A dual-inducible control system for multistep biosynthetic pathways

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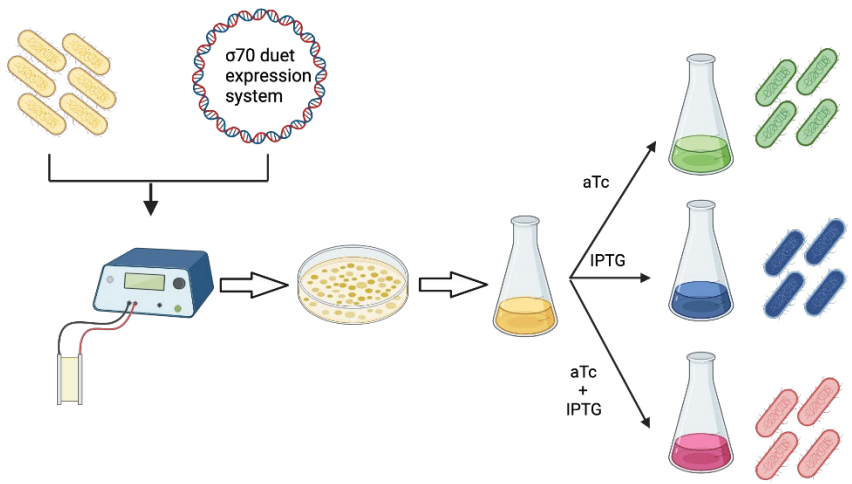
Keywords: inducible promoters, dual expression, reporter system, lycopene, β-carotene, luciferase, *Escherichia coli*, *Pseudomonas putida*, *Vibrio natriegens*

11 **GRAPHICAL ABSTRACT**



12

ABSTRACT

Background

The successful production of industrially relevant natural products hinges on two key factors: the cultivation of robust microbial chassis capable of synthesizing the desired compounds, and the availability of reliable genetic tools for expressing target genes. The development of versatile and portable genetic tools offers a streamlined pathway to efficiently produce a variety of compounds in well-established chassis organisms. The σ^{70} *lac* and *tet* expression systems – adaptations of the widely used *lac* and *tet* regulatory systems developed in our laboratory – have shown effective regulation and robust expression of recombinant proteins in various Gram-negative bacteria. Understanding the strengths and limitations of these regulatory systems in controlling recombinant protein production is essential for progress in this area.

Results

To assess their capacity for combinatorial control, both the σ^{70} *lac* and *tet* expression systems were combined into a single plasmid and assessed for their performance in producing fluorescent reporters as well as the terpenoids lycopene and β-carotene. We thoroughly characterized the induction range, potential for synergistic effects, and metabolic costs of our dual σ^{70} *lac* and *tet* expression system in the well-established microorganisms *Escherichia coli*, *Pseudomonas putida*, and *Vibrio natriegens* using combinations of fluorescent reporters. The dynamic range and basal transcriptional control of the σ^{70} expression systems were further improved through the incorporation of translational control mechanisms via toehold switches. This improvement was assessed using the highly sensitive luciferase reporter system. The improvement in control afforded by the integration of the toehold switches enabled the accumulation of a biosynthetic intermediate (lycopene) in the β-carotene synthesis pathway.

Conclusion

This study presents the development and remaining challenges of a set of versatile genetic tools that are portable across well-established gammaproteobacterial chassis and capable of controlling the expression of multigene biosynthetic pathways. The enhanced σ^{70} expression systems, combined with toehold switches, facilitate the biosynthesis and study of enzymes, recombinant proteins, and natural products, thus providing a valuable resource for producing a variety of compounds in microbial cell factories.

41 **BACKGROUND**

42 Modern sequencing techniques have unveiled a myriad of unknown compounds and proteins
43 encoded by microorganisms with potential industrial applications ranging from biotherapeutics to
44 sustainable manufacturing [1, 2]. However, these microorganisms are not always easy to cultivate, and
45 manipulating their genes poses a central challenge in unlocking their full potential [3]. *Escherichia coli* is
46 typically the primary choice for expressing proteins from other organisms due to its robust genetic toolkit
47 and well-established cultivation parameters [4]. In some cases, *E. coli* may struggle to produce toxic genes,
48 membrane proteins, or other complex proteins necessary for the synthesis of natural products [5, 6]. To
49 overcome these limitations of *E. coli*, alternative hosts like *Pseudomonas putida* and *Vibrio natriegens*
50 have become appealing chassis for producing recombinant proteins. They offer standard laboratory
51 techniques akin to *E. coli*, along with robust genetic and metabolic backgrounds, and rapid growth
52 characteristics [7, 8]. Importantly, these microorganisms have successfully produced natural products
53 such as non-ribosomal peptides, polyketides, terpenoids, fatty acids, and polymers [9-13], and can be
54 genetically engineered with a diverse set of genetic tools [14, 15].

55 The *lac* and *tet* inducible promoters have been workhorses of molecular biology for decades [16-
56 19]. Many studies have been completed to optimize their performance [20, 21] and expand their
57 functionality beyond *E. coli* [14, 22]. Our recent work contributed to this body of research by optimizing
58 the inducible *tet* and *lac* promoters for σ^{70} recognition, showing precise regulation and robust gene
59 overexpression in *E. coli*, *P. putida* and *V. natriegens* [23]. However, we have only assessed our genetic
60 tools for their efficacy in producing the reporter protein *mCardinal* and the cocaine esterase *CocE* [23].
61 Multiple biological processes necessitate the coordinated activity of numerous genes, such as those
62 involved in synthesizing natural products encoded by biosynthetic gene clusters (BGCs) [24, 25]. To
63 facilitate multi-gene expression, the pET system was modified to concurrently express various genes
64 within the same *E. coli* BL21 strain using the pETDuet system [26]. However, in pETDuet, both multiple

65 cloning sites are induced by IPTG, preventing independent or sequential control [27]. To overcome this
66 limitation, a dual inducible plasmid was developed to independently control gene expression with the *lac*
67 and P_{BAD} promoters [28]. In addition to the single-plasmid dual-expression system, the *E. coli* "marionette"
68 strain was developed with twelve inducible systems [25]. The "marionette" strain contains the
69 transcriptional regulators integrated in the chromosome, including *lacI*, *tetR* and *araC*, and the target gene
70 is deployed in a replicative plasmid under the control of the respective inducible promoter [25]. Further,
71 the synergistic effect of combined promoters to produce a single target recombinant protein in *E. coli* has
72 demonstrated differing levels of success thus far; while positive synergy has been observed in the
73 production of specific recombinant proteins, the opposite trend has also been reported [29]. A dual-
74 inducible duet expression system was also engineered in *P. putida* using a multicopy plasmid and utilizing
75 the original configuration of the *lac*, *tet* and P_{BAD} promoters, resulting in the successful production of
76 fluorescent proteins and an inducible CRISPR interference system [30, 31]. Despite these significant
77 achievements, the native promoters utilized in these studies lack the strength and control of the
78 optimized σ^{70} *lac* and *tet* promoter, which we demonstrated enable strong production of recombinant
79 proteins upon induction while maintaining low basal levels in the uninduced state in *E. coli*, *P. putida* and
80 *V. natriegens* [23]. We believe our tools could be useful in improving control and production titer in
81 industrial biotechnology applications, however their practical utility, strengths, and limitations for such
82 applications has not yet been measured.

83 In our prior work, adapting the consensus sequences recognized by the σ^{70} factor to the *lac* and
84 *tet* promoters significantly enhanced their strength, surpassing the efficiency of expression from the pET
85 system in *E. coli* [23]. Nevertheless, the uninduced state of the optimal σ^{70}V2LacI expression system was
86 scarcely repressed by its transcriptional regulator *LacI*, thereby negating the advantages of having an
87 inducible system [23]. One strategy to enhance the fold-change and minimize the leaky expression of the
88 target gene, thereby reducing unnecessary consumption of cellular resources required for cell growth,

89 involves the adaptation of toehold switch riboregulators [32]. In the toehold-mediated strand
90 displacement (TMSD) strategy, the toehold sequesters the ribosome binding site (RBS) of an mRNA
91 molecule through the formation of a stem loop, and translation is only activated in the presence of the
92 trigger RNA complementary to the toehold sequence that linearizes the stem loop [33]. Toehold switches
93 have been shown to significantly enhance the mean dynamic range of reporter expression by over 400-
94 fold [34]. Thus, incorporating toehold switches into strong inducible promoters, like the σ^{70}V2LacI, could
95 effectively reduce leakage in the uninduced state, improving the usefulness of this promoter in controlling
96 recombinant protein expression.

97 In this study, we showed that the strong inducible promoters σ^{70}V2Tc and σ^{70}V2/3Lac, recognized
98 by the σ^{70}, can be adapted into a dual-inducible duet expression system, facilitating the overexpression
99 of multiple genes in well-studied microorganisms such as *E. coli*, *P. putida*, and *V. natriegens*. The efficacy
100 of this duet expression system was confirmed through the expression of the reporter systems *mCardinal*
101 and *sfGFP*, as well as the production of terpenoids lycopene and β-carotene. Furthermore, we enhanced
102 the dynamic range and minimized the leakiness of these expression systems by incorporating toehold
103 switches, a refinement validated by the sensitive multigene reporter *lux* operon (*luxCDABE*). Our toehold-
104 controlled dual inducible system permitted partial accumulation of lycopene as a pathway intermediate
105 in the biosynthesis of β-carotene, which was fully converted to β-carotene with the second inducer,
106 providing proof-of-concept that such control is possible. In summary, our findings illustrate that the σ^{70}
107 duet *lac/tet* expression system offers a versatile gene tool for activating multiple genes with various
108 inducers across different bacterial chassis. Additionally, the incorporation of a toehold switch effectively
109 reduces the expression of target genes in the uninduced state, a crucial feature for sensitive or toxic
110 applications.

111 **METHODS**

112 **Bacterial strains and culture conditions**

113 *E. coli* DH10B, *P. putida* JE90 [35] and *V. natriegens* [8] were cultured in LB media supplemented
114 with Kanamycin when required (50 µg/mL for *E. coli* and *P. putida*, and 200 µg/mL for *V. natriegens*). *E.*
115 *coli* and *P. putida* cells were transformed by electroporation, and *V. natriegens* by chemical
116 transformation with the plasmids listed in Table 1. *E. coli* was cultivated at 37ºC and *P. putida* and *V.*
117 *natriegens* at 30ºC at 220 rpm.

118 **Plasmid construction**

119 Phusion® High Fidelity Polymerase (Thermo Scientific) and primers synthesized by Integrated DNA
120 Technologies (IDT) were used in all PCR amplifications for plasmid construction. Plasmids were
121 constructed using Gibson Assembly EX Cloning Kit or T4 DNA ligase (Thermo Scientific) according to
122 manufacturer's instructions. Plasmids were transformed into ElectroMAX DH10B Cells (Thermo Scientific)
123 according to manufacturer's instructions. Transformants were selected on LB (Miller) agar plates
124 containing 50 mg/L kanamycin sulfate for selection and incubated at 37°C. Plasmids were constructed
125 using a combination of the ligation of phosphorylated oligonucleotides, and Gibson Assembly. Sequences
126 of all plasmids were confirmed using Sanger sequencing performed by Quintarabio, and/or whole plasmid
127 sequencing by Plasmidsaurus. The details of the molecular cloning steps are available in the
128 supplementary information. The complete list of plasmids used and generated in this study is presented
129 in Table 1. Primers and DNA sequences used in this study are presented in the supplementary information.

130 **Measurement of the reporter systems in the plate reader**

131 Growth curves and fluorescence measurements were performed in 96-well clear bottom
132 microplates (Corning Incorporated, NY) using the BioTek Synergy H1 Hybrid Multi-Mode Reader following

133 the experimental approach described in [36]. Briefly, overnight cultures of the recombinant strains were
134 diluted into fresh LB medium supplemented with kanamycin to reach OD_{600} 0.05. *E. coli* experiments were
135 performed at 37ºC, and *P. putida* and *V. natriegens* at 30ºC, shaking at 355 cycles per minute (cpm). Cell
136 density (OD_{600}) and fluorescence (*sfGFP* excitation 485nm, emission 510nm; *mCardinal* excitation 604nm,
137 emission 659nm) were monitored every hour for 16 hours. *E. coli* cultures were induced at 3 hours with
138 0.2 mM IPTG and/or 0.1 µg/mL aTc, *P. putida* at 3 hours with 5 mM IPTG and/or 1 µg/mL aTc, and *V.*
139 *natriegens* at 2 hours with 0.2 mM IPTG and/or 0.1 µg/mL aTc when required.

140 **Lycopene and β-carotene determination**

141 Lycopene and β-carotene were measured by UHPLC following the procedure described in [37]
142 using a Shimadzu Nexera UHPLC. Lycopene and β-carotene were extracted with acetone as described in
143 [38] with the following modifications. 5% of overnight cultures of the recombinant *E. coli*, *P. putida* and
144 *V. natriegens* strains containing either the LYC operon or the *crtEBIY* genes were inoculated in 50 mL
145 culture tubes in 5 mL of fresh LB media supplemented with kanamycin and grown at 220 rpm and 37ºC
146 for *E. coli* and 30ºC for *P. putida* and *V. natriegens*. The cultures were induced at OD_{600} 0.7 with aTc, IPTG,
147 or both accordingly. After the induction the cultures were grown for 4 hours, 2 mL samples were collected,
148 centrifuged at 14,000 rpm, the supernatant was discarded, and the cell pellets were stored at -80ºC. The
149 cell pellets were resuspended with 200 µL acetone and incubated at 55ºC for 15 min in the dark followed
150 by centrifugation at 14,000 rpm, then the supernatant was filtered through a 0.22 µm pore-size nylon
151 membrane for UHPLC analysis.

152 **Bioluminescence assay**

153 *E. coli* or *P. putida* overnight cultures supplemented with kanamycin (LB, 37°C with shaking, 220 rpm)
154 were diluted 1/200 in fresh LB in 96-well black microplates (Corning Incorporated, NY) shaking at 355
155 cycles per minute (cpm) and induced after three hours of growth with 0.2/ 5 mM IPTG and 0.1/1.0 µg/mL

156 aTc accordingly. Bioluminescence (gain: 100) and cell density (OD_{600}) were monitored every 20 minutes

157 with the BioTek Synergy H1 Hybrid Multi-Mode Reader instrument for 20 hours.

158 **Table 1.** List of plasmids evaluated in this study.

Name	Expression system 1	ORF1	Expression system 2	ORF2	Source
pσ^{70} V2TcR-mCardinal	V2TcR	*mCardinal*			[23]
pσ^{70} V2lacI-mCardinal	V2lacI	*mCardinal*			[23]
pσ^{70} V3LacI-mCardinal	V3LacI	*mCardinal*			[23]
pσ^{70} V2TcR-sfGFP	V2TcR	*sfGFP*			[23]
pσ^{70} V2lacI-sfGFP	V2LacI	*sfGFP*			[23]
pσ^{70} V3LacI-sfGFP	V3LacI	*sfGFP*			[23]
pJH0204F					This study
pσ^{70} V2TcR-mCardinal/V2LacI-mCardinal	V2TcR	*mCardinal*	V2LacI	*mCardinal*	This study
pσ^{70} V2TcR-mCardinal/V3LacI-mCardinal	V2TcR	*mCardinal*	V3LacI	*mCardinal*	This study
pσ^{70} V2TcR- sfGFP/V2LacI-mCardinal	V2TcR	*mCardinal*	V2LacI	*sfGFP*	This study
pσ^{70} V2TcR- sfGFP/V3LacI-mCardinal	V2TcR	*mCardinal*	V3LacI	*sfGFP*	This study
pUC19-crtEBI	-	*crtEBI*			This study
pσ^{70} V2TcR-crtEBI	V2TcR	*crtEBI*			This study
pσ^{70} V2TcR-crtEBIY	V2TcR	*crtEBIY*			This study
pσ^{70} V2TcR-crtEBI/V2LacI-crtY	V2TcR	*crtEBI*	V2LacI	*crtY*	This study
pσ^{70} V2TcR-crtEBI/V3LacI-crtY	V2TcR	*crtEBI*	V3LacI	*crtY*	This study
pσ^{70} V2TcR-THS-mCardinal	V2TcR	mCardinal			This study
pσ^{70} V3-2LacI-THS-mCardinal	V3-2LacI	mCardinal			This study
pσ^{70} V2TcR-THS-luxCDABE	V2TcR	*luxCDABE*			This study

pσ^{70} V3-2LacI-THS-luxCDABE	V3-2LacI	*luxCDABE*			This study
pσ^{70} V2TcR -luxCDABE	V2TcR	*luxCDABE*			This study
pσ^{70} V2LacI-luxCDABE	V2LacI	*luxCDABE*			This study
pSC101-Kn					This study
pSC101Kn σ^{70} V2TcR -luxCDABE	V2TcR	*luxCDABE*			This study
pSC101Kn σ^{70} V2LacI-luxCDABE	V2LacI	*luxCDABE*			This study
pSC101Kn σ^{70} V2TcR-THS-luxCDABE	V2TcR	*luxCDABE*			This study
pSC101Kn σ^{70} V3-2LacI-THS-luxCDABE	V3-2LacI	*luxCDABE*			This study
pSC101Kn σ^{70} V2TcR-THS-crtEBI	V2TcR	*crtEBI*			This study
pSC101Kn σ^{70} V2TcR-THS-crtEBIY	V2TcR	*crtEBIY*			This study
pSC101Kn σ^{70} V3-2LacI-THS-crtEBI	V3-2LacI	*crtEBI*			This study
pSC101Kn σ^{70} V3-2LacI-THS-crtEBIY	V3-2LacI	*crtEBIY*			This study
pσ^{70} V2TcR-THS-crtEBI	V2TcR	*crtEBI*			This study
pσ^{70} V2TcR-THS-crtEBIY	V2TcR	*crtEBIY*			This study
pσ^{70} V3-2LacI-THS-crtEBI	V3-2LacI	*crtEBI*			This study
pσ^{70} V3-2LacI-THS-crtEBIY	V3-2LacI	*crtEBIY*			This study
pSC101Kn σ^{70} V2TcR-THS-crtY V3-2LacI-THS-crtEBI	V2TcR	*crtY*	V3-2LacI	*crtEBI*	This study

162 **RESULTS AND DISCUSSION**

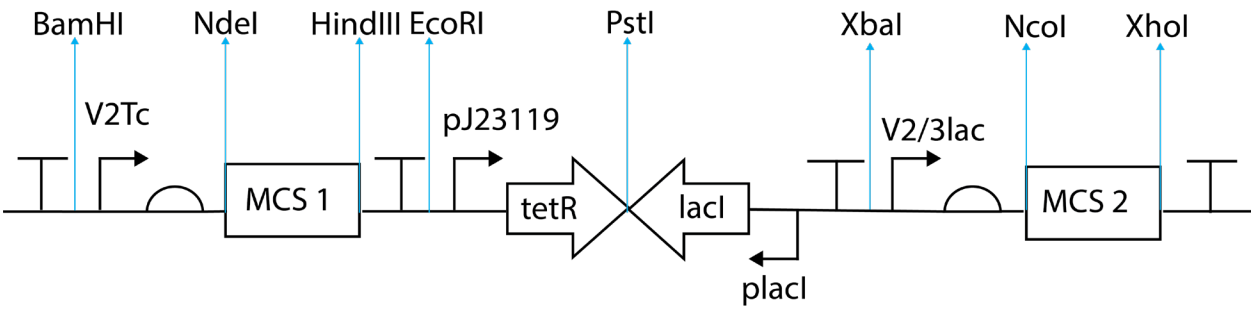
163 **Development of the dual expression system**

164 Duet vectors are available for *E. coli* and *Pseudomonas fluorescens*, but they are solely inducible
165 by either IPTG or arabinose [26, 39], whereas dual-inducible duet expression vectors have been developed
166 specifically for *E. coli* [28], *Corynebacterium glutamicum* [40] and *P. putida.* Our previous results indicated
167 that the σ^{70}V2TcR and σ^{70}V3LacI expression systems exhibited tight regulation and robust induction in *E.*
168 *coli* in the presence of anhydrotetracycline (aTc) and IPTG, respectively, while in *P. putida* and *V.*
169 *natriegens*, the σ^{70}V2TcR and σ^{70}V2LacI promoters exhibited enhanced performance as inducible
170 promoters when compared to the original *lac* and *tet* promoters [23]. These newly engineered promoters
171 not only yielded higher amounts of recombinant proteins upon induction but also maintained lower
172 expression levels in the absence of the inducers, thereby providing a more controlled and efficient
173 production system [23]. We developed the dual expression system using the pJH0204 vector as a
174 backbone, which contains the pColE1 origin or replication that replicates in *E. coli* and *V. natriegens*, and
175 the *attB* sites specific for the bxb1 recombinase, to integrate it into the chromosome of *P. putida* [41].
176 Each transcriptional unit of the dual expression system was insulated by a terminator [42]. The
177 transcriptional regulators *tetR* and *lacI* with their corresponding constitutive promoters pJ23119 and placI
178 were positioned in-between the inducible promoters σ^{70}V2Tc and σ^{70}V2Lac or σ^{70}V3Lac (σ^{70}V2/3Lac) and
179 in opposite direction to block any undesired transcription triggered by the constitutive promoters.
180 Additionally, two different multiple cloning sites were placed after the inducible promoters σ^{70}V2Tc and
181 σ^{70}V2/3Lac to facilitate the incorporation of target genes (Fig. 1).

182

183

184 The dual expression system was assessed in *E. coli*, *P. putida*, and *V. natriegens* to evaluate: i)
185 independent control of two different fluorescent proteins, ii) potential synergy (expression in excess of
186 additive transcription) of both promoters when expressing the same protein, and iii) control of the
187 biosynthetic gene cluster (BGC) responsible for the biosynthesis of lycopene and β-carotene. Expression
188 of various recombinant proteins was achieved by placing *sfGFP* under the control of σ^{70}V2TcR and
189 *mCardinal* under the control of σ^{70}V2LacI and V3LacI (Fig. 2C). Synergy was gauged using the reporter
190 system *mCardinal*, which was incorporated into both inducible promoters of the dual expression system
191 (Fig. 3B). Finally, production of the terpenoids lycopene and β-carotene by the dual expression system
192 was achieved by the assembly of the *crtEBIY* genes from *Pantoea ananatis* [11], which were synthesized
193 with the codon usage for *E. coli.* The *crtEBI* (LYC operon) was assembled in the pUC19 vector via Gibson
194 assembly, adapting the ribosome binding site (RBS) of J23119 [43] to *crtB* and the RBS pJL1 [44] to *crtI,*
195 then the synthetic construct was transferred to the σ^{70}V2TcR expression system to evaluate lycopene
196 production (Fig. 4A). β-carotene production was evaluated with the incorporation of the *crtY* gene with
197 the J23119 RBS to σ^{70}V2TcR-*crtEBI* yielding σ^{70}V2TcR-*crtEBIY* (Fig. 4B). The dual control of the *crtEBI*
198 operon together with the *crtY* gene to produce β-carotene was achieved by adapting the *crtEBI* to the
199 σ^{70}V2TcR expression system and the *crtY* gene to σ^{70}V2/3LacI expression system present in the dual vector
200 (Fig. 4E).



201

202 **Figure 1.** Design of the dual expression systems σ^{70}V2TcR-V2LacI and σ^{70}V2TcR-V3LacI

203

204 **Independent and concurrent control of two fluorescent proteins with the dual expression system**

205 We have previously demonstrated the strength and portability of the σ^{70}V2TcR, σ^{70}V2LacI and
206 σ^{70}V3LacI promoters separately in *E. coli, P. putida*, and *V. natriegens* [23]. To test the independent and
207 concurrent regulation of two proteins simultaneously from these promoters, we introduced two different
208 reporter proteins into the dual-inducible σ^{70}V2TcR/V2LacI and σ^{70}V2TcR/V3LacI expression systems (Fig.
209 2C) and assessed their production in *E. coli*, *P. putida*, and *V. natriegens*. Specifically, the transcriptional
210 unit V2TcR controlled the expression of *sfGFP*, while the V2LacI and V3LacI unit regulated *mCardinal*
211 production (Fig. 2C). This configuration was chosen because the σ^{70}V2TcR expression system exhibited
212 similar performance across the three bacterial species under study [23]. However, the σ^{70}V2LacI system
213 showed strong recombinant protein production in *E. coli* even in the uninduced state, undermining the
214 benefits of an inducible system [23]. As a result, the σ^{70}V3LacI system was selected for *E. coli*, while the
215 σ^{70}V2LacI system was further evaluated in *P. putida* and *V. natriegens*. Due to the endogenous
216 autofluorescence in the yellow-green spectrum of these bacteria [23], which could interfere with *sfGFP*
217 measurement, the σ^{70}V2TcR promoter was selected to regulate *sfGFP* expression. This promoter provides
218 tighter control in the OFF state, allowing for a more accurate and sensitive estimation of reporter system
219 production. On the other hand, the σ^{70}VLacI systems, which tend to leak [23], control the expression of
220 the far-red fluorescent protein *mCardinal*. Importantly, at this wavelength (excitation 604 nm, emission
221 659 nm), the cells do not produce endogenous fluorescence [45].

222 To create a direct comparison, the induction of the individual expression systems σ^{70}V2TcR-*sfGFP*
223 was measured in *E. coli* (Fig. 2D), *P. putida* (Fig. 2F) and *V. natriegens* (Fig. 2H), while the σ^{70}V3LacI-
224 *mCardinal* was evaluated in *E. coli* (Fig. 2E) and the σ^{70}V2LacI-*mCardinal* in *P. putida* (Fig. 2G) and *V.*
225 *natriegens* (Fig. 2I). The dual-inducible σ^{70}V2TcR/V2LacI and σ^{70}V2TcR/V3LacI expression systems were
226 also measured in the same species (Fig. 2J-O). Under the control of σ^{70}V2TcR, *sfGFP* production was higher
227 in the dual system by 1.2-fold in *E. coli* and *P. putida*, and 1.7-fold in *V. natriegens* at 16 hours of induction

228 (Fig. 2J, L, N). However, an opposite trend was observed in the dual expression system for production of
229 *mCardinal* under the control of σ^{70}V2/3LacI, which was significantly reduced, resulting in 6.2-fold, 2.4-fold,
230 and 2.0-fold less red emission in *E. coli*, *P. putida*, and *V. natriegens*, respectively (Fig. 2K, M, O).

231 Interestingly, when the cultures containing the dual expression system were co-induced with aTc
232 and IPTG, both green and red fluorescence reached comparable levels compared to cultures induced with
233 only one inducer. So, while the maximum expression from each promoter was distinct from the maxima
234 achieved in the single promoter systems, concurrent induction of both promoters did not further impact
235 their transcriptional output. This suggests that the promoters (σ^{70}V2TcR and σ^{70}V2/3LacI) are minimally
236 affected by the activation of the other, reaching almost their maximum activity either upon sole activation
237 or concurrent activation (Fig. 2J-O). The three bacterial species investigated, each containing the dual
238 expression system, did not display detectable green fluorescence in the uninduced state compared to
239 their wild type counterparts, indicating tight regulation of the σ^{70}V2TcR expression system (Fig. 2D, F &
240 H). However, as we previously observed [23], some *mCardinal* leakage was observed originating from the
241 σ^{70}V2LacI-*mCardinal* (Fig. 2E, G & I).

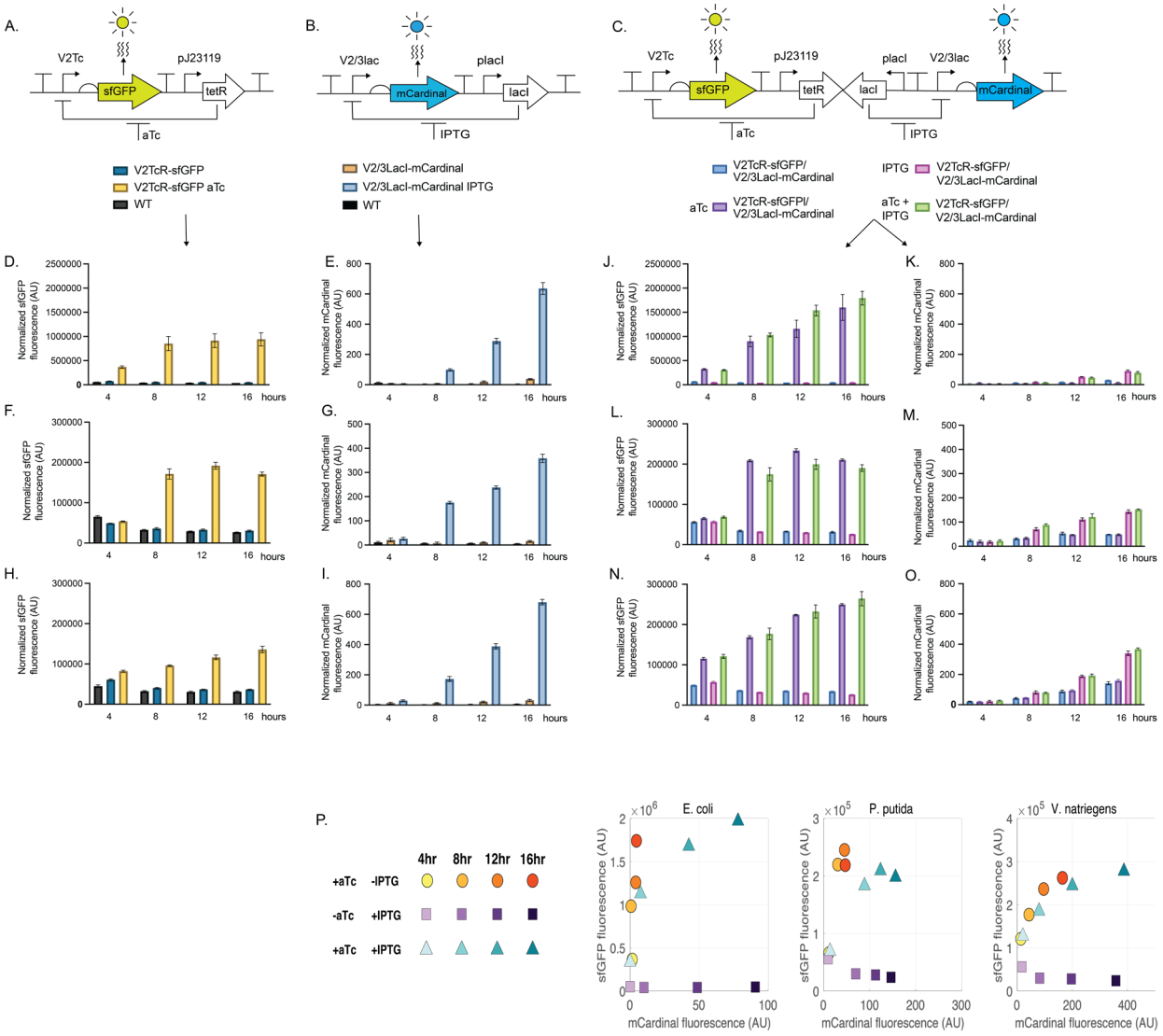
242 The dual expression system constitutively expresses the transcriptional regulators *TetR* and *LacI*,
243 both of which have been reported to exhibit toxicity at high concentrations [46-48]. Each sensor requires
244 cellular resources (for example, ribosomes) to function, and the activation of one sensor can influence
245 another indirectly due to resource competition [49]. We anticipated a lower performance for both
246 promoters in the dual expression system, due to the metabolic burden imposed by the simultaneous
247 presence of *TetR* and *LacI*. However, this scenario was only observed with σ^{70}V2/3LacI, which exhibited
248 reduced production of *mCardinal*, which was particularly pronounced in *E. coli* (Fig. 2K, M & O), thus
249 suggesting that the presence of both transcriptional regulators might affect other cellular processes that
250 indirectly affect the strength of the σ^{70}Lac promoter. Conversely, the levels of *sfGFP* appeared to be
251 enhanced by σ^{70}V2TcR in the dual expression system. One hypothesis for this observation is that our

252 transcriptional systems are stressing the cells with their metabolic load. To mitigate the adverse effect of
253 stresses, some cells produce endogenous fluorophores and/or metabolic byproducts increasing the
254 emission of green light [50]. For instance, *P. putida* secretes pyoverdine, a soluble fluorescent yellow-
255 green pigment, under iron-limiting conditions [51]. We predict that σ^{70}V2TcR in the dual expression
256 system did not in fact display increased *sfGFP* production. Instead, it is possible that the presence of
257 endogenous molecules interfering in the green spectrum [52, 53], not originating from the recombinant
258 protein, amplified the *sfGFP* signal due to activation of cellular stress responses. Similarly, we would
259 predict that the cells, in addition to increasing their autofluorescence, would display changes in growth
260 that reflect this additional metabolic load.

261 To corroborate these hypotheses, we examined the growth rate of the parent *E. coli* strain
262 (DH10B) and the recombinant strains carrying the dual expression system. Addition of IPTG or aTc shows
263 minimal effect in the cell growth (Fig. S1A-B), however the combined exposure to both inducers imparts
264 a modest effect on the growth rate (Fig. S1C). A major impact on growth is observed when the dual
265 expression system is incorporated into *E. coli* in pColE1 plasmid, reaching the beginning of the exponential
266 phase 4 hours after the wild type (Fig. S1D). Addition of IPTG does not delay the growth rate further in
267 the recombinant strains (Fig. S1E), however, aTc (Fig. S1F) or aTc with IPTG (Fig. S1G) supplementation
268 prolongs the exponential phase.

269 The emission of green fluorescence was assessed to identify any potential endogenous molecules
270 or proteins that might interfere with the readings of the recombinant sfGFP. The wild-type DH10B strain
271 does not produce any additional green fluorescence when exposed to IPTG, aTc, or a combination of both
272 (Fig. S2A-C). However, the recombinant strain with the dual expression system, without *sfGFP* and
273 containing only mCardinal, exhibits significantly higher green fluorescence compared to the wild type,
274 showing a maximum 5-fold increase after 4 hours of growth (Fig. S2D). Induction with IPTG does not
275 increase green fluorescence emission (Fig. S2E). However, the addition of aTc (Fig. S2F) and aTc combined

with IPTG (Fig. S2G) results in approximately a 2-fold increase in green emission compared to the uninduced state. It is tempting to conclude that perhaps aTc is itself the source of the fluorescence, however these data are inconsistent with that conclusion because additional green fluorescence is not observed in the parent DH10B strain when aTc is added. Therefore, the results are consistent with the idea that the cells carrying the dual expression system are generating a fluorescent metabolite of some kind, which correlates with a growth defect.



284 **Figure 2. *sfGFP* and *mCardinal* production in *E. coli*, *P. putida*, and *V. natriegens* containing the single and dual expression**
285 **systems**. For all samples, the fluorescence mean of *sfGFP* signal (excitation 485, emission 510) and *mCardinal* signal (excitation
286 605, emission 659) was normalized by the cell density (OD_{600}). Gene circuits of **A.** σ^{70}V2TcR-*sfGFP* **B.** σ^{70}V2/3LacI-*mCardinal* & **C.**
287 σ^{70}V2TcR-*sfGFP*/σ^{70}V3LacI-*mCardinal*. Normalized *sfGFP* emission produced by σ^{70}V2TcR-*sfGFP* in **D.** *E. coli* **F.** *P. putida* & **H.** *V.*
288 *natriegens*. Normalized *mCardinal* emission produced by σ^{70}V2/3LacI-*mCardinal* in **E.** *E. coli* **G.** *P. putida* & **I.** *V. natriegens*.
289 Normalized *sfGFP* and *mCardinal* emission produced by the dual expression system σ^{70}V2TcR-*sfGFP*/σ^{70}V2/3LacI-*mCardinal* in **J.K**
290 *E. coli* **L.M.** *P. putida* & **N.O.** *V. natriegens*. (Error bars are +/- S.D., n=3. **P.** Matrix display of both *sfGFP* and *mCardinal* production
291 of individual and concurrent inductions of the dual expression systems.

292

293 **Evaluating synergistic potential and metabolic cost of the dual expression system**

294 The combinatorial use of two promoters that control transcription of the same gene, or double
295 promoter expression systems, have the potential to increase the titer of recombinant protein production
296 [29, 54]. However, from our first dual expression system experiments, we anticipated a metabolic cost to
297 the cell (Fig. S3). We observed that while *sfGFP* fluorescence from the σ^{70}V2Tc promoter increased relative
298 to a single expression system, *mCardinal* fluorescence from the σ^{70}V2/3Lac promoters was significantly
299 decreased. If, as we hypothesized, the increased *sfGFP* was an artifact of cellular stress caused by the
300 combination of metabolic load and aTc exposure, then one would predict that driving the same protein
301 from both promoters would have no synergistic effect on expression over a single promoter system.

302 Previous results indicated that recombinant protein production by σ^{70}V2TcR expressed in pJH0204
303 (pColE1 derived plasmid with 30 copies per cell) was enhanced by increasing the copy number by adapting
304 the expression system to pUC19 vector (250 copies per cell). However, the toxicity associated with the
305 transcriptional regulator *LacI* prevented the evaluation of the σ^{70}V2/3LacI expression system in a high
306 copy plasmid like pUC19 [23]. Hence, to test the synergistic potential of the duet expression system
307 σ^{70}V2TcR/V3LacI, a version was made where both promoters regulate *mCardinal* production (Fig. 3B). The
308 *E. coli* strain harboring the duet expression system σ^{70}V2TcR/V3LacI exhibited 2-fold and 4-fold lower red
309 fluorescence compared to strains containing σ^{70}V2TcR and σ^{70}V3LacI individually when induced with aTc
310 and IPTG, respectively (Fig. 3C, D). This observation supports the hypothesis that the increased green
311 fluorescence signal in the σ^{70}V2TcR/V3LacI was an artifact of some kind, possibly a product of cellular

312 stress. Synergy was observed by the addition of IPTG and aTc to the σ^{70}V2TcR/V3LacI *E. coli* strain
313 improving *mCardinal* production by 1.5-fold against the induction obtained with aTc alone (Fig. 3D).
314 However, the *mCardinal* production in the duet system reached about the same total amount of
315 *mCardinal* as the σ^{70}V2TcR expression system alone (Fig. 3C, D). These results indicate that the tandem
316 version of the synthetic promoters σ^{70}V2TcR/V3LacI does not offer a real synergy in *E. coli*, but rather an
317 alternative to co-express different genes at high levels using different inducers.

318 In *P. putida* the σ^{70}V2TcR expression system exhibited similar *mCardinal* performance in the single
319 and dual version (Fig. 3E & F). Conversely, induction of the dual system with IPTG resulted in a 3-fold
320 decrease relative to the σ^{70}V2lacI system alone (Fig. 3E, F). The simultaneous addition of both inducers
321 (aTc and IPTG) increased *mCardinal* expression by 1.1-fold over the σ^{70}V2TcR and σ^{70}V2LacI expression
322 systems in the dual system, demonstrating a minimal, statistically insignificant synergistic effect in this
323 host. The duet expression system σ^{70}V2TcR/V2LacI markedly reduced the efficiency of the synthetic
324 promoters in *V. natriegens* compared to their single expression system counterparts, with reductions of
325 12-fold and 2-fold for σ^{70}V2LacI and σ^{70}V2TcR after induction with IPTG and aTc, respectively (Fig. 3G &
326 H). Co-induction of the dual expression system with IPTG and aTc did not yield a synergistic effect in
327 *mCardinal* production, but achieved similar yields as obtained by induction with aTc alone (Fig. 3H).

328 This information collectively demonstrates that synergy cannot be attained with the dual
329 expression system in the three microorganisms under study. In *E. coli* and *V. natriegens*, the pJH0204
330 plasmid containing the pColE1 origin of replication was used, rendering 30 copies per cell in *E. coli* and
331 300 copies per cell in *V. natriegens*. [14, 55]. On the contrary, the duet expression system appears to
332 attenuate the activity of each promoter. Rewiring the cell metabolism imposes additional stress which
333 affects protein synthesis and growth rate [56]. Consequently, the constitutive expression of the
334 transcriptional regulators *TetR* and *LacI*, as well as competition for the σ^{70} factor by both promoters, may
335 pose a significant metabolic burden. This triggers energetic inefficiency within the cell, leading to

336 undesirable physiological changes and placing hidden constraints on host productivity [57]. In *P. putida*,
337 the dual expression system was directly integrated into the chromosome, thereby reducing the quantities
338 of the transcriptional regulators *TetR* and *LacI* and thus lowering the metabolic cost of the synthetic
339 expression systems. This resulted in similar *mCardinal* production with σ^{70}V2TcR, while the σ^{70}V2LacI
340 expression system showed reduced expression and a lack of synergy.

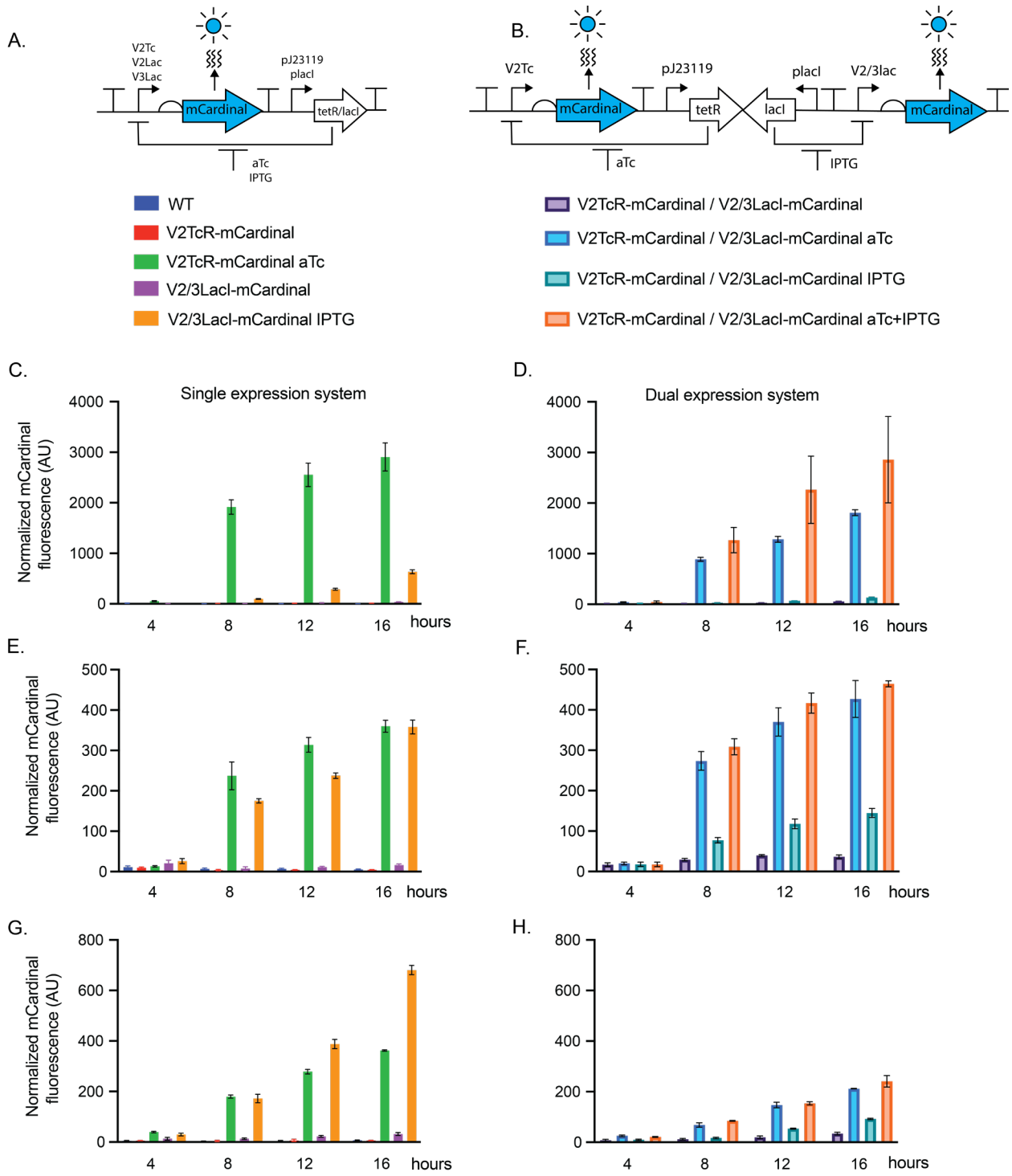


Figure 3. Assessing potential synergy between *lac* and *tet* promoters. Gene circuits of the single expression system **A.** σ^{70}V2TcR-*mCardinal*, σ^{70}V2LacI-*mCardinal* or σ^{70}V3LacI-*mCardinal* & the dual expression system **B.** σ^{70}V2TcR-*mCardinal*-V2/3LacI-*mCardinal*. Time course of *mCardinal* production from the single expression systems in **C.** *E. coli* **E.** *P. putida* & **G.** *V. natriegens* Vs *mCardinal* production in the double expression system in **D.** *E. coli* **F.** *P. putida* & **H.** *V. natriegens*. For all samples, the fluorescence mean of *mCardinal* signal (excitation 605 nm, emission 659 nm) was normalized by the cell density (OD_{600}). Error bars are +/- S.D., n=3.

348 **Production of lycopene and β-carotene in *E. coli*, *P. putida* and *V. natriegens* using the σ^{70}V2TcR single**
349 **expression system**

350 To assess the capability of the dual expression system to produce natural products, we tested the
351 ability of the σ^{70}V2TcR expression system to control the synthesis of multiple genes in a BGC. To do so, we
352 evaluated the production of lycopene and β-carotene. Lycopene production has already been
353 demonstrated in *E. coli* and *P. putida* through heterologous expression of the lycopene-producing operon
354 (LYC), which contains the *crtEBI* genes from *Pantoea ananatis* [11]. Lycopene serves as the biosynthetic
355 precursor of β-carotene. Therefore, the expression of the LYC operon together with *crtY* leads to β-
356 carotene production. This process has also been demonstrated in *E. coli* and *V. natriegens* [13, 58]. The
357 LYC and *crtEBIY* operons were incorporated into the σ^{70}V2TcR expression system (Fig. 4A, B) and the
358 production of lycopene and β-carotene was quantified using UHPLC [37] after 4 hours of induction with
359 aTc. In *E. coli*, production of both lycopene and β-carotene was achieved. However, the induced culture
360 only yielded 1.7-fold more lycopene and 1.2-fold more β-carotene compared to the uninduced culture
361 when induced for four hours (Fig. 4C, D). A decrease in lycopene production was noted with longer
362 induction times which may be due to the documented toxicity of high lycopene production in *E. coli* that
363 inhibits cell growth and productivity, thereby decreasing titer per volume [59]. The results demonstrate
364 that the tight control of the σ^{70}V2TcR system is still unable to completely repress the expression of the
365 *crtEBI* and *crtEBIY* BGCs in *E. coli*. A similar observation has been previously published, where lycopene
366 biosynthesis in *E. coli* was controlled using the pET expression system, and also showed significant leakage,
367 with production reaching only 1.1-fold more after 40 hours of induction than the uninduced culture [38].
368 Clearly, the tight regulation of terpenoid biosynthesis in *E. coli* remains a significant challenge.

369 The σ^{70}V2TcR expression system produced a maximum 0.8 mg/L of lycopene in *E. coli* cultivated
370 in a culture tube after only 7 hours of growth (4 hours with the inducer aTc) (Fig. 4C), and a maximum of
371 0.46 mg/L β-carotene (Fig. 4D). Notably, no media optimizations were performed, nor any strain

372 engineering beyond the addition of the *crtEBI* or *crtEBIY* BGCs. While higher titers of lycopene and β-
373 carotene have been reported in *E. coli* by rewiring its metabolic network, and utilizing optimal media and
374 bioreactors [38], our goal here was to optimize control, rather than titer. Still, the titers obtained with the
375 σ^{70}V2TcR expression are comparable to those generated in some previous reports, even in the absence of
376 further optimizations [11, 13, 38, 58]. These titers could be further increased through media and strain
377 optimization in future studies.

378 In recombinant strains of *P. putida* and *V. natriegens* containing the σ^{70}V2TcR-*crtEBI* and
379 σ^{70}V2TcR-*crtEBIY* transcriptional units, little to no lycopene or β-carotene production was detected
380 without induction. In both organisms, induction of the BGC by aTc led to the robust production of the
381 terpenoids (Fig. 4C, D). *P. putida* was reported to produce 1.233 µg/L of lycopene after 24 hours of growth
382 with the expression of *crtEBI* from pSEVA421 vector, in the absence of further strain engineering [11].
383 With the σ^{70}V2TcR expression system, lycopene production reached 170 µg/L after only 4 hours of
384 induction (Fig. 4C), marking a substantial increase in reported titer. Additionally, β-carotene production
385 by *P. putida* reached 496 µg/L, the highest titer among the three species in our study (Fig. 4D). *V.*
386 *natriegens* was reported to produce 500 µg/L of β-carotene cultivated in the rich medium LBv2 for 20
387 hours [13], while in the σ^{70}V2TcR expression system achieved 286 µg/L of lycopene (Fig. 4C) and 353 µg/L
388 of β-carotene (Fig. 4D) in the standard LB medium and four hours of induction with no particular
389 optimization. These titers achieved here suggest that significantly increased titers would be possible in
390 the future with the combination of our expression system and other optimizations. The overall results
391 demonstrate that the σ^{70}V2TcR expression system can be efficiently used to drive production of BGCs in
392 *E. coli*, *P. putida* and *V. natriegens*.

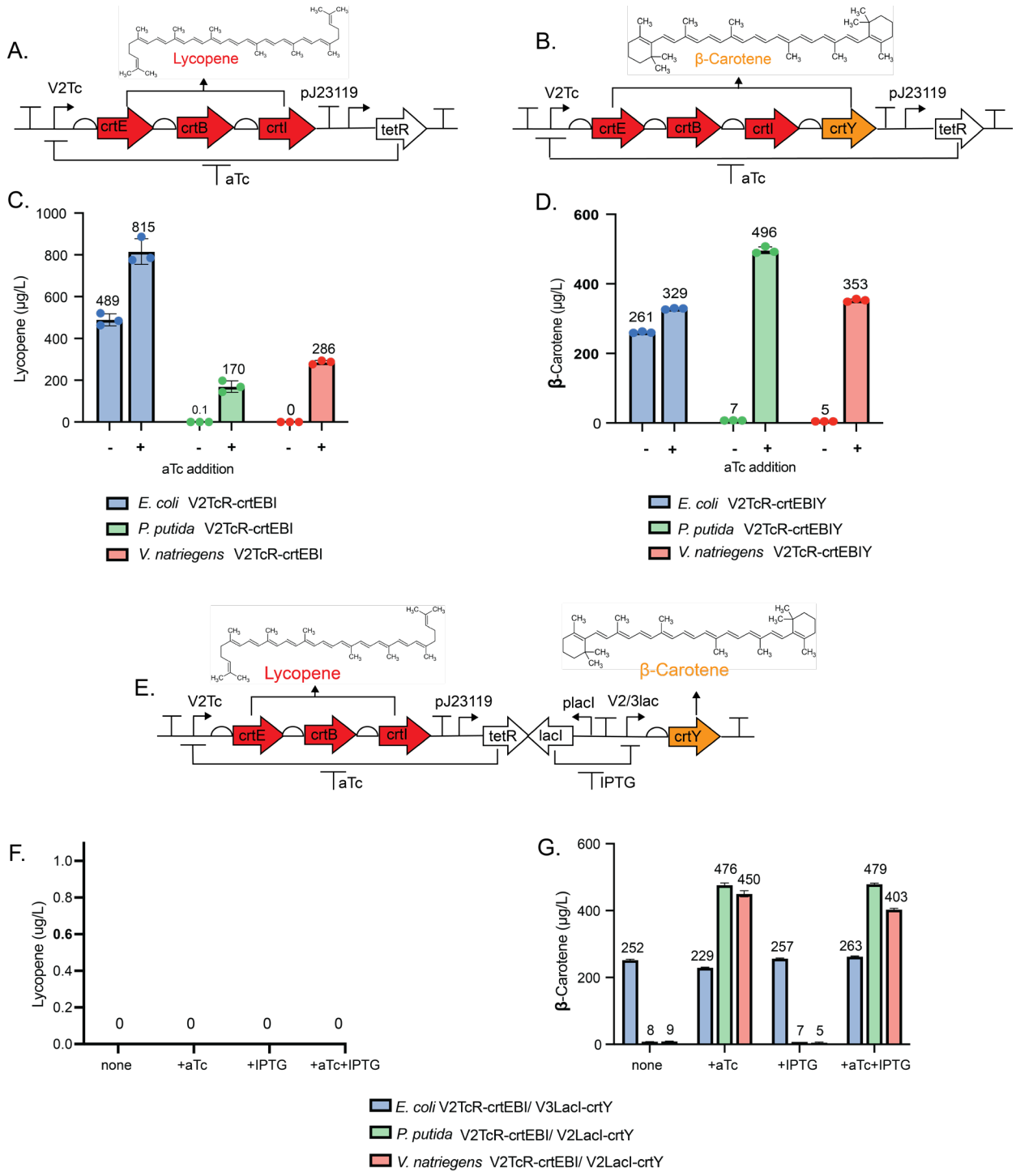


Figure 4. Production of lycopene and β-carotene in *E. coli, P. putida,* and *V. natriegens* with the single and dual expression systems. Gene circuit of **A.** σ^{70}V2TcR-*crtEBI* and **B.** σ^{70}V2TcR-*crtEBIY*. Production of **C.** lycopene and **D.** β-carotene by the single σ^{70}V2TcR expression system in *E. coli, P. putida* and *V. natriegens*. Gene circuit of **E.** the dual expression system σ^{70}V2TcR-*crtEBI*-V2/3LacI-*crtY*. Production of **F.** lycopene and **G.** β-carotene by the dual expression system in *E. coli, P. putida* and *V. natriegens*. Cultures were induced with aTc, IPTG or both inducers at OD_{600}. 0.7 for 4 hours and terpenoids titers were measured by UHPLC. Error bars are +/- S.D., n=3.

400 **Coordinated expression of the lycopene and β-carotene BGCs with the dual expression system**

401 Natural products are often encoded by BGCs, and the ability to activate each gene at different
402 time points with different inducers can be advantageous, particularly when the genes are toxic or the
403 metabolites produced by the BGC affect the growth of the heterologous host [60]. Such control may also
404 be useful in industrial bioprocess settings, permitting independent optimization and control of different
405 enzymatic steps in a pathway. In this study, we have demonstrated that the σ^{70}V2TcR expression system
406 can effectively activate the production of lycopene and β-carotene. As a next step, we attempted to exert
407 control of β-carotene at different points in the production pathway using two different inducers, aTc and
408 IPTG. The LYC operon was integrated into the σ^{70}V2TcR expression system, while the *crtY* gene was
409 controlled by the σ^{70}V2/3LacI expression system (Fig. 4E). The plasmid pσ^{70}V2TcR-*crtEBI*/V3LacI-*crtY* was
410 transformed into *E. coli*, and the plasmid pσ^{70}V2TcR-*crtEBI*/V2lacI-*crtY* was transformed into *P. putida* and
411 *V. natriegens*. In all three bacterial species, no lycopene accumulation was observed upon sole induction
412 by aTc despite the *crtEBI* genes being controlled by the σ^{70}V2TcR expression system (Fig. 4F). This result
413 in *E. coli* can be explained by the leakage of the *crtY* under the control of the synthetic promoter σ^{70}V3LacI.
414 The high processivity of the lycopene cyclase CrtY to metabolize lycopene directly into β-carotene requires
415 tighter regulation of the promoter to completely repress this gene, and the σ^{70}V3LacI expression systems
416 lacks this control. On the other hand, while the σ^{70}V2TcR-*crtEBI* system showed no measurable leakage in
417 *P. putida* and *V. natriegens* using fluorescent reporters, we previously showed that the σ^{70}V2lacI
418 expression system does leak in these strains [23]. Consequently, the lycopene produced after the addition
419 of aTc was directly processed into β-carotene by the CrtY present in the cells due to leakage of σ^{70}V2LacI.

420 Production of β-carotene in *E. coli* was observed regardless of the absence or presence of the
421 inducers aTc and IPTG (Fig. 4G), demonstrating that the dual σ^{70}V2TcR/ σ^{70}V3LacI expression system would
422 require tighter regulation in the OFF state to effectively downregulate the target gene in the absence of
423 the inducer. We believe the leaky transcription we are observing in the lycopene and β-carotene BGCs,

424 which appears more severe than was observed for similar promoter designs expressing fluorescent
425 proteins, is not due to an increase in leaky transcription from these promoters under carotenoid
426 biosynthesis conditions, but rather to the processivity of enzymes versus fluorescent proteins. In *P. putida*
427 and *V. natriegens*, production of β-carotene was only achieved when the cultures were induced with aTc
428 or with aTc + IPTG (Fig. 4G). IPTG induction alone did not lead to β-carotene production because the *crtEBI*
429 genes were not transcribed by the σ^{70}V2TcR expression system. Addition of aTc resulted in β-carotene
430 levels similar to the ones obtained when the cells were induced with aTc + IPTG (Fig. 4G), thus indicating
431 that the minimal leakage of the σ^{70}V2lacI-*crtY* in *P. putida* and *V. natriegens* is sufficient to metabolize all
432 lycopene produced by the σ^{70}V2TcR-*crtEBI* immediately into β-carotene upon induction by aTc. The
433 varying yields of β-carotene between *E. coli*, *P. putida*, and *V. natriegens* highlight the differences in their
434 suitability as hosts for controlled natural product production.

435

436 **Adaptation of toehold switch to the synthetic σ^{70} expression systems and fold-change evaluation with**
437 **the reporter system *mCardinal***

438 The assessment of σ^{70} expression systems using the reporter *mCardinal* suggested that the
439 σ^{70}V2TcR promoter displays tight regulation, leading to undetectable levels of red fluorescence in the
440 uninduced state [23]. However, when the lycopene and β-carotene operons were incorporated into this
441 synthetic promoter, the terpenoids were produced by *E. coli* in the uninduced state, indicating that the
442 promoter was not completely repressed. The processivity of enzymes, unlike static fluorescent proteins,
443 amplifies the effect of leaky transcription. In the σ^{70} V2/3LacI expression systems the leakage was even
444 more evident, as we were unable to repress the production of CrtY in the dual expression system
445 sufficiently to permit the intermediate accumulation of lycopene (Fig. 4F). Maintaining low basal
446 expression levels in the absence of the inducer is crucial to minimize unwanted effects on cell growth and

447 to prevent potential damage caused by the target protein, including issues like self-aggregation, inclusion
448 body formation [61], or as evidenced in this study, uncontrolled biosynthesis of a downstream product. If
449 independent control of steps within a multistep biosynthetic pathway is to be achieved, it will require
450 significant improvements in the control of inducible promoter systems.

451 One solution to this problem involves the adaptation of RNA-based gene regulation to reduce the
452 impact of basal expression [32, 33]. The current design of the synthetic σ^{70} expression system comprises
453 transcriptional control performed by the transcriptional regulators TetR and LacI. Introducing an
454 additional translational control mechanism, such as a toehold switch (THS), alongside the existing
455 transcriptional control, could enhance the precision of the σ^{70} expression systems. This added layer of
456 regulation could provide finer control over gene expression, potentially reducing leakiness and improving
457 inducibility. To this end, the σ^{70}V2TcR and σ^{70}V2lacI expression systems were modified with the
458 incorporation of the three components required to add toehold-mediated translational control: the THS
459 trigger, the THS target and the cognate ribosome binding site (RBS) [34]. For the σ^{70}V2TcR expression
460 system, two σ^{70}V2Tc promoters were incorporated into the system, the first σ^{70}V2Tc promoter regulates
461 the transcription of the THS trigger, while the second promoter is fused to the THS target, which masks
462 the RBS (Fig. 5A). The σ^{70}V2lacI follows the same architecture, except the THS trigger is regulated by the
463 V3Lac promoter due its reduced leakage compared to the V2Lac, which controls the THS target that hides
464 the RBS (Fig. 5B). This arrangement combines transcriptional and translation regulation, therefore, the
465 absence of the inducers (aTc or IPTG) results in both transcriptional repression (via the transcriptional
466 regulators *TetR* and *LacI*) and translational repression (via the THS trigger) of the target gene (*mCardinal*).
467 Upon addition of the inducers, the constitutively expressed transcriptional regulators are removed from
468 the synthetic σ^{70} promoters, activating the transcription of the THS trigger, which binds to the THS target
469 inducing the conformational change that relieves the translational repression of *mCardinal* resulting in its
470 subsequent expression.

471 The comparison between the uninduced and induced states of the σ^{70}V2TcR expression system
472 with and without the toehold switch revealed very similar induction dynamics (Fig. 5C). It was previously
473 determined that the leakiness of σ^{70}V2TcR could not be observed with a fluorescent reporter, thus no
474 change in the performance of this reporter was expected. The system may exhibit leakage even with the
475 addition of the THS, but it would not be detectable in this assay. The addition of aTc induced *mCardinal*
476 production by promoters with and without the THS. However, the promoter with the THS exhibited a 2-
477 fold reduction in the dynamic range compared to the promoter without the switch, although it still
478 maintained a satisfactory level of recombinant protein production (Fig. 5C, E, F). On the other hand, the
479 addition of the THS had a significant effect on suppressing leaky transcription from the σ^{70}V2lacI
480 expression system (Fig. 5D). The dynamic range in the σ^{70}V3-2lacI THS system improved by 5.7-fold, but
481 still, leaky transcription was significant. However, the THS did reduce the leakiness by 7-fold compared to
482 the original version (Fig. 5E). Importantly, *mCardinal* production after IPTG induction reached similar
483 levels in both σ^{70}V2lacI expressions, with the variant lacking the THS producing only 1.2-fold more
484 *mCardinal* fluorescence (Fig. 5D).

485 These results demonstrate that THSs enhance the regulation of gene expression by modulating
486 translation initiation (Fig. 5E, F). This feature is particularly effective in inducible expression systems prone
487 to leakage. However, while fluorescent proteins like *mCardinal* offer valuable insights, they might not
488 capture the subtle nature of basal transcription. For instance, the evaluation of the σ^{70}V2TcR expression
489 system using fluorescent proteins indicated complete lack of fluorescent protein synthesis in the OFF state
490 when uninduced. However, the results presented for the production of lycopene in the dual expression
491 system revealed that the promoter leaks to some extent. Therefore, to truly assess any basal
492 transcriptional activity, the use of enzymatic reporter systems will be necessary, with higher sensitivity
493 for accurately evaluating gene expression dynamics.

494

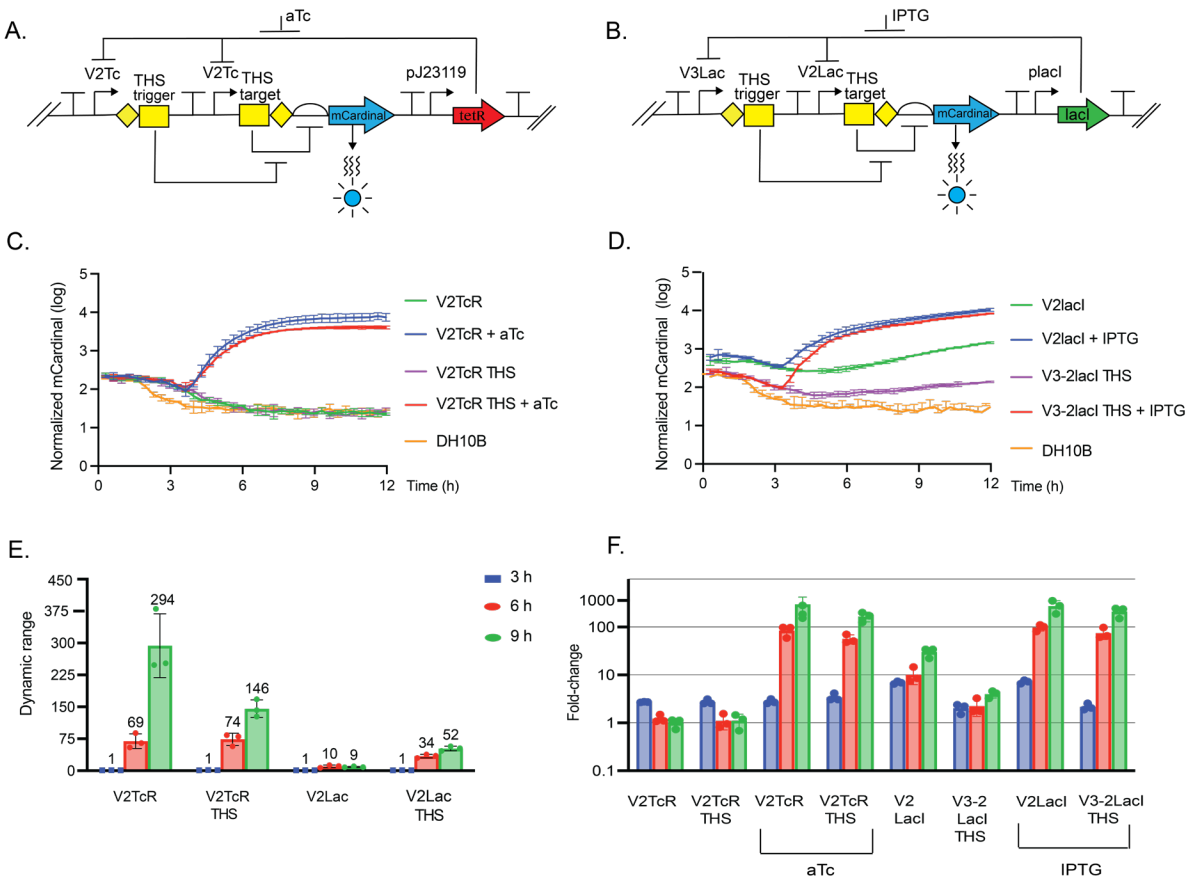


Figure 5. Comparison of the σ^{70} expression systems to the THS versions controlling the expression of the reporter system *mCardinal* in *E. coli*. Architecture of the synthetic gene circuits with the toehold switches (THSs) in **A.** σ^{70}V2TcR & **B.** the σ^{70}V3-2LacI system. **C**. Time course of *mCardinal* production by pσ^{70}V2TcR THS. **D**. Time course of *mCardinal* production by σ^{70}V3-2LacI THS. **E.** Difference in *mCardinal* production between the ON and OFF states. **F**. Fold-change difference in the red-light emission of the recombinant cells vs the wild type DH10B. The cultures were induced after 3 hours of growth with aTc or IPTG accordingly. For all samples, the fluorescence mean of *mCardinal* signal (excitation 605 nm, emission 659 nm) was normalized by the cell density (OD_{600}). Error bars are +/- S.D., n=3.

Evaluation of the *σ^{70}* expression systems using luciferase as a reporter

The selection of the red fluorescent protein *mCardinal* to estimate the efficiency of the *σ^{70}* expression systems was a strategic choice, to circumvent intrinsic issues associated with *sfGFP* and the potential interference of green autofluorescence from cells, which could lead to inaccurate quantification of the recombinant protein produced by the synthetic promoters [23]. However, upon assessing the synthetic promoters for terpenoid production, it became evident that the *σ^{70}*V2TcR expression system

510 does display leaky transcription, a phenomenon not discernible with *mCardinal* as the reporter. The
511 integration of the THS into σ^{70}V2lacI effectively mitigated basal expression. However, this effect was not
512 evident in THS-σ^{70}V2TcR-*mCardinal*, as undetectable fluorescence (undetectable above the background
513 autofluorescence) in the OFF state in this expression system was achieved even without the THS. The
514 terpenoids lycopene and β-carotene, aside from their nutraceutical properties, can serve as reporter
515 systems due to their ability to induce a color change in the cell biomass upon production [62]. Indeed, the
516 biosynthesis of these pigments was instrumental in detecting the leakage of the σ^{70}V2TcR system.
517 However, accurate estimation of terpenoid yields necessitates laborious compound isolation techniques
518 and complex measurements. Consequently, their use as a reporter system may not be the ideal choice for
519 evaluating the metrics of an inducible system. To comprehensively grasp the behavior of the synthetic σ^{70}
520 expression systems and address the challenges posed by the limited sensitivity of *mCardinal* and the
521 intricate evaluation of terpenoid production, we opted to employ luciferase as a reporter.

522 The bacterial luciferase, encoded by the lux operon (*luxCDABE*), initiates the emission of
523 bioluminescence upon its expression, without requiring the addition of an external substrate [63]. To
524 amplify the detection of leaky basal transcription, the luciferase reporter was integrated into the σ^{70}
525 expression systems and their corresponding THS variants in the pColE1-containing plasmid pJH2024 (Fig.
526 6A - D). We expected these constructs would allow us to better measure leaky transcription, particularly
527 in the σ^{70}V2TcR system where the uninduced state appears completely OFF when using *mCardinal*, yet
528 shows activity with lycopene and β-carotene (Fig. 4C, D). The time course evaluation of luciferase
529 production permitted the visualization of the leakage of the σ^{70}V2TcR, and also confirmed that the THS
530 version of this promoter diminished the leakage and amplified the signal of bioluminescence in *E. coli* (Fig.
531 6E, G, I). Interestingly, bioluminescence production with the THS tends to diminish slightly over time,
532 suggesting that translational regulation favors the repression of the lux operon after the mid-log phase of
533 growth (Fig. S4). In contrast, the original σ^{70}V2TcR system maintains its pronounced leakage over time

534 (Fig. 6E). The σ^{70}V2lacI variant, exhibited high basal leakage under both induced and uninduced
535 conditions, with only a modest improvement in induction by the integration of the THS (Fig. 6F).
536 Interestingly, induction with IPTG in the σ^{70}V2lacI variant slightly suppressed bioluminescence production
537 (Fig. 6F, blue bars versus red bars). This phenomenon may be attributed to the potency of this promoter,
538 whose activation could exceed the metabolic capacity of the cells.

539 These results demonstrate that the reporter luciferase provides a more sensitive assessment of
540 the expression levels in the uninduced state compared to fluorescent proteins like *mCardinal*. Importantly,
541 the translational regulation provided by the toehold switches efficiently amplifies the fold change and
542 reduces the basal expression levels of the σ^{70} expression systems, thereby improving the tight regulation
543 of the promoters while maintaining their strength (Fig 6G - J). However, total repression was not achieved
544 by the adaptation of the toehold switch since the σ^{70}V2TcR THS expression system showed minimal (Fig.
545 6E), and the σ^{70}V3-2LacI THS pronounced (Fig. 6F) leakage, thus prohibiting total control of the target
546 recombinant genes.

547 Total gene expression control is crucial for regulating enzymatic reactions involved in the
548 biosynthesis of natural products, as demonstrated with the production of terpenes presented in this
549 study. The synthetic expression systems evaluated in this study were evaluated in the pJH0204 plasmid
550 which contains the pColE1 origin of replication [23]. This plasmid has the advantage to integrate in the
551 chromosome of *P. putida* and replicate in *E. coli* and *V. natriegens*, therefore it can be evaluated in
552 multiple chassis without further manipulation [23]. However, one potential disadvantage of this plasmid
553 in *E. coli* is the copy number. The pColE1 origin of replication produces 25–30 copies per cell in *E. coli* [55].
554 Therefore, to reduce basal expression and achieve tighter control over gene expression, we transferred
555 the synthetic expression systems to the low copy plasmid pSC101, which has approximately 3 copies per
556 cell [64]. In this configuration, both σ^{70}V2TcR expression systems (with and without the THS) achieved
557 total repression of the lux operon in the uninduced state (Fig. 6E). Activation by aTc successfully induced

558 bioluminescence production. However, the THS-version produced ~2-fold less light, indicating that the
559 translational control provided by the THS may be incompletely relieved in the induced state (Fig. 6G, I). A
560 similar trend was observed with the σ^{70}V2LacI and σ^{70}V3-2LacI expression systems in pSC101 (Fig. 6F, H,
561 J). Although total repression was not achieved, the translational control provided by the toehold
562 significantly reduced the basal expression levels and amplified the dynamic range of the strong σ^{70}V2Lac
563 promoter (Fig. 6H, J). We conclude that the combination of translational regulation and reduced plasmid
564 copy number significantly improved the problem of leaky transcription from our promoters in *E. coli*.

565 In *P. putida*, the pJH0204 plasmid integrates at one specific location in the chromosome [35]. In
566 this configuration, using *mCardinal* as the reporter system, the σ^{70}V2TcR showed no leakage, while the
567 σ^{70}V2LacI exhibited minimal red fluorescence in the uninduced state [23]. However, traces of lycopene
568 and β-carotene were detected in the uninduced state of the σ^{70}V2TcR (Fig. 4C, D), and only β-carotene
569 was produced in the dual system (Fig. 4F, G), thus corroborating the leakage of σ^{70}V2LacI in this host. Since
570 the transcriptional control provided by the regulatory proteins TetR and/or LacI proved insufficient to fully
571 repress the σ^{70} synthetic promoters, even at one copy per cell, we decided to incorporate translation
572 repression in *P. putida*. The luciferase reporter system confirmed the leakage of the σ^{70} expression systems
573 in this host (Fig. 7A-B, Fig. S5), and the addition of the THS amplified the dynamic range and reduced the
574 luminescence emission in the uninduced state by ~2-fold in both expression systems (Fig. 7C, D). These
575 results demonstrate that the transcriptional control provided by the regulatory proteins can be enhanced
576 by translational control via THS σ^{70}V2TcR in *P. putida*. As in *E. coli*, these two layers of control are not
577 sufficient to completely turn off the σ^{70}V2LacI expression system in *P. putida*. Unlike in *E. coli*, plasmid
578 copy number is not a parameter that could be further optimized in *P. putida.*

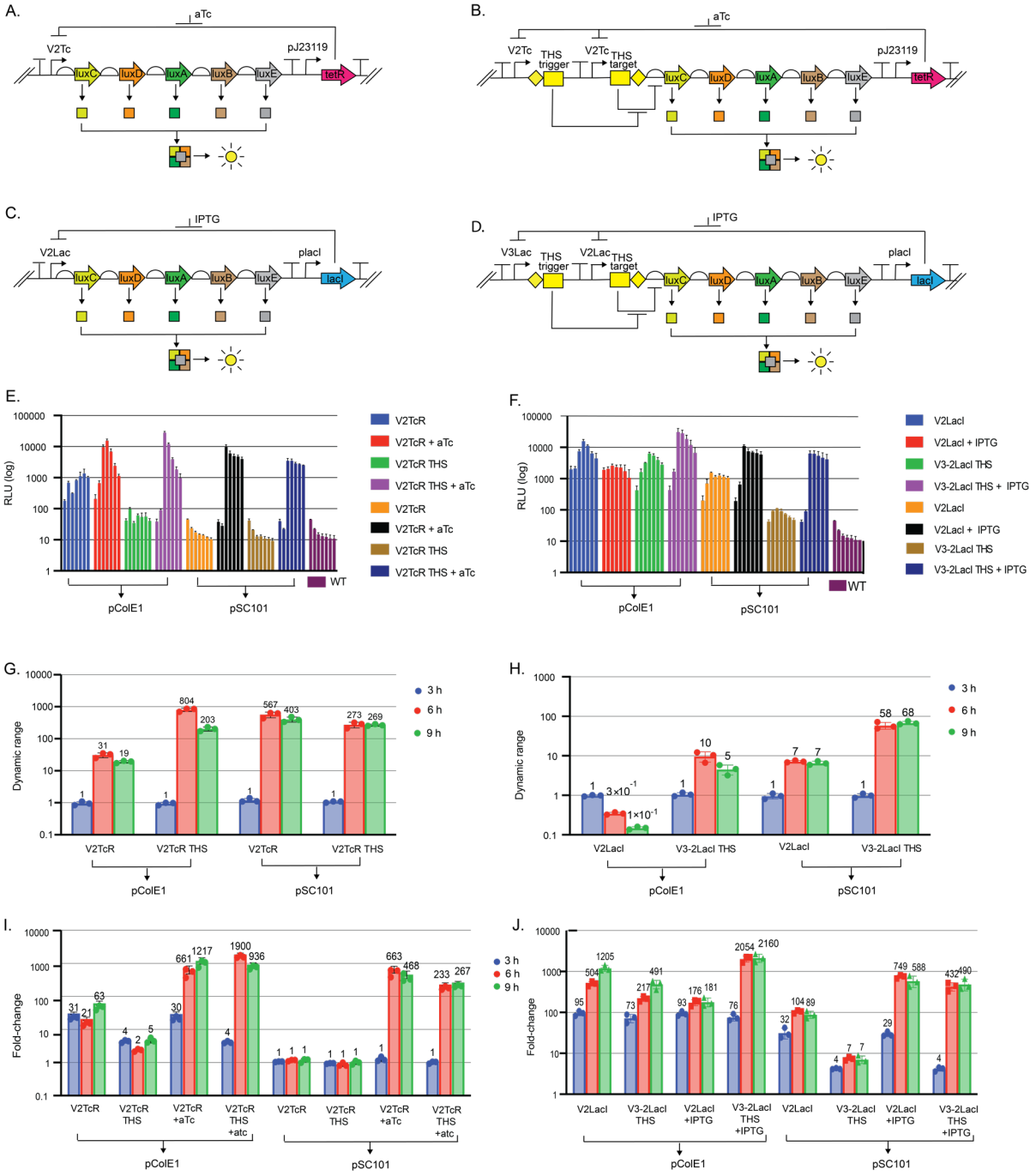
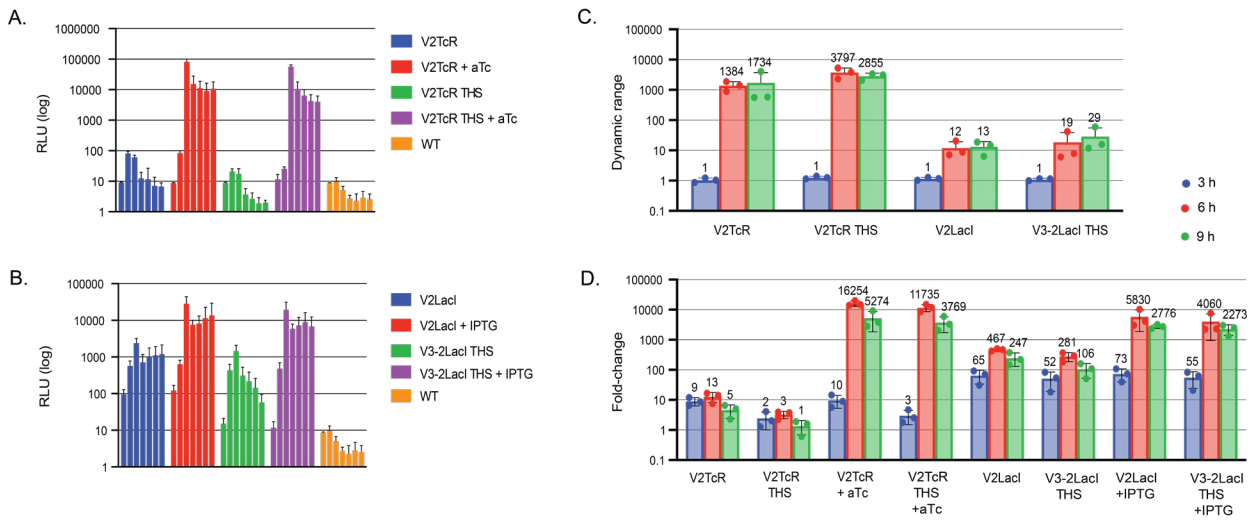


Figure 6. Comparison of the σ^{70} expression systems to the toehold versions controlling the expression of the lux operon in *E. coli* using pCOLE1 and pSC101 derived plasmids. Architecture of the synthetic gene circuits controlling the *luxCDABE* genes with and without the THS in **A-B**. σ^{70}V2TcR system & **C-D.** σ^{70}V3-2LacI system. Time course of bioluminescence production by the **E.** pσ^{70}V2TcR & **F.** pσ^{70}V3-2LacI expression systems; each bar represents the bioluminescence measurement every 3 hours from time 0 to 18 hours (bars in each set, left to right, are 0h, 3h, 6h, 9h, 12h, 15h, and 18h). Difference in bioluminescence production between the ON and OFF states in **G.** pσ^{70}V2TcR & **H.** pσ^{70}V3-2LacI expression systems. Fold-change difference in the luminescence emission of the recombinant cells containing the **I.** pσ^{70}V2TcR and **J.** pσ^{70}V3-2LacI expression systems vs the wild type DH10B. The cultures were induced after 3 hours of growth with aTc or IPTG accordingly. For all samples, the bioluminescence mean was normalized by the cell density (OD_{600}). Error bars are +/- S.D., n=3. Relative Luminescence Units (RLU).

589



590
591 **Figure 7. Comparison of the σ^{70} expression systems to the toehold versions controlling the expression of the lux operon in *P.***
592 ***putida***. Time course of bioluminescence production by the **A.** pσ^{70}V2TcR and **B.** pσ^{70}V3-2LacI expression systems; each bar
593 represents the bioluminescence measurement every 3 hours from time 0 to 18 hours. Difference in bioluminescence production
594 between the ON and OFF states in **C.** pσ^{70}V2TcR and pσ^{70}V3-2LacI expression systems. Fold-change difference in the light emission
595 of the recombinant cells containing the **D**. pσ^{70}V2TcR or and pσ^{70}V3-2LacI expression systems vs the wild type *P. putida*. The
596 cultures were induced after 3 hours of growth with aTc or IPTG accordingly. For all samples, the bioluminescence mean was
597 normalized by the cell density (OD_{600}). Error bars are +/- S.D., n=3. Relative Light Unites (RLU).

598

599 **Production of lycopene and β-carotene using the *σ^{70}* THS control systems in *E. coli***

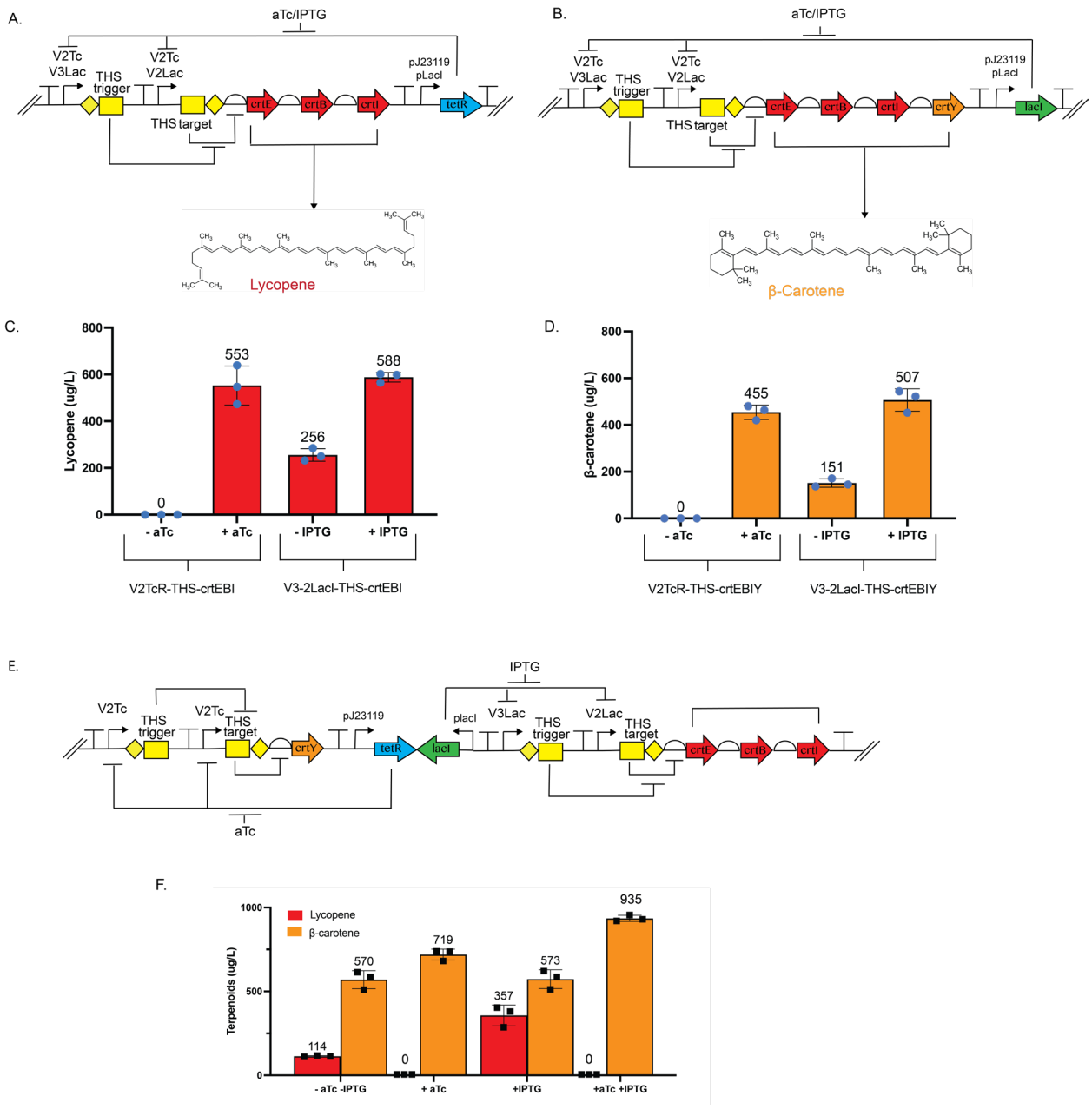
600 The data collected using the luciferase reporter system demonstrated that adapting the THS to
601 the *σ^{70}* expression systems effectively suppressed the basal expression of *σ^{70}*V2TcR and significantly
602 reduced the leakage of *σ^{70}*V3-2LacI when tested in a low-copy plasmid (Fig. 6I, J). These findings enabled
603 a re-evaluation of lycopene and β-carotene production, with the goal of independently controlling
604 different steps within a multistep BGC. Previously, in *E. coli* expressing *crtEBI* and *crtEBIY* under the control
605 of *σ^{70}*V2TcR in a medium copy plasmid (pJH0204), the system essentially behaved as a constitutive
606 promoter, with significant production of lycopene and β-carotene in the uninduced state, and less than
607 2-fold increase in product upon induction (Fig. 4C, D). To assess the ability of the THS designs to better
608 regulate terpenoid production, lycopene and β-carotene production were evaluated separately using the

609 σ^{70}V2TcR-THS and σ^{70}V3-2LacI-THS expression systems in the pSC101 vector (Fig. 8 A, B). With the
610 σ^{70}V2TcR-THS system, terpenoid production was completely abolished in the uninduced state. Upon the
611 addition of aTc, lycopene levels reached titers of 553 µg/L, while β-carotene titers reached 455 µg/L (Fig.
612 8C, D). Thus, we were able to achieve complete OFF state control of these BCGs in *E. coli*.

613 The performance of the σ^{70}V2/3LacI systems were not assessed in the pColE1 vector in our first
614 set of experiments, due to its well documented leakage [23]. However, given the improvements made by
615 the integration of the THS and measurements with the *lux* reporter, the σ^{70}V3-2LacI-THS system was
616 evaluated for terpenoid production. We still observed considerable levels of lycopene and β-carotene
617 production in the uninduced state using σ^{70}V3-2LacI-THS (Fig. 8C, D, and Fig. S6), though titers (256 µg/L
618 for lycopene, and 151 µg/L for β-carotene) were approximately 1.8-fold lower than those observed in the
619 uninduced state for σ^{70}V2TcR in pColE1 (Fig. 4C, D). The addition of IPTG activated the terpenoid
620 production reaching similar titers (588 µg/L for lycopene, and 507 µg/L for β-carotene) to the ones
621 obtained with the σ^{70}V2TcR-THS (Fig. 8C, D). This result represents a significant improvement in the
622 performance of our *lac* promoter system in controlling a BGC.

623 The next step was to evaluate the capabilities of the dual expression system for producing both
624 β-carotene and its intermediate, lycopene. In the earlier setup (Fig. 4E), the LYC operon (*crtEBI*) was
625 controlled by σ^{70}V2TcR, and *crtY* by σ^{70}V3LacI. However, in this configuration, only β-carotene was
626 produced due to significant leakage from both expression systems. This leakage prevented lycopene
627 accumulation in the absence of *crtY* induction (Fig. 4F), as the high processivity of CrtY meant that the
628 leaky expression was sufficient to produce enough enzyme to rapidly converted any lycopene produced
629 into β-carotene (Fig.4G). To prevent a similar outcome in the new dual expression system, the *crtY* was
630 placed under the control of the tight σ^{70}V2TcR-THS expression system, and the LYC operon regulated by
631 the more relaxed σ^{70}V3-2LacI-THS expression system (Fig. 8E). The uninduced state of σ^{70}V2TcR-THS-crtY

632 / σ^{70}V3-2LacI-THS-crtEBI revealed production of both terpenoids (Fig. 8F). We expected to see lycopene
633 being produced, because we saw leaky lycopene from this promoter in Fig. 8C. However, we were
634 surprised by the amount of β-carotene produced, as we expected *crtY* transcription and translation to be
635 tightly controlled based on the results in Fig. 8C-D. Nonetheless, we observed a new behavior in this circuit
636 that was not observed in the first iteration in Fig. 4G, that is, the accumulation of lycopene and β-carotene
637 in the same cell (Fig. 8F). Similarly, when IPTG was added to induce *crtEBI*, the amount of lycopene
638 accumulation increased. Addition of aTc to induce *crtY*, either alone or with IPTG, resulted in the rapid
639 and complete conversion of all lycopene to β-carotene. The dual circuit seems to be producing carotenoids
640 more efficiently, reaching a titer of 935 µg/L under dual induction conditions. The results suggest that
641 leaky expression of *crtY* cannot be entirely eliminated in this configuration of our genetic circuit. However,
642 the amount of CrtY enzyme produced has been decreased by the THS significantly so as to be incapable
643 of metabolizing all available lycopene to β-carotene.



644

645 **Figure 8. Production of lycopene and β-carotene in *E. coli* using the single and dual expression systems with THS transformed**
646 **with pSC101 vector.** Architecture of the σ^{70}V2TcR σ^{70}V3-2LacI-THS controlling the **A.** LYC operon and **B.** *crtEBIY* genes. Production
647 of **C.** lycopene and **D.** β-carotene by the single expression systems. **E.** Gene circuit of the dual expression system with THSs **F.**
648 Lycopene and β-carotene production by the dual expression system. Cultures were induced with aTc, IPTG or both inducers at
649 OD_{600}. 0.7 for 4 hours and terpenoids titers were measured by UHPLC. Error bars are +/- S.D., n=3.

650

651

652

653 **CONCLUSION**

654 The development of various bacterial chassis has garnered attention in pursuit of the goals of
655 next-generation industrial biotechnology (NGIB) [65]. NGIB aims to utilize low-cost mixed substrates,
656 minimize freshwater consumption, save energy, and implement long-lasting, continuous, intelligent
657 processing methods [65]. Despite the numerous advantages of *E. coli*, such as its robust genetic toolbox
658 and standardized cultivation parameters [66, 67], it falls short of achieving the goals of NGIB due to its
659 sensitivity to harsh environments and limited metabolic capabilities [68, 69]. Alternative hosts may be
660 more attractive to accomplish complex tasks. For example, the metabolic versatility of *P. putida* has
661 demonstrated tolerance to organic solvents and stresses associated with industrial-scale cultivations, the
662 ability to utilize lignin-derived compounds, sustain difficult redox reactions, and a relatively high GC-
663 content similar to that of actinomycete-derived natural product BGCs [15, 70, 71]. The marine bacterium
664 *V. natriegens*, inherently halophilic, offers interesting advantages as well, such as rapid growth and a
665 versatile metabolism capable of utilizing a variety of substrates as carbon and energy sources [72-74].
666 Other non-model Gram-negative bacterial chassis also demonstrate the potential to outperform *E. coli*
667 [75, 76]. However, each chassis necessitates the adaptation of distinct genetic tools. The σ^{70} expression
668 system showcased in this study, whether used individually or in combination, offers another tool to the
669 ongoing challenge of developing numerous host organisms. It provides a versatile and robust gene
670 expression platform with a broad host range. Consequently, proteins or BGCs of interest can be readily
671 cloned into each σ^{70} expression system and evaluated across various chassis, thereby expediting the
672 optimization process.

673 In this study, we selected chassis bacteria commonly used by the scientific community and,
674 through the straightforward incorporation of synthetic gene pathways for the biosynthesis of lycopene
675 and β-carotene, achieved relatively high titers of these terpenoids using the σ^{70} expression system.
676 Notably, no media optimization was necessary to complete this task, highlighting a remarkable advantage

677 offered by the σ^{70} expression system. However, the production of terpenoids using the first generation of
678 the σ^{70} expression system revealed leakage in the OFF state, a feature imperceptible when using
679 fluorescent proteins as a reporter system.

680 In this instance, the characterization of the regulatory systems with a multi-step enzymatic
681 reporter (*lux*), was essential in our task to minimize leaky transcription. We acknowledge that differences
682 in culture conditions between the *lux* study (performed in 96-well plates) and our terpene studies (grown
683 in 5 mL cultures in 50 mL culture tubes) may account for some of the differences in performance between
684 these experiments. Still, the general approach of employing an enzymatic reporter led to insights that
685 improved our dual circuit performance when expressing a BGC. Approaches like decreasing plasmid copy
686 number, weakening the RBS sequences, and introducing mutations in the consensus promoter sequences
687 have demonstrated the ability to minimize leakage in the basal state, albeit at the cost of reducing the
688 induced expression levels [77]. The strength and portability of the σ^{70} expression systems reside in the
689 specific nucleotide arrangement of the promoter and RBS DNA sequence [23]; therefore, altering these
690 sequences undermines the value of the system. Instead, the adaptation of the riboswitch toehold,
691 together with the use of a low-copy plasmid in *E. coli*, reduced the leakage of the σ^{70}V2TcR THS to non-
692 detectable levels and nearly abolished the basal expression levels of the σ^{70}V3-2LacI. This approach not
693 only amplified the dynamic range but also maintained the portability of the synthetic expression systems.
694 This was demonstrated in *P. putida*, where the DNA was integrated into the chromosome, and both THS
695 σ^{70} expression systems maintained strong induction, with the σ^{70}V2TcR THS reaching very low *lux* reporter
696 signal. The second generation of the σ^{70} expression system (with THS) remains digital, with ON and OFF
697 state only, however, the emergence of plasmids like TULIP (TUnable Ligand Inducible Plasmid) with
698 controllable copy number [64] could allow an analogue behavior of the versatile σ^{70} expression systems.

699 While our dual inducible system remains imperfect, we did achieve the accumulation of a pathway
700 intermediate (lycopene) using a combination of transcriptional and translational control. We were able to

701 convert that intermediate to a final product (β-carotene) upon specific induction of the downstream
702 enzyme (CrtY). Complementary engineering approaches, such as decreasing the processivity of CrtY, could
703 be used to further tune the system and achieve complete and independent inducible control of
704 intermediate substrates and products.

705 In conclusion, the expression system developed in this study expands the genetic toolkit of various
706 promising Gram-negative chassis, thereby enhancing their capabilities to address fundamental biological
707 questions and bolster their biotechnological applications. Ultimately, the σ^{70} expression system has the
708 potential to facilitate the expression of biosynthetic gene clusters (BGCs) that have previously been
709 inaccessible or suboptimal due to the lack of appropriate gene tools or the limitations of model organisms
710 such as *E. coli*.

711

712 **DECLARATIONS**

713 **Ethics approval and consent to participate:** Not applicable.

714 **Consent for publication:** Not applicable.

715 **Availability of data and materials:** The raw datasets used and/or analyzed during the current study are
716 available from the corresponding author at reasonable request. All processed data generated or
717 analyzed during this study are included in this published article and its supplementary information files.

718 **Competing interests:** A.F.C.R and N.G.F have filed a U.S. patent application for the pσ^{70} *lac* and *tet*
719 promoters described in this work.

720 **Funding:** This work was supported by new faculty start-up funds from Worcester Polytechnic Institute to
721 N.G.F. & the Sandooq Al Watan grant PRJ-SWARD-256 to A.G.

722 **Authors' contributions**

723 **Andres Felipe Carrillo Rincón:** Conceptualization (lead); data curation (lead); formal analysis (lead);
724 investigation (lead); methodology (lead); resources (equal); validation (lead); visualization (lead); writing
725 – original draft (lead); writing – review and editing (equal).

726 **Alexandra J. Cabral**: Methodology (supporting); writing – review and editing (supporting).

727 **Andras Gyorgy** Conceptualization (supporting); funding acquisition (lead); project administration (lead);
728 resources (lead); supervision (supporting); writing – original draft (supporting); writing – review and
729 editing (supporting).

730 **Natalie Gilks Farny:** Conceptualization (supporting); funding acquisition (lead); project administration
731 (lead); resources (lead); supervision (lead); writing – original draft (equal); writing – review and editing
732 (equal).

Acknowledgements: We thank Adam Guss of Oak Ridge National Laboratory for *P. putida* strains and plasmids; Kevin Keating, Emma Tobin, and Eric Young of WPI for assistance with UHPLC experiments and for the gift of *crtEBIY* genes; Shimshon Belkin of Hebrew University of Jerusalem for the *luxCDABE* operon.

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