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3 **BST-2019-0059C**  
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9 **Exploitation of the *Escherichia coli lac*-operon promoter for**  
10 **controlled recombinant protein production**  
11

12  
13  
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32 **Abstract**

33 The *Escherichia coli lac* operon promoter is widely used as a tool to control recombinant  
34 protein production in bacteria. Here we give a brief review of how it functions, how it is  
35 regulated, and how, based on this knowledge, a suite of *lac* promoter derivatives has been  
36 developed to give controlled expression that is suitable for diverse biotechnology  
37 applications.

38

39 **Key words:** *Escherichia coli*, bacterial transcription initiation, *lac* promoter, *tac* promoter,  
40 Lac repressor, recombinant protein production

41

42 **Abbreviations Used:** RPP, recombinant protein production; RNAP, RNA polymerase; bp,  
43 base pairs; CRE, Core-recognition element; CRP, cyclic AMP receptor protein; LacI, lactose  
44 operon repressor protein; IPTG, isopropyl  $\beta$ -D-1-thiogalactopyranoside; PAR, promoter  
45 activity rating; GFP, green fluorescent protein; hGH, human growth hormone; Tat, twin  
46 arginine translocon pathway; LB: lysogeny broth; ONPG, o-nitrophenyl- $\beta$ -D-galactopyranose.

47

48

49 **Introduction**

50 Most of the recombinant protein production (RPP) systems used for expressing proteins in  
51 bacteria were constructed in the last century [1-4]. High level RPP provided by these  
52 systems enables the synthesis and purification of large amounts of soluble recombinant  
53 protein. However, the expression of difficult protein targets (*e.g.* membrane proteins or  
54 proteins secreted out of the cytoplasm), using these RPP systems, may be too high for cells  
55 to cope and adequately fold protein, resulting in substantial target degradation or the  
56 production of insoluble aggregates (*i.e.* inclusion bodies) [5-8]. Many “tricks of the trade” can  
57 be employed to slow down RPP expression and increase the level of soluble product, *e.g.*  
58 lowering the growth temperature, decreasing the inducer concentration or using a weaker  
59 promoter [6-9]. Although such tinkering can be very successful, determining the correct  
60 combination of refinements can be time-consuming. This can also be very “hit-and-miss”,  
61 being dependent on the particular target protein in question [6, 9], and for some induction  
62 regimes, for example when using low inducer concentrations, only a proportion of the cells in  
63 a culture may in fact express recombinant protein [10-12].

64

65 The *E. coli lac* operon promoter was one of the first bacterial promoters to be adopted by  
66 biotechnologists for RPP, and it is still used today, especially when *Escherichia coli* is used  
67 as the host [6]. Here, we give a brief update of our current understanding of transcript  
68 initiation in bacteria, emphasising special features of the *lac* promoter and its regulation. We  
69 then review how, based on this information, many *lac* promoter derivatives have now been  
70 engineered in order to facilitate controlled RPP expression and avoid the problems that are  
71 concomitant with high level overexpression.

72

73 **Transcript initiation and regulation at the *E. coli lac* operon promoter**

74 Transcript initiation in bacteria takes place when the multisubunit DNA-dependent RNA  
75 polymerase holoenzyme (RNAP) interacts with a DNA promoter sequence (Figure 1A). In  
76 brief, the RNAP first interacts with double-stranded DNA to form a ‘closed complex’ in which  
77 determinants in different RNAP subunits interact with different promoter sequence elements  
78 (Figure 1B) [13]. Thus a determinant in the RNAP  $\alpha$  subunit C-terminal domain interacts with  
79 the promoter UP element, a determinant in Domain 4 of the RNAP  $\sigma$  subunit interacts with  
80 the promoter -35 element, and a determinant in Domain 3 of the RNAP  $\sigma$  subunit interacts  
81 with the extended -10 element (Figure 1B). Following this, Domain 2 of the RNAP  $\sigma$  subunit  
82 drives the local unwinding of 13-15 base pairs (bp) of promoter DNA to form the ‘open  
83 complex’, in which the single-stranded DNA template strand is positioned in the active site of  
84 the RNAP, such that initiation of DNA-templated RNA synthesis can take place (Figure 1C)  
85 [14]. Formation of the initiation-competent ‘open complex’ from the ‘closed complex’ is driven  
86 by further specific interactions between other RNAP determinants and promoter sequences

87 [15-17]. Thus, determinants in Domain 2 of the RNAP  $\sigma$  subunit interact with single-stranded  
88 bases of the promoter -10 element and with the promoter discriminator element. These  
89 interactions involve only the non-template strand of the locally unwound segment of  
90 promoter DNA, thereby permitting the single-stranded template strand to access the RNAP  
91 active site. The exact position of the template strand in the active site, and the location of the  
92 downstream junction between single-stranded and double stranded DNA, is set by other  
93 contacts involving amino-acid side-chains of the RNAP  $\beta$  and  $\beta'$  subunits (which interact with  
94 the promoter Core-recognition element, CRE) (Figure 1C) [16].

95  
96 The activity of any bacterial promoter is set by the formation of the 'closed complex' and the  
97 ensuing isomerisation to, and escape from, the 'open complex', as the RNAP copies the  
98 template strand to elongate its transcript. In the case of the *E. coli lac* operon promoter,  
99 defects in the UP element, -35 element and extended -10 element hinder 'closed complex'  
100 formation but this is remedied by binding of an activatory factor, the cyclic AMP receptor  
101 protein (CRP) to a target sequence centred between bp 61 and 62 (referred to as  
102 position -61.5) upstream of the transcript start (denoted as +1; see Figure 1D) [18, 19]. A  
103 second regulator protein, the lactose operon repressor protein (LacI) binds to a high affinity  
104 target sequence, known as operator O1, centred at position +11 (Figure 1D) [19, 20]. LacI  
105 binding to its target effectively shuts down *lac* promoter activity, but repression can be  
106 broken by the presence of allolactose (a breakdown product of lactose) or by the sugar  
107 analogue IPTG (isopropyl  $\beta$ -D-1-thiogalactopyranoside), which both bind to the LacI  
108 repressor and cause it to release operator DNA [20]. LacI-dependent repression of the *lac*  
109 promoter is supported by LacI binding to two secondary weaker operators, O3, located at  
110 position -82 (Figure 1D), and far downstream, O2 [19-23]. Here, we review how the starting  
111 *lac* promoter has been engineered to make it fit for different biotechnology purposes. We  
112 focus on constructions that release the requirement for CRP, base changes in key promoter  
113 elements, and the exploitation of the O3 operator.

### 114 115 **Activator-independent *lac* promoter derivatives**

116 Figure 2A illustrates the organisation of the *E. coli lac* operon promoter, showing key  
117 promoter elements, the DNA site for CRP, and the location of LacI-binding operators, O1  
118 and O3. Figure 2B shows the base sequence of a typical DNA fragment carrying the *lac*  
119 promoter that might be used in any biotechnology application. The fragment, which carries a  
120 useful restriction site at each end, is denoted *lac* O3O1. Potentially, regulation by CRP might  
121 be exploitable but, since CRP activity is difficult to control by external cues, many  
122 biotechnology applications that use the *lac* promoter have sought to eliminate CRP effects  
123 and focussed on regulation by LacI. One way to do this is by the use of the *lac* UV5 mutant  
124 promoter. This mutant promoter carries a 2 bp change in the promoter -10 hexamer element  
125 that creates a consensus -10 promoter element (Figure 2B) [19, 24]. The alternative is to  
126 replace *lac* promoter upstream DNA sequences and this has been done in the *tac* promoter,  
127 which is a chimeric fusion between the upstream elements of the *E. coli trp* promoter and the  
128 downstream elements of the *lac*UV5 promoter [1, 2]. Figure 2A illustrates both the *lac*UV5  
129 and *tac* promoters, and Figure 2B shows the base sequence of DNA fragments carrying  
130 these promoters. Note that the *tac* promoter carries consensus -35 and -10 promoters  
131 elements and a single operator for LacI, O1. For *E. coli* promoters that are dependent on the  
132 housekeeping sigma factor  $\sigma^{70}$  the optimal spacing between the -35 and -10 promoter  
133 elements is 17 bp and deviation from this leads to a reduction in promoter activity [25, 26].  
134 Thus, it worth noting that both the *tac* and *lac* promoters are in fact suboptimal, having  
135 spacers of 16 and 18 bp, respectively (Figure 2B).

136  
137 Figure 3A-C illustrates the results of simple assays to compare the activities of the *tac*,  
138 *lac*UV5 and *lac* promoters. To perform these assays, the *tac* O1 fragment, the *lac*UV5 O3O1  
139 fragment and the *lac* O3O1 fragment were each cloned into a plasmid expression vector  
140 (pRW50) such that the promoters controlled transcription of the gene (*lacZ*) encoding  $\beta$ -  
141 galactosidase [27]. Recombinant plasmids were then transformed into a  $\Delta$ *lac* *E. coli* host

142 strain and  $\beta$ -galactosidase expression was measured. As expected the hierarchy of  
143 promoter activity was *tac* > *lacUV5* > *lac* (Figure 3).

144

### 145 **Modulation of promoter activities using upstream *lac* operator sequences**

146 A key feature of LacI-dependent repression of the *E. coli lac* promoter is the contribution of  
147 the auxiliary upstream operator, O<sub>3</sub>, and this has been exploited to tailor promoter activity  
148 levels. Data in Figure 3A illustrate how the introduction of certain *lac* operator sequences (*i.e.*  
149 O<sub>1</sub> or the high affinity “ideal” *lac* operator O<sub>id</sub>, see Figure 2A [21, 28, 29]) at position -82 of  
150 the *tac* O<sub>1</sub> promoter fragment reduced the high activity of the *tac* promoter. Thus, expression  
151 from the starting *tac* O<sub>1</sub> promoter fragment and each of the *tac* O<sub>3</sub>O<sub>1</sub>, *tac* O<sub>1</sub>O<sub>1</sub> and *tac*  
152 O<sub>id</sub>O<sub>1</sub> derivatives was induced by IPTG but the introduction of the O<sub>1</sub> and O<sub>id</sub> operator  
153 sequences decreased IPTG-induced expression levels. Similarly, data in Figures 3B and 3C  
154 illustrate how the introduction of higher affinity *lac* operators into the upstream region of the  
155 *lacUV5* promoter (*i.e.* in the *lacUV5* O<sub>1</sub>O<sub>1</sub> and *lacUV5* O<sub>id</sub>O<sub>1</sub> promoter fragments) or the *lac*  
156 promoter (*i.e.* in the *lac* O<sub>1</sub>O<sub>1</sub> and *lac* O<sub>id</sub>O<sub>1</sub> promoter fragments) decreased promoter  
157 activity.

158

### 159 **The power of *lac*: combinations make anything possible**

160 In addition to controlling promoter activity by upstream-bound LacI, activity can be  
161 modulated by point mutations in different promoter elements. Data in Figure 3D illustrate  
162 how the p34G, p14G, p9A or p8A substitutions (at positions -34, -14, -9 or -8), which make  
163 the -35 element, the extended -10 or the -10 element more similar to the respective  
164 consensus, can be combined with different operator combinations to produce a suite of  
165 IPTG-inducible promoters with a wide range of activities.

166

167 To illustrate the use of these promoters in RPP, we selected a subset of 8 promoters from  
168 the above suite and gave each a promoter activity rating (PAR) value of PAR<sub>1</sub> to PAR<sub>8</sub>  
169 (Figure 4A). Our rationale for this is that, depending on the target protein being expressed,  
170 specific IPTG-induced expression levels could be achieved. To examine this, some of the  
171 promoter constructs were introduced into the low-copy-number vector pTorA-GFP [30] and  
172 the high-copy-number vector pHAK1 [31], using standard techniques [32-34]. For pTorA-  
173 GFP derivatives, each PAR promoter drives the expression of a *torA-GFP-6his* fusion (GFP,  
174 green fluorescent protein), whilst, for pHAK1, each construct expresses a *torA-hGH-6his*  
175 fusion (hGH, human growth hormone). Note that the *torA* signal sequence in each case will  
176 direct the recombinant protein to the Tat (twin arginine translocon) system for periplasmic  
177 targeting [7, 30, 31]. Plasmid constructs were transferred into *E. coli* BL21 cells and RPP  
178 was induced in bacterial cultures by the addition of 1 mM IPTG for 3 hrs [7]. Normalised total  
179 cellular protein from cells, expressing either TorA-GFP-6His or TorA-hGH-6His, were then  
180 analysed by Western blotting. Results in Figures 4B and 4C show that both GFP and hGH  
181 were expressed using the PAR promoter constructs. Note that, in some instances, two  
182 product bands can be observed, in each case the species with the higher molecular weight  
183 still carries the TorA signal sequence, whilst the smaller species has been processed and  
184 lacks the TorA moiety [7, 30, 31, 35]. Importantly, using this expression system, inducible  
185 expression of different target proteins can be set to specific levels when using both low- and  
186 high-copy-number vectors.

187

### 188 **Perspectives**

189 The *lac* promoter and its derivatives have been widely used to express many recombinant  
190 proteins to high levels in *E. coli* [1, 2, 7, 36] and many currently used vectors have been  
191 designed to optimise expression. However, there are situations where expression must be  
192 moderated. For example, the secretion of recombinant biopharmaceuticals out of the *E. coli*  
193 cytoplasm into the periplasm is often a preferred industrial strategy, as this minimises  
194 downstream processing costs, since the target protein can be purified from the periplasmic  
195 contents, with minimal cellular and DNA contamination [37]. For this to be successful, RPP  
196 needs to be slowed down so that product is not degraded before it is transported [7].

197

198 In this review, we have sought to show how knowledge of the *E. coli lac* promoter can be  
199 exploited to produce a suite of derivative promoter fragments to cope with any situation.  
200 Previous promoter engineering had focused on altering the *lac* promoter -10 and -35  
201 elements to change the basal promoter expression of constructs [1, 38]. Recent advances in  
202 our understanding of transcript initiation in bacteria and its regulation now allow *lac*  
203 promoters to be constructed with different operator sequences to alter the induced level of  
204 expression. This is possible because, even in the presence of inducer, LacI has some affinity  
205 for its operator sequence and, thus, in the induced state the LacI repressor can still remain  
206 bound to DNA, as has been observed in single molecule studies [39].

207

208

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213

#### 214 **Author contributions**

215 D.F.B., S.J.W.B. and C.R. conceived and designed the research programme. D.F.B, R.E.G  
216 and K.L.R. performed the experiments. D.F.B. wrote the manuscript with input from all  
217 authors.

218

#### 219 **Competing Interests**

220 The Authors declare that there are no competing interests associated with the manuscript.

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328

329 **FIGURE LEGENDS**

330

331 **Figure 1. Transcript initiation at bacterial promoters and regulation at the *lac* promoter**

332 **(A)** Bacterial promoter elements. A sketch showing the organization of different promoter  
333 elements at a model bacterial promoter with respect to the transcript start site (+1). Elements  
334 are denoted by coloured rectangles with their consensus sequence motifs indicated. Note  
335 that bacterial promoters usually contain a selection of these elements but rarely contain all of  
336 them [40].

337 **(B)** Sketch illustrating the key interactions between different domains of the RNAP sigma  
338 and alpha subunits and different promoter elements in the initial “closed” complex at a  
339 promoter. Note that the DNA duplex remains double-stranded and that the contacts result in  
340 Sigma Domain 2 being positioned adjacent to the promoter -10 element.

341 **(C)** Sketch illustrating the key interactions between different RNAP determinants and  
342 different promoter elements in the transcriptionally-competent “open” complex at a promoter.  
343 Note that the DNA duplex around position +1 is unwound, with Sigma Domain 2 making  
344 specific base contacts with the single-stranded non-template strand of the promoter -10  
345 element, thereby permitting the single-stranded template strand to enter the RNAP active  
346 site.

347 **(D)** A schematic representation of the *E. coli lac* operon promoter. The -35 and -10 promoter  
348 elements are shown as green and yellow boxes, respectively, the *lac* operator sequences  
349 (O1 and O3) are red boxes and the CRP binding site is shown by inverted arrows. The  
350 position of each element is given with respect to the start site of transcription (+1). The LacI  
351 tetramer binds to the O1 and O3 operators to form a repression loop, silencing *lac* operon  
352 expression (-ve), whilst CRP activates transcription (+ve).

353

354

355 **Figure 2. Organisation of *lac*, *tac* and *lacUV5* promoter constructs**

356 **(A)** The panel shows schematic representations of the *lac* O3O1, *lacUV5* O3O1 and *tac* O1  
357 promoters and the important elements involved in their regulation. All numbering is in  
358 relation to the promoter transcription start (+1), which is indicated by a bent arrow. The O1  
359 and O3 operator sequences, which bind the LacI repressor, are indicated by red boxes,  
360 the -35 promoter elements by green boxes, and the CRP site, within the *lacUV5* and *lac*  
361 promoters, is represented by inverted arrows. The *plac* -10 promoter element is shown as a  
362 yellow box, whilst the *placUV5* and *ptac* consensus -10 elements are gold. The sequence of  
363 the O3, O1 and Oid operators, and the site at which different operator sequences were  
364 introduced into the *tac* O1, *lacUV5* O3O1 and *lac* O3O1 promoters is shown by an arrow.

365 **(B)** The panel shows the base sequence of DNA fragments carrying the *lac* O3O1, *lacUV5*  
366 O3O1 and *tac* O1 promoters. All promoter fragments carry the relevant sequences from -92  
367 to +38, in relation to the transcription start, and possess terminal EcoRI and HindIII sites for  
368 cloning into the *lacZ* expression vector, pRW50 [27]. The O1 and O3 operator sequences  
369 are highlighted in grey, CRP binding targets are underlined and the -35 and -10 promoter  
370 elements are in bold and underlined, having also been aligned to the relevant consensus  
371 sequence for each element [40]. Note that the *lacUV5* promoter only differs from the wild-  
372 type *lac* promoter by carrying a consensus -10 element (*i.e.* the p8A and p9A substitutions)  
373 [24].

374

375 **Figure 3. Expression levels of engineered *ptac* and *plac* promoter derivatives**

376 The figure illustrates measured  $\beta$ -galactosidase activities of *E. coli* K-12 strain JM109 ( $\Delta lac$ ,  
377 *lac<sup>+</sup>*) cells carrying pRW50 [27, 41] containing different promoter fragments. Promoter  
378 fragments were generated using PCR and cloned into pRW50 to create *lacZ* transcriptional  
379 fusions, using standard techniques [27, 33]. Panels **(A)**, **(B)** and **(C)** show the effect of  
380 introducing the O3, O1 and Oid operator sequences into the upstream region of the *tac* O1,  
381 *lacUV5* O3O1 and *lac* O3O1 promoter fragments, respectively, as displayed in Figure 2. **(D)**  
382 The panel details the effect of introducing point mutations into the -10 and -35 elements of  
383 various *lac* promoter derivatives (*i.e.* *lac* O3O1, *lac* O1O1, *lacUV5* O3O1, *lacUV5* O1O1) to

384 improve these regions in relation to the extended -10 and -35 consensus sequences (see  
385 Figure 2) [42]. By convention, locations are labelled in relation to the *plac* transcript start  
386 point (+1). Note that the *lac* O1O1 D19 promoter carries a base pair deletion at position -19.  
387 The fold increase in expression, in comparison to the weakest promoter (*lac* O1O1) is  
388 indicated for each promoter in the presence of IPTG. In all panels, JM109 cells were grown  
389 in LB medium until mid-exponential phase, in the presence or absence of 1 mM IPTG.  $\beta$ -  
390 galactosidase activities were determined using o-nitrophenyl- $\beta$ -D-galactopyranose (ONPG)  
391 and a Miller protocol, as in our previous work [43], and are expressed as nmol of ONPG  
392 hydrolysed  $\text{min}^{-1} \text{mg}^{-1}$  dry cell mass. Each activity is the average of three independent  
393 determinations and the error bars represent the standard deviation of values.

394  
395

396 **Figure 4. The expression of TorA-protein fusions can be set to different levels with**  
397 **PAR promoter constructs**

398 **(A)** Expression from the PAR promoter constructs. The panel shows the  $\beta$ -galactosidase  
399 activities measured in the *E. coli* K-12 strain JM109 ( $\Delta lac$ , *lacI<sup>q</sup>*) containing the *lacZ*  
400 expression vector pRW50 carrying various PAR promoters. PAR promoters (PAR1 to PAR8)  
401 were selected from the various *tac* and *lac* promoters detailed in Figure 3.  $\beta$ -galactosidase  
402 activities are expressed as nmol of ONPG hydrolysed  $\text{min}^{-1} \text{mg}^{-1}$  dry cell mass. Each activity  
403 is the average of three independent determinations and standard deviations are shown for  
404 all data points. **(B)** Expression of a TorA-GFP-6His protein fusion in BL21 cells (Novagen),  
405 using pGFP-TorA based vectors [30]. PAR promoters, PAR4 to PAR6 and PAR8 were  
406 introduced into the low-copy-number vector pGFP-TorA, such that each promoter drives the  
407 expression of a *torA-gfp-6his* fusion. Note that pGFP-TorA is a derivative of expression  
408 vector pEXT22 [44], which has been included as an empty vector control. **(C)** Expression of  
409 a TorA-hGH-6His protein fusion in BL21 cells, using pHAK1 based vectors [31]. PAR  
410 promoters, PAR5 to PAR8 were introduced into the high-copy-number vector pHAK1 such  
411 that each promoter drives the expression of a *torA-hGH-6his* fusion. Note that the *torA* signal  
412 sequence directs the GFP-6His and hGH-6His moieties to the Tat translocon for periplasmic  
413 targeting [7, 30, 31]. Cells were grown in LB medium until an  $\text{OD}_{600}$  of  $\sim 0.4$  when RPP was  
414 induced by the addition of 1 mM IPTG for 3 hrs. In **(B)** and **(C)** expression of TorA-GFP-6His  
415 and TorA-hGH-6His was analysed by Western blotting (upper panel), using either anti-GFP  
416 antibody (Sigma) or polyclonal anti-hGH antibody, respectively, and a Coomassie blue  
417 stained SDS-PAGE gel (lower panel), using normalised total cell protein from BL21 cells, as  
418 detailed in [45]. Samples were calibrated by loading Page Ruler Plus prestained markers  
419 (Thermo Scientific). Note that, in the Western blot in panel **(B)**, all lanes were from the same  
420 blot and have only been separated to aid presentation of the data.

Figure 1

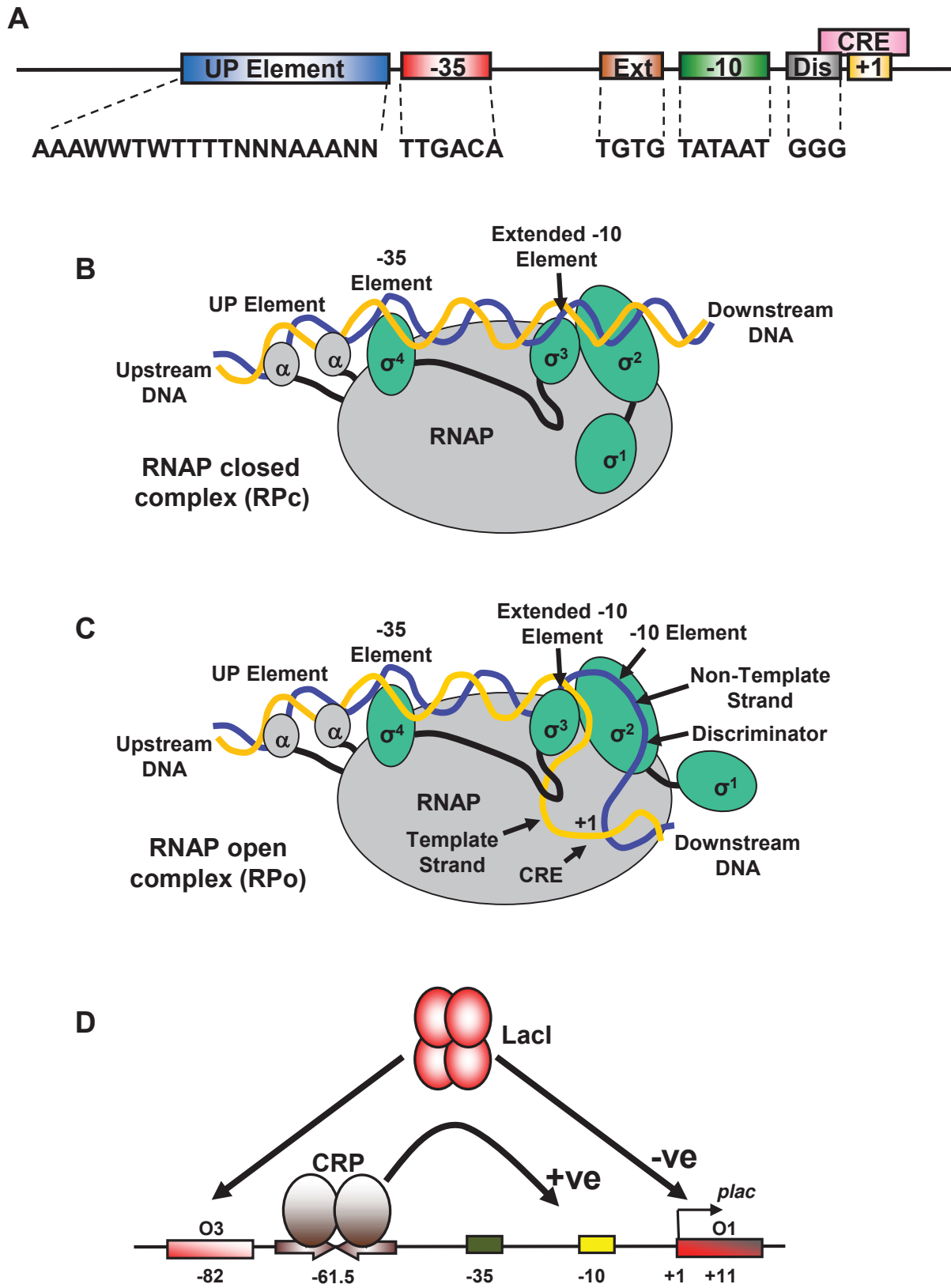
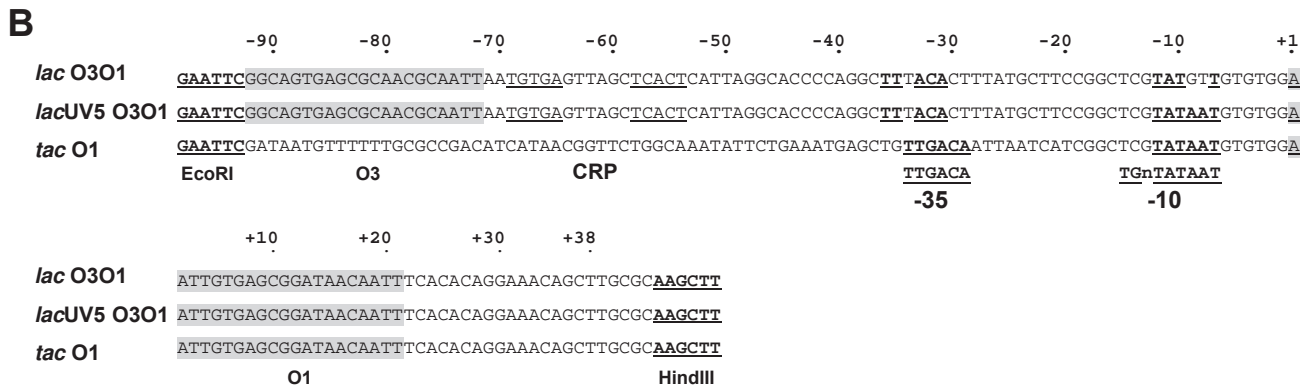
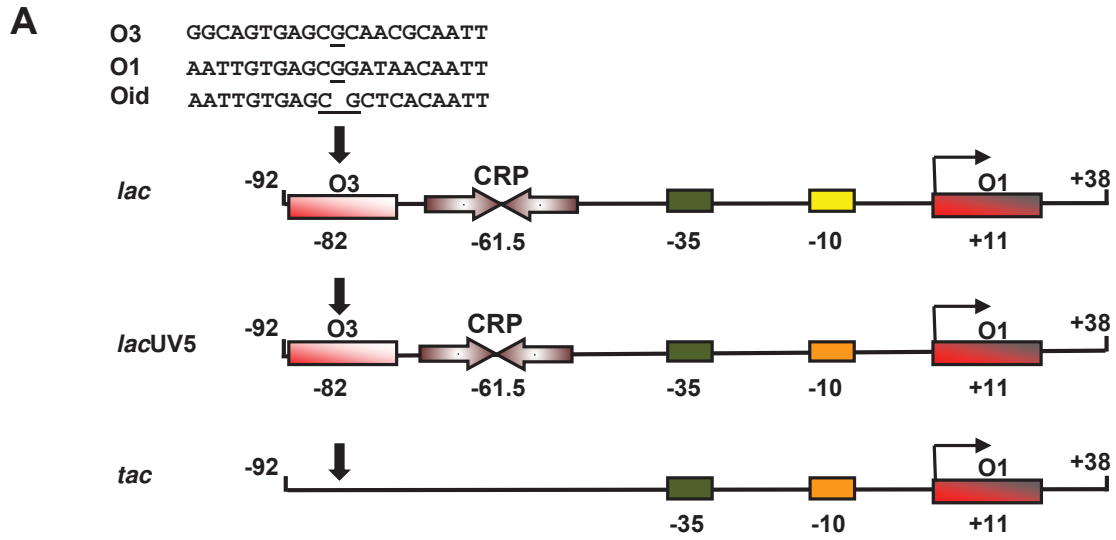
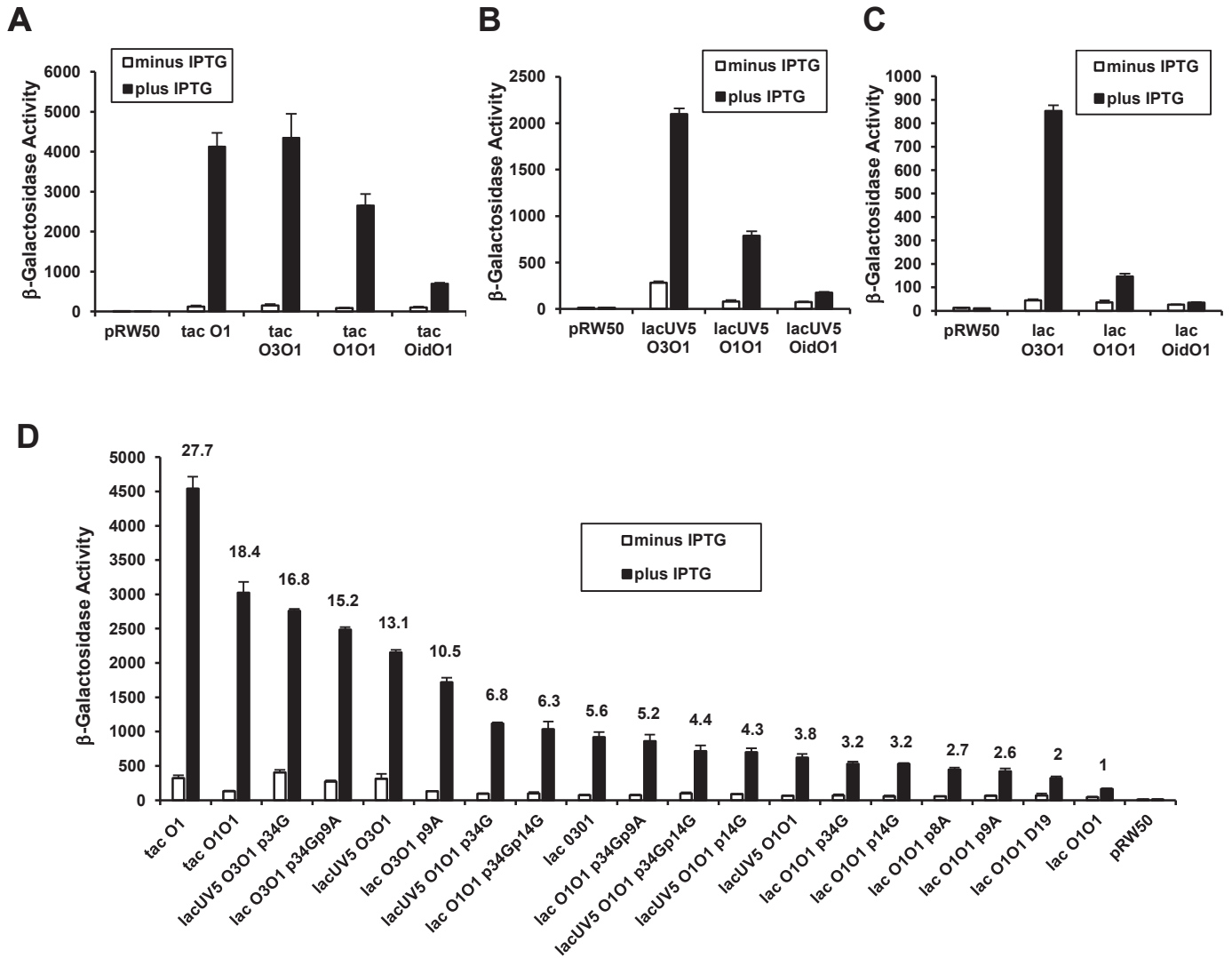


Figure 2

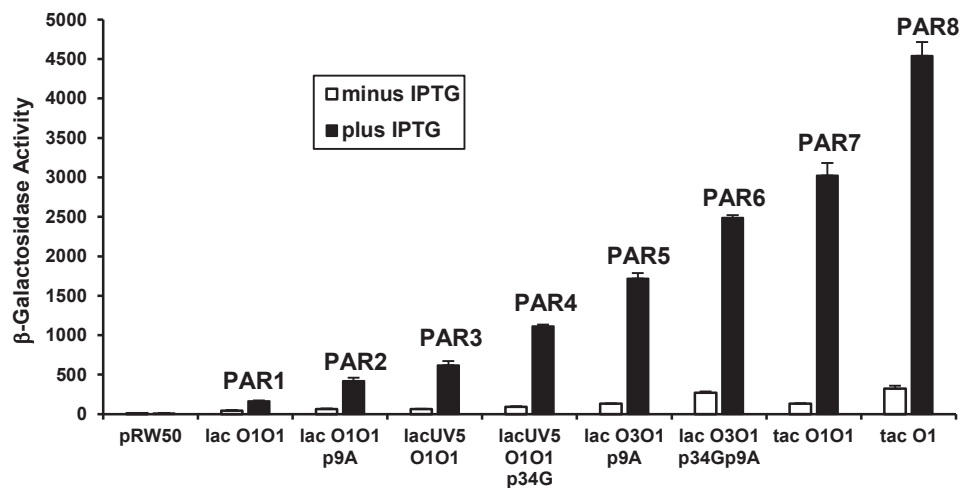


**Figure 3**

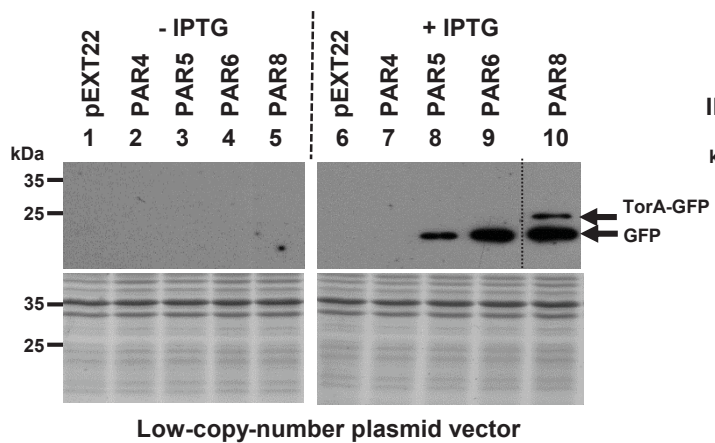


**Figure 4**

**A**



**B**



**C**

