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Exploring the non-conserved sequence space of synthetic expression modules in *Bacillus subtilis*

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TOC Figure

Bacillus subtilis, FlowSeq, synthetic expression module, promoter library, GFP, xylanase

ABSTRACT

Increasing protein expression levels is a key step in the commercial production of enzymes. Predicting promoter activity and translation initiation efficiency based solely on consensus sequences have so far met with mixed results. Here, we addressed this challenge using a “brute-force” approach by designing and synthesizing a large combinatorial library comprising ~12,000 unique synthetic expression modules (SEMs) for *Bacillus subtilis*. Using GFP fluorescence as a reporter of gene expression, we obtained a dynamic expression range that spanned five orders of magnitude, as well as a maximal 13-fold increase in expression compared with that of the already strong *veg* expression module. Analyses of the synthetic modules indicated that sequences at the 5'-end of the mRNA were the most important contributing factor to the differences in expression levels, presumably by preventing formation of strong secondary mRNA structures that affect translation initiation. When the *gfp* coding region was replaced by the coding region of the *xynA* gene, encoding the industrially relevant *B. subtilis* xylanase enzyme, only a 3-fold improvement in xylanase production was observed. Moreover, the correlation between GFP and xylanase expression levels was weak. This suggests that the differences in expression levels between the *gfp* and *xynA* constructs were due to differences in 5'-end mRNA folding and consequential differences in the rates of translation initiation. Our data show that the use of large libraries of SEMs, in combination with high-throughput technologies, is a powerful approach to improve the production of a specific protein, but that the outcome cannot necessarily be extrapolated to other proteins.

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43 **INTRODUCTION**

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45 The expression of native protein encoding genes is optimized by evolutionary pressures that
46 maximize an organism's fitness in its natural environment. In contrast, industrial
47 bioprocesses aim to achieve maximum productivity of a specific target protein or enzyme in
48 a defined (artificial) environment, irrespective of the impact on fitness. For these industrial
49 processes, transcriptional behavior is a key element for successful strain construction and
50 process development. However, predicting how upstream and downstream elements of a
51 synthetic expression module will function *in vivo* remains a challenge. While empirical
52 mutational studies have shown the importance of single nucleotides for modulating promoter
53 strength and translation initiation efficiency ¹⁻⁶, algorithms, such as the RBS calculator, have
54 been developed that predict translation initiation rates for diverse bacterial species including
55 *Bacillus subtilis* ⁷⁻⁹. Traditional approaches such as combining different transcriptional and
56 translational elements, and optimizing their sequences, are time-consuming processes, and
57 are prone to unforeseen outcomes, including heterogeneous expression across a population
58 of cells ¹⁰. Consequently, identifying expression modules that maximize transcription and
59 translation for a protein of interest can be achieved by trial and error processes in
60 combination with experimental design, build, test and learn cycles ¹¹⁻¹³.

61 The use of comprehensive synthetic DNA libraries, combined with systematic and
62 rational engineering design principles, provides an alternative solution for current challenges
63 in the optimization of protein expression for biotechnological purposes ¹⁴⁻¹⁶. In this study, we
64 employed a combinatorial design approach using a library of ~12,000 synthetic expression
65 modules (SEMs) to improve the production of heterologous proteins in *B. subtilis*, a
66 biotechnological important host. This Gram-positive bacterium is both a well-established
67 model organism and an important industrial workhorse ^{17, 18}, used for the production of
68 various metabolites and industrial enzymes ^{10, 19}.

69 We used the native expression module upstream of the *veg* gene as a basis for the
70 construction of a synthetic expression module library containing ~12,000 unique sequences,

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3 71 with modifications to the upstream, downstream and intervening promoter regions. The *veg*
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5 72 promoter is one of the strongest native expression modules in *B. subtilis* and commonly
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7 73 used for protein expression in *B. subtilis*^{20, 21}. The resulting synthetic expression module
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9 74 library was cloned upstream of a GFP reporter gene and analyzed by FlowSeq, a technique
10
11 75 that combines fluorescence-activated cell sorting and high-throughput DNA sequencing²²⁻²⁴.
12
13 76 The resulting library had a dynamic range of GFP production spanning five orders of
14
15 77 magnitude, and maximally showed a 13-fold improvement over that of the native *veg*
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17 78 expression module. Following in-depth sequencing, we quantified the influence of different
18
19 79 elements of the expression module with respect to their influence on GFP expression.
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21 80 However, when a selection of the synthetic expression modules was used to drive
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23 81 expression of the *B. subtilis xynA* gene encoding a secretory xylanase²⁵, the maximum
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25 82 increase in productivity was only 3-fold. Moreover, the correlation between GFP expression
26
27 83 and XynA production was weak. Here, we discuss how sequences at the 5' end of the
28
29 84 coding sequence of both genes affects the mRNA secondary structure and the translation
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31 85 initiation rate, and compare our measurements with the prediction of the RBS calculator²⁶.

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89 Design of a synthetic expression module (SEM) library

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97 The -35 and -10 regions of P_{veg} promoter and the Shine-Dalgarno (SD) sequence of the RBS
98 are similar to the consensus for σ^A recognition and pairing with 16S rRNA, respectively.
99 However, the -10 region was optimized to better match the consensus sequence of σ^A , and
100 changed from 5'-TACAAT-3' to 5'-TATAAT-3', and the SD sequence from 5'-AGGTGG-3' to
101 5'-AGGAGG-3' ²⁹. For the combinatorial design and synthesis, the expression module was
102 divided into four elements (Elements A-D, Figure 1B): Element A; the AT rich UP-element
103 located upstream of the -35 region, Element B; between the conserved “promoter elements”

-35 and -10, Element C; the sequence between the -10 sequence and SD, and Element D; the spacer between the SD and the translational start site (ATG). A higher AT content upstream of the -35 region can increase the interaction between the UP-element and the α -subunits of RNA polymerase, thereby stimulating promoter binding and transcription³⁰⁻³³. Therefore, the 24-base pair long UP-element was randomized and twenty different sequences were generated, five times four sequences with 25, 50, 75 or 100% AT contents (Figure 1B). For the 17-base pair spacer between the -35 and -10 regions, 5 different randomized nucleotide orders were generated³⁴⁻³⁶. Translation initiation can be tuned by reducing the folding of transcribed mRNAs into stable secondary structures, since strong secondary structures can impede ribosome binding and therefore lower the translation initiation^{37, 38}. By generating fifteen variants of the spacing between the -10 region and the SD, mRNAs were obtained with different folding energies (secondary structures) at their 5'-end. Additionally, by varying the length from 10, 20 to 30 nucleotides, the effect of local distance between transcription (RNA polymerase binding) and translation initiation (ribosome binding) could be analyzed. Five variants of the spacer between the SD and start codon were designed to provide potential variations in translation initiation efficiency. The distance of 8 nucleotides has been shown to be optimal and kept constant¹. The different elements, including those of the native P_{veg} promoter, were combined to generate a library of ~12,000 synthetic expression units (Supporting Information Table S1). The library was synthesized on a chip and pooled (see Methods for details).

Quantification of the SEM library

The library of synthetic expression modules was cloned upstream of a *gfp* coding region and integrated as single copy at the chromosomal *amyE* locus (Figure 2A). Production of GFP was used as a measure of expression efficiency. To monitor possible effects on the metabolic load imposed by the activity of the GFP expression module, a constitutively expressed mCherry reporter was used as internal reference. The mCherry expression module was driven by the constitutive P_{infC} promoter³⁹ and integrated into the *nprE* locus of

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B. subtilis (Figure 2A). A library of ~152,000 clones was generated, reflecting a ~13-fold clonal coverage of the ~12,000 variants in the synthetic library. The total library was grown in LB to early exponential phase ($OD_{600} \sim 0.25$) and subjected to fluorescence-activated cell scanning (FACScan) to determine the variations in SEM activity (Figure 2B). The GFP intensities ranged over five orders of magnitude, with a profile showing a bimodal distribution. In contrast, the mCherry fluorescence intensity showed a single peak indicating that the protein expression capacity of individual cells was not affected at high GFP expression levels.

Figure 2

The wide range of GFP expression levels was confirmed by analyzing a sample containing the entire library during the exponential growth phase using fluorescence microscopy (Figure 2C). In addition, 135 random colonies were grown overnight and their GFP over mCherry fluorescence ratio was determined with a plate reader. These ratios spanned a 500-fold difference in GFP expression with ratios from 10 to 0.02 (Supporting Information Figure S1).

FACS was subsequently used to divide the library into 12 non-adjacent log-spaced bins of increasing GFP intensities (Figure 2B, blue bins from I to XII). The population of each bin was reanalyzed by FACScan (Figure 3A-B), confirming the expected GFP signal range, although all bins, and especially bins X and XI, showed a long tail of cells with lower GFP intensities (Figure 3A). The mCherry signal was comparable to that of cells before sorting (Figure 3B), but now the mCherry signal in some cells became visible in the lower intensity values, possibly caused by cell differentiation^{40, 41}.

Figure 3

Identification and ranking of elements

DNA was extracted from the binned populations and SEM sequences were amplified by PCR adding unique sequence barcodes for each of the twelve bins. The barcoded fragments were pooled and subjected to high-throughput sequencing (Supporting Information Figure S2). Of the 12,000 designed variants, 91% were identified in the sequence data. Bin I

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3 162 contained many (~60%) shorter sequences which represented non-functional SEM
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5 163 sequences, presumably caused by errors during DNA synthesis. In addition, it appeared that
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7 164 the 100% AT variants of element A were highly underrepresented, probably reflecting
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9 165 difficulties in synthesis, and therefore these variants were not used in further analyses. To
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11 166 access the sorting stringency, the distribution of nine arbitrary sequences over all bins was
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13 167 investigated. Indeed, each of the unique SEMs was overrepresented in only one bin
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15 168 (Supporting Information Figure S3). To evaluate SEMs, a score was calculated by summing
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17 169 the scores of each design sequence in each bin. This score was obtained by counting the
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19 170 presence of each SEM in a specific bin and dividing it by the total number of that SEM
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21 171 across all bins, multiplying the value obtained with a log10 weighted average fluorescence of
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23 172 each bin (Supporting Information Table S2 and Dataset S1). For instance, SEM169 has 271
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25 173 sequence reads across all bins, divided over bins I-XII in the following order 4, 3, 2, 12, 7,
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27 174 116, 85, 21, 12, 1, 3, 5, with most reads in bins VI and VII. The relative presence in bin I
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29 175 (4/271) was amplified with the log10 weighted average fluorescence of bin I (2.81). This was
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31 176 done for all twelve bins and these values were summed up to come to an average log10
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33 177 GFP for SEM169 of 4.01. By calculating ten to the power of 4.01 the GFP expression score
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35 178 of 10253,82 is obtained, which falls between the average GFP fluorescence of bins VI and
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37 179 VII. The average GFP expression score for each SEM in the library was calculated and used
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39 180 for the analysis of the SEM library (Supporting Information Table S2 and Dataset S1).
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42 182 **Contribution of individual elements**

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44 183 To analyze the influence of each element on promoter strength and translation capacity, the
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46 184 abundance of the different elements within the twelve bins were examined. As shown in
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48 185 Figure 4A, the variants of Element A, located upstream of the -35 region (Figure 1), have
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50 186 only a small effect on the expression levels. It was mainly the constructs with 50 and 75%
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52 187 AT content (A1, A8, A12 or A13) that were overrepresented in the bins with high GFP
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54 188 expression levels. This suggests that the sequence upstream of the -35 region do not
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56 189 necessarily require an AT-rich content for an optimal interaction with the RNA polymerase.
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59
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Figure 4

Analyses of Element B variants (Figure 4B), located between the -35 and -10 region (Figure 1), show little impact on expression levels. Nevertheless, B1 (native) and B2 are reflected in bins with high GFP expression levels, while the B3 variant is found more dominantly in bins with lower GFP fluorescence levels. Interestingly, B6 contains the sequence TATG from -17 to -14, also called the -15 region or TGn motif, which has been shown to positively influence the promoter activity in both *B. subtilis* and *E. coli*^{3, 33}. However, in our experiments this element does not necessarily lead to an improved promoter activity (Figure 4B & 5). Element C variants, located between the -10 region and the SD (Figure 1), have a dominant influence on GFP expression levels, with variants C1, C2, C4 and C15 being overrepresented in bins VIII and higher. C15 is found in more than 20% of the cases in the higher bins. In contrast,

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3 202 variant C5, C6, C8, C11 and C16 are found predominantly in bins with lower GFP intensities
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5 203 with C11 clearly overrepresented in more than 40% of the cases in bin I, reflecting a poor
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7 204 RBS sequence. Among the Element D variants, D3 is most dominant in the high expressing
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9 205 bins, whereas, the native sequence (element D1) is dominant in the low expressing bins.
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11 206 The native variant of Element D, containing a 5-bp spacer, is predominant in SEMs with low
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13 207 expression scores, confirming that spacings of 5 bp between SD and start codon is less
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15 208 optimal for translation initiation than an 8-bp spacer ¹. To illustrate the distribution of element
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17 209 variants over the twelve bins in relation to the other elements, a heatmap was created to
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19 210 facilitate the selection of variants for low, medium or high levels of GFP production (Figure
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21 211 5). Bin numbers are shown on the bottom with low fluorescence in Bin I and highest
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23 212 fluorescence in Bin XII. White to yellow tiles illustrate a relative high abundance of reads and
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25 213 red indicates low abundance or absence. The first 14 elements (A5-B4) are over
26
27 214 represented in the low expressing SEMs, the elements C11-B5 in the middle of the figure
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29 215 are most abundant on the medium expressing SEMs while elements C2-B6 are the most
30
31 216 abundant in Bins IX-XII containing high GFP expressing SEMs.

Figure 5

The most optimal expression system is presumably a combination of the four elements that are most abundant in bins with the highest GFP levels. To confirm this, we filtered our library *in silico* for SEMs that contained combinations of Element A1, A8, A12 or A13, Element B1 or B2, Element C1, C2, C4 or C15 and Element D3, thirty-two in total ($4 \times 2 \times 4 \times 1 = 32$). These thirty-two SEMs exhibited a high level of GFP expression with an average of 4.6 (log10 scale), and a calculated score of 10.8 (Supporting Information Dataset S2). The highest GFP expression level was observed with a SEM composed of Elements A13, B2, C15, D3, which had a GFP level of expression of 4.7 (log10 scale) and a score of 11.5. This illustrates the benefits of a high-throughput combinatorial approach versus optimization of individual parts of an expression module.

It has been shown that weak secondary structures at the 5'-end of the RBS region

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3 230 and the first codons of the coding sequence ⁴² correlate with expression levels ^{13, 24, 43}. The
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5 231 SEM library combines sixteen C elements with six D elements and this resulted in 96 unique
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7 232 mRNA sequences. Transcriptional starts were set at the 7th nucleotide after the -10 promoter
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9 233 region followed by the RBS and the first 35 nucleotides of the *gfp* coding region. The total
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11 234 free energy exchange between ribosome and mRNA and translation initiation rates of these
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13 235 96 mRNA sequences were calculated with the online RBS calculator predict program ²⁶
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15 236 (Supporting Information Dataset S3). The measured average GFP fluorescence of each
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17 237 mRNA sequence was plotted against the predicted translation initiation calculated with the
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19 238 RBS calculator program (Figure 6A). Both values have a moderate positive correlation of R
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21 239 ²= 0.56, implying some predictive value of the RBS calculator for GFP, which can still differ
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23 240 easily by 10-fold for the same translation initiation prediction.

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25 241 The SEM library combines twenty-one A elements with six B elements, resulting in
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27 242 126 unique promoter sequences. The R^2 above was calculated with the average measured
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29 243 GFP fluorescence of one mRNA behind all possible 126 promoter sequences. Notably, the
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31 244 correlation between measured GFP and predicted translation initiation is higher or lower for
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33 245 certain promoter sequences. Therefore, this correlation was calculated for each of the 126
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35 246 individual promoter sequences (Figure 6B, Dataset S4). The promoters containing variants
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37 247 B1, B2, B5 & B6 have a similar correlation, with R^2 averages of 0.53, 0.48, 0.47 and 0.53
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39 248 respectively (Figure 6B, blue dots). However, the promoter containing variants B3 & B4
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41 249 (Figure 6B, red dots), show significantly lower R^2 values (average: B3 ~0.29, B4 ~0.36). For
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43 250 variant B3 this lower correlation can be explained by its negative effect on transcription,
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45 251 since SEMs with this sequence are predominantly found in Bins I-III. Increases in translation
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47 252 initiation and gene expression observed for C and D elements will not become visible in this
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49 253 lower range, due to the experimental set up of the bin size and distribution. For Element B4,
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51 254 the lack of correlation cannot be explained by a dominant negative effect on transcription
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53 255 since it is more evenly distributed across the bins (Figure 5).
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55 256

Figure 6

Expression in log and stationary growth

The initial FACS analysis was performed with cells grown to early exponential phase. To determine whether the strongly expressing SEMs show increased GFP levels throughout the exponential and stationary phases of growth, we selected 16 SEMs with high GFP expression levels on agar plates. Using a robotic QPix™ 450 colony picker, 16 colonies, out of a total of ~2000 colonies, were selected that displayed the highest fluorescence signal. These clones were sequenced (Supporting Information Dataset S5), and indeed they more frequently included the high expression elements, C2, C4 and D3 (44%, 31% and 69%, respectively).

The 16 clones were grown in a BioLector microbioreactor, monitoring biomass and fluorescence over time. A clone containing the native *veg* expression module was included as a reference. As shown in Figure 7A and B, growth rates, final cell densities and mCherry

levels were comparable for all 16 strains. GFP expression levels peaked towards the end of exponential phase (~3 h) with a wide range of expression levels, and decreased when the stationary phase commenced (Figure 7C). Nevertheless, the differences in expression between the 16 SEM variants were observed throughout the growth phases, indicating that these promoter variants are not subjected to strong transcriptional regulation under these growth conditions. The expression levels of the highly expressing variants 2, 9 and 16 were approximately 10 to 13-fold higher in log phase compared to the native *veg* expression module (Figure 7C). The GFP expression values after 2, 4 and 20 hours of these 16 constructs, are provided in the supporting information Dataset S5.

Figure 7

Application of strong SEMs for xylanase production

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3 285 To establish whether the combinatorial synthetic library approach yielded SEMs that
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5 286 would increase production of other proteins besides GFP, we examined whether the 16
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7 287 isolated SEM variants could increase the production of the commercially relevant secreted
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9 288 enzyme xylanase of *B. subtilis*. This was achieved by replacing the coding sequence of the
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11 289 *gfp* reporter gene downstream of the 16 SEMs with that of the commercially significant
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13 290 xylanase gene (*xynA*). The enzymatic activity of secreted xylanase was quantified at three
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15 291 time points during growth in the BioLector microbioreactor (2 h, 4 h and 20 h). The XynA
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17 292 activity at these three time points and the DNA sequences of these 16 constructs are
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19 293 provided in the Supporting Information Dataset S5. Growth rate, biomass production and
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21 294 mCherry expression was again similar for all strains (Supporting Information Figure S4).
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23 295 Xylanase was produced primarily during the logarithmic phase of growth (until 4 h), after
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25 296 which the activity presumably remained constant between 4 and 20 hours. Most of the
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27 297 selected SEMs resulted in a substantially increase in xylanase production over the native
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29 298 *veg* expression module, although the highest yielding variant (variant 7) was only 3-fold
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31 299 higher (Figure 8A). Furthermore, the expression level ranking of the SEM variants was
32
33 300 different for XynA and GFP. To determine the correlation between the GFP and XynA
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35 301 production levels during logarithmic growth (2 hours), when the rate of synthesis of both
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37 302 proteins was highest, GFP and XynA expression levels were normalized to the mCherry
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39 303 signal and plotted against each other (Figure 8B). Only a weak correlation was observed
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41 304 when both genes are expressed with the same SEM ($R^2 = 0.20$). Since only the gene of
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43 305 interest has changed, the most likely explanation for these differences is the interactions
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45 306 between the 5' untranslated region and the coding region. This weak correlation cannot be
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47 307 explained by post-translational events such as protein secretion or extracellular degradation
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49 308 by proteases since these are identical for all *xynA* constructs⁴⁴. This is supported by the
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51 309 wide range of XynA activities in Figure 8A, whereas identical expression levels would be
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53 310 expected when limitations occur in the secretion capacity or other post-transcriptional
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55 311 processes.
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Figure 8

Conclusion

We have designed and used a large library of synthetic expression modules to improve protein expression in *B. subtilis*. By combining the native *veg* promoter and RBS with synthetic sequences modulating transcription and translation, we were able to generate GFP levels with a dynamic range of over five orders of magnitudes, and a maximal 13-fold increase in expression compared with the native *veg* sequence on which the variants were based. A large part of the expression increase results from improving of the eight nucleotides between the ribosome binding site and the ATG start codon. Especially for *B. subtilis*, shorter SD space regions reduce the translation rate ¹. The extrapolation of our intracellular GFP expression based data to that of a secreted protein (xylanase) yielded a poor correlation. Our results confirm that optimizing and predicting gene expression levels of a specific target gene cannot simply be achieved using a single reporter gene. Furthermore, the coding sequence itself strongly influences the expression efficiency of the encoded protein, due to the formation of secondary mRNA structures that influence, in particular,

translation initiation^{6, 23}. This 5'mRNA folding is impacted by the first codons of the coding region and can affect mRNA stability and translation initiation^{23, 24, 43}. The translation initiation rate predictions with the RBS calculator includes these mRNA folding and ribosome binding interactions²⁶. Correlating the predicted and measured GFP expression levels of the entire library demonstrated a moderate positive correlation with a ten-fold window of possible expression levels, demonstrating that the RBS calculator can partially explain the different expression levels of SEM library. Previous studies found a higher correlation $R^2=0.81$ for a *B. subtilis* RBS library in combination with RFP^{7, 8}, illustrating that the predictive power of the RBS calculator is context dependent, and will differ with different genes of interest. The strong regulating influence of translation initiation on gene expression is supported by the finding that Elements C and D, downstream of the -10 region, had the strongest effect on GFP expression. The influence of the coding region on 5' mRNA folding also explains why xylanase and GFP production differed when the same expression module was used. Our data illustrate that the predictive value of algorithms that predict mRNA folding and its consequences for ribosome binding and translation initiation are context dependent and differ greatly with each gene of interest, growth condition and sequence design method. Therefore, the use of large libraries of SEMs, in combination with high-throughput screening technologies, provides an effective strategy to optimize the expression and production of specific proteins.

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3 349 **METHODS**

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6 351 **Bacterial Strains and Growth Conditions**

8 352 For cloning purposes, NEB® 10-beta competent *E. coli* cells (#C3019H, NEB®) were used.
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10 353 Vector pCS66 was transferred to *dam⁻dcm⁻* competent *E. coli* cells (#C2925H) to increase
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12 354 its transformation efficiency. All analyses of the library were performed in *B. subtilis* strain
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14 355 168 (*nprE::P_{infC}-mcherry*, *kan^R*; *amyE::SEM-gfp*, *spec^R*). Cells were grown in Luria–Bertani
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16 356 (LB) medium with the addition of 10 µg/mL kanamycin or 100 µg/mL spectinomycin in
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18 357 overnight cultures. No antibiotics were added to the medium when cultures were assayed for
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20 358 expression, since the expression modules are stably integrated into the chromosome. For
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22 359 monitoring biomass, GFP activity and XynA production, strains containing identical
23
24 360 expression modules were cultured in a BioLector® microbioreactor (m2p labs).

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28 362 **Plasmid and Strain Construction**

30 363 All PCR primers are listed in the Supporting Information (Table S3). Vector pCS78, used for
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32 364 the integration of the *P_{infC}-mcherry* reporter at the *nprE* locus of 168, was constructed in the
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34 365 following manner. Vector pAPNC213 ⁴⁵ was amplified with primers oCS001/002 to excise
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36 366 *lacI* and *P_{spac}*, resulting in pCS01. *gfp* was amplified from pPG49 ⁴⁶ with primers oCS003 and
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38 367 oCS004 and ligated via in pCS01 which resulted in pCS02. The kanamycin resistance
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40 368 cassette was amplified from pKT25N (Euromedex France SA) with primers oCS007 and
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42 369 oCS008 and ligated in the *EcoRI* and *BamHI* sites of pCS02, which resulted in pCS04. This
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44 370 vector was amplified with primers oCS009 and oCS010 and re-circularized which resulted in
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46 371 pCS05. The *amp^R* and *ColE1* region was amplified with primers oCS027 and oCS028 and
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48 372 ligated, resulting in pCS08. Vector pCS08 was digested with *XbaI* and *BamHI* and the
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50 373 upstream flanking region of *nprE* was amplified with primers oCS023 and oCS024; the
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52 374 ligated product resulted in pCS09. The downstream flanking region of *nprE* was amplified
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54 375 with primers oCS025 and oCS026 and ligated into pCS09 digested with *NotI* and *SacII*, this
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56 376 resulting in pCS10. Vector pCS10 was amplified with primers oCS045 and oCS069 to excise

the *gfp* gene and circularized, resulting in pCS25. Vector pCS25 was amplified with primers oCS048 and oCS049 to excise the *XhoI* site and circularized, resulting in pCS28. Vector pCS28 was amplified with oCS062 and oCS071 to create pCS29. Vector pCS29 was used with primers oCS077 and oCS069 to create a StarGate vector integrating in the *nprE* locus, which resulted in pCS31. The *ery^R* gene of pCS31 was replaced with the *kan^R* gene resulting in pCS41. The *P_{infC}*-mCherry from pDOW10⁴⁷ was fused to the *infC* promoter and *mobU* SD. This fragment was amplified with primers oCS523 and oCS524 and inserted in pCS41 which resulted in pCS78.

Vector pCS66 was used to integrate the SEM-*gfp* library into the *amyE* locus of strain CS335. Vector pCS66 was constructed in the following manner. Vector pBest4 was amplified by PCR using primers oCS592 and oCS593 and digested with *SbfI* and *NotI*. The chloramphenicol acetyltransferase gene flanked with *BsmBI* restriction sites was amplified with primers oCS590 and oCS591. The *amyM* terminator was amplified from *B. stearothermophilus* C599⁴⁸ with the primers oCS586 and oCS587. The resulting PCR products were fused using oCS590 and oCS586 and the resulting 1-kb fragment was digested with *HindIII* and *NotI*. The spectinomycin selection marker flanked with *lox66* and *lox71* sites⁴⁹ was amplified with primers oCS588 and oCS589 and digested with *SbfI* and *HindIII*. Both inserts and the vector backbone were ligated, resulting in pCS75. The *gfp* gene was amplified from pPG49 with primers oCS594 and oCS595 and subsequently *BsmBI* restriction sites were introduced to allow StarGate® cloning. This resulted in vector pCS66 that was used for the insertion of SEMs to create the *gfp* based library and integration in the *amyE* locus using spectinomycin selection.

The Xylanase expression vector pCS80 was created by using Gibson Assembly® with pCS75 and the *xynA* gene amplified with primers oCS545 and oCS546 from the chromosomal DNA of 168.

StarGate® and Library Cloning

The oligonucleotide pool was synthesized on a DNA microarray by the BROAD

Technology Lab (Cambridge, MA 02142). The resulting oligonucleotide pool was quantified using a Qubit® fluorometer with a measured concentration of 1.44 ng/μl. The oligo pool was enriched for full-length synthesis products. This step was carried out using 25 μl NEBNext® high-fidelity 2× PCR master mix (cat. no. M0541S, NEB®), 19 μl MilliQ water, 2.5 μl of each primer (10 μM; oCS492/oCS493) and 1 μl of the 1:100 diluted oligonucleotide pool. The following PCR conditions were applied: initial denaturation at 98°C for 30 s, 25 cycles consisting of denaturation at 98°C for 10 s, annealing at 58.4°C for 20 s and an elongation at 72°C for 15 s followed by a final extension at 72°C for 300 s.

Afterwards, StarGate® cloning with *Bsm*BI overhangs (IBA GmbH) was performed to assemble the expression vectors and the SEM library. For a simple StarGate® reaction, 3 μl of both vector (4 nM) and insert (4 nM – 8 nM) were combined with 5 μl 5×T4 DNA ligase buffer (Thermo Fisher Scientific), 1 μl T4 DNA ligase (1 U/μl) (cat. no. 15224-017, Thermo Fisher Scientific), 0.5 μl *Esp*3I (*Bsm*BI) (10 U/μl) (cat. no. ER0451, Thermo Fisher Scientific), made up to 25 μl with 12.5 μl MilliQ water. This mixture was incubated for 3 h at 30°C, another hour at 37°C and then used for transformation of *B. subtilis*. For the SEM library, the reaction volume was doubled to 50 μl and contained 12 nM of unmethylated pCS66, 24 nM of amplified SEM DNA and 1 μl *Esp*3I (*Bsm*BI). 950 μl of competent cells were transformed using a 14 mL Falcon tube shaking at 37°C for 1 h before plating on 2×PY selective agar plates (BioAssay dishes, low profile, cat. no. 240845, Thermo Fisher Scientific) and incubated overnight at 30°C wrapped in plastic foil. This cloning procedure led to the creation of a library of 152,000 CFUs with ~13-fold clonal coverage. The resulting colonies were scrapped off the plates, combined in a flask, and stored at -80°C.

Xylanase assay

Xylanase activities were assayed with the EnzChek® ultra xylanase assay kit (cat. no. E33650, Thermo Fisher Scientific) following the suppliers protocol. The assay is based on the substrate 6,8-difluoro-4-methylumbelliferyl beta-D-xylobioside, its hydrolysis of which the fluorescent compound, 6,8-difluoro-4-methylumbelliferone, is released. The xylanase-

containing samples were diluted 1:100 and analyzed using a plate reader (Infinite 200 Pro, Tecan® or FLUOstar OPTIMA, BMG LABTECH®) with excitation/emission wavelengths of 358/455 nm after an incubation period at room temperature in the dark for 15 or 30 min.

Microscopy and data analysis

Samples for fluorescence microscopy were grown in LB medium or on LB agar and, following collection, washed immediately with PBS buffer. A sample of 0.3 µl was applied to a GeneFrame (AbGene, Surrey, UK) containing 1.25% agarose in PBS. For visualization, a Zeiss 200M microscope with following excitation/emission filters and timings were used: GFP (470/525 nm) for 1000 ms; mCherry 550-600/615-665 nm for 1000 ms.

Bioinformatics design

For the SEM library design, sequence parts of the native P_{veg} were synthetically modified with randomized sequence and were combined afterwards leading to a library of 12,000 different variants (Supporting Information Dataset S6). However, specific regulatory elements were conserved to ensure optimal SD and sigma factor recognition sequences. The elements of the synthetic library included: (1) Element A 1-20: the UP element upstream the -35 region of sigma factor recognition (TTGACA) was modified by altering the percentage of AT content (25%, 50%, 75% and 100%), (2) Element B 1-5: the spacer between the -35 and -10 (TATAAT) regions was randomized with the restriction that it maintained a high AT content of 75%, (3) Element C 1-15: the spacer between position -10 and the SD, was randomized by using an increasing spacer length in steps of 10 from 10 bp to 30 bp with no restriction on the %AT content, and (4) Element D 1-5: the 8-bp-spacer between SD and start codon was modified while maintaining a %AT content of 75%. The associated combinatorial design, resulting in a synthetic expression module library, containing 12,000 oligonucleotides, was created using the programming language Python version 2.7.6.

461 Validation SEM Library

462 The experimental design was validated by generating a limited number of
463 transformants containing SEMs. A total of 192 colonies were picked at random and
464 sequenced to verify the library. This revealed that only ~34% of the expression module
465 sequences matched that of the bioinformatics design. The other sequences carried point
466 mutations or deletions leading to a more diverse expression library than had previously been
467 anticipated. After removing duplicates and non-active modules due to deletions, 135
468 colonies were grown overnight and subjected to end point measurements of GFP and
469 mCherry to verify the dynamic range of the expression modules, as shown in the Supporting
470 Information (Figure S1). The strengths of the individual expression modules were measured
471 by determining the GFP:mCherry ratio. The data showed GFP:mCherry ratios from ~10,
472 reflecting strong expression modules, to ~0.02, revealing weak expression units. The
473 average GFP:mCherry ratio was around 1.8 and the ~500-fold difference in expression
474 strength of this limited validation set reflected an enormous dynamic expression range and a
475 large numbers of variations in the synthetic module sequences (Supporting Information
476 Figure S1), demonstrating the effectiveness of the design concept.

478 Fluorescence-activated cell sorting (FACS)

479 An overnight culture of the SEM library was prepared by using 5 mL of LB medium
480 containing the appropriate antibiotics with shaking at 165 rpm in an Innova 2300 platform
481 shaker (New Brunswick Scientific) at 30°C. The next day, 0.33 mL of overnight culture was
482 added into 100 mL of LB (without antibiotics) with shaking for 3 h. After reaching an OD₆₀₀ of
483 ~0.25, the flask was immediately placed on ice to prevent further growth and protein folding.
484 The cells were centrifuged at 3,000 × g for 10 min at 4°C. The supernatant was removed
485 and the cells were resuspended in 15 mL of ice-cold PBS and cell chaining was reduced by
486 ultrasonication using the lowest power for 10 s. This suspension was immediately analyzed
487 with a FACSAria™ Fusion flow cytometer system (BD Biosciences) at 4°C using the

excitation/emission spectrum of 488/530-30-A nm for GFP and 561/610-20-A nm for mCherry. Additionally, the forward and side scatter parameter was applied to facilitate the measurement of cell volume and morphology, respectively, and excluding any dead or doublet cells. For the SEM library, 12 non-adjacent bins were set up with a 43-channel gap to reduce cross contaminations between the bins. The bins collected different numbers of cells as follows: bin I, one million, bins II through VIII, 250,000 cells, bins IX and X, 100,000 cells and bins XI and XII, 25,000 cells. After sorting, the individual bins were amplified by culturing in LB medium at room temperature overnight followed by 8 h at 30°C. All bins were finally subjected to FACScan and DNA extraction for subsequent sequencing.

Fluorescence-activated cell scan (FACScan)

The FACScan technology is based on flow cytometry but only screens for the fluorescence activity of cells. For suitable FACScan analyses, cells were pelleted and subsequently resuspended in ice-cold PBS and diluted 100-fold to remove any remaining auto fluorescence of the medium. For the scanning of 50,000 events, the LSRFortessa™ X-20 (BD Biosciences) was applied using the excitation/emission spectrum and the forward and side scatter as done before using FACS (see above). MATLAB R2014b (MathWorks®) was used to analyze generated data.

Amplification and barcoding of the SEM library

After sorting via flow cytometry and cultivation, chromosomal DNA was extracted from the cells in each of the 12 fluorescence intensity bins. The extracted DNA was subjected to PCR using primer oCS568 which binds on the 5'end of the *amyE* flanking region and primer oCS569 which binds on the first 4-11 codons of *gfp*, to amplify the complete expression modules and a few nucleotides of *amyE* and *gfp*. The resulting PCR products were analyzed using the Agilent DNA 1000 kit (Agilent Technologies), and amplified with forward primers oCS596 until oCS607 containing a 5' Illumina adapter (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG), bin-specific barcodes followed by the

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amyE generic sequence (5'-GTTCCAATACGGAGAAATCG). Primer oCS608 was used as reverse primer containing the Illumina adapter (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG) followed by the GFP generic sequence (5'-TCTTCTCCTTTGCTCATCTG).

Sequencing of SEM library

PCR products with bin-specific adapters were sequenced by Baseclear (Leiden, The Netherlands) using an Illumina HiSeq 2500 as described previously²³. This resulted in more than 9.8 million reads of 126-bp fragments and between 151-235 Mbp of data per bin (Supporting Information Figure S2). For all bins, at least 98% of the data could be matched and merged into longer pseudo-reads using overlapping paired-end reads, generating complete SEM sequences using FLASH version 1.2.11. FastQC version 0.11.3 (Andrews, 2010: www.bioinformatics.babraham.ac.uk/projects) was used for quality inspection of the data and MATLAB version R2014b was used for data processing. The sequences were filtered by size, with a minimum of 163 bp matching the shortest design. The second filter removed the 5'-*amyE*, GFP and bin adaptor sequences, reducing the sequences by an additional 7%. The remaining sequences were subjected to de-duplicating using MATLAB. The SEM designs were converted to a FASTA file format using FASTAwrite in MATLAB. This FASTA file was used in the CLC genomics workbench as reference. The design sequences were trimmed to remove the generic sequences using CLC genomics workbench version 8.0. Reads were mapped with 0.97 similarity over 0.9 of their length, which means that 90% of the read could have a maximum of three mismatches to be mapped to the reference. The mapping file (.sam) was loaded into MATLAB and for each unique read, a table was generated to which a reference was mapped, *i.e.* several unique reads can map to one library design due to the similarity constraint. An expression score for each SEM design was calculated by summing the scores of each design variant in each bin and removing the unique reads that are not matched to the design library. This score was calculated as

follows: $\frac{n_{bin}^i}{\sum_{bin} n} \times bin$. The count (n) of a specific design in a specific bin was divided by the total count of that specific design across all bins and multiplied with the bin number. Therefore, the highest score is 12 for a design only being present in bin XII. In addition, we calculated the average GFP signal per SEM and for each RBS and promoter sequence. First, we estimated the GFP average per bin by retrieving the GFP value at the peaks as shown in the GFP histograms in Figure 3A using function “density” from the stats package in R. We then calculated the GFP average per SEM using a weighted average with relative bin frequencies as weights: $\frac{n_{bin}^i}{\sum_{bin} n} \times \log_{10}(GFP_bin)$. Finally, we calculated the average GFP for each RBS and promoter sequence by averaging all SEMs that contain the same RBS or promoter. In total, we have 96 unique RBS sequences (combination of Elements C and D) and 126 promoter sequences (Elements A and B). We used cor() and lm() functions from the stats package in R to explore the relations between the observed GFP average, promoter and the predicted translation initiation rates for each RBS. Relative abundance of specific designs in a bin and the average of relative abundance for each element was calculated. To generate the heatmap in Figure 5, correlation was used as distance measure, hierarchical clustering was performed with complete linkage as agglomeration method (function heatmap.2 from library gplots) and bins were normalized by z-score.

Prediction of translation rates and total free energy exchange between ribosome and mRNA

The RBS calculator v2.0 was used to predict translation rates of RBS sequences and total free energy exchange between ribosome and mRNA using Version 2.1^{7, 26}. The mRNA folding energies (ΔG) were calculated starting from the 7th nucleotide (the transcription start point) after the -10 promoter region, followed by the RBS and the first 35 nucleotides of the *gfp* gene. The one way ANNOVA statistical analyses were performed with Minitab software version 18.

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570 **Supporting Information**

- 571 Table S1: Synthetic expression elements used for the SEM library.
- 572 Table S2: Average fluorescence intensities of the twelve bins.
- 573 Table S3: PCR primers used in this study.
- 574 Figure S1: GFP/mCherry ratio of library variants after overnight growth.
- 575 Figure S2: Illumina HiSeq 2500 sequence statistics of twelve bins containing SEMs.
- 576 Figure S3: Relative abundance of nine arbitrary SEMs over the twelve bins.
- 577 Figure S4: Growth and mCherry production of selected SEMs over time.
- 578 Dataset S1: SEM constructs
- 579 Dataset S2: 32 SEMs (high GFP)
- 580 Dataset S3: RBS
- 581 Dataset S4: Promoters
- 582 Dataset S5: 16 SEMs fused with *xynA/gfp*
- 583 Dataset S6: Oligo designs
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594 REFERENCES

595

596 [1] Vellanoeweth, R. L., and Rabinowitz, J. C. (1992) The influence of ribosome-binding-site
597 elements on translational efficiency in *Bacillus subtilis* and *Escherichia coli* in vivo,
598 *Molecular microbiology* 6, 1105-1114.

599 [2] Jensen, P. R., and Hammer, K. (1998) The sequence of spacers between the consensus
600 sequences modulates the strength of prokaryotic promoters, *Applied and*
601 *environmental microbiology* 64, 82-87.

602 [3] Liu, M., Tolstorukov, M., Zhurkin, V., Garges, S., and Adhya, S. (2004) A mutant spacer
603 sequence between -35 and -10 elements makes the Plac promoter hyperactive and
604 cAMP receptor protein-independent, *Proceedings of the National Academy of*
605 *Sciences of the United States of America* 101, 6911-6916.

606 [4] Tuller, T., Carmi, A., Vestsigian, K., Navon, S., Dorfan, Y., Zaborske, J., Pan, T., Dahan,
607 O., Furman, I., and Pilpel, Y. (2010) An evolutionarily conserved mechanism for
608 controlling the efficiency of protein translation, *Cell* 141, 344-354.

609 [5] Davis, J. H., Rubin, A. J., and Sauer, R. T. (2011) Design, construction and
610 characterization of a set of insulated bacterial promoters, *Nucleic acids research* 39,
611 1131-1141.

612 [6] Liebeton, K., Lengefeld, J., and Eck, J. (2014) The nucleotide composition of the spacer
613 sequence influences the expression yield of heterologously expressed genes in
614 *Bacillus subtilis*, *Journal of biotechnology* 191, 214-220.

615 [7] Espah Borujeni, A., Channarasappa, A. S., and Salis, H. M. (2014) Translation rate is
616 controlled by coupled trade-offs between site accessibility, selective RNA unfolding
617 and sliding at upstream standby sites, *Nucleic acids research* 42, 2646-2659.

618 [8] Farasat, I., Kushwaha, M., Collens, J., Easterbrook, M., Guido, M., and Salis, H. M.
619 (2014) Efficient search, mapping, and optimization of multi-protein genetic systems in
620 diverse bacteria, *Molecular systems biology* 10, 731.

621 [9] Espah Borujeni, A., and Salis, H. M. (2016) Translation Initiation is Controlled by RNA
622 Folding Kinetics via a Ribosome Drafting Mechanism, *Journal of the American*
623 *Chemical Society* 138, 7016-7023.

624 [10] Ploss, T. N., Reilman, E., Monteferrante, C. G., Denham, E. L., Piersma, S., Lingner, A.,
625 Vehmaanpera, J., Lorenz, P., and van Dijl, J. M. (2016) Homogeneity and
626 heterogeneity in amylase production by *Bacillus subtilis* under different growth
627 conditions, *Microbial cell factories* 15, 57.

628 [11] Mijakovic, I., Petranovic, D., and Jensen, P. R. (2005) Tunable promoters in systems
629 biology, *Curr Opin Biotechnol* 16, 329-335.

630 [12] Brockmeier, U., Caspers, M., Freudl, R., Jockwer, A., Noll, T., and Eggert, T. (2006)
631 Systematic screening of all signal peptides from *Bacillus subtilis*: a powerful strategy
632 in optimizing heterologous protein secretion in Gram-positive bacteria, *Journal of*
633 *molecular biology* 362, 393-402.

634 [13] Mutalik, V. K., Guimaraes, J. C., Cambray, G., Lam, C., Christoffersen, M. J., Mai, Q. A.,
635 Tran, A. B., Paull, M., Keasling, J. D., Arkin, A. P., and Endy, D. (2013) Precise and
636 reliable gene expression via standard transcription and translation initiation elements,
637 *Nature methods* 10, 354-360.

638 [14] Rooke, J. (2013) Synthetic biology as a source of global health innovation, *Syst Synth*
639 *Biol* 7, 67-72.

640 [15] Lienert, F., Lohmueller, J. J., Garg, A., and Silver, P. A. (2014) Synthetic biology in
641 mammalian cells: next generation research tools and therapeutics, *Nat Rev Mol Cell*
642 *Biol* 15, 95-107.

643 [16] Slomovic, S., Pardee, K., and Collins, J. J. (2015) Synthetic biology devices for in vitro
644 and in vivo diagnostics, *Proceedings of the National Academy of Sciences of the*
645 *United States of America* 112, 14429-14435.

- [17] Harwood, C. R. (1992) *Bacillus subtilis* and its relatives: molecular biological and industrial workhorses, *Trends Biotechnol* 10, 247-256.
- [18] van Dijk, J. M., and Hecker, M. (2013) *Bacillus subtilis*: from soil bacterium to super-secreting cell factory, *Microbial cell factories* 12, 3.
- [19] Kulkarni, N., Shendye, A., and Rao, M. (1999) Molecular and biotechnological aspects of xylanases, *FEMS microbiology reviews* 23, 411-456.
- [20] Radeck, J., Kraft, K., Bartels, J., Cikovic, T., Durr, F., Emenegger, J., Kelterborn, S., Sauer, C., Fritz, G., Gebhard, S., and Mascher, T. (2013) The *Bacillus* BioBrick Box: generation and evaluation of essential genetic building blocks for standardized work with *Bacillus subtilis*, *Journal of biological engineering* 7, 29.
- [21] Guiziou, S., Sauveplane, V., Chang, H. J., Clerle, C., Declerck, N., Jules, M., and Bonnet, J. (2016) A part toolbox to tune genetic expression in *Bacillus subtilis*, *Nucleic acids research* 44, 7495-7508.
- [22] Sharon, E., Kalma, Y., Sharp, A., Raveh-Sadka, T., Levo, M., Zeevi, D., Keren, L., Yakhini, Z., Weinberger, A., and Segal, E. (2012) Inferring gene regulatory logic from high-throughput measurements of thousands of systematically designed promoters, *Nature biotechnology* 30, 521-530.
- [23] Kosuri, S., Goodman, D. B., Cambray, G., Mutalik, V. K., Gao, Y., Arkin, A. P., Endy, D., and Church, G. M. (2013) Composability of regulatory sequences controlling transcription and translation in *Escherichia coli*, *Proceedings of the National Academy of Sciences of the United States of America* 110, 14024-14029.
- [24] Goodman, D. B., Church, G. M., and Kosuri, S. (2013) Causes and effects of N-terminal codon bias in bacterial genes, *Science* 342, 475-479.
- [25] Paice, M. G., Bourbonnais, R., Desrochers, M., Jurasek, L., and Yaguchi, M. (1986) A xylanase gene from *Bacillus subtilis*: nucleotide sequence and comparison with *B. pumilus* gene, *Archives of microbiology* 144, 201-206.
- [26] Salis, H. M., Mirsky, E. A., and Voigt, C. A. (2009) Automated design of synthetic ribosome binding sites to control protein expression, *Nature biotechnology* 27, 946-950.
- [27] Lam, K. H., Chow, K. C., and Wong, W. K. (1998) Construction of an efficient *Bacillus subtilis* system for extracellular production of heterologous proteins, *Journal of biotechnology* 63, 167-177.
- [28] Fukushima, T., Ishikawa, S., Yamamoto, H., Ogasawara, N., and Sekiguchi, J. (2003) Transcriptional, functional and cytochemical analyses of the *veg* gene in *Bacillus subtilis*, *J Biochem* 133, 475-483.
- [29] Osada, Y., Saito, R., and Tomita, M. (1999) Analysis of base-pairing potentials between 16S rRNA and 5' UTR for translation initiation in various prokaryotes, *Bioinformatics* 15, 578-581.
- [30] Aiyar, S. E., Gourse, R. L., and Ross, W. (1998) Upstream A-tracts increase bacterial promoter activity through interactions with the RNA polymerase alpha subunit, *Proceedings of the National Academy of Sciences of the United States of America* 95, 14652-14657.
- [31] Caramori, T., and Galizzi, A. (1998) The UP element of the promoter for the flagellin gene, *hag*, stimulates transcription from both SigD- and SigA-dependent promoters in *Bacillus subtilis*, *Molecular & general genetics : MGG* 258, 385-388.
- [32] Meijer, W. J., and Salas, M. (2004) Relevance of UP elements for three strong *Bacillus subtilis* phage phi29 promoters, *Nucleic acids research* 32, 1166-1176.
- [33] Phan, T. T., Nguyen, H. D., and Schumann, W. (2012) Development of a strong intracellular expression system for *Bacillus subtilis* by optimizing promoter elements, *Journal of biotechnology* 157, 167-172.
- [34] Moran, C. P., Jr., Lang, N., LeGrice, S. F., Lee, G., Stephens, M., Sonenshein, A. L., Pero, J., and Losick, R. (1982) Nucleotide sequences that signal the initiation of transcription and translation in *Bacillus subtilis*, *Molecular & general genetics : MGG* 186, 339-346.

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60

[35] Helmann, J. D. (1995) Compilation and analysis of *Bacillus subtilis* sigma A-dependent promoter sequences: evidence for extended contact between RNA polymerase and upstream promoter DNA, *Nucleic acids research* 23, 2351-2360.

[36] Jarmer, H., Larsen, T. S., Krogh, A., Saxild, H. H., Brunak, S., and Knudsen, S. (2001) Sigma A recognition sites in the *Bacillus subtilis* genome, *Microbiology* 147, 2417-2424.

[37] de Smit, M. H., and van Duin, J. (1990) Secondary structure of the ribosome binding site determines translational efficiency: a quantitative analysis, *Proceedings of the National Academy of Sciences of the United States of America* 87, 7668-7672.

[38] Na, D., Lee, S., and Lee, D. (2010) Mathematical modeling of translation initiation for the estimation of its efficiency to computationally design mRNA sequences with desired expression levels in prokaryotes, *BMC Syst Biol* 4, 71.

[39] Nicolas, P., Mader, U., Dervyn, E., Rochat, T., Leduc, A., Pigeonneau, N., Bidnenko, E., Marchadier, E., Hoebeke, M., Aymerich, S., Becher, D., Bisicchia, P., Botella, E., Delumeau, O., Doherty, G., Denham, E. L., Fogg, M. J., Fromion, V., Goelzer, A., Hansen, A., Hartig, E., Harwood, C. R., Homuth, G., Jarmer, H., Jules, M., Klipp, E., Le Chat, L., Lecointe, F., Lewis, P., Liebermeister, W., March, A., Mars, R. A., Nannapaneni, P., Noone, D., Pohl, S., Rinn, B., Rugheimer, F., Sappa, P. K., Samson, F., Schaffer, M., Schwikowski, B., Steil, L., Stulke, J., Wiegert, T., Devine, K. M., Wilkinson, A. J., van Dijl, J. M., Hecker, M., Volker, U., Bessieres, P., and Noirot, P. (2012) Condition-dependent transcriptome reveals high-level regulatory architecture in *Bacillus subtilis*, *Science* 335, 1103-1106.

[40] Veening, J. W., Smits, W. K., and Kuipers, O. P. (2008) Bistability, epigenetics, and bet-hedging in bacteria, *Annual review of microbiology* 62, 193-210.

[41] Grimbergen, A. J., Siebring, J., Solopova, A., and Kuipers, O. P. (2015) Microbial bet-hedging: the power of being different, *Current opinion in microbiology* 25, 67-72.

[42] Bentele, K., Saffert, P., Rauscher, R., Ignatova, Z., and Bluthgen, N. (2013) Efficient translation initiation dictates codon usage at gene start, *Molecular systems biology* 9, 675.

[43] Plotkin, J. B., and Kudla, G. (2011) Synonymous but not the same: the causes and consequences of codon bias, *Nat Rev Genet* 12, 32-42.

[44] Westers, H., Westers, L., Darmon, E., van Dijl, J. M., Quax, W. J., and Zanen, G. (2006) The CsrRS two-component regulatory system controls a general secretion stress response in *Bacillus subtilis*, *The FEBS journal* 273, 3816-3827.

[45] Morimoto, T., Loh, P. C., Hirai, T., Asai, K., Kobayashi, K., Moriya, S., and Ogasawara, N. (2002) Six GTP-binding proteins of the Era/Obg family are essential for cell growth in *Bacillus subtilis*, *Microbiology* 148, 3539-3552.

[46] Gamba, P., Jonker, M. J., and Hamoen, L. W. (2015) A Novel Feedback Loop That Controls Bimodal Expression of Genetic Competence, *PLoS genetics* 11, e1005047.

[47] Detert Oude Weme, R. G., Kovacs, A. T., de Jong, S. J., Veening, J. W., Siebring, J., and Kuipers, O. P. (2015) Single cell FRET analysis for the identification of optimal FRET-pairs in *Bacillus subtilis* using a prototype MEM-FLIM system, *PloS one* 10, e0123239.

[48] Diderichsen, B., and Christiansen, L. (1988) Cloning of a maltogenic alpha-amylase from *Bacillus stearothermophilus*, *FEMS microbiology letters* 56, 53-60.

[49] Yan, X., Yu, H. J., Hong, Q., and Li, S. P. (2008) Cre/lox system and PCR-based genome engineering in *Bacillus subtilis*, *Applied and environmental microbiology* 74, 5556-5562.

FIGURE LEGENDS

Figure 1: Design of Synthetic Expression Module (SEM) library. (A) Sequence of the *veg* native expression module. (B) SEM design with four variable elements: Element A (UP-element), Element B (spacer between -35 and -10 region [red]), Element C (spacer between -10 region and SD) and Element D (spacer between SD and start codon). The elements were modified as follows: Element A reflecting the AT content (blue) of 25%, 50%, 75%, 100% (5 variants each), Element B (5 variants), Element C (15 variants) including the +1 region (red), and Element D, the 8 NT spacer (5 variants). Nucleobases (n) are reflected by adenine, thymine, guanine or cytosine.

Figure 2: Fluorescence analysis of the SEM library. (A) The *mcherry* gene under the control of the *infC* promoter (P_{infC}) and the kanamycin selection marker (*kan*) were integrated in the *nprE* locus. The *gfp* gene under control of the SEM library and the spectinomycin (*spec*) selection marker were integrated in the *amyE* locus. Terminators (T) prevent read-through into adjacent genes. *BsmBI* restriction sites indicate the insert location of the SEMs upstream of *gfp*. Disrupted integration loci (*amyE*; *nprE*) are indicated with dashed lines. (B) GFP and mCherry fluorescence levels of the SEM library. The expression library of 152,000 clones was grown until early exponential phase. mCherry fluorescence levels are shown in red and GFP fluorescence levels in green. Cells were sorted into 12 non-adjacent log-spaced bins (I to XII) with increasing GFP intensities indicated by blue shades (top). (C) Microscopic analysis of the unsorted SEM library during exponential growth. Showing from left to right: phase contrast (PC), homogeneous red fluorescence (mCherry) and heterogeneous green fluorescence (GFP). Scale bar is 5 μ m.

Figure 3: GFP and mCherry fluorescence of bins I to XII. The populations of the 12 bins and the parent strain 168 were cultured and 50,000 events of each population were analyzed by FACScan. (A) The cell counts of GFP fluorescence and (B) mCherry

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3 778 fluorescence intensities are shown.
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7 780 **Figure 4: Abundance of SEM Elements in bins I to XII.** The abundance of Element A,
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9 781 Element B, Element C and Element D variants over the twelve bins were quantified and
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11 782 normalized against their total counts in the SEM library, respectively. Bin I represents the
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13 783 lowest GFP fluorescence and bin XII the highest GFP fluorescence. The frequencies of (A)
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15 784 16 Element A variants with different %AT content, (B) 6 Element B variants, (C) 16 Element
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17 785 C variants with different spacer length and (D) 6 Element D variants are shown. Variant 1
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19 786 represents the native *veg* expression module. The probabilities of appearance are color
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21 787 coded and shown in panel A.
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25 789 **Figure 5: Heat map of all element variants distributed over the 12 bins.** Hierarchical
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27 790 clustering of the elements using correlation as distance metric and complete linkage as
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29 791 agglomeration method. Bins are normalized by Z-scores. High relative abundance is white
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31 792 and low relative abundance is red.
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35 794 **Figure 6: Measured GFP levels and predicted translation initiation.** (A) The log10 of the
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37 795 average GFP expression level of the 96 RBS modules (sixteen C elements times six D
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39 796 elements = 96) including their standard deviation was plotted against the log10 of the
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41 797 predicted translation initiation rate (TIR) calculated by the RBS calculator V2.0 ²⁶ and resulted
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43 798 in a moderate positive correlation ($R^2 = 0.56$). (B) Correlating GFP expression and predicted
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45 799 translation initiation rate (TIR) for each of the 126 promoter sequences on the x-axes
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47 800 (twenty-one A elements times six B elements = 126). First 32 promoters with Elements B1
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49 801 and B2 are colored blue, promoters 33-64 with Elements B3 and B4 are colored red and
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51 802 promoters 65-96 with Elements B5 and B6 are colored blue.
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55 804 **Figure 7: Growth, mCherry and GFP over time.** Strains containing sixteen selected SEMs
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57 805 and control strains carrying no expression unit (empty) or native expression module (*veg*)
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3 806 were analyzed during growth. (A) Biomass increase, (B) production of mCherry over
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5 807 biomass and (C) SEM activity reflected by GFP fluorescence over biomass are shown.
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7 808 Strains were grown in quadruplicate in a BioLector® microbioreactor for 20 h in LB medium
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9 809 and the average values are shown.

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13 811 **Figure 8: Xylanase production of selected SEMs and analysis of interaction between**
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15 812 **GFP and XynA expression.** Strains containing 16 expression modules and control strains
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17 813 carrying no expression module (empty) or the native expression module (veg) were analyzed
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19 814 for (A) XynA activity in the culture medium during exponential (2 hours), transition (4 hours)
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21 815 and stationary phase (20 hours). (B) Correlation analysis between XynA and GFP production
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23 816 levels with respect to mCherry expression during logarithmic growth phase (2 hours) ($R^2 =$
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25 817 0.20). Error bars indicate the standard deviation of four biological replicates.

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Figure 1: Design of Synthetic Expression Module (SEM) library. (A) Sequence of the veg native expression module. (B) SEM design with four variable elements: Element A (UP-element), Element B (spacer between -35 and -10 region [red]), Element C (spacer between -10 region and SD) and Element D (spacer between SD and start codon). The elements were modified as follows: Element A reflecting the AT content (blue) of 25%, 50%, 75%, 100% (5 variants each), Element B (5 variants), Element C (15 variants) including the +1 region (red), and Element D, the 8 NT spacer (5 variants). Nucleobases (n) are reflected by adenine, thymine, guanine or cytosine.

116x95mm (300 x 300 DPI)

Figure 2: Fluorescence analysis of the SEM library. (A) The mcherry gene under the control of the infC promoter (P_{infC}) and the kanamycin selection marker (kan) were integrated in the nprE locus. The gfp gene under control of the SEM library and the spectinomycin (spec) selection marker were integrated in the amyE locus. Terminators (T) prevent read-through into adjacent genes. BsmBI restriction sites indicate the insert location of the SEMs upstream of gfp. Disrupted integration loci (amyE; nprE) are indicated with dashed lines. (B) GFP and mCherry fluorescence levels of the SEM library. The expression library of 152,000 clones was grown until early exponential phase. mCherry fluorescence levels are shown in red and GFP fluorescence levels in green. Cells were sorted into 12 non-adjacent log-spaced bins (I to XII) with increasing GFP intensities indicated by blue shades (top). (C) Microscopic analysis of the unsorted SEM library during exponential growth. Showing from left to right: phase contrast (PC), homogeneous red fluorescence (mCherry) and heterogeneous green fluorescence (GFP). Scale bar is 5 μm.

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Figure 3: GFP and mCherry fluorescence of bins I to XII. The populations of the 12 bins and the parent strain 168 were cultured and 50,000 events of each population were analyzed by FACScan. (A) The cell counts of GFP fluorescence and (B) mCherry fluorescence intensities are shown.

111x115mm (300 x 300 DPI)

Figure 4: Abundance of SEM Elements in bins I to XII. The abundance of Element A, Element B, Element C and Element D variants over the twelve bins were quantified and normalized against their total counts in the SEM library, respectively. Bin I represents the lowest GFP fluorescence and bin XII the highest GFP fluorescence. The frequencies of (A) 16 Element A variants with different %AT content, (B) 6 Element B variants, (C) 16 Element C variants with different spacer length and (D) 6 Element D variants are shown. Variant 1 represents the native veg expression module. The probabilities of appearance are color coded and shown in panel A.

177x155mm (300 x 300 DPI)

Figure 5: Heat map of all element variants distributed over the 12 bins. Hierarchical clustering of the elements using correlation as distance metric and complete linkage as agglomeration method. Bins are normalized by Z-scores. High relative abundance is white and low relative abundance is red.

84x131mm (300 x 300 DPI)

Figure 6: Measured GFP levels and predicted translation initiation. (A) The log10 of the average GFP expression level of the 96 RBS modules (sixteen C elements times six D elements = 96) including their standard deviation was plotted against the log10 of the predicted translation initiation rate (TIR) calculated by the RBS calculator V2.0 26 and resulted in a moderate positive correlation ($R^2 = 0.56$). (B) Correlating GFP expression and predicted translation initiation rate (TIR) for each of the 126 promoter sequences on the x-axis (twenty-one A elements times six B elements = 126). First 32 promoters with Elements B1 and B2 are colored blue, promoters 33-64 with Elements B3 and B4 are colored red and promoters 65-96 with Elements B5 and B6 are colored blue.

116x160mm (300 x 300 DPI)

Figure 7: Growth, mCherry and GFP over time. Strains containing sixteen selected SEMs and control strains carrying no expression unit (empty) or native expression module (veg) were analyzed during growth. (A) Biomass increase, (B) production of mCherry over biomass and (C) SEM activity reflected by GFP fluorescence over biomass are shown. Strains were grown in quadruplicate in a BioLector® microbioreactor for 20 h in LB medium and the average values are shown.

84x135mm (300 x 300 DPI)

Figure 8: Xylanase production of selected SEMs and analysis of interaction between GFP and XynA expression. Strains containing 16 expression modules and control strains carrying no expression module (empty) or the native expression module (veg) were analyzed for (A) XynA activity in the culture medium during exponential (2 hours), transition (4 hours) and stationary phase (20 hours). (B) Correlation analysis between XynA and GFP production levels with respect to mCherry expression during logarithmic growth phase (2 hours) ($R^2 = 0.20$). Error bars indicate the standard deviation of four biological replicates.

99x118mm (300 x 300 DPI)

Figure S1: GFP/mCherry ratio of library variants after overnight growth. 135 colonies containing the synthetic expression modules were picked randomly from agar plates and grown overnight. Subsequently, end point measurements of GFP and mCherry were performed in a plate reader to determine expression strength, as reflected by the GFP/mCherry ratio.

84x54mm (300 x 300 DPI)

Figure S2: Illumina HiSeq 2500 sequence statistics of twelve bins containing SEMs. The yellow bars represent the total number of reads, followed by the length filtering >163 bp (purple) and the filtering for after trimming 5' amyE and gfp sequences (green). The cyan bars represent the total number of reads that were mapped to the design library. 91.37% of the 12,000 designed SEMs were identified in the sequence data.

84x60mm (300 x 300 DPI)

Figure S3: Relative abundance of nine arbitrary SEMs over the twelve bins. The differential sequence distribution of nine arbitrary sequences over all bins is shown.

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Figure S4: Growth and mCherry production of strains expressing XynA over time. Strains containing sixteen selected SEMs, no expression module (empty) or the native expression module (veg) were analyzed during growth. (A) Biomass increase and (B) production of mCherry per unit biomass are shown. Strains were grown in quadruplicate in a BioLector® for 20 hours in LB medium and the average values are shown.

84x90mm (300 x 300 DPI)