Transient heterogeneity in extracellular protease production by Bacillus subtilis

Veening, Jan-Willem; Igoshin, Oleg A.; Eijlander, Robyn T.; Nijland, Reindert; Hamoen, Leendert W.; Kuipers, Oscar P.

Published in: Molecular Systems Biology

DOI: 10.1038/msb.2008.18

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Document Version
Publisher's PDF, also known as Version of record

Publication date: 2008

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Veening, J-W., Igoshin, O. A., Eijlander, R. T., Nijland, R., Hamoen, L. W., & Kuipers, O. P. (2008). Transient heterogeneity in extracellular protease production by Bacillus subtilis. Molecular Systems Biology, 4(6), 184 - 184. [184]. https://doi.org/10.1038/msb.2008.18

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Supplemental supporting material

Supplemental Results

Environmental signals cause DegS to autophosphorylate and transfer its phosphate to the regulator protein DegU enabling it to activate aprE gene transcription (for reviews see Msadek et al., 1993; Kunst et al., 1994; Msadek, 1999). DegS however, also acts as a phosphatase on DegU~P (Dahl et al., 1992). Remarkably, artificial induction of DegU was shown to lead to activation of aprE in a degS mutant background, indicating that other phosphate donor(s) can phosphorylate DegU as well (Ogura et al., 2001). To see whether aprE expression (and thus DegU phosphorylation) is completely DegS dependent under our experimental conditions, we introduced a degS mutation in our aprE reporter strain. The resulting strain aprE-gfp/hy/ΔS was induced with IPTG (500 µM) during the logarithmic growth phase and compared to the parental strain and a strain mutant for both degS and degU by flow cytometry. As shown in Fig. S5, the aprE response in the degS mutant upon DegUhy induction is significantly lower compared to the wildtype after 1 h of induction (P<0.001, Student T-test) and similar to a degSU mutant. This shows that under these experimental conditions, DegS is required to phosphorylate DegU and commence degU autoactivation. After prolonged induction however (3h), the difference in aprE-gfp between wild-type and degS is not significant anymore (P = 0.13, Student T-test). The degSU mutant strain however shows significantly higher aprE-gfp expression at prolonged induction with DegUhy (P<0.001, Student T-test). This is likely caused by the fact that in this strain unphosphorylated DegU cannot accumulate and repress
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\textit{degU} transcription in contrast to the wild-type and the \textit{degS} mutant strains (also see Fig. 4).

To demonstrate that in principle all non-endospore forming cells are able to exhibit fluorescence from production of a fluorescent protein under these conditions, we constructed a strain that harbors both the P_{aprE}gfp construct and an inducible copy of the cyan fluorescent protein. As shown in Fig. S6, all vegetative cells express CFP but not all vegetative cells highly express GFP again demonstrating that heterogeneity in \textit{aprE} expression is regulated.

\section*{Materials and Methods}

\subsection*{Recombinant DNA techniques and oligonucleotides}

Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis and transformation of \textit{E. coli} were carried out as described before (Sambrook et al., 1989). Enzymes were obtained from Roche (Mannheim, GER). Oligonucleotides were purchased from Biolegio BV (Malden, NL). \textit{B. subtilis} was transformed as described before (Harwood and Cutting, 1990).

\subsection*{Construction of plasmids}

To construct plasmids pGFP-aprE, pGFP-bpr, pGFP-acoA, pGFP-srfA, pGFP-hag, pGFP-sunA, pGFP-degS and pGFP-‘degSU, PCR’s with primer pairs aprE-F/aprE-R, bpr-F/bpr-R, acoA-F/acoA-R, srfA-F/srfA-R, hag-F/hag-R, sunA-F/sunA-R, degU-F+HindIII/degS-R+RBS+EcoRI and degU-F+HindIII_inside/degU-R-R+RBS+EcoRI
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(Table S3) were performed, respectively, using chromosomal DNA of *B. subtilis* 168 as a template. The resulting amplified fragments contained the promoter regions of the genes *aprE, bpr, acoA-L, srfAA, hag, sunA, degS* and *degU*, respectively. The amplified fragments were subsequently cleaved with *HindIII* and *EcoRI*, and ligated into the corresponding sites of pSG1151, in that way generating fusions with the *gfpmut1* gene (Lewis and Marston, 1999), resulting in plasmids pGFP-*aprE*, pGFP-*bpr*, pGFP-*acoA*, pGFP-*srfA*, pGFP-*hag*, pGFP-*sunA*, pGFP-*degS* and pGFP-*‘degSU*, respectively (Table S2).

To construct plasmid pDG-PdegSU-gfp, a PCR’s with primer pairs degU-F+*HindIII* and gfp-R+*BglII* (Table S3) was performed, using chromosomal DNA of strain ‘degSU-gfp’ as a template. The 2.5kb product was digested with *HindIII* and *BglII* and ligated into the *HindIII/BamHI* sites of pDG1664 (Guerout-Fleury et al., 1996). To construct plasmids pDG1730-PdegS-gfp and pDG1730-PdegSU-gfp, PCR’s with primer pairs degU-F+*HindIII* and gfp-R+*BglII* (Table S3) were performed, using chromosomal DNA of strains degS-gfp and ‘degSU-gfp’ as a template, respectively. The products were digested with *HindIII* and *BglII* and ligated into the *HindIII/BamHI* sites of pDG1730 (Guerout-Fleury et al., 1996).

To construct plasmid pDG-hy, carrying the *B. subtilis degU32(Hy)* allele fused behind the IPTG inducible *Pspac* promoter, a PCR with the primers degU-F-hy (carrying at the 12th codon the nucleotide sequence ‘CTT’ coding for a lysine instead of the wildtype codon ‘CAT’ coding for a histidine) and degU-R+SalI was performed, using chromosomal DNA of *B. subtilis* 168 as a template. The amplified fragment was subsequently cleaved with *HindIII* and *SalI*, and ligated into the corresponding sites of pDG148 (Stragier et al., 1988).
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To construct plasmid pDR-hy, carrying the *B. subtilis* *degU32(Hy)* allele fused behind the IPTG inducible $P_{\text{hyper spank}}$ promoter, in between flanking regions coding for the non-essential *amyE* gene, a *HindIII/SalI* fragment from plasmid pDG-hy, containing the *degU32(Hy)* allele, was ligated into the corresponding sites of pDR111 (kind gift of David Rudner).

**Construction of bacterial strains**

*B. subtilis* strains aprE-gfp, bpr-gfp, acoA-gfp, srfA-gfp, hag-gfp, sunA-gfp, degS-gfp and ‘degSU-gfp, respectively, were obtained by Campbell-type integrations of plasmids pGFP-aprE, pGFP-bpr, pGFP-acoA, pGFP-srfA, pGFP-hag, pGFP-sunA, pGFP-degS and pGFP-‘degSU, respectively, into the chromosome of *B. subtilis* 168 (Kunst et al., 1997). Transformants were selected on TY agar plates containing chloramphenicol (5 µg/ml), after overnight incubation at 37°C. Note that strains acoA-gfp, srfA-gfp and hag-gfp have also recently been described elsewhere (Smits et al., 2007; Lulko et al., 2007; Veening et al., 2008). *B. subtilis* strain degSU-gfp was obtained by a double crossover recombination event between the *thrC* regions located on pDG-degSU and the chromosomal *thrC* gene of strain 168. Transformants were selected on TY agar plates containing erythromycin and lincomycin, after overnight incubation at 37°C. Correct integration into the *thrC* gene was tested and confirmed by lack of growth without threonine and spectinomycin sensitivity. Strains A:degS-gfp and A:degSU-gfp were obtained by a double crossover recombination event between the *amyE* regions located on the pDG1730-PdegS-gfp and pDG1730-PdegSU-gfp plasmids and the chromosomal *amyE* gene of strain 168, respectively. Transformants were selected on TY agar plates containing spectinomycin after overnight incubation at 37°C. Correct integration into the *amyE* gene was tested and
confirmed by lack of amylase activity upon growth on plates containing 1% starch. Strains degS-gfp/hy, degU-gfp/hy and aprE-gfp/hy were obtained by a double crossover recombination event between the *amyE* regions located on pDR-hy and the chromosomal *amyE* gene of strains degS-gfp, degSU-gfp and aprE-gfp, respectively. Transformants were selected on TY agar plates containing spectinomycin after overnight incubation at 37°C. Correct integration into the *amyE* gene was tested and confirmed by lack of amylase activity upon growth on plates containing 1% starch. Correct DegUhy overproducing clones were tested and confirmed by increased halos upon growth on plates containing 1% skim milk and IPTG. Construction of the other strains used in this study is explained in Table S1.

**Microscopy**

Cells were prepared for fluorescence microscopy and applied to agarose slides as described before (Glaser *et al.*, 1997), and images were acquired using an Zeiss Axiovert M200 equipped with a CoolSNAP HQ (Princeton Instruments, Trenton, New Jersey, United States). Metamorph (Universal Imaging, Doningtown, Pennsylvania, United States) was used for image capturing and figures were prepared for publication using ImageJ (http://rsb.info.nih.gov/ij/) and Corel Graphics Suite 11 (Corel Corporation). In all cases exposure times were 1 sec, but for Fig.3, exposure times were 2 sec. It should be noted that the low amount of GFP molecules that are produced from the *degU* promoter makes it difficult to accurately quantify the signal. Image analyses and quantification were performed using ImageJ. For time-lapse microscopy, cells were grown on a semi-solid chemically defined medium (CDM). A detailed description of this technique is described elsewhere (Veening *et al.*, 2008). In brief, CDM-agarose contained 0.4 µg/ml of the membrane dye FM5-95 (Invitrogen,
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Paisley, UK) and microscope slides were mounted on a Deltavision RT automated temperature controlled (30 °C) microscope (Applied Precision, Marlborough, UK). Images were obtained up to 20 h with a CoolSNAP HQ (Princeton Instruments, Trenton, New Jersey, United States) at 60X magnification. Fluorescent images were recorded at each field every 15 min.

**Flow cytometric analyses**

Cells were 100x diluted in 0.2 μM filtered minimal medium and directly measured on a Coulter Epics XL-MCL flow cytometer (Beckman Coulter, Mijdrecht, NL) operating an argon laser (488 nm) essentially as described (Veening et al., 2005). For each sample, at least 20,000 cells were analyzed. Data containing the green fluorescent signals were collected by a FITC filter and the photomultiplier voltage was set between 700 and 800 V. Data was captured using System II software (Beckman Coulter) and further analyzed using WinMDI 2.8 software (http://facs.scripps.edu/software.html). In all FACS figures, fluorescence is plotted on a logarithmic scale. Figures were prepared for publication using WinMDI 2.8 and Corel Graphics Suite 11.

**Western blot analysis and immunodetection**

Cells were separated from the growth medium by centrifugation (14,000 RPM, 1 min, room temperature). Pelleted cells were resuspended in protoplast buffer (20 mM potassium phosphate pH 7.5, 15 mM MgCl₂, 20% sucrose and 1 mg/ml of lysozyme) and incubated at 37°C for 30 min. The resulting protoplasts were diluted with 2xSDS-sample buffer, incubated at 95°C for 5 min and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described (Sambrook et al., 1989). Next, proteins
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were transferred to a polyvinylidene difluoride (PVDF) membrane (Roche) as described (Sambrook et al., 1989). DegU was detected with polyclonal DegU antibodies at a 1:450 dilution (Kind gift of Teruo Tanaka; Hata et al., 2001) and horseradish peroxidase-anti-rabbit-IgG conjugate (Amersham Biosciences, Little Chalfont, UK) according to the manufacturer’s instructions.

Model equations and parameter analysis

Governing equations

Kinetics of chemical reactions (1)-(9) was modeled by both a stochastic and a continuous (deterministic) approach. Stochastic simulations were done by direct sampling of master equations using Gillespie algorithm (Gillespie, 1977). Deterministic equations corresponding to the reactions for concentrations of proteins and their complexes are given below:

\[
\frac{d[mDegU]}{dt} = k_{i_{U}} - k_{degM}[mDegU] \\
\frac{d[DegU]}{dt} = k_{r}[mDegU] - k_{deg}[DegU] - k_{ph}[DegU] + k_{deph}[DegU-P] \\
\frac{d[DegU-P]}{dt} = k_{ph}[DegU] + k_{d}[Dim] - k_{deg}[DegU-P] - k_{deph}[DegU-P] - k_{b}[DegU-P]^{2} \\
\frac{d[Dim]}{dt} = k_{h}[DegU-P]^{2} - k_{d}[Dim] - k_{deg}[Dim] \\
\frac{d[mAprE]}{dt} = k_{r}[mAprE] - k_{degM}[mAprE] \\
\frac{d[AprE]}{dt} = k_{r}[mAprE] - k_{deg}[AprE]
\]
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Here expression rates $k_U$ and $k_E$ are defined as in Eqs. (8)-(9) in the main text.

**Parameters that support bistability**

In steady state, right-hand sides of Eqs. (S.1)-(S.6) are zero. Using (S.1), (S.3) and (S.4) to solve for $[mDegU]$, $[DegU-P]$ and $[Dim]$ respectively and using the result in (S.2) we obtain:

$$0 = \frac{k_T}{k_{degM}} I_0 \frac{K_i}{K_i + [Dim]} + I_m \frac{[Dim]}{K_i + [Dim]} - k_{deg} ([DegU] + [DegU-P] + [Dim]) \quad (S.7)$$

Assuming that biochemical processes are faster than protein degradation and that $[Dim] \ll ([DegU-P] + [DegU]$ we can simplify Eq. (S.7) to get a closed-form equation for $[DegU]$

$$0 = \frac{k_T}{k_{degM}} I_0 \frac{K_i}{k_i + k_d} \cdot \left(\frac{k_{ph}[DegU]}{k_{deph}}\right)^2 - k_{deg}[DegU](1 + \frac{k_{ph}}{k_{deph}}) \quad (S.8)$$

Or, equivalently,

$$0 = \frac{\alpha c_1^2 + [DegU]^2}{c_1^2 + [DegU]^2} - \frac{[DegU]}{c_2} \quad (S.9)$$

Where three new parameters are introduced

$$\alpha = \frac{I_0}{I_m} \equiv g^{-1}; \quad c_1^2 = \frac{k_{degM} k_d}{k_i} \left(\frac{k_{deph}}{k_{ph}}\right)^2; \quad \frac{1}{c_2} = \frac{k_{deg}}{I_m k_T} \frac{k_{degM}}{(1 + \frac{k_{ph}}{k_{deph}})} \quad (S.10)$$

These parameters are easy to interpret: $g > I$ is a dimensionless parameter that represents a fold gain from basal level to a full activation of the feedback loop and $0 < \alpha < 1$ is inverse of that; concentration $c_1$ denote the concentration of DegU at which there is a 50% chance that the $degU$ promoter is bound by dimer; and concentration $c_2$ is the DegU concentration with saturated positive feedback (for maximal expression level). To determine the parameter range that supports bistability
we need to determine when Eq. (S.9) has three positive solutions, or equivalently, polynomial below has three positive roots: $\beta \equiv c_1^2 / c_2^2 = \frac{K_d}{k_b} \left[ \frac{k_{deg}}{k_{M}} \frac{k_{degM}}{k_{T}} \left( \frac{k_{dep}}{k_{ph}} + 1 \right) \right]^2$

and $x = [\text{DegU}] / c_2$:

$$x^3 - x^2 + \beta x - \alpha \beta = 0$$ (S.11)

Alternating signs of coefficient indicates that real roots of this polynomial are positive (left-hand side of Eq. (S.11) is always negative for $x<0$). To get the condition for 3 real root we use a cubic discriminant formula, $\Delta = -4\alpha \beta - \beta^2 + 18\alpha \beta^2 + 27\alpha^2 \beta^2 + 4\beta^3 < 0$. Using a quadratic formula to solve for $\alpha$, we obtain the following conditions on the dimensionless parameters:

$$\frac{9\beta - 2 - 2(1-3\beta)^{3/2}}{27\beta} < \alpha < \frac{9\beta - 2 + 2(1-3\beta)^{3/2}}{27\beta}$$ (S.12)

This inequality obviously only holds when the expressions are real, i.e. $\beta < 1/3$.

Figure S7 shows the region of bistability shaded in gray. Two conclusions are straightforward from the analysis: (i) The gain, $g = 1/\alpha$, of the feedback should be at least 9-fold for the bistability to exist regardless of other parameter values. (ii) The maximal level of DegU synthesis must result in concentration saturating the feedback.

Choosing the parameters for numerical simulations

Our modeling predictions are most sensitive to the kinetic parameters that characterize the DegU/DegS system and positive feedback in degU transcription. Unfortunately, little data is available in the literature on biochemical parameters characterizing this system. However, our experimental results and measurements on other two-component system allow us to estimate some of the parameters (Table 1).

1 [http://mathworld.wolfram.com/PolynomialDiscriminant.html](http://mathworld.wolfram.com/PolynomialDiscriminant.html)
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The other parameters used are of the same order as in better characterized two-component systems (Wright et al., 1993; Fisher et al., 1996; Silversmith et al., 1997; Mayover et al., 1999; Jiang et al., 2000; Kato and Groisman, 2004; Kremling et al., 2004; Stewart and Van Bruggen, 2004).

- The typical lifetime of mRNA in bacteria is about 1-2 min. We therefore choose mRNA degradation rate to be $10^{-2} \text{ s}^{-1}$.
- Rate of translation was chosen to be relatively low given comparatively weak GFP signal from the degU-promoter fusion. We chose a translational rate that results in about 4 proteins translated per message. Note that we chose the same rate for aprE translation as well. This assumption by no means limits the generality of our conclusions because any increase in this rate will mainly affect absolute levels of AprE in the cell but not the distribution of AprE.
- We chose the rate of protein degradation to correspond to about 40 min lifetime. The same rate is assumed for all proteins and complexes. Our conclusions are not very sensitive to this assumption (data not shown).
- A relatively weak signal of our degU-GFP reporter strain leads us to assume relatively low intracellular DegU concentrations. The basal level of degU expression is chosen to be $10^{-3} \text{ #/s}$ resulting in about 40 DegU proteins per cell prior to feedback activation. The maximal basal level of DegU expression (with feedback saturated) is chosen to be 12x the basal level, 0.048 #/s, resulting in about 500 DegU proteins per cell.
- Low concentration of DegU in cells led us to model DegU phosphorylation and dephosphorylation reactions as first order processes without saturation. These assumptions do not affect our conclusions; furthermore, saturatable
Michaelis-Menten kinetics to model these reactions may even enhance bistability. The rates for these processes were measured for some *in vitro* conditions (Tanaka *et al.*, 1991). These measurements indicate a ~1 min timescale of both phosphorylation and dephosphorylation reactions in approximately stoichiometric proportions. However, caution has to be taken to extrapolate such measurements to *in vivo* data because both the phosphorylation and dephosphorylation reaction may be strongly affected by metal ion concentrations. Moreover, in many two-component system phosphorylation or dephosphorylation rates serve as the system input and are therefore, affected by the environmental conditions to dephosphorylation rate.

We therefore hypothesized that the ratio of phosphorylation to dephosphorylation rate serves as a signal to activate DegU production. We set the phosphorylation rate to be 0.004 s$^{-1}$ and chose the dephosphorylation rate to be 0.05 s$^{-1}$ so that the DegU system has only one (ON) steady state but is close to the bistability threshold to ensure long and noisy response times (see discussion below).

- For the association rate constant of phosphorylated DegU dimers, we chose a diffusion-limit value for typical sized proteins $k_a \approx 15$ µM$^{-1}$s$^{-1}$=0.025 s$^{-1}$/# (cell volume of 1e-15 l is used for conversion). For the dissociation rate constant we used $k_d \approx 0.1$ s$^{-1}$ so that the dissociation constant corresponds to about 5# DegU–P per cell.

- Equilibrium constant of DegU dimer binding to DNA are chosen such to insure that the dimer first binds to the *degU* promoter activating the positive feedback and only then binds to the *aprE* promoter.
The selection of the parameter values for the model was mainly driven by the experimental distributions of DegU and AprE. Running our model simulations with different values of parameter values we see several general trends:

(1) We find that the low copy number of $\text{degU}$ mRNA per cell is the major source of the extrinsic noise in the system.

(2) Existence of bistability for some intermediate rates of phosphorylation (dephosphorylation) is essential for slow and noisy response of the system. Without the bistability, the system responds much faster and homogeneous expression of DegU should be expected after overnight incubation.

(3) If the rates of phosphorylation and dephosphorylation used for modeling are within the bistability range and some parameter values support the transitions between two stable states (only from OFF to ON or both OFF to ON and ON to OFF) but the transitions are faster than the time the system spends in each state, bimodal distribution of DegU is observed.

(4) When the rates of phosphorylation and dephosphorylation used for modeling are in the region where only the ON state is stable but close to the bistability range, the transition from initial OFF to ON state is slow and noisy and often results in unimodal DegU distribution. We observe that as long as this condition is satisfied, model predictions qualitatively match the observed results regardless of the specific values for the kinetic parameters.
**Supporting figures**

| Category  | Size | Down | Not | Up |
|-----------|------|------|-----|-----|
| AbrB      | 29   | 11   | 5   | 1   |
| CcpA      | 40   | 16   | 9   | 1   |
| ComA      | 41   | 13   | 6   | 1   |
| DegU      | 66   | 3    | 13  | 1   |
| SigE      | 69   | 14   | 14  | 1   |
| SigH      | 23   | 4    | 2   | 1   |
| SinR      | 6    | 2    |     |     |
| Spo0A     | 13   | 6    | 2   |     |

**Description of category**
- **transcriptional regulator**
- **two-component response regulator**
- **RNA polymerase sporulation-specific sigma-29 factor (sigma-E)**
- **RNA polymerase sigma-30 factor (sigma-H)**

**Fig. S1.** Transcriptome analysis using FIVA software (Blom et al., 2007). The total number of genes that were shown to belong to a certain regulon (category) is indicated by blue numbers (size). The number of genes identified to be significantly down-regulated in the sporulating fraction from each category is indicated in the ‘down’ column, and the number of genes identified being up-regulated in the sporulating fraction is shown in the ‘up’ column. The total number of genes that were identified in both fractions, and were not significantly different, are indicated in the ‘not’ column. The darker the color of the category, the more over-represented and significant this category is. This analysis shows that non-sporulating cells express 31 members of the DegU regulon (containing a total of 66 members Ogura et al., 2001; Mader et al., 2002) more than twofold higher compared to endospore-formers.
**Fig. S2.** Spo0A modulates aprE gene expression. Strains aprE-gfp/hy (top) and aprE-gfp/hy/Δ0A (bottom) were grown in TY medium and induced with IPTG at mid-exponential growth. Cells were collected for flow cytometric analyses after 3.5h of induction.
Fig. S3. Total DegU protein levels. Cells from Fig. 6A-B were subjected to Western blot analysis using a DegU specific antibody as described in the supplemental materials and methods.
Fig. S4. Time dependent heterogeneity in *aprE* gene expression. Cells were grown overnight in TY medium (strain aprE-gfp) and diluted into fresh TY to an OD600 of 0.05. Samples were taken for flow cytometric analyses at the indicated times after dilution.
Fig. S5. DegS is required for degU autostimulation. Strains aprE-gfp/hy, aprE-gfp/hy/ΔS and aprE-gfp/hy/ΔSU were grown in TY medium and induced with 500 µM of IPTG at mid-exponential growth. After 1h of induction, cells were collected for flow cytometric analyses. Representative flow profiles are depicted.
**Fig. S6.** Heterogeneity in *aprE* expression is regulated. An overnight culture of strain *aprE*-gfp/X-cfp (P*_aprE*-gfp, amyE::P*_xyl*-cfp_bs) was induced with 1% xylose. Cells were collected for light and fluorescence microscopy 4 hours after induction. All cells, except for endospore containing cells, express CFP from the xylose inducible promoter but only a small subpopulation expresses GFP from the *aprE* promoter.
Fig. S7. The range of parameters supporting bistability for Eq. (S.12). The model parameters are aggregated into two dimensionless numbers. * denoted the value used in our simulation. For this value the system is monostable resulting in unimodal distribution of DegU but the switching kinetics are slow and noisy because of the close proximity to the bistable region.
Table S1. Bacterial strains used in this study.

| Strains | Description                      | Relevant genotype                                      | Reference                                      |
|---------|----------------------------------|--------------------------------------------------------|-----------------------------------------------|
| TG90    | TG1 derivative, maintains plasmids at low copy numbers | trpC2                                                   | (Lopilato et al., 1986)                        |
| B. subtilis |                                |                                                        |                                               |
| 168     | Parental                        | degS::aphA3, Km<sup>f</sup>                           | (Kunst et al., 1997)                          |
| QB4277  | ∆degS                            | degSU::aphA3, Km<sup>f</sup>                           | (Msadek et al., 1991)                         |
| QB4238  | ∆degSU                           | degSU::aphA3, Km<sup>f</sup>                           | (Msadek et al., 1990)                         |
| QB4487  | ∆degU                            | degU::erm, Em<sup>f</sup>                              | (Dartois et al., 1998)                        |
| SWV215  | ∆spoA                           | spoO::kan, Km<sup>f</sup>                              | (Xu and Strauch, 1996)                        |
| RL1265  | ∆sigF                           | sigF::kan, Km<sup>f</sup>                              | (Branda et al., 2001)                         |
| abrB::Em | ∆abrB                           | abrB::erm, Em<sup>f</sup>                              | (Smits et al., 2005)                          |
| aprE-gfp | pGFP-aprE→168                   | P<sub>apE-gfp</sub>, Cm<sup>f</sup>, amyE::P<sub>hyper-spank</sub>degUhy, Sp<sup>f</sup> | This study                                    |
| aprE-gfp | pDR-hy→aprE-gfp                 | P<sub>apE-gfp</sub>, Cm<sup>f</sup>, amyE::P<sub>hyper-spank</sub>degUhy, Sp<sup>f</sup> | This study                                    |
| aprE-gfp/∆SU | QB4238→aprE-gfp/∆SU       | P<sub>apE-gfp</sub>, Cm<sup>f</sup>, amyE::P<sub>hyper-spank</sub>degUhy, Sp<sup>f</sup> | This study                                    |
| aprE-gfp/∆S | QB4277→aprE-gfp/∆S       | P<sub>apE-gfp</sub>, Cm<sup>f</sup>, amyE::P<sub>hyper-spank</sub>degUhy, Sp<sup>f</sup> | This study                                    |
| aprE-gfp/pDG-hy | pDG-hy→aprE-gfp  | P<sub>apE-gfp</sub>, Cm<sup>f</sup>, amyE::P<sub>hyper-spank</sub>degUhy, Sp<sup>f</sup> | This study                                    |
| aprE-gfp/∆0A | SWV215→aprE-gfp/∆0A    | P<sub>apE-gfp</sub>, Cm<sup>f</sup>, amyE::P<sub>hyper-spank</sub>degUhy, Sp<sup>f</sup> | This study                                    |
| aprE-gfp/X-cfp | P1726-cfp<sub>barb2right</sub>→aprE-gfp | P<sub>apE-gfp</sub>, Cm<sup>f</sup>, amyE::P<sub>hyper-spank</sub>degUhy, Sp<sup>f</sup> | This study                                    |
| degS-gfp | pGFP-degS→168                   | P<sub>degS-gfp</sub>, Cm<sup>f</sup>                   | This study                                    |
| 'degSU-gfp | pGFP·''degSU→168            | P<sub>degSU-gfp</sub>, ∆degU<sub>U</sub>, Cm<sup>f</sup> | This study                                    |
| degSU-gfp | pDG-PdegSU-gfp→168             | thC::P<sub>degSU-gfp</sub>, Em<sup>f</sup>            | This study                                    |
| A:degS-gfp | pDG1730·PdegS-gfp→168 | amyE::P<sub>degS-gfp</sub>, Sp<sup>f</sup>            | This study                                    |
| A:degSU-gfp | pDG1730·PdegSU-gfp→168 | amyE::P<sub>degSU-gfp</sub>, Sp<sup>f</sup>            | This study                                    |
| degS-gfp/∆SU | pDR-hy→degS-gfp         | P<sub>degS-gfp</sub>, Cm<sup>f</sup>, amyE::P<sub>hyper-spank</sub>degUhy, Sp<sup>f</sup> | This study                                    |
| degU-gfp/∆SU | pDR-hy→degU-gfp          | thC::P<sub>degU-gfp</sub>, Em<sup>f</sup>, amyE::P<sub>hyper-spank</sub>degUhy, Sp<sup>f</sup> | This study                                    |
| degU-gfp/∆degU | pDR-hy→'degU-gfp'     | P<sub>degU-gfp</sub>, ∆degU<sub>U</sub>, Cm<sup>f</sup>, amyE::P<sub>hyper-spank</sub>degUhy, Sp<sup>f</sup> | This study                                    |
| bpr-gfp | pGFP-bpr→168                   | P<sub>bpr-gfp</sub>, Cm<sup>f</sup>                   | This study                                    |
| acO-gfp | pGFP-acO→168                   | P<sub>acO-gfp</sub>, Cm<sup>f</sup>                   | This study                                    |
| srfA-gfp | pGFP-srfA→168                  | P<sub>srfA-gfp</sub>, Cm<sup>f</sup>                   | This study                                    |
| sunA-gfp | pGFP-sunA→168                  | P<sub>sunA-gfp</sub>, Cm<sup>f</sup>                   | This study                                    |
| hag-gfp | pGFP-hag→168                   | P<sub>hag-gfp</sub>, Cm<sup>f</sup>                   | This study                                    |

* Arrows (→) indicate construction by transformation with either plasmid or chromosomal DNA.
### Table S2. Plasmids used in this study.

| Plasmids            | Relevant genotype                                      | Reference                                      |
|---------------------|--------------------------------------------------------|-----------------------------------------------|
| pSG1151             | bla, Ap<sup>i</sup>, cat, Cm<sup>r</sup>, gfpmut1     | (Lewis et al., 1999)                          |
| pDG148              | lacI, bla, Ap<sup>i</sup>, kan, Km<sup>r</sup>, Pspac | (Stragier et al., 1988)                       |
| pDG148-degU         | lacI, bla, Ap<sup>i</sup>, kan, Km<sup>r</sup>, Pspac-degU | (Ogura et al., 2001)                         |
| pDG1664             | thrC, Sp, Sp<sup>i</sup>, bla, Ap<sup>i</sup>, ery, Em<sup>i</sup> | (Guerout-Fleury et al., 1996)                |
| pDG1730             | amyE, Sp, Sp<sup>i</sup>, bla, Ap<sup>i</sup>, ery, Em<sup>i</sup> | (Guerout-Fleury et al., 1996)                |
| pDG-hy              | lacI, bla, Ap<sup>i</sup>, kan, Km<sup>r</sup>, Pspac-degUhy | This study                                   |
| pDR111              | amyE, lacI, bla, Ap<sup>i</sup>, Sp, Sp<sup>i</sup>, Phyper-spank | Gift of D. Rudner                           |
| pDR-hy              | amyE, lacI, bla, Ap<sup>i</sup>, Sp, Sp<sup>i</sup>, Phyper-spank-degUhy | This study                                   |
| P1726-cfp_bs        | amyE, bla, Ap<sup>i</sup>, Sp, Sp<sup>i</sup>, PxyI-cfp<sub>bs</sub> | Gift of L. Wu                               |
| pGFP-aprE           | bla, Ap<sup>i</sup>, cat, Cm<sup>r</sup>, PaprE-gfpmut1 | This study                                   |
| pGFP-bpr            | bla, Ap<sup>i</sup>, cat, Cm<sup>r</sup>, Pbpr-gfpmut1 | This study                                   |
| pGFP-acoA           | bla, Ap<sup>i</sup>, cat, Cm<sup>r</sup>, PacoA-gfpmut1 | This study                                   |
| pGFP-sunA           | bla, Ap<sup>i</sup>, cat, Cm<sup>r</sup>, PsunA-gfpmut1 | This study                                   |
| pGFP-srfA           | bla, Ap<sup>i</sup>, cat, Cm<sup>r</sup>, Psrfa-gfpmut1 | This study, (Smits et al., 2007)              |
| pGFP-hag            | bla, Ap<sup>i</sup>, cat, Cm<sup>r</sup>, Phag-gfpmut1 | This study, (Lulko et al., 2007)              |
| pGFP-degS           | bla, Ap<sup>i</sup>, cat, Cm<sup>r</sup>, PdegS-gfpmut1 | This study                                   |
| pGFP-‘degSU         | bla, Ap<sup>i</sup>, cat, Cm<sup>r</sup>, P’degSU-gfpmut1 | This study                                   |
| pDG1730-PdegS-gfp   | amyE, Sp, Sp<sup>i</sup>, bla, Ap<sup>i</sup>, ery, Em<sup>i</sup>, PdegS-gfpmut1 | This study                                   |
| pDG1730-PdegSU-gfp  | amyE, Sp, Sp<sup>i</sup>, bla, Ap<sup>i</sup>, ery, Em<sup>i</sup>, PdegSU-gfpmut1 | This study                                   |
| pDG-PdegSU-gfp      | thrC, Sp, Sp<sup>i</sup>, bla, Ap<sup>i</sup>, ery, Em<sup>i</sup>, PdegSU-gfpmut1 | This study                                   |
Table S3. Oligonucleotides used in this study.

| Oligo       | Sequence (5’ to 3’)* | Description; position |
|-------------|----------------------|------------------------|
| degU-F-hy   | CCCAAGCTTAGGAGGAGGAATGACTAAAGTAAAACA TTGAATTATCAGCACGCTTTTG | HindIII; 5’ end of degU, A35→C
| degU-R+SalI | GCCAGAGTCGAGCTTACTTACATCGTACACCCAGCCATT | SalI; 3’ end of degU
| gfp-R+BglII | CGGAAGATCTTTATTAGAGTAGCTCAGCATCAGCATT | BglII; 3’ end of gfpmut1
| aprE-F      | CCCAAGCTTACGCGGCGCAATGCGCCCGCTTACGAG | HindIII; 5’ end of PaprE
| aprE-R      | CGGAATCTTTACGCTCAGCATCAGCATCAGCATT | EcoRI; 3’ end of PaprE
| bpr-F       | GGCTAGCAGGCAAAGCTTAACTC | HindIII; 5’ end of Phpr
| bpr-R       | CGGAATCTTTACGCTCAGCATCAGCATCAGCATT | EcoRI; 3’ end of Phpr
| acoA-F      | CCCAAGCTTACGCGCAGCTATGAGCATACGAGTACGAG | HindIII; 5’ end of PacoA-L
| acoA-R      | CGGAATCTTTACGCTCAGCATCAGCATCAGCATT | EcoRI; 3’ end of PacoA-L
| srfA-F      | CCCAAGCTTACGCGCAGCTATGAGCATACGAGTACGAG | HindIII; 5’ end of PsrFA
| srfA-R      | CGGAATCTTTACGCTCAGCATCAGCATCAGCATT | EcoRI; 3’ end of PsrFA
| sunA-F      | CCCAAGCTTACGCGCAGCTATGAGCATACGAGTACGAG | HindIII; 5’ end of PsunA
| sunA-R      | CGGAATCTTTACGCTCAGCATCAGCATCAGCATT | EcoRI; 3’ end of PsunA
| hag-F       | GGATCAAGTGAAGCTTACGCGGAAATTGACG | HindIII; 5’ end of Phag
| hag-R       | CGGAATCTTTACGCTCAGCATCAGCATCAGCATT | EcoRI; 3’ end of Phag
| degU-F+HindIII | CCCAAGCTTACGCGCAGCTTACGCTCAGCATCAGCATCAGCATT | HindIII; 5’ end of PdegS
| degS-R+RBS+RcoRI | GCCGAGTTATGACGCTTACGCTCAGCATCAGCATCAGCATT | EcoRI; 3’ end of PdegS
| degU-F+HindIII_inside | CCCAAGCTTACGCGCAGCTTACGCTCAGCATCAGCATCAGCATT | HindIII; 5’ end of P’ddegSU
| degU-R+RBS+RcoRI | CGGAATCTTTACGCTCAGCATCAGCATCAGCATT | EcoRI; 3’ end of P’ddegSU

* Relevant restriction sites are underlined.

b The A35→C base pair mutation, causing a H12→L amino acid change, is indicated in italics.
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