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Supplementary Methods

1. Strains, media and reagents

The bacterial strain used for cloning and construct assembly was *E. coli* DH5α unless stated otherwise. For the purification of plasmid DNA, cells were cultured in LB media supplemented with suitable antibiotics at 37°C and DNA was purified from culture using standard kits (Qiagen, Germany). All primers were synthesized by Sigma Aldrich, Israel (Appendix A – primers table). PCR reactions were performed using Phusion polymerase (Finnzymes, Finland). Cloning procedures were designed using Clone manager professional suite (Scientific & Educational Software, State Line, US-PA). Restriction enzymes were purchased from New England BioLabs (Beverly, US-MA) unless stated otherwise. Ligation reactions were performed using T4 DNA ligase (Fermentas, Lithuania).

2. “No-Background” Assembly: general cloning scheme

We developed a “No-Background” assembly strategy, which is aimed to facilitate the serial assembly of multiple DNA sequences into a single construct. All DNA constructs were generated according to the methodology described below. “No-Background” assembly adheres to the principles described in “Idempotent Vector Design for Standard Assembly of Biobrick” (http://dspace.mit.edu/handle/1721.1/21168), also known as the BioBrick standard. In addition to preserving the main features described in the BioBrick system we added a feature which eliminates the need to screen and validate that intermediate constructs were correctly assembled throughout a multi-step assembly process.

The key feature of the method is the concatenation of a chloramphenicol resistance marker (CmR) : a constitutive promoter followed by a Chloramphenicol Acetyltransferase coding sequence, to each of the DNA sequences designated for assembly. The CmR cassette is paired to the DNA sequence using PCR overlap extension (see figure 1 in the cited article). prior to the assembly process. When the target DNA sequence (now paired with the CmR) is assembled into a vector using a standard restriction-ligation process, only clones that were properly assembled are able to form colonies on agar plates supplemented with Cm.

Since the resistance cassette is flanked by restriction sites (figure S1), it can be easily removed when preparing the vector for the next assembly cycle. In this manner, it is possible to perform multiple assembly rounds while using a single resistance marker. A general scheme of the “No-Background” Assembly strategy is described in figure S1.
Fig. S1. A modular cloning strategy for combinatorial assembly of multi-gene constructs. We developed a method of eliminating false-positive clones by a direct selection for correctly assembled constructs. Each gene of interest was joined with a chloramphenicol (Cm) resistance cassette flanked by NheI (‘N’) and PciI (‘P’) restriction sites. The sequence designated for assembly contains an upstream SpeI (‘S’) restriction site. To assemble the first target sequence into a vector we first digest the DNA using SpeI and PciI, followed by ligation and transformation. Cells are plated on a selective agar supplemented with Cm. Since the backbone vector does not include Cm resistance, only clones which were properly assembled (i.e. contain the designated insert sequence attached to the resistance marker) will be able to form colonies. To incorporate the next target sequence, we extract the assembly product from the cells and digest it with NheI and PciI, effectively discarding the resistance marker from the construct. The second target sequence, as the first one, is digested using SpeI and PciI and assembled to the vector. Importantly, the sticky ends of NheI and SpeI restriction sites are compatible and joined together to form a scar, a sequence that cannot be cleavage by either NheI or SpeI (‘x’). After the second assembly round the new construct now contains both target sequences and the Cm resistance, enabling once more a direct selection for positive constructs. This sequence of events can be repeated to assemble multi-gene operons.
2.1 Construction of chloramphenicol resistance cassette

The resistance cassette contains a constitutive promoter and a chloramphenicol acetyltransferase gene as the resistance marker. The cassette was amplified via PCR using the pSB3C5 plasmid as a template (BioPart: BBa_P1004). Restriction sites were added so the resistance cassette is flanked by NheI site at the 5’ and XhoI and PciI sites in its 3’.

2.2 Pairing resistance cassette with a target sequence

We join each of the DNA sequences designated for assembly with the Cm resistance cassette using a standard assembly PCR reaction. An insulator sequence (see section 3.2) containing an NsiI site is added upstream to the target sequence while the sequence GCTAGCGTTGATCGGGCACGTAAGAG is added downstream. The latter sequence contains a NheI (underlined) site and a homology region of 20bp to the beginning of the Cm resistance cassette (bold). The homology sequence enables overlap extension PCR between the target sequence and the cassette, effectively enabling to pair the sequence of interest with the resistance marker. The PCR reaction is conducted using a sequence specific forward primer and a generic reverse primer (Cm-R, primers table). The resulting PCR product (i.e. the target sequence concatenated to the resistance cassette) is gel purified and can be sub-cloned into a vector or digested directly with suitable restriction enzymes.
3. Ribosome Binding Site as protein expression modulator

3.1 Choosing a compact set of RBS sequences to span expression space

To find a small set of RBS sequences that span a large fraction of the expression space we utilize the forward engineered RBS series experimentally analyzed in the work of Salis et al. (2009). First, we computationally calculated the expected translation rate of each of the RBS sequences attached to various genes (https://salis.psu.edu/software/). We choose RBS sequences whose strength seems to be the least affected from the downstream sequence. From this limited set we picked 5 RBS sequences which span the largest expression space experimentally. These RBS sequences were:

#8 (RBS-A): AGGAGGTGTGGA
#1 (RBS-B): AACAAAAATGAGGAGGTACTGAG
#17 (RBS-C): AAGTTAAGGGAAG
#27 (RBS-D): TTCGCAGGGGGAAG
#20 (RBS-E): TAAGCAGGACCGGCGGCG

RBS-F was adopted from the Central Dogma BIOFAB initiative (http://biofab.jbei.org/services/studio/dac):
“Dead-RBS” (RBS-F): CACCATAACTG

3.2 Flanking “Insulator” sequences

Since the sequences flanking the RBS can affect expression levels, we decided to place insulator sequences – a constant sequence of ~20bp located upstream and downstream of each of the RBS. Such isolation sequences have been previously reported to be effective in reducing the effect of flanking sequences in the case of promoters. The upstream insulator sequence was taken to be 19 base pairs, not natively found in E. coli: TAATAGAAATAATTTTGTTTA while the downstream insulator sequence was taken to be ATGCATCATCACCATCACCA, a sequence coding for a 6His-tag which can be also be utilized for a variety of downstream applications.

3.3 RBS modulation of a target ORF

In order to clone a target coding sequence for RBS modulation we first amplified it via PCR and paired it to Cm resistance marker as described above (“No-Background” cloning, see section 2.1). Once the target gene is paired to the resistance cassette, we ligate the product into a linearized BlueScript KS+ plasmid. The target ORF is then excised from the plasmid using either NsiI and PciI (non-barcoded assembly, see section 4.1) or NsiI and XhoI (barcoded assembly, see section 4.2). The resulting fragment, containing the target sequence and the resistance marker is then assembled upon an RBS backbone vector (RBS backbone, see section 3.5) containing an RBS sequence upstream to the insertion site. The resulting construct contains the desired RBS followed by the target ORF and the resistance marker, as described in figure S2.
The desired coding sequence is paired with the resistance cassette and assembled upon a vector containing an RBS upstream to the insertion site. Barcoded and non-barcoded assemblies (as described in section 4) rely on the same logic but differ in the restriction sites due to technical reasons.

3.4 **pNiv – the backbone plasmid**

The backbone plasmid was constructed using pBluescript Ks+ as a base. We eliminated the LacZ gene and swapped the original multiple cloning site (MCS) with a new site that contains the following restriction sites: EcoRI, SpeI and PciI (figure S3). In order to minimize leaky gene expression throughout the assembly process, we placed the strong RRNB terminator (amplified by PCR using pENTER11-Gateway as a template) upstream to MCS. All DNA sequence modifications were accomplished using PCR overhang extension method followed by self-ligation to circularize the linear PCR product.
Fig. S3. pNiv backbone plasmid. RRNB terminator was placed upstream to the multiple cloning site to minimize leaky expression throughout the assembly process.

3.5 pNiv:RBS-A-YFP to pNiv:RBS-F-YFP – plasmid Set

Each of the six core RBS sequences (see section 3.1) were purchased as synthesized oligodeoxynucleotides. Each of the core RBS sequences was flanked with the up- and downstream insulation sequences (see section 3.2) and fused to a YFP reporter gene in an assembly PCR reaction (see primers table). The resulting six RBS-YFP reaction products (namely RBS-A to RBS-F), were restricted and ligated into the pNiv backbone plasmid to yield the six designated plasmids – pNiv:[RBS-A to RBS-F]-YFP (see section 2 for a detailed description of the cloning procedure). The RBS-YFP unit design is described in figure S4.
The composition of the flanking sequences has been shown to affect the expression level of a given RBS. In an effort to minimize such secondary effects, a constant spacer and tag sequences were introduced up- and downstream to the RBS sequence. A resistance marker is concatenated to each RBS-ORF unit to facilitate downstream assembly process (as described in section 2).

3.6 Expression plasmids

DNA manipulation and assembly processes were conducted on the pNiv backbone plasmids which contain no designated promoter. Once the assembly process was completed, the resulting product was sub-cloned into an expression plasmid - pSB4K5:Ptac. This plasmid was derived from pSB4K5, a BioBrick standard vector with low copy pSC101 replication origin (BioPart : BBa_I50042) and kanamycin antibiotic resistance marker (BioPart : BBa_P1003). LacIq Brick (BioPart : BBa_C0012) and a Tac promoter (BioPart : BBa_K301000) were assembled on pSB4K5 upstream to the multiple cloning site using standard assembly methods to yield pSB4K5:Ptac (figure S5).
Fig. S5. pSB4K5:Ptac - the expression plasmid. Hybrid Ptac promoter was placed on a pSB4K5 plasmid backbone, upstream to the multiple cloning site.

3.7 Experimental measurements of RBS expression modulation using flow cytometry

In order to quantify the effect of the RBS sequence on the expression level \textit{in-vivo}, we placed a reporter gene (either YFP or mCherry) upstream to each of the RBS sequence on a pSB4K5:Ptac plasmid using the cloning strategies described previously. \textit{E. coli} MG1655 cells were transformed with the pSB4K5:Ptac-[RBS-A to RBS-F]-XFP plasmids and incubated at 37°C in minimal media supplemented with 0.2 % glucose until mid exponential phase (OD$_{600}$=0.3). Fluorescence was quantified using BD LSR II Flow Cytometer. A blue laser (488 nm) and a 530±30 nm emission filter were used to measure YFP fluorescence and a yellow laser (560 nm) and a 610±20 nm emission filter were used to measure mCherry fluorescence. ~100,000 cells were recorded in each experiment (figure S6).
Fig. S6. Flow cytometry fluorescence measurements of the RBS set. YFP and mCherry reporters were RBS modulated using the RBS set and placed under the control of Ptac promoter. The six YFP constructs (RBS A-F) and the three mCherry constructs (RBS A,C,E) were transformed into E. coli cells and the fluorescence of each reporter was measured with and without IPTG induction (0.5mM). AF represents the measured fluorescence of cells when no fluorescent protein is expressed. (auto-fluorescence).
4. Combinatorial assembly of RBS modulated genes

4.1 Non barcoded RBS mixture

4.1.1 pNiv-RBS mixture preparation

Each of the six pNiv-RBS-YFP plasmids was digested separately with NsiI and PciI, hence removing YFP from the backbone vector. Digestion products were treated with Calf Intestinal Alkaline Phosphatase (CIP) and gel purified. We then prepared an equimolar mixture of the six linearized vectors, each differing only in the RBS sequence upstream to the cloning site. This vector mixture (pRBS mix) was used to perform one-tube combinatorial assembly with any target (figure S7).

Fig S7. Single tube combinatorial assembly. An equi-molar mixture of pNiv:RBS plasmids with six distinct RBS sequence is used in the assembly reaction. The target coding sequence is ligated to the plasmid mix to yield six distinct products. All containing the same coding sequence but with a different RBS located upstream to it.

4.1.2 Combinatorial Assembly of RBS mixture

For any coding sequence of interest, we first cloned the coding sequence as described in section 2. In order to combinatorially pair the coding sequence with the RBS set, we sub-cloned the coding sequence into the linearized pRBS vector mixture, resulting in a mixture of ligation products: all containing the same coding sequence but with a variety of RBS (RBS-A to RBS-F) sequences upstream (figure S7).
4.1.3 Assembling a RBSmodulated synthetic operon

The resulting library, containing a mixture of constructs, all with an identical coding sequence but with a variety of RBS sequences upstream (RBS-A to RBS-F) can be used either as a vector or as an insert for downstream assembly steps. First, by restricting the mixture using NheI and PciI, the resistance cassette is removed and the plasmid library can be used as a vector into which we assemble more RBS modulated coding sequences. Alternatively, by digesting the mixture with SpeI and PciI, it is possible to excise the coding sequence (along with the upstream RBS) and use it as an insert for further assembly rounds (figure S8).

In order to assemble a library of operons – where each variant contains the same combination of genes but with a different combination of RBS, we construct in parallel the RBS modulated mixture for each of the desired genes (as described in section 4.1). We then perform iterative assembly steps, where at each step an additional RBS modulated coding sequence is added along the operon. At every step, the product of the previous round is digested with NheI and PciI as shown in figure S8, removing the Cm resistance cassette. The reaction product is treated with CIP and is gel purified. The purified product, a linearized vector without Cm resistance, serves as a vector in the next assembly step.

To assemble an additional RBS modulated coding sequence to the operon, we digest the RBS modulated mixture of the designated insert using SpeI and PciI, resulting in a DNA fragment which contains a mixture of RBS sequences upstream to the coding sequence and the resistance cassette. This fragment is ligated into the operons library (already harboring the first RBS modulated coding sequences). Such assembly process results in a combinatorial mixture RBS modulated coding sequences, where each variant has a distinct RBS composition upstream to the coding sequences. The library is transformed into *E. coli* DH5α and plated on LB agar plates supplemented with Cm. The resistance cassette which is paired to the last incorporated gene ensures that only constructs which contain the newly added RBS modulated sequence will continue for further assembly rounds.

Plasmid DNA from the newly constructed operon library is recovered from the plate by scraping the colonies directly from the plate and extracting the plasmids encoding for the operons library. Therefore, by repeating this process for N rounds, where in each round an additional RBS modulated coding sequence is added to the combinatorial operon library, we sequentially assemble a combinatorial mixture of plasmids containing the same N coding sequences in a pre-defined order and driven by a varying combination of the six RBS.
Fig. S8. Assembling a synthetic operon of RBS modulated genes. Synthetic operons contacting several RBS modulated genes are assembled in an iterative process. First, the vector is digested and the first RBS-gene pair is ligated to it. The resulting construct contains additional restriction sites which can be used in order to excise the resistance marker, therefore recycling the vector for an additional assembly of RBS modulated gene. The process can be repeated in order to generate a synthetic operon containing multiple RBS modulated genes.
4.2 Barcoded RBS mixture – approach

Since the operons library of RBS modulated coding sequences is built in a combinatorial manner, it is required to sequence all of the RBS sequences across the entire operon in order to determine the RBS composition of a specific clone. To facilitate this process, and eliminate the need to sequence all of the RBS sequences spread across an operon, we assigned a 3 base pair barcode sequence to each RBS. These barcodes enable us to easily determine the complete RBS composition of each clone using a single sequencing reaction at the 3’ end of stacked barcodes as described in figure S9.

First we assigned numeric values to each of the DNA bases in order to compute the barcode which is assigned to each RBS sequence. These values were determined arbitrary in order to assign a numeric value to each DNA base as seen on table S1.

| Base | Numeric Value |
|------|---------------|
| A    | 0             |
| G    | 1             |
| T    | 2             |
| C    | 3             |

*Table S1.* Numeric values assigned to each of DNA base.

Next we computed the barcodes as follows: each RBS (A-F) was assigned to a 2-letter DNA code. A given 2-letter code word (b₁b₂ = AT, for example) maps to a particular number by the formula b₁ + 4 * b₂, where b₁ and b₂ are arbitrary numeric values which are assigned to the bases of the code word (see table S1). For example, assuming G=1, GG becomes 1 + 4*1 = 5. We then add a third base as a check-base. This base is computed from the previous two bases as follows: b₃ = ( b₁ + 5 * b₂) % 4, where '%' represents the modulus operator. It is simple to show that this check-base allows detection of any single base mutation or sequencing error. The table below displays the barcode values for each RBS. Note that, though we employ only 6 RBS sequences here, we can potentially encode up to 16 RBS sequences total using a 3 b.p scheme. The approach is also scalable to much higher library sizes with a logarithmically scaled increase in code length.
|       | Code [with Check-Base] | Numeric Value |
|-------|------------------------|---------------|
| RBS-A | AA[A]                  | 0             |
| RBS-B | GA[G]                  | 1             |
| RBS-C | TA[T]                  | 2             |
| RBS-D | CA[C]                  | 3             |
| RBS-E | AG[G]                  | 4             |
| RBS-F | GG[T]                  | 5             |

*Table S2.* Barcode values for each of the RBS sequences, the numeric value of the barcode is calculated by the first two letters while the third letter (in brackets) represents the check base letter.

### 4.2.1 Addition of barcodes to the pNiv-RBS set
Each of the six pNiv plasmid containing an RBS-YFP insert (RBS-A to RBS-F) served as template for a PCR reaction in which barcode bases and restriction sites were added using designated primers (primers table). XhoI restriction site was added upstream of the barcode area while SalI and PstI sites were added downstream. Each of the six pNiv-RBS-YFP-barcoded plasmids was separately digested with NsiI and XhoI, removing the YFP coding sequence. An equimolar mixture of the six resulting linearized vectors (namely, pRBS-Barcode mixture) was prepared as described in section 4.1.1.

### 4.2.2 Single tube combinatorial assembly using barcoded RBS mixture
The use of the barcoded RBS plasmid set relies on the same logic as described in section 4.1.2 except a few technical changes resulting from the different use of restriction enzymes. The target coding sequence is first cloned as described in section 2 and digested using NsiI and XhoI. The insert is ligated with the pRBS-Barcode mixture and transformed in DH5α cells. A schematic description of the process is described below in figure S9.
Fig. S9. Single tube combinatorial assembly of barcoded RBS mixture. The target gene for RBS modulation is ligated with pRBS-barcoded mixture. The resulting library contains the desired coding sequence with different RBS sequence upstream to it. The barcode located downstream to the resistance marker is later used to generate the operon barcode tag during the operon assembly process (see 4.2.3).

4.2.3 Assembling a synthetic operon of RBS modulated genes (barcoded version)
A schematic representation of the barcoded assembly process is described at figure S10 below. For simplicity the assembly process shown here contains only two coding sequences assembled into an operon, each with a specific RBS. The process can be iteratively extended by additional assembly cycles. Moreover, a combinatorial RBS mixture can be used instead of specific ones (as shown in figure S9).
4.3 Subcloning into an expression plasmid

After the assembly process has been completed, the resulting operon is sub-cloned into an expression vector containing a designated promoter. This is obtained by using the designated restriction sites flanking the final operon. Moreover, since the expression plasmid has a resistance marker differing from the Cm marker paired to the operon, while selecting with both antibiotics, only positive colonies can grown while clones transformed with either the self ligated expression plasmid or the library donor plasmid could not.

5. RGB – tricolor reporter system

5.1 Bacterial strains and growth conditions

The bacterial strain used for the cloning and construct assembly process was *E. coli DH5α*. For fluorescence measurements plasmids were transformed into *E. coli K12 MG1655*, grown in minimal media supplemented with 0.2% glucose and chloramphenicol (34 ug/ml).
5.2 **Genes**

mYFP, mCFP and mCherry were amplified by PCR from the following plasmids pRSETB-YFP, pRSETB-CFP and pRSETB-mcherry\textsuperscript{28}. PstI restriction site in the mCherry gene was eliminated by introducing a single silent mutation (see primers table).

5.3 **Assembly process**

mYFP, mCherry and CFP were first paired with resistance cassette as explained in section 2. The operon was assembled using the barcoded RBS set as described in section 4.2.2. pRBS-mYFP-barcode1 was digested with NheI & XhoI restriction enzymes in order to use it as a vector, while pRBS-mCherry-barcode2 was digested with SpeI & SalI in order to use it as an insert (figure S10). The two restriction products were ligated, resulting in new product pRBS-mYFP-RBS-mCherry-barcode2-barcode1 (Bi-color reporter operon). For the assembly of the third gene (CFP), pRBS-mYFP-RBS-mCherry-barcode2-barcode1 was digested as insert with SpeI & SalI, while pRBS-mCFP-barcode3 was digested as a vector with NheI and SalI, the resulting products were ligated to assemble the following operon in pNiv plasmid: pRBS-mCFP-mYFP-mcherry-barcode3-barcode2-barcode1. Next, the operon was digested sub-cloned into an expression plasmid as described in section 4.3. The final construct plasmid map is described below in figure S11.
**Fig. S11. Tricolor reporter operon – plasmid map.** Each of the reporter genes contain an RBS sequence (not shown) upstream to the coding sequence.

### 5.4 Measurements of RGB fluorescence library

#### 5.4.1 Automated fluorescence measurements

Cells transformed with a reporter operon were grown in a 96 well plate containing M9 + 0.2% Glucose in an automated robotic platform (Evoware II, Tecan). Every 15 minutes the plate was transferred by a robotic arm into a multi-well fluorimeter (Infinite M200-pro, Tecan). In each measurement OD was sampled at 600 nm, mCherry was sampled by excitation at 587nm and emission measurement at 620nm and YFP was sampled by excitation at 520nm and emission measurement at 555nm.

#### 5.4.2 Calculating the protein accumulation rate

Raw data of OD and fluorescence was background corrected by subtracting wells containing medium with no cells.
Because of the large required dynamic range, we could not analyze wells with weak RBS at low bacteria concentrations. We therefore choose to work at mid to late exponential phase. We analyzed cells around an OD600 value of 0.1 as measured by the plate reader after media subtraction, equivalent to OD600 of ~0.2 with standard 1 cm path length. For each measurement point, the protein accumulation rate, $A(T)$, was defined as the increase of fluorescence during a time window of one hour centered at the measurement’s time divided by the total OD measured during that time (assumed proportional to the biomass producing the fluorescent signal):

$$A(T) = \frac{F(T + \tau) - F(T - \tau)}{\int_{T-\tau}^{T+\tau} OD(t) \, dt}$$

Where $A$ is the protein accumulation rate, $F$ is the fluorescence measurement and $\tau = 30$ minutes. The result reflects the increase in fluorescence during one hour divided by the total OD (which is proportional to the biomass). Mean protein accumulation rate was calculated by averaging over 5 measurements around OD 0.1 for each sample:

$$\bar{A} = \frac{\sum_{i=1}^{5} A(t_i)}{5}$$

All analysis steps were performed using custom Haskell software.

5.4.3 Fluorescence microscopy of bacterial colonies

Fluorescence images were taken using a Nikon ECLIPSE E800 microscope equipped with a Nikon Intensilight (C-HGFI) for illumination. Chroma filter cubes set was used to image fluorescence proteins: mCherry (excitation filter 530–560 nm, emission filter 590–650, 30 ms exposure), cyan fluorescent protein (mCFP) (excitation filter 426–446 nm, emission filter 460–500 nm, 60 ms exposure) and yellow fluorescent protein (mYFP) (excitation filter 490–510 nm, emission filter 520–550 nm, 800 ms exposure). Images were captured with a camera and NIS-Elements BR3.22 software. Different channels were overlaid to give the figures shown.
6. **RBS modulation of the carotenoid biosynthesis pathway**

6.1 **Bacterial strains and growth conditions**

The bacterial strain used for the cloning and construct assembly process was *E. coli DH5α*. For carotenoids expression, transformed cells were grown in LB media supplemented with chloramphenicol (34 ug/ml) at 37°C.

6.2 **Genes for the astaxanthin biosynthetic pathway**

For the astaxanthin synthesis we used the following genes:

- Geranylgeranyl pyrophosphate synthase (crtE) from *Pantoea agglomerans* - GenBank: AAA21260.1
- Prephytoene pyrophosphate synthase (crtB) from *Pantoea agglomerans* - GenBank: AAA21264.1
- Phytoene dehydrogenase (crtI) from *Pantoea agglomerans* - GenBank: AAA21263.1
- Isopentenyl pyrophosphate (idi) from *Haematococcus pluvialis* - GenBank: AAC32208.1
- Beta-lycopene cyclase (lcy-B) from *Solanum lycopersicum* - GenBank: ABR57232.1
- Beta-carotene hydroxylase (crtZ) from *Pantoea ananatis* - Swiss-Prot: P21688.1
- Beta-carotene ketolase (crtW) from *Nostoc sphaeroides* - GenBank: BAB74888.1
- 1-deoxyxylulose-5-phosphate synthase (dxs) from *Escherichia coli* - Swiss-Prot: A7ZX72.1

Plasmids containing CrtB,CrtI,Idi,Lyc-B and CrtW kindly provided by Prof. J. Hirschberg of the Hebrew university of Jerusalem. CrtZ was synthesized by using assembly PCR, the primers for the assembly PCR were calculated using Johnson Lab Oligo maker. The restriction sites EcoRI, SpeI, NsiI, NheI, PstI and PciI were eliminated from the listed genes by introducing silent mutations.

6.3 **Assembly process**

idi,crtE,crtB,crtI,lcy-B,crtW,crtZ and dxs were amplified by PCR and then paired with resistance cassette as explained in section 2.2. RBS was added to each gene as described in section 4.1.2. We assembled the library in an iterative process (as described in section 4.1.3) according to the order of the genes along the biosynthetic pathway. The complete operon was subcloned into an expression plasmid as described in section 4.3.
Fig.S12. Carotenoids biosynthesis operon – plasmid map. Each of the carotenoid biosynthesis genes contain an RBS sequence (not shown) upstream to the coding sequence.

6.4 Carotenoid analysis

6.4.1 Carotenoid extraction

E. coli cells carrying a plasmid with the biosynthetic genes of the carotenoid pathway were grown in suspension cultures in shake flasks containing 100 ml of LB medium. Cultivation was carried out in 37°C. 20 ml samples were withdrawn from the culture after 48 hours and cells were harvested by centrifugation. Cell pellet was washed with cold water and carotenoids were extracted by vigorous shaking with acetone (20 ml). Insoluble components of the extract were removed by centrifugation (15,000 g) and supernatant was transferred into a glass round-bottom flask and was evaporated using a rotary evaporator. Dried extract was re-solvated in 1.5 ml acetone and 50-ul samples were taken for HPLC analysis. To measure dry cell weight, additional 20 ml from the cell culture were taken from each sample. Cells were centrifuged, and cell pellet was transferred into pre-weighted tubes. To evaporate residual water, cell pellet was lyophilized for 24 hours before weighting.
6.4.2 Carotenoid analysis by HPLC

HPLC analysis was performed on Jasco platform with high pressure mixing installed with a Borwin software, P4987 pumps and a MD-915 photodiode array detector. Samples were analyzed by injecting 50 ul on a YMC pack ODS-A column (250x4.6mm, 5 um, 12nm). Solvent A: 75% aqueous methanol, Solvent B: ethylacetate. Solvent flow rate of 0.6 ml/min was used with the following gradient: 15-85% of B (0-24 min), 85% (24-30 min), 85-15% (30-34 min), 15% (34-745 40 min). The spectra of the eluted carotenoids were recorded online with the photodiode array detector (300- 900 nm). Carotenoid compounds were identified by co-chromatography with authentic standard compounds and by analysis of their UV-Vis spectra. For the quantification of the carotenoid compounds the integrated peak areas were compared to those of authentic standards. The concentration of the standard solutions was determined spectrophotometrically (Jasco V-570 instrument)\(^{15}\). For additional identification, the peaks isolated by HPLC were collected and directly injected into a mass-spectrometer (Micromass Quattro Ultima tandem quadrupole instrument equipped with a Z-spray ESI interface and Waters Masslynx v4.1 software). The corresponding masses were analyzed from obtained full-scan (ESI(+), m/z 100-1000) mass spectra\(^{16}\).

6.5 Wide field images of bacterial colonies

Images of colonies appearing in Figure 3-B in main text were taken using a binocular microscope (WILD M8; Heerbrugg, Switzerland) with Schott Ace Fiber Optic Light Source 150W Microscope Illuminator. Images were captured using a Nikon Digital Sight Camera System. Stitching of adjacent fields was done using AutoStitch software (http://www.cs.bath.ac.uk/brown/autostitch/autostitch.html).
Supplementary Results

1. Two step metabolic pathway described by a reversible Michaelis-Menten kinetics

We consider a simple two-enzyme pathway leading from a substrate to an intermediate (via reaction v1) and further to a product (via reaction v2). As shown in Figure S13, the pathway is connected to the rest of the metabolic network through the compounds S*, Biomass, and P*. In the model, the concentrations of these metabolites are assumed to be fixed. The substrate is produced from the precursor S* (“supply”) and mostly converted into biomass (“growth”). The product is further converted into P* (“usage”). All reactions have the uni-uni form A \rightarrow B and are described by reversible Michaelis-Menten rate laws:

\[
v(a, b) = E \frac{k^+ \frac{a}{K_A^M} - k^- \frac{b}{K_B^M}}{1 + \frac{a}{K_A^M} + \frac{b}{K_B^M}}
\]

with substrate concentration a, product concentration b, and enzyme level E. Since we are interested in the generic behavior of pathways, all quantities are expressed in arbitrary units. For simplicity, all Michaelis constants \(K^M\) as well as the forward and reverse catalytic constants \(k^\pm\) are set to 1. The enzyme levels of the supply, growth, and usage reaction are assumed to be constant and are set to values of E=1. With these rate laws, the model equations read:

\[
\begin{align*}
\dot{a} &= v_{\text{supply}} - v_{\text{growth}} - v_1 \\
\dot{b} &= v_1 - v_2 \\
\dot{c} &= v_2 - v_{\text{usage}}
\end{align*}
\]

By setting the left-hand-side equations to zero, we obtain the steady state concentrations and fluxes, which depend on the enzyme levels \(E_1\) and \(E_2\). In practice, steady states for many combinations of enzyme levels were systematically obtained by varying the enzyme levels between 0.01 and 100 integrating the systems equations for each of these choices. The results are shown in Figure S14 and lead to the feasible region shown Figure 1 (main article) and Figure S13, right.
**Fig. S13: Enzyme levels in a two-enzymes pathway.** Left: Structure of the pathway. Reactions are described by reversible Michaelis-Menten kinetics with parameter values given in the text. Concentrations (circle colors) and fluxes (arrow sizes) represent a stationary state with enzyme levels $E_1 = E_2 = 1$. Right: Feasible region in the space of enzyme levels $E_1$ (x-axis) and $E_2$ (y-axis), shown on logarithmic scale. Many combinations of $E_1$ and $E_2$ are problematic because they imply a low pathway flux (shaded in magenta, threshold values 0.06 and 0.07), a high sum of enzyme levels (shaded in blue, thresholds 15 and 20), or a high intermediate concentration (shaded yellow, thresholds 0.32 and 0.33). The remaining white region contains feasible enzyme levels, which satisfy all three criteria.
**Fig.S14: Evaluating the levels of enzymes E1 and E2.** Top row: metabolic flux as a function of E1 (x-axis) and E2 (y-axis). Both enzyme levels were varied between 0.1 to 100 (arbitrary units, axis in logarithmic scale). The graphics show numerical results (left) and smoothed contour lines (right). Center row: the same, for the sum of enzyme levels. Bottom row: the same, for the concentration of the intermediate compound V.
2. Translational Coupling

The translation of sequential genes within a single operon was previously shown to be dependent on upstream genes, a phenomenon termed translational coupling.\textsuperscript{18,30} Specifically, the expression level of a gene is modulated by the expression level of the gene preceding it. Translational coupling was observed for various operons in \textit{E. coli} as well as other prokaryotes. While translational coupling has been known for many years, it is only crudely quantified and its underlying mechanism is under debate.\textsuperscript{31,32}

The RGB grid shown in figure 2 (main text) demonstrates translational coupling. YFP’s YFP accumulation rate depends only on the strength of the RBS controlling it, while mCherry’s accumulation rate depends both on the RBS controlling it and on YFP’s production. To further analyze this effect we utilized clones where YFP was controlled by one of the six RBS (A-F) while mCherry was controlled by either a weak RBS (E), a moderate strength RBS (C), or a strong RBS (A). We then measured the accumulation rate of YFP and mCherry for each library variant, and plotted them against each other. The grid we got (figure S1) clearly demonstrates translational coupling. Clones that share the same RBS for mCherry show different mCherry accumulation rates depending on the upstream YFP activity. We have ruled out cross fluorescence as the cause for such an effect: clones containing a single fluorescence protein (either YFP or mCherry) do not give a signal at the reciprocal fluorescence channel even for high levels of expression. The maximal translation enhancement of mCherry by YFP expression level in our system was found to be ~6 fold. The dependency between YFP and mCherry levels shows a linear dependence in log space (Fig. S1). Linear regression gives a slope of ~ \( \frac{1}{3} \) (95% confidence intervals 0.26-0.36).
**Fig. S15. Translational coupling.** Dependence of the second gene in the operon (mCherry) on first gene (YFP). The RBS sequence controlling mCherry is denoted using colors (blue for RBS A, red for RBS C, and green for RBS E), and RBS sequences controlling YFP correspond to shapes (asterisk for RBS A, circle for RBS B, triangle for RBS C, square for RBS D, cross for RBS E, and stars for RBS F). The effect of translational coupling is evident, where the protein accumulation rate of YFP modulates the accumulation rate of mCherry. The dependency between YFP and mCherry levels follows a linear trend in log space with a slope of $\sim \frac{1}{3}$. 

$rbsA$-mCherry$=0.29X+9.41$ R$^2=0.96$

$rbsC$-mCherry$=0.31X+8.25$ R$^2=0.96$

$rbsE$-mCherry$=0.31X+5.52$ R$^2=0.88$
3. RBS modulation of the carotenoid biosynthesis pathway – fitness

To clarify the possible fitness effects we conducted the following experiments: several clones from our carotenoid pathway library (each containing a distinct RBS composition) were grown in a 96 well plate containing LB media in an automated robotic platform. Every 30 minutes the plate was transferred by a robotic arm into a multi-well spectrophotometer and cell density was measured (OD at 600nm). Measurements points during the logarithmic growth phase (0.015 – 0.15 OD) were used to calculate the growth rate. For biomass yield measurements, cells were grown in a shaking incubator (220rpm) at 37 degrees for 48 hours. OD (600nm) was measured using a 1cm cuvette.

| Astaxanthin [mg/gCDW] | RBS composition  | Growth rate [mins] | Yield [OD] |
|------------------------|------------------|--------------------|------------|
| 2640                   | {D,D,B,F,A,B,E}  | 45 ± 3             | 3.5 ± 0.1  |
| 2450                   | {E,B,F,D,A,A,A}  | 50 ± 1             | 3.4 ± 0.5  |
| 640                    | {E,E,E,A,A,A}    | 43 ± 4             | 2.3 ± 0.1  |
| 610                    | {D,D,F,E,D,B,A}  | 48 ± 3             | 3.1 ± 0.1  |
| 410                    | {F,E,E,B,A,B,F}  | 49 ± 2             | 3.0 ± 0.3  |
| 160                    | {E,E,A,C,B,D,F}  | 56 ± 2             | 2.5 ± 0.2  |
| 140                    | {E,E,E,E,E,E}    | 43 ± 2             | 3.1 ± 0.2  |
| 90                     | {E,D,A,C,F,C,D}  | 51 ± 3             | 1.9 ± 0.6  |

*Table S3.* Astaxanthin productivity and RBS composition do not correlate with growth rate or biomass yield. RBS composition can be inferred according to gene order described on figure 5 in the main text.
4. RBS modulation of the carotenoid biosynthesis pathway – “predesigned” constructs

**Fig.S16: “pre-designed” constructs.** We constructed “pre-designed” operons, composed of either strong or weak RBS sequences. Circle area indicates the production yield of major carotenoid intermediates (>10% of total carotenoids) according to the metabolic pathway on the right. In several cases we find that some RBS combinations (those which contain strong RBS sequences) lead to non-viable clones. While we were able to assemble these constructs on a promoter-less assembly vector, we did not succeed in sub-cloning them into our expression vector. Carotenoid profile and astaxanthin productivity is compared to a variant isolated from the combinatorial RBS library (blue).
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