 categories, which were then utilized as the prediction labels: (1) mutations seen in less than 5 natural GDH sequences (classified as 'not present', 66% of mutations), (2) mutations observed in 5 - 49 sequences ('rare', 19%) and (3) mutations present in ≥50 sequences ('frequent', 15%). Introducing class weights into the loss function compensated the unbalanced nature of the dataset. For each feature combination of a varied length, we built an SVM classification model and assessed its accuracy using 3-fold cross validation. Additionally, in order to reduce noise, assuming that our data belong to linear space, we extracted the first ten principal components of the feature matrix and used them as the new feature vectors for a model construction. To examine if our relatively high (>0.6) model accuracy was distributed uniformly across different classes, for each model and genotype, we recorded the predicted values during 3-fold cross-validation. Moreover, for each condition combination, and for each kernel, we built 100 different models and recorded the number of times each of the genotypes was predicted correctly.

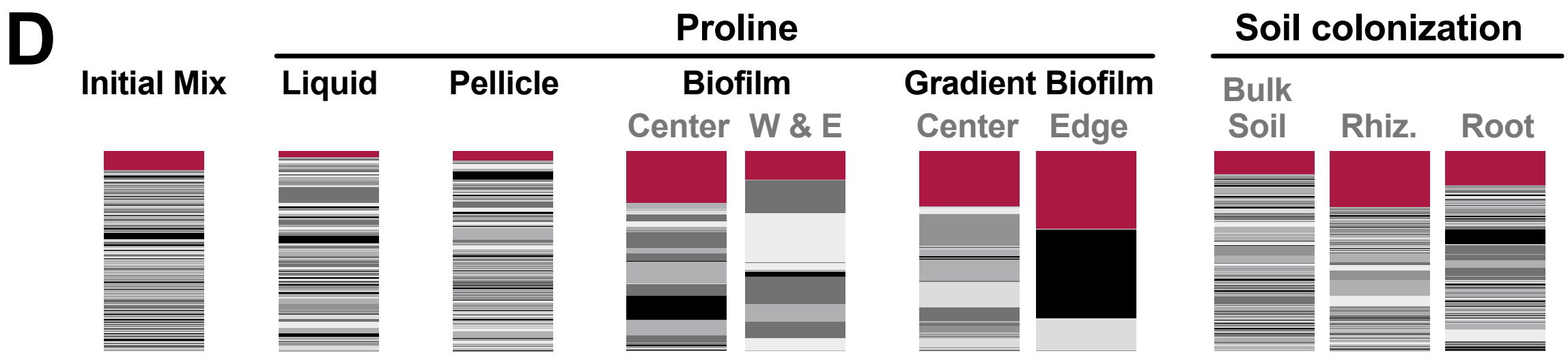

A
S.D. = 0.1
FC 2
FC 3
FC 1
B
S.D. = 1.35
FC 2
FC 3
FC 1
C
SD (Log FC)
D
SDsyn (log FC)
Liquid
Pellicles
Spores
Germinated
Biofilms
Gradient biofilms
Soil

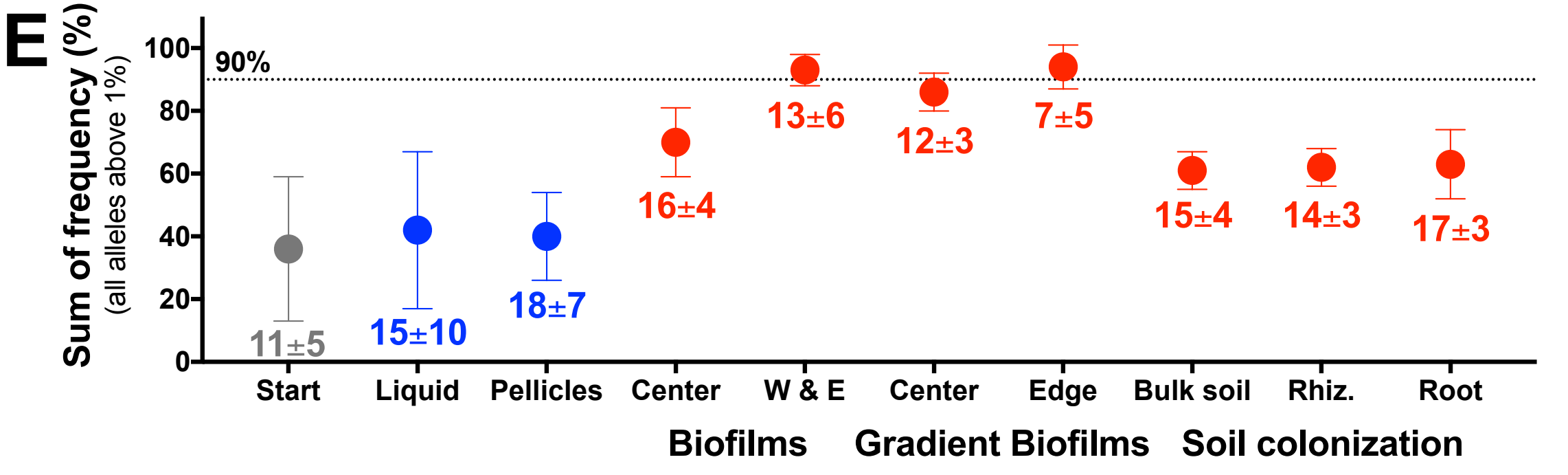

A
FC (Arginine / Proline)
FC (Arginine)
FC (Proline)
100
10
1
0.1
0.01
0.001
B
Pearson
1
0
-1
Spores
L-GA
L-GG
L-P
P-GG
P-P
L-AP
L-A
P-A
1
0.3 1
0.6 0.6 1
-0 0.3 0.3 1
0.2 0.6 0.7 0.3 1
-0 0.5 0.3 0.6 0.7 1
-0.2 0 -0.3 0.1 -0.1 -0.1 1
-0.1 0 0.2 0.4 0.4 0.4 0.1 1
-0.5 -0.1 -0.3 0.2 -0.1 0.1 0.2 0.6 1
C
36%
70%
2%
Percentage
100
80
60
40
20
0
Liquid & Pellicles (8 conditions)
Liquid, Pellicles & Spores (9 conditions)
Control

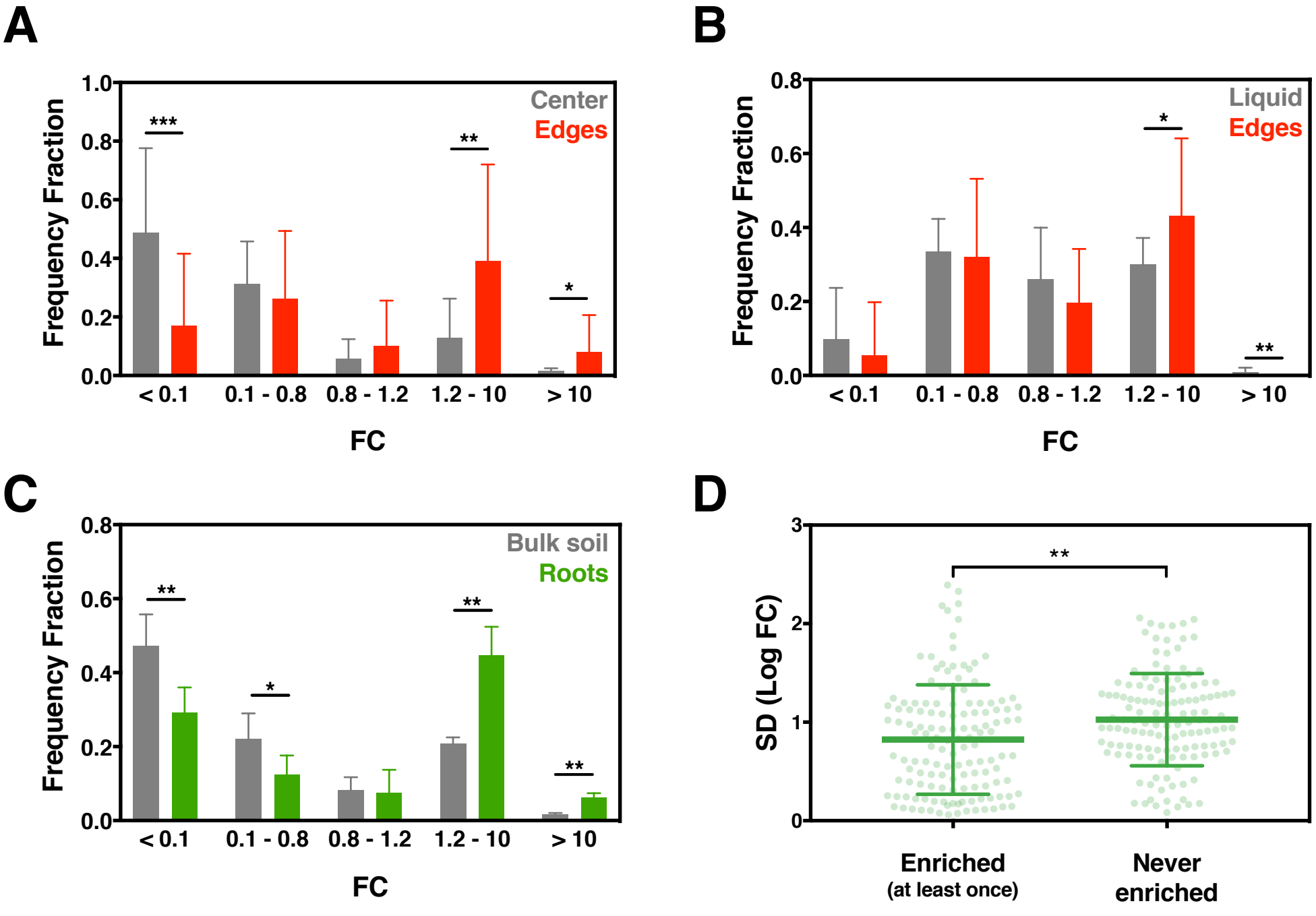


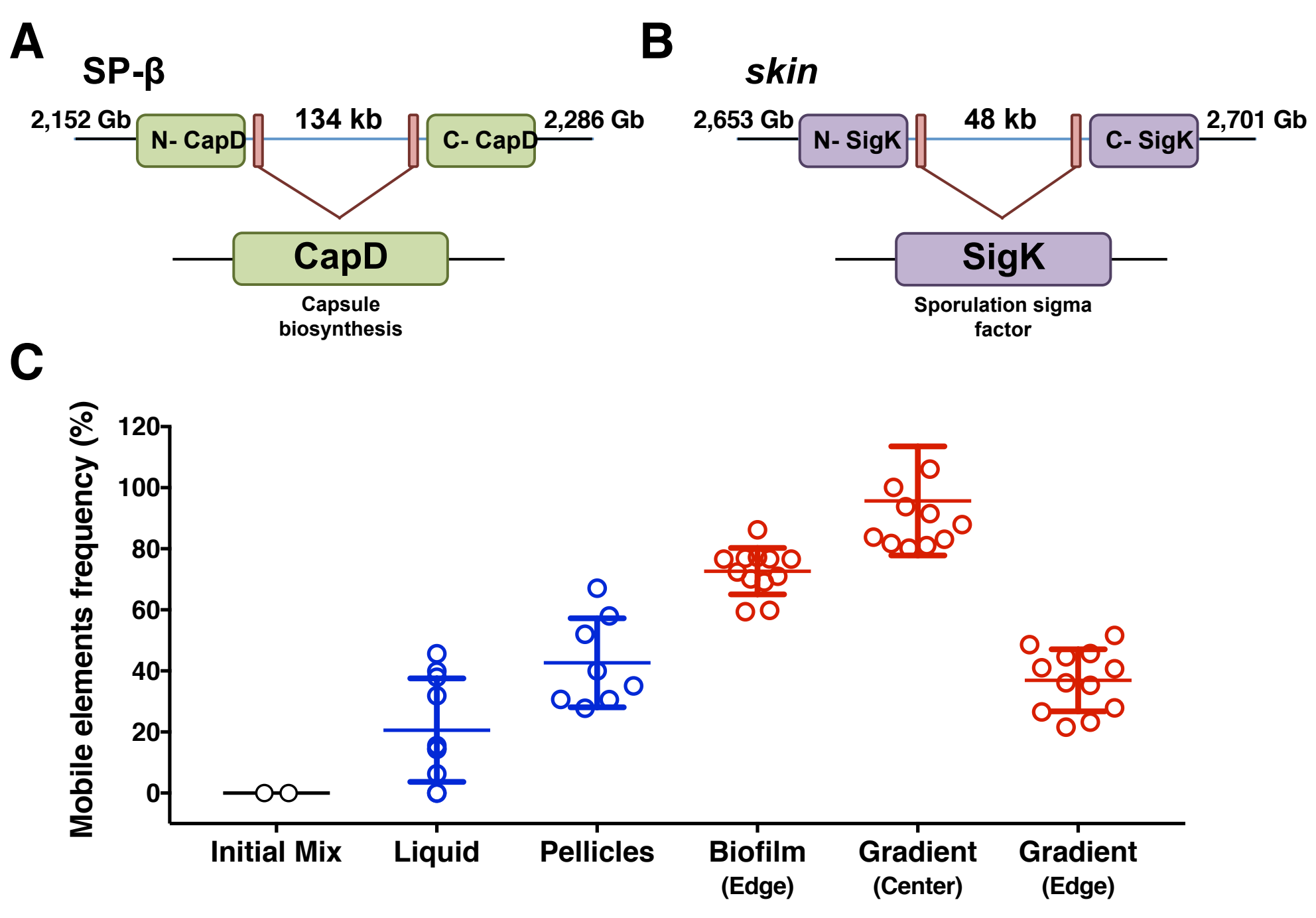









A
Frequency Fraction
1.0
0.8
0.6
0.4
0.2
0.0
Center
Edges
***
**
*
< 0.1
0.1 - 0.8
0.8 - 1.2
1.2 - 10
> 10
FC
B
Frequency Fraction
0.8
0.6
0.4
0.2
0.0
Liquid
Edges
*
**
< 0.1
0.1 - 0.8
0.8 - 1.2
1.2 - 10
> 10
FC
C
Frequency Fraction
0.8
0.6
0.4
0.2
0.0
Bulk soil
Roots
**
*
**
**
< 0.1
0.1 - 0.8
0.8 - 1.2
1.2 - 10
> 10
FC
D
SD (Log FC)
3
2
1
0
**
Enriched
(at least once)
Never
enriched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2,152 Gb
N- CapD
134 kb
C- CapD
2,286 Gb
CapD
Capsule biosynthesis
B
skin
2,653 Gb
N- SigK
48 kb
C- SigK
2,701 Gb
SigK
Sporulation sigma factor
C
Mobile elements frequency (%)
120
100
80
60
40
20
0
Initial Mix
Liquid
Pellicles
Biofilm (Edge)
Gradient (Center)
Gradient (Edge)