Supplemental Information

Different resource allocation in a bimodal *Bacillus subtilis* population displaying bimodal motility

Simon Syvertsson, Biwen Wang, Jojet Staal, Yongqiang Gao, Remco Kort, Leendert W. Hamoen

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Strain construction

Strains, plasmids and oligonucleotides used are listed in Table S1, S2 and S3, respectively. Strain bSS339, containing the P*hag-gfp* reporter was constructed by transforming chromosomal DNA from DS901 to wild type background 168CA.

The *gfp-ssrA* vector plasmid pSS52 was created by cutting pSG1164 with *Spe*I, blunting the ends with Klenow fragment, then cutting again with *Bsr*GI (which cuts in the middle of *gfp*). An insert was created by amplifying *gfp* from pSG1164 using primer pair oSS63/oSS106, carrying an *ssrA* overhang. The insert was cut with *Bsr*GI and ligated blunt/sticky ends into the vector. An IPTG-inducible P*hyper-hy-gfp-ssrA* construct was created with vector plasmid pDR111 using *Hind*III and *Sph*I. A *gfp-ssrA* insert was amplified from pSS52 using primer pair oSS159/oSS158, and a gfp insert was amplified from pSG1164 (oSS159/oSS160). *gfp-ssrA* and *gfp* were ligated into pDR111, creating pSS59 and pSS60, respectively.

To create versions of the *gfp-ssrA* construct carrying weaker versions of *ssrA*, pSS59 was mutagenized using PCR with the linearizing primer pairs oSS173/oSS174 or oSS175/oSS176 to create mutations 'DAV' and 'AAV', respectively (plasmids pSS67 and pSS68, respectively). The PCR product was treated with *Dpn*I and purified using a PCR clean up kit to remove template product. The linearized plasmid was kinated using T4 PNK, then ligated using T4 DNA Ligase (Roche), before being transformed into competent DH5α E. coli.

The P*rpsD-gfp-ssrA* reporter plasmid pSS102 for integration at the *amyE* locus was created using CloneJet (Thermo Scientific). 550 bp PCR products of the 3' and 5' ends of *amyE* were amplified using primer pairs oSS243/oSS244 and oSS251/oSS252, respectively. A spectinomycin resistance cassette was amplified

from pSG1154 using oSS245/oSS246, a 333 bp region of P*rpsD* extending until the start codon of *rpsD*, from 168CA chromosomal DNA using oSS247/oSS248. *sfGFP* was amplified from pSG1729-sfGFP using oSS249/oSS250, with oSS250 providing part of the SsrA tag in an non-complementary overhang sequence. Using overlap extension PCR, the fragments were joined in the following order: *amyE* 5′, *spcR*, P*rpsD*, *sfGFP-ssrA*, *amyE* 3′, and joined using the terminal primer pair oSS243/oSS252. The resulting 3.3 kbp fragment was cloned into PJET1.2Blunt using the protocol and T4 DNA ligase provided by the supplier, then transformed into DH5α *E. coli*. A P*rpsD-gfp* reporter construct (lacking *ssrA*) was created from pSS102 by linearizing the plasmid with primer pair oSS190/oSS429 by PCR. The linearized plasmid was treated with *Dpn*I, PNK and ligase as described above, resulting in plasmid pSS164. The SsrA tag variants 'DAV' and 'AAV' were constructed using primer pairs oSS424/oSS425 and oSS424/oSS426, respectively, and plasmid pSS102 as template, resulting in integration vectors plasmids pSS160 and pSS161, respectively.

A P*hag-mCherry* reporter construct was made based on the P*hag-gfp* construct from (1). A 377 bp P*hag* fragment was amplified from 168CA chDNA using primers oSS411/oSS370, and a 749 bp *mCherry* fragment from DS1675 chDNA using primers oSS412/oSS413. The promoter and reporter were joined by overlap extension PCR using 1 µl of each purified fragment as template, and oSS370/oSS413 as primers. The resulting 1103 bp P*hag-mCherry* fragment was cut using *Eco*RI and ligated into pAPNC213Cat linearized using the same enzyme and treated with calf intestine alkaline phosphatase (Roche), resulting in pSS153.

To block autoregulation of *rpsD* expression, the S4 binding region between +21

to +156 was removed using primers oSS332/oSS333 from pSS102, resulting in integration vector pSS114, after digestion of the PCR product with *Bam*HI and self-ligation.

B. subtilis strains containing the different recombined plasmids and deletions were constructed using standard protocols for inducing natural competence (2).

RNA extraction and RNA-seq analysis

Total RNA extraction was performed according to (3). Briefly, cell pellets were resuspended in 0.4 ml ice-cold PBS and added to a screw cap tube containing 1.5 g glass beads (0.1 mm), 0.4 ml 25:24:1 phenol/chloroform/isoamyl alcohol mixture (P/C/I) and 50 µl 10 % SDS, followed by fortexing to mix the suspension thoroughly. Cell disruption was achieved by bead beating (Precellys 24). After centrifugation, the RNA in the upper aqueous phase was ethanol-precipitated, washed twice with 70 % ethanol, dried and dissolved in water. DNA was removed by DNaseI (New England Biolabs) treatment. RNA was then extracted by a second-round of P/C/I cleaning, followed by ethanol-precipitation and washing. After dissolving the RNA pellet in water, the concentration was measured using Qubit™ RNA HS Assay Kit (Invitrogen™).

In order to better quantifying the abundance of transcripts in the GFP-on/off cells, we made use of External RNA Controls Consortium (ERCC) synthesized control RNAs as internal ruler. Briefly, 1 µl 1:1000 diluted ERCC RNA Spike-In mix 1 (Invitrogen) was added to 50 ng RNA sample and mixed thoroughly. The mixed RNA was subjected to sequencing library construction using the NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina® (New England Biolabs), according to

the manufacturer's instructions. As control, RNA of unsorted bSS339 cells was also mixed with ERCC RNA and sequenced. Importantly, all libraries with the ERCC spike-in controls have undergone 9 PCR-amplification cycles. The libraries were sequenced by Illumina NextSeg 550 System using NextSeq 500/550 High Output v2.5 kit (75-bp read length), and the raw data were analyzed on the web-based platform Galaxy. Reads of different libraries were firstly normalized based on sequencing depth, then the total reads of ERCC spike-in control RNAs in each library were calculated and used to further normalize the transcripts in respective libraries. After ERCC normalization, the reads of each genes were compared between the GFP-on and the GFP-off libraries. An Excel file of the results is available in the supplementary information.

Table S1. Strains used in this study. *Spc*, *cat* and *tet* refers to spectinomycin, chloramphenicol and tetracycline resistance cassettes, respectively.

Strain	**Genotype**	**Construction or reference**
168CA	*trpC2* wild-type *B. subtilis*	Laboratory strain
CB100	*sigD::cat*	(4)
DS901	PY79 *amyE::(Phag-gfp cat)*	(1)
bSS135	168CA *amyE::Pspac(hy)-gfp-ssrA spc*	168CA x pSS59
bSS136	168CA *amyE::Pspac(hy)-gfp spc*	168CA x pSS60
bSS161	168CA *amyE::Pspac(hy)-gfp-ssrADAV spc*	168CA x pSS67
bSS162	168CA *amyE::Pspac(hy)-gfp-ssrAAAV spc*	168CA x pSS68
bSS277	168CA *amyE::PrpsD-sfGFP-ssrA spc*	168CA x pSS102
bSS322	168CA amyE::(PrpsD*Δ(21-156)*-sfGFP-ssrA spc)	168CA x pSS114
bSS385	168CA *aprE::Phag-mCherry cat*	168CA x pSS153
bSS387	168CA *amyE::PrpsD-sfGFP-ssrA spc aprE::Phag-mCherry cat*	bSS277 x bSS385
bSS410	168CA *amyE::PrpsD-sfGFP-ssrAAAV spc*	168CA x pSS161
bSS411	168CA *amyE::PrpsD-sfGFP-ssrADAV spc*	168CA x pSS160
bSS421	168CA *amyE::PrpsD-sfGFP spc*	168CA x pSS164
bSS429	168CA *amyE::PrpsD-sfGFP spc aprE::Phag-mCherry cat*	bSS421 x bSS385
bSS432	168CA *amyE::PrpsD-sfGFP-ssrAAAV spc aprE::Phag-mCherry cat*	bSS385 x bSS410
bSS485	168CA *amyE::Pspac(hy)-gfp-ssrA spc clpX ::cat*	bSS135 x JK47
bSS492	168CA *amyE::PrpsD-sfGFP-ssrADAV spc aprE::Phag-mCherry cat*	bSS385 x bSS411
bSS339	168CA *amyE::Phag-gfp cat*	168CA x DS901
bSS407	168CA *amyE::PrpsD-sfGFP-ssrA spc aprE::Phag-mCherry tet sigD::cat*	bSS402 x CB100

Table S2: Plasmids used in this study

plasmid	**Genotype**	**reference**
pDR111	*amyE::(Phyper-spank lacI spc) bla*	(5)
pSG1164	*Pxyl-gfpmut1 bla cat*	(6)
pSG1154	*amyE::(Pxyl-MCS-gfp spc) bla*	(7)
pJet1.2/blunt	*bla rep placUV 5-eco47IR' (blunt) 'eco47IR*	GenBank: EF694056
pSG1729-sfGFP	*amyE::(sfGFP spc) bla*	F. Bürmann, unpublished
pAPNC213Cat	*aprE::(MCS lacI cat) bla*	(8)
pSS52	*amyE::(gfp-ssrA spc) bla*	this work
pSS59	*amyE::(Phyper-spank-gfp-ssrA spc) bla*	this work
pSS60	*amyE::(Phyper-spank-gfp spc) bla*	this work
pSS67	*amyE::(Phyper-spank-gfp-ssrADAV spc) bla*	this work
pSS68	*amyE::(Phyper-spank-gfp-ssrAAAV spc) bla*	this work
pSS102	*amyE::(PrpsD-sfGFP-ssrA spc) bla*	this work
pSS114	*amyE::(PrpsDΔ(21-156)-sfGFP-ssrA spc) bla*	this work
pSS153	*aprE::(Phag-mCherry cat) bla*	this work
pSS160	*amyE::(PrpsD-sfGFP-ssrADAV spc) bla*	this work
pSS161	*amyE::(PrpsD-sfGFP-ssrAAAV spc) bla*	this work
pSS164	*amyE::(PrpsD-sfGFP spc) bla*	this work

Table S3: Oligonucleotides used in this study.

Olig name	**Sequence (5' to 3')** (CAPITALS = complementary)
oSS63	GAATTCCTGCAGATGAGTAAAGG
oSS106	gcgcgcaagcttgcgcttaggcagctaatgctacgttttggttaaaactgttagttttgcccgcggatctTTTGTATAG TTCATCCATGCC
oSS158	gcgccggcatgcGCGCTTAGGCAGCTAATGC
oSS159	gcgcgcaagcttTAAGGAGGCCTGCAGATGAGTAAAGGAG
oSS160	gcgccggcatgcAGTGGATCTGAAGTCTGGAC
oSS173	CCACCGAATTAGCTTGCATGCGCGCTTAaaCAGCgtcTGCTACGTTTTGGTTAAAACTGTT
oSS174	AACAGTTTTAACCAAAACGTAGCAgacGCTGttTAAGCGCGCATGCAAGCTAATTCGGTGG
oSS175	CCACCGAATTAGCTTGCATGCGCGCTTAaaCAGCggcTGCTACGTTTTGGTTAAAACTGTT
oSS176	AACAGTTTTAACCAAAACGTAGCAgccGCTGttTAAGCGCGCATGCAAGCTAATTCGGTGG
oSS190	ATTCTGCGTGACATCCCATC
oSS243	GTCTAGCCTTGCCCTCAATG
oSS244	gattaataattACTGACGATTACCTTGCGTG
oSS245	gtaatcgtcagTAATTATTAATCTGTAGACAAATTGTG
oSS246	GATAAAAACATGGTCTTTGAAGCATGCAAATGTCAC
oSS247	gcttcaaagACCATGTTTTTATCACCTAAAAG
oSS248	ctttgctcatctgcaggaattccATAATGTGACTCCTCCTTTG
oSS249	gaattCCTGCAGATGAGCAAAGGAGAAGAACTTTTC
oSS250	ctaatgctacgttttggttaaaactgttagttttgcccgcTTTGTAGAGCTCATCCATGC
oSS251	gttttaaccaaaacgtagcattagctgcctaataaGTGTATTTTGTGTATTCCAGTC
oSS252	ATGTTTGCAAAACGATTCAAAAC
oSS332	gcgcggatccAAGGCTGCAATATTTCACAC
oSS333	gcgcggatccAATAAAACCAAAGGAGGAGTC
oSS370	cgcggaattcTTATCGCGGAAAATAAACGAAG
oSS411	gtagttcctccttatgtgctagcGAATATGTTGTTAAGGCACGTC
oSS412	GCTAGCACATAAGGAGGAACTACTATGGTCAGCAAGGGAGAGG
oSS413	gcgcgaattcATCCTTATTTGTATAATTCGTCC
oSS424	TGCTACGTTTTGGTTAAAACTG
oSS425	gacGCTGttTAATAAGTGTATTTTGTGTATTCC
oSS426	gccGCTGttTAATAAGTGTATTTTGTGTATTCC
oSS429	tcattaTTTGTAGAGCTCATCCATGCC
	q-RT-PCR primers
GapA-Fw	ATTGGACGTAACGTATTCCGCG
GapA-Rv	CTTCAGCGTCTAATTTTCCGTGTA
rpsJ-Fw	GGTCCGATTCCGTTGCCAACTG
rpsJ-Rv	TGTGGTGTTGGGTTCACAATGTCG
rplC-Fw	GCGCTTCGTTAAAGAATTACGCGGA
rplC-Rv	GATCGCACCTTGGAAACCTTTACCT

Fig. S1

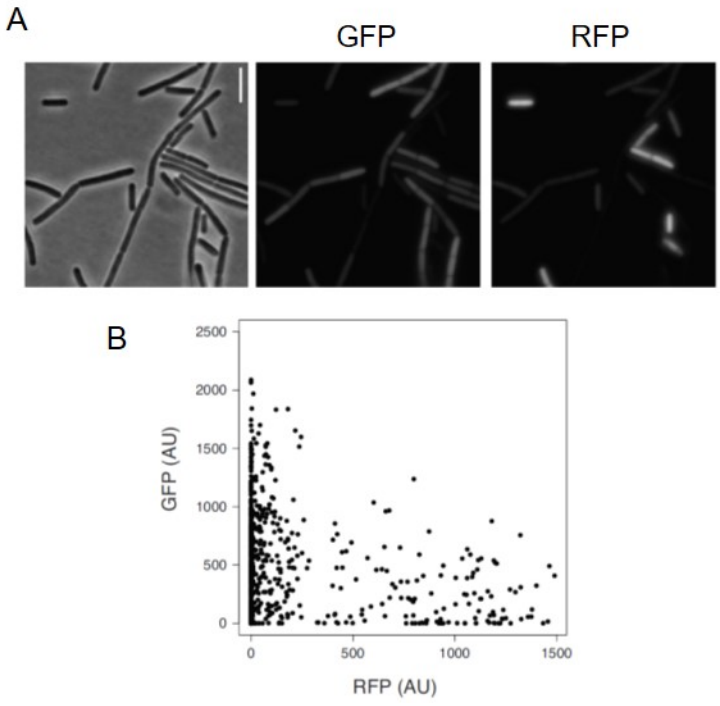
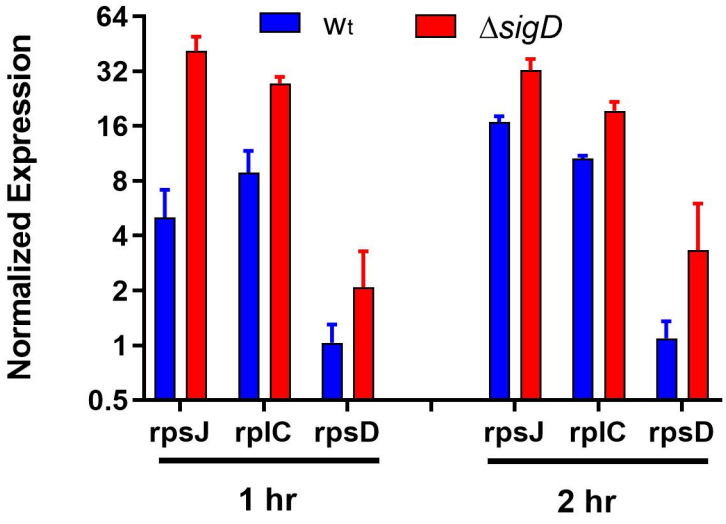


Fig. S1: Effect of blocking *rpsD* autoregulation on cellular expression. The rpsD promoter, lacking the binding site for ribosomal protein S4 binding (region +21 to +156) was fused to *gfp-ssrA* (strain bSS399, which also contains the *Phag-mCherry* reporter fusion). This prevents autoregulation of expression by the product of *rpsD*, which is ribosomal protein S4 (9). Cells were grown in CH medium at 37 ^{0}C and sampled after 60 min in exponential growth. (A) Phase contrast, GFP and mCherry (RFP) signals. Scale bar is 5 μm. (B) Scatter-histogram plots of *PrpsD-gfp-ssrA* (GFP) and *Phag-mCherry* (RFP) activities of 887 cells.

120 Fig. S2



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122 Fig. S2: q-RT-PCR of *rpsJ, rplC and rpsD* mRNA in wild type and Δ*sigD* cells. Cultures
123 were sampled from an exponentially growing culture at 1 hr and 2 hr, corresponding
124 to the first two time points of Fig. 5. Averages are based on 5 biological replicates.

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