

Pr is a member of a restricted class of σ^{70} -dependent promoters that lack a recognizable -10 element

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ABSTRACT

The Pr promoter is the first verified member of a class of bacterial σ^{70} -promoters that only possess a single match to consensus within its -10 element. In its native context, the activity of this promoter determines the ability of *Pseudomonas putida* CF600 to degrade phenolic compounds, which provides proof-of-principle for the significance of such promoters. Lack of identity within the -10 element leads to non-detection of Pr-like promoters by current search engines, because of their bias for detection of the -10 motif. Here, we report a mutagenesis analysis of Pr that reveals strict sequence requirements for its activity that includes an essential -15 element and preservation of non-consensus bases within its -35 and -10 elements. We found that highly similar promoters control plasmid- and chromosomally- encoded phenol degradative systems in various *Pseudomonads*. However, using a purpose-designed promoter-search algorithm and activity analysis of potential candidate promoters, no *bona fide* Pr-like promoter could be found in the entire genome of *P. putida* KT2440. Hence, Pr-like σ^{70} -promoters, which have the potential to be a widely distributed class of previously unrecognized promoters, are in fact highly restricted and remain in a class of their own.

INTRODUCTION

The sequential steps of bacterial transcriptional initiation begin with the binding of the multi-subunit RNA polymerase (RNAP; subunit composition $\sigma\alpha_2\beta\beta'\omega$) to specific DNA elements within a promoter sequence to form a closed DNA promoter complex. Subsequent steps to form the transcriptionally competent open complex

involve multiple conformational changes in both the DNA and RNAP that alter their interaction—with each step presenting a potential target for regulation of the initiation process (reviewed in 1 and 2). In bacteria, initiation of transcription from promoters requires a σ -factor. All bacteria possess a housekeeping σ -factor (σ^{70} in *Escherichia coli* and *Pseudomonas putida*) that controls transcription from the majority of promoters, although most also employ additional alternative σ -factors for recognition of other classes of promoters (reviewed in 3).

RNAP is directed to promoter regions by the σ -factors, which provide sequence-specific recognition determinants for promoter motifs. For σ^{70} -promoters, the major motifs that determine promoter recognition are classically considered to be the -35 (consensus $^{-35}\text{TGACA}^{-30}$) and -10 (consensus $^{-12}\text{TATAAT}^{-7}$) elements that are contacted by the σ_4 and σ_2 subregions of σ^{70} , respectively (4). Within some promoters, the spacer region between the -35 and -10 elements (ranging between 15 and 19 bp, with 17 bp being the most optimal) contains an extended -10 element (consensus $^{-15}\text{TGn}^{-13}$) for contact with the σ_3 subregion of σ^{70} . An additional feature of some promoters is a discriminator that provides contact through subregion $\sigma_{1,2}$ of σ^{70} and the non-template G at -5 (reviewed in 5). In addition to these σ -mediated contacts, the α -subunits of RNAP can also interact with AT-rich UP elements within DNA upstream of promoters (2 and references therein). UP elements, the -35 element and the extended -10 element are all bound by σ^{70} -RNAP as double-stranded DNA (dsDNA) and thus may be used for promoter recognition and formation of the initial closed complex. On the other hand, the -10 element is recognized as both dsDNA (-12T) and as single-stranded DNA (ssDNA of the non-template strand, positions -11 to -7). The single-stranded sequences at positions -11 to -7 as well as the -5 G contribute to later steps in transcription initiation that involve isomerization (melting) and separation of the promoter DNA around the transcription start site (2).

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Natural promoters do not necessarily possess all these DNA elements and the relative contribution of each for RNAP binding to form the initial closed promoter complex differs from promoter to promoter. Recently, a re-delineation of σ^{70} -promoter elements has been proposed based on recognition as either dsDNA or ssDNA and on involvement in closed and/or open complex formation (2). These considerations introduce the concept of the -15 element (from -15 to -12 ; consensus $^{-15}\text{TGGT}^{-12}$) and the so-called short -10 element (from -11 to -7 , consensus $^{-11}\text{ATAAT}^{-7}$) (2,6). Within this classification system, the -35 and -15 elements are directly related to σ^{70} -dsDNA interactions and thus closed complex formation, whereas the short -10 element is directly related to σ^{70} -ssDNA interactions and thus open complex formation.

The σ^{70} -Pr promoter controls transcription of the master DmpR regulator of the plasmid-encoded *dmp*-system for (methyl)phenol catabolism by *P. putida* CF600. Previous deletion and footprinting analysis have delineated the Pr promoter region (-35 to $+1$, relative to the transcriptional start) and demonstrated that it functions independently of any UP element (7,8). The Pr promoter is intrinsically weak and requires the action of ppGpp and DksA on σ^{70} -RNAP to stimulate efficient activity in both *P. putida* and *E. coli* (7,8 and references therein). Previous work traced the poor performance of Pr to its extremely suboptimal -10 element that compromises both binding of σ^{70} -RNAP and the rate of open complex formation in the absence of ppGpp and DksA (8). The Pr promoter is unusual in possessing only one out of six matches to the -10 consensus sequence and represents the first verified bacterial σ^{70} -promoter that lacks a recognizable -10 element. As such, Pr provides proof-of-principle that such promoters can function in a biologically significant context (8).

The lack of identity with the consensus -10 element leads to mis-predictions or non-detection of the Pr promoter using promoter search engines. Therefore, it seems plausible that other ' -10 -less' σ^{70} -promoters exist but have eluded detection. Such promoters could potentially comprise a widely distributed class of previously unrecognized promoters (8) that might, e.g., be responsible for the frequently observed transcripts originating from within bacterial operons (9). To address this issue, we have undertaken extensive mutagenesis of Pr to define the requirements for its activity. Based on these results, a flexible algorithm was developed to search for similar promoters within bacterial genomes. Our results show that similar ' -10 -less' promoters are utilized in other chromosomally- and plasmid-encoded systems for degradation of phenolics; however, no other functional candidate ' -10 -less' promoter could be identified within the *P. putida* KT2440 genome. Hence, Pr (and Pr-like promoters of other phenol degradative systems) remains in a class of its own.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Escherichia coli DH5 (10) and *P. putida* KT2440 (11) were cultured in Luria-Bertani/Lennox medium (AppliChem

GmbH) at 37°C and 30°C , respectively. Cultures were supplemented with carbenicillin (Cb, $100\ \mu\text{g ml}^{-1}$ for *E. coli* or $1000\ \mu\text{g ml}^{-1}$ for *P. putida*) to select for the resident plasmid.

Plasmid constructions

All luciferase transcriptional reporter plasmids (Supplementary Table S1) are based on pVI928 that carries the promoter-less *luxAB* genes of *Vibrio harveyi* downstream of a poly-cloning site (7). Reporter plasmids were constructed by inserting synthetic double-stranded linkers with the desired promoter sequence bounded by SmaI and BglII/BamHI compatible ends (Supplementary Table S2) between the SmaI and BglII sites of pVI928. The fidelity of the promoter regions generated by insertion of linkers was confirmed by DNA sequencing.

Luciferase assays

For screening promoter activity, individual colonies of *E. coli* DH5 or *P. putida* KT2440 harbouring different transcriptional reporter plasmids were streaked on solid medium and incubated overnight at 30°C . After addition of $100\ \mu\text{l}$ of 1:1 diluted decanal (luciferase substrate) to the lid of inverted plates, light emission was documented using an X-ray film. Quantitative luciferase assays were performed on cultures grown and assayed at 30°C essentially as previously described (12). To ensure balanced growth, overnight cultures were diluted 1:50 and grown into exponential phase before a second dilution to an OD_{600} of 0.05–0.08 and initiation of the experiment. Light emission from $100\ \mu\text{l}$ of whole cells using a 1:2000 dilution of decanal was measured using an Infinite M200 (Tecan) luminometer. Specific activity is expressed as relative luciferase units per OD_{600} of 1.0.

User-specified algorithm for promoter search within bacterial genomes

A program was developed and implemented in the language R version 12.1 to identify user-defined promoter motifs. The program uses a flexible algorithm that can be applied to identify a wide range of pre-defined promoter motifs in any bacterial genome. The program—available with instructions at snovit.math.umu.se/promotersearch.html—contains three components: the Input, Identification and Output components. Within the Input component, the user uploads the genome sequence of interest and, if available, a file describing the locus tags and activities encoded by the open reading frames (ORFs). The user specifies the minimum length of the ORFs. Within the Identification component, the type of 'candidate' promoter motifs to be searched for is specified. In order to reduce the number of false positives, the program also contains the option to additionally search for alternative 'competitive' promoter motifs that are close(er) to the consensus motif. The user can choose to ignore this option. In addition to user-defined candidate and competitive motifs, criteria (rules) for the length of the sequence upstream of the ORFs to be searched, the maximum/minimum spacing between -35 and -10 promoter elements and maximum/minimum spacing of

the -10 element from the start codon can be varied. Logical AND and OR commands can be used to combine several rules. Within the Output component, an Excel file is generated in which the following information is provided for each identified candidate promoter: (i) the start and end positions of the associated ORF; (ii) the start and end of the candidate promoter; (iii) the sequence of the candidate promoter; (iv) the distance between the -35 and -10 motifs; (v) the distance between the -10 element and the ORF's start codon; (vi) a list of potential competitive motifs [with similar information as specified in (i)–(v)] and (vii) the locus tag and activity encoded by the ORF if the information was supplied.

Our searches for Pr-like promoters within the *P. putida* KT2440 genome (NC_002947.3) employed the following criteria. The minimum ORF length was set as 30 codons with a search region of 8–350 bp upstream of the start codon (ATG or GTG). Candidate promoters were defined by the criteria: (i) at least five matches to the -35 consensus sequence and only one match to the -10 consensus element ($-7T$, i.e. XXXXT) and (ii) spacing between the -35 and -10 elements should be 17 bp. Competitive promoter motifs within the same search region were defined by the criteria: (i) at least 8 out of 12 matches to the classical σ^{70} -35 and -10 consensus sequences ($^{-35}\text{TGACA}^{-30}/^{-12}\text{TATAAT}^{-7}$) or at least 6 out of 8 matches to the extended -10 consensus ($^{-15}\text{TGnTATAAT}^{-7}$) and (ii) the spacing between the -35 and -10 sequences could be 16, 17 or 18 bp.

RESULTS

Mutagenesis of Pr reveals strict requirements for promoter activity

As outlined in the 'Introduction' section, Pr is the only verified σ^{70} -dependent promoter that possesses only one out of six matches to the -10 element consensus sequence ($^{-12}\text{CTGGCT}^{-7}$, consensus $^{-12}\text{TATAAT}^{-7}$), which is below random chance (8). This extremely suboptimal -10 element is separated by 17 bp from a near-perfect -35 element ($^{-35}\text{TGACT}^{-30}$, consensus $^{-35}\text{TGACA}^{-30}$). To rationally search for other potential members of this new promoter class, we first analysed the sequence requirements for Pr activity. To this end, we generated a series of luciferase transcriptional reporter plasmids in which different variants of the -38 to $+8$ Pr region control transcription of the *luxAB* genes. The activities of the different Pr derivatives, which carry mutations within the -10 element, the spacer or the -35 element (Figures 1A and 2A), were screened in *E. coli* DH5 using a simple luciferase plate test assay as described in 'Materials and Methods' section.

Within the context of Pr, the presence of a T in the -7 position is an absolute requirement, because exchange of the $-7T$ to A, G or C (which removes any similarity to the -10 element consensus sequence) abolished promoter activity (Figure 1B, 80 min exposure) (8). Moreover, no other single match to consensus resulted in promoter activity in the absence of $-7T$ (Figure 1C, 80 min exposure). Promoter variants carrying two out of six matches ($-7T$ and one more additional consensus base)

resulted in varying degrees of hyperactivity. Increased promoter activity mediated by the additional consensus base lay in the order: $-11A > -9A > -10T > -8A = -12T$ (Figure 1D, 2 min exposure). Note that the short exposure time used to visualize the hyperactivity of these derivatives leads to non-detection of the activity from the wild-type Pr promoter. The most powerful of these hyperactive derivatives ($^{-12}\text{CAGGCT}^{-7}$) was previously identified by a genetic approach and quantitatively mediates ~ 40 -fold higher output than the counterpart wild-type Pr reporter (8). Furthermore, we found that the spacing requirement between -10 and -35 elements was absolute, because derivatives with 16- or 18-bp spacing retained no promoter activity (Figure 1E).

Similar analysis of the near-perfect -35 element of Pr ($^{-35}\text{TGACT}^{-30}$, consensus $^{-35}\text{TGACA}^{-30}$) was performed by exchanging native bases for complementary bases. The results revealed that promoter variants with different combinations of only four (rather than five) out of six matches to the -35 consensus sequence exhibited no promoter activity (Figure 2B). Similarly, no combination of five out of six matches other than that found in the -35 element of wild-type Pr showed any activity either (Figure 2C). The only exchange we found to be tolerated was that of the non-consensus $-30T$ to a consensus A, which results in a perfect consensus -35 element. This exchange did not significantly increase promoter activity when comparing it with native Pr (Figure 2D); however, to our surprise, exchange of the non-consensus $-30T$ to C or G renders Pr non-functional (Figure 2D). Thus, based on these data, we conclude that the requirements for Pr activity are very strict, with only the following variations tolerated within the -35 element: $^{-35}\text{TGACT/a}^{-30} < 17 \text{ bp} >^{-12}\text{XXXXXT}^{-7}$.

Pr-like promoters function in phenol degradation systems of different *Pseudomonas* strains

The Pr promoter controls transcription of the specific regulator of phenol catabolism encoded by the pV1150 plasmid. A blast of the DNA sequence surrounding Pr highlighted the existence of potential promoters, either identical or very similar to Pr, located upstream of genes encoding cognate transcriptional activators belonging to other phenol degradation systems (Figure 3A). We found a sequence identical to Pr upstream of the chromosomal *pheR* gene of *P. putida* BH. We also identified sequences that are almost identical to Pr upstream of *phlR* of the catabolic pPGH1 plasmid of *P. putida* H and the chromosomally- encoded *phhR* gene of *P. putida* P35X. Both these potential promoters show only one match to consensus within their -10 elements ($-7T$ as in Pr), although, unlike Pr, they have a C rather than a T in the -11 position. In the case of the sequence detected upstream of *phlR*, there is one more additional difference—the G in the -4 position of Pr is exchanged to an A. In contrast, the potential promoter identified upstream of chromosomal *capR* of *P. putida* KCTC 1452 has two matches to the -10 consensus ($^{-12}\text{TCGGCT}^{-7}$ in *PcapR* as compared with $^{-12}\text{CTGGCT}^{-7}$ in Pr; Figure 3A).

To test if these are functional promoters, we constructed analogous transcriptional reporter plasmids where the

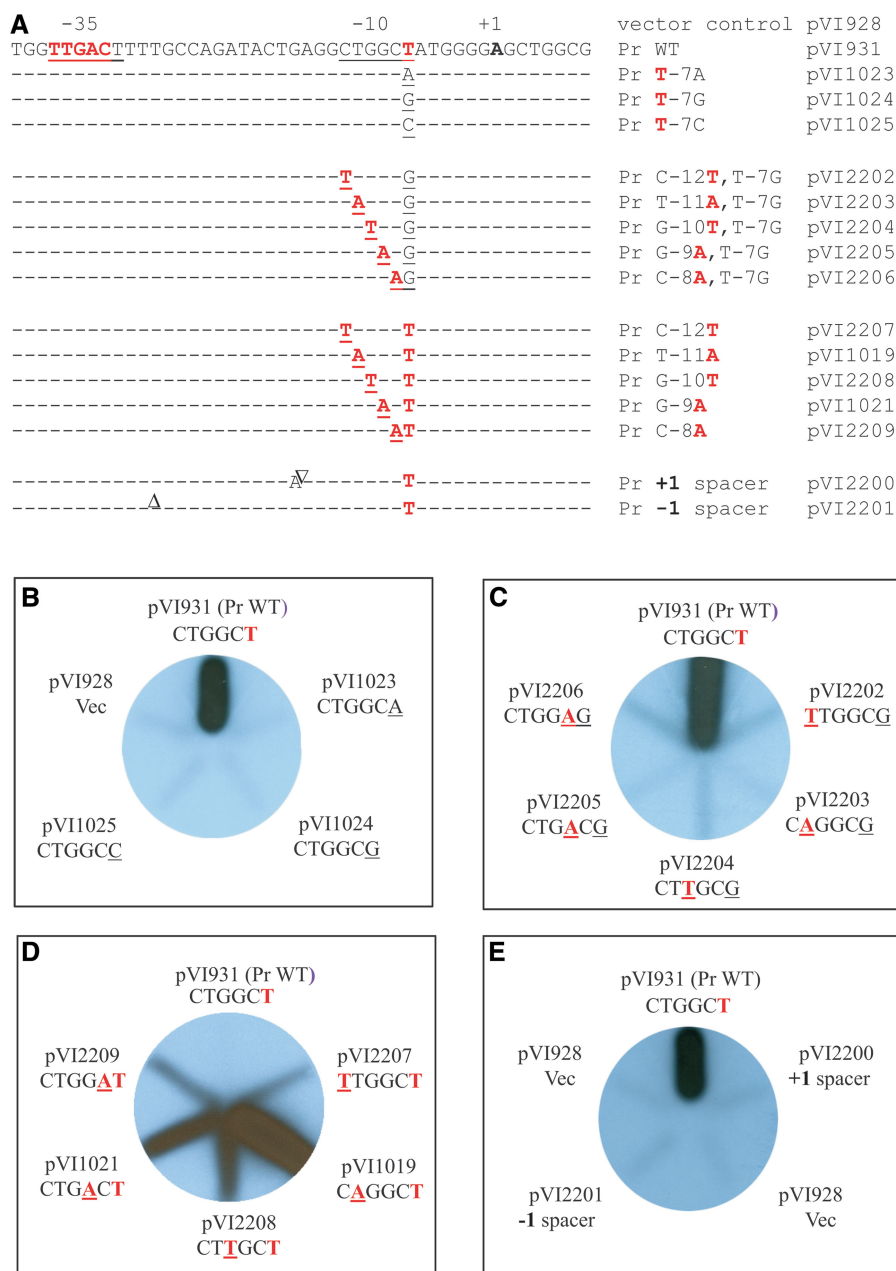


Figure 1. Analysis of the -10 element and spacer of Pr. (A) Sequence of the -38 to $+8$ region of Pr present in the indicated reporter plasmids used in panels B–E. The -35 and -10 elements are underlined with matches to their consensus sequences shown in red bold-case letters. The $+1$ start of the mRNA is indicated in bold-case letter. Insertion or deletion of 1 bp in spacer variants pVI2200 and pVI2201 is represented by an inverted and upright triangle, respectively. (B–E) Luciferase plate test assays employing *E. coli* DH5 carrying different transcriptional reporter plasmids with variations from the Pr sequence underlined. The pVI931 (Pr WT) and pVI928 (the promoter-less vector) are used as positive and negative controls, respectively. (B) Activities of variants carrying zero out of six matches to the -10 element consensus exposed for 80 min. (C) Activities of variants carrying one out of six matches to the -10 element consensus exposed for 80 min. (D) Activities of hyperactive variants carrying two out of six matches to the -10 element consensus—note that short exposure time (~ 2 min) leads to non-detection of Pr WT activity. (E). Activities of variants carrying a ± 1 bp alteration in the spacer between the -35 and -10 elements exposed for 80 min.

-38 to $+8$ sequence of these potential promoters control transcription of *luxAB*. Promoter activity was monitored in both *E. coli* DH5 and *P. putida* KT2440 strains by comparison with the wild-type Pr promoter (pVI931) and the promoter-less vector negative control (pVI928). The results from luciferase plate test assays show that all are functional in both *E. coli* DH5 (Figure 3B) and

P. putida KT2440 (Supplementary Figure S1B). However, all variants that differ from Pr are less active than Pr (Figure 3A and B and Supplementary Figure S1B). This is even the case for the *PcapR* promoter that has two matches to consensus within its -10 element, an exchange that renders Pr hyperactive (6.8-fold over wild type; Figure 3A). Thus, the presence of a non-consensus C

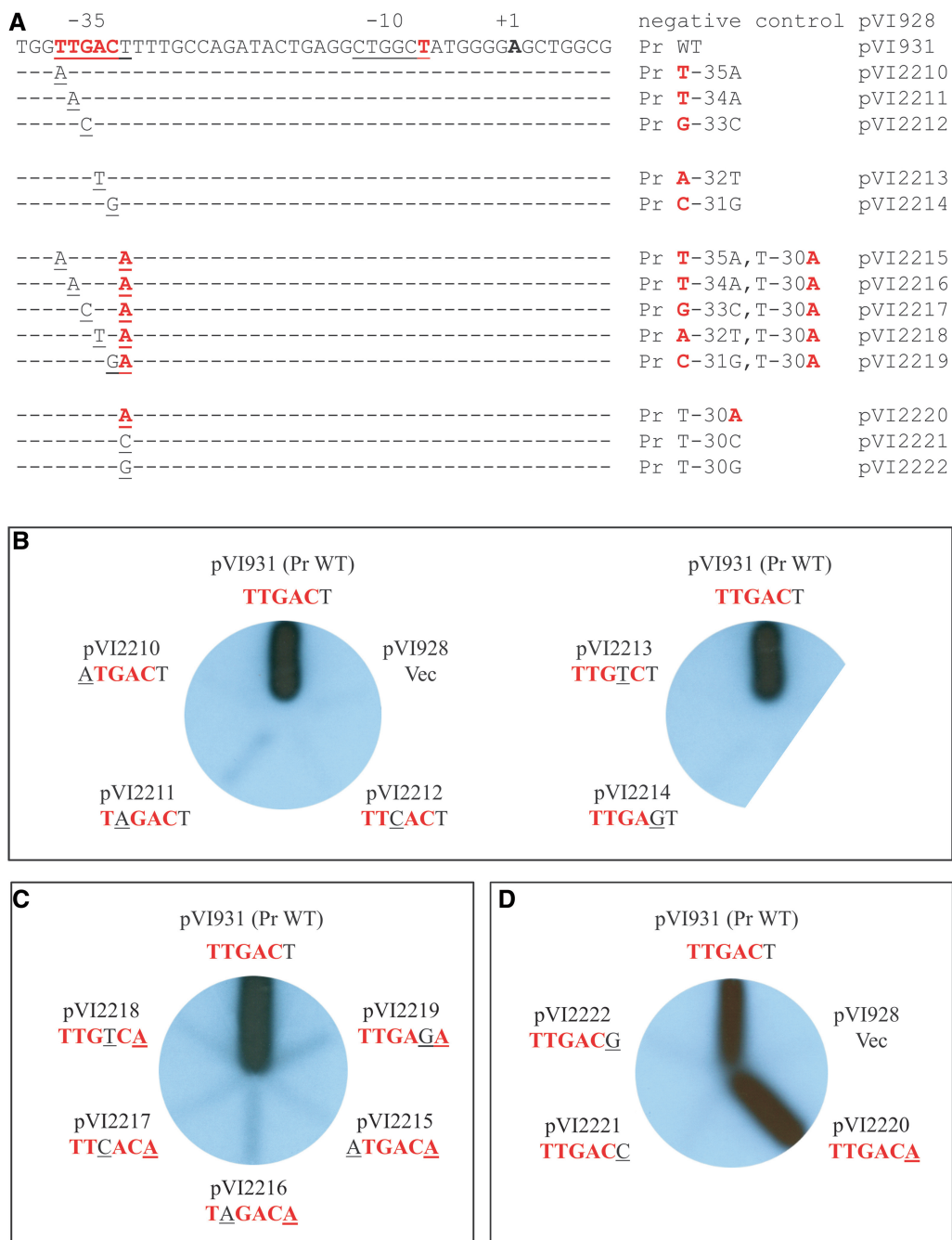


Figure 2. Analysis of the -35 element of Pr. (A) Sequence of the -38 to $+8$ region of Pr present in the indicated reporter plasmids used in panels B–D as described under Figure 1. (B–D) Luciferase plate test assays employing *E. coli* DH5 carrying different transcriptional reporter plasmids with variations from the Pr sequence underlined. pVI931 (Pr WT) and pVI928 (the promoter-less vector) are used as positive and negative controls, respectively, and an exposure time of 80 min was used in all cases. (B) Activities of variants carrying four out of six matches to the -35 element consensus (i.e. the non-conserved -30 T and one more mismatch). (C) Activities of variants carrying five out of six matches to the -35 element consensus. (D) Activities of variants carrying alternative bases in the -30 position.

instead of a non-consensus T at the -11 position of these promoters has a detrimental effect on promoter output, reducing activity to 23% of wild-type Pr. Similarly, a G-substitution at the -11 T position reduces activity, to 16% of wild-type Pr levels (Figure 3A). The additional G-4A substitution of the *phlR* promoter has a minor beneficial effect on activity, resulting in 30% activity as compared with the 23% activity of the *phhR* promoter

(Figure 3A), which may be due to increased ease of DNA melting afforded by the G-4A substitution.

The *PpheR*, *PphhR* and *PcapR* promoters have not previously been described. However, Burchhard *et al.* have proposed a different *PphlR* promoter than the one we suggest even though the experimentally determined $+1$ start sites of *PphlR* and Pr are identical (7,13). As illustrated in Figure 3C, the promoter proposed by

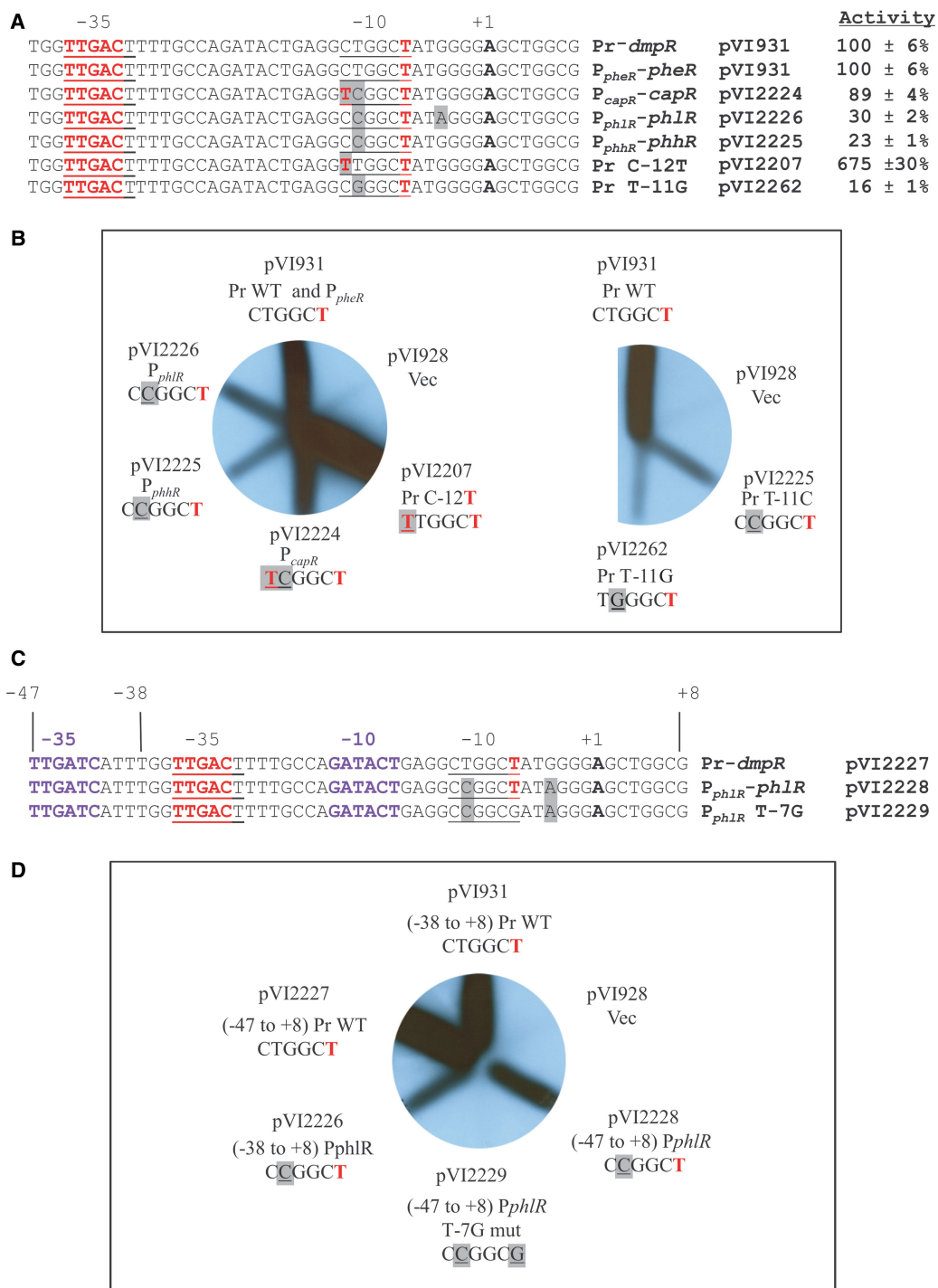


Figure 3. Activities of Pr-like promoters from other phenol degradation systems. (A) Sequence of Pr (*P. putida* CF600, plasmid pVI150), *PpheR* (*P. putida* BH, genome), *PcapR* (*P. putida* KCTC 1452, genome), *PphIR* (*P. putida* H, plasmid pPGH1), *PphhR* (*P. putida* 35X, genome) and the Pr C-12T and T-11G variants with the -35 and -10 elements underlined. Matches to the -35 and -10 consensus sequence are shown in red bold-case letters. The experimentally verified +1 of Pr and *PphIR* and the inferred +1 of other promoters are indicated in bold. Differences from the Pr sequence are highlighted in grey. Values for promoter activity are given relative to that of Pr (set as 100%) and are the average of duplicate determinations for at least two independent cultures ± SD. (B) Luciferase plate test assays with *E. coli* DH5 containing the indicated transcriptional reporter plasmids exposed for 80 min. pVI931 (Pr WT) and pVI928 (the promoter-less vector) are used as positive and negative controls, respectively. (C) Sequence of the -47 to +8 region of Pr, *PphIR* and its *PphIR* T-7G variant as in panel A. The -35 and -10 elements proposed by Burchhardt *et al.* 1997 (13) are shown in purple boxes. (D) Luciferase plate test assay with *E. coli* DH5 carrying the indicated transcriptional reporter plasmids exposed for 80 min.

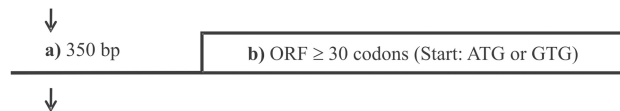
Burchhard *et al.* has a $^{-47}\text{TTGATC}^{-42}$ -35 element suboptimally spaced 19 bp away from a $^{-22}\text{GATACT}^{-17}$ -10 element. Hence, this proposed promoter lies 16 bp away from the +1 start site. In order to test which of the two is the real promoter, we generated different reporter variants (Figure 3C) and assayed promoter functionality as previously described. Two findings verify the *bona fide* *PphlR* promoter as a Pr-like promoter. First, reporter variants carrying the -47 to $+8$ regions of Pr (pVI2227) and *PphlR* (pVI2228) show only slightly higher activity than -38 to $+8$ counterparts that would lack the -35 element of the alternative promoter (pVI931 for Pr and pVI2226 for *PphlR*) in *E. coli* DH5 and *P. putida* KT2440 (Figure 3D and Supplementary Figure S1D). Second, substitution of the Pr-like -7T to G within the -47 to $+8$ *PphlR* variant completely abolished detectable promoter activity (pVI2229 in Figure 3D and Supplementary Figure S1D).

Pr-like promoters are a restricted class of σ^{70} -promoters

Pr-like σ^{70} -promoters have the potential to be a widely distributed class of previously unrecognized promoters (8). Having ascertained the requirements for Pr activity and that Pr-like promoters function in other systems, we next set out to search for additional Pr-like promoters. Because Pr is originally derived from a *P. putida* strain, we chose to search the genome of *P. putida* KT2440. For this, we developed a program that employs a flexible algorithm for identifying user-defined candidate promoters within bacterial genomes, as detailed in ‘Materials and Methods’ section. The program extraction and search rules are schematically summarized in Figure 4. Criteria based on the requirements of the Pr promoter for activity, as detected through our mutagenesis approach, were used to search for candidate Pr-like promoters within a 350-bp region upstream of ORFs greater than 30 codons, i.e. with a -35 element having five out of six matches to consensus and spaced 17 bp from a -10 element with only a -7T match to the consensus. Likely false positives, i.e. those with potential classical $-35/-10$ or extended -10 promoters with higher homology to consensus located downstream of candidate Pr-like promoters, were eliminated from consideration by must-not-have rules. The criteria for these must-not-have elimination rules were determined empirically by varying parameters to find the most permissive criteria that allowed the program to detect the wild-type Pr promoter sequence within the 15-kb DNA sequence of the *dmp*-system region. The final criteria chosen for our search are shown in Figure 4.

Analysis of *P. putida* KT2440 genome with the criteria as outlined in Figure 4 identified 81 Pr-like candidate promoters upstream of annotated ORFs. Of these, only two possessed features identified by our mutational analysis of Pr that would predict activity, i.e. a $^{-35}\text{TTGACT}/\text{a}^{-30}$ $\langle 17\text{bp} \rangle$ $^{-12}\text{XXXXXT}^{-7}$ configuration, while a further 10 candidates had a $^{-35}\text{TTGACg}/\text{c}^{-30}$ $\langle 17\text{bp} \rangle$ $^{-12}\text{XXXXXT}^{-7}$ promoter configuration (Table 1). All other candidates had an alternative combination of five out of six matches to the -35 consensus. Of these, we chose 9 for further analysis alongside the 12 candidates

I] Regulatory region extraction – rules:



II] Candidate identification – rules: “*must have*”

- c) -35 element: match ≥ 5 to consensus
- d) spacer 17 bp
- e) -10 element: specified match XXXXXT (1 match to consensus)
- f) distance between the -10 element and the start codon ≥ 8

III] False positive elimination – rules: “*must not have*”

- Downstream:
- g) extended -10 promoter element: match ≥ 6 to consensus
 - h) classical σ^{70} $-35/-10$ elements: total match ≥ 8 to consensus

Figure 4. Algorithm for genome searches of Pr-like promoters. The schematic shows the specifications used with the program to search for potential Pr-like promoter candidates in the *P. putida* KT2440 genome. All rules (a)–(h) can be varied by the user.

that had the Pr-like $^{-35}\text{TTGACX}^{-30}$ $\langle 17\text{bp} \rangle$ $^{-12}\text{XXXXXT}^{-7}$ configuration (Table 1). The additional nine were chosen because they either lay upstream of a gene encoding a transcriptional activator (as is the case for Pr), or contain additional homology within a potential -15 element (see next section). Upon testing the functionality of these 21 potential candidates in both *E. coli* DH5 and *P. putida* KT2440 using reporter plasmids, only two showed any activity (Supplementary Figures S2 and S3). The slight activity of these two candidate promoters in pVI2243 and pVI2244 was only observed in the *P. putida* KT2440 background. However, derivatives of these candidates carrying a T-7G substitution showed the same slight activity as their wild-type counterparts, eliminating them as potential Pr-like promoters (pVI2260 and pVI2261, respectively, Supplementary Figure S3).

The results above demonstrate that no Pr-like promoter is in primary control of transcription from any of the genes of *P. putida* KT2440. However, it is still possible that the false-positive elimination criteria used may have eliminated one or more genuine Pr-like promoters. Therefore, we re-ran the analysis without elimination criteria, resulting in detection of 19 additional candidates with a $^{-35}\text{TTGACT}/\text{a}^{-30}$ $\langle 17\text{bp} \rangle$ $^{-12}\text{XXXXXT}^{-7}$ configuration predictive of potential activity. However, a similar analysis as described above did not detect any functional Pr-like promoter (Supplementary Figure S4). Taken together, these data strongly suggest that no *bona fide* Pr-like promoters exist in the genome of *P. putida* KT2440. Therefore, we conclude that Pr-like promoters constitute a restricted class of σ^{70} -dependent promoters.

Pr possesses a -15 element

The proposal to delineate σ^{70} -promoter DNA motifs into three elements, two involved in recognition by σ^{70} -RNAP as dsDNA—namely, a -35 element ($^{-35}\text{TTGACA}^{-30}$) and a -15 element ($^{-15}\text{TGGT}^{-12}$)—and a short -10 element ($^{-11}\text{ATAAT}^{-7}$) involved in interactions with σ^{70} -RNAP as ssDNA prompted us to examine the

Table 1. List of selected Pr-like candidates from *P. putida* KT2440 genome

Sequence (5'–3') ^a	Plasmid	Activity encoded by the downstream gene
TTGACT TTTTGCCAGATACTGAG GGCTGGCT ATGGGG AGCTGG TTGACT/a <17 bp> XXXXXT candidates	pVI931	DmpR
TTGACA AGAAAGTAGCTGCCAAC AGCCCT GCGAAGACCAAC TTGACA ACGTTCCTCCATTGCCAAC CGACGT GGCCAAATACTT	pVI2234 pVI2239	PP_1129: pyridoxamine 5'-phosphate oxidase PP_4159: potassium-transporting ATPase subunit C
TTGACc/g <17 bp> XXXXXT candidates		
TTGACC GAGCTGGAGCTGGAA AGCGGGT CAGCAATGAAGC TTGACC GGGGCCGCTCCTGCAAA AGCCTGT GCTGCCTTTTGT TTGACC AGCGCATCGAAGACGA AGCCCGT GCCAGCTGCAG TTGACC GAGGTGATGCGGGGCT TCGGCCT CCCTTCGCTTTC TTGACC ATCGCCAGGTGCTGAC CGGGCT GGCGGCTGTTGC TTGACC CCAAACGCTGCGCAT GGGGCT TGGCGACAATGT TTGACC CAGATCCTGAAG TTGGCTCGGT GTATCCGTTCCA TTGACC CAAACCGTGTGGGAGC GGCCCT TGGGTGACTGAAA TTGACC CCCATGCGGGGGCG GAACCTGT GGGAGCGGGCTT TTGACC CGGGCCATCAGTT TCGGGT GAACGCTTGCC	pVI2231 pVI2232 pVI2233 pVI2235 pVI2236 pVI2237 pVI2238 pVI2240 pVI2241 pVI2242	PP_0070: Sua5/YciO/YrdC/YwlC family protein PP_0568: hypothetical protein PP_0691: gamma-glutamyl kinase PP_1610: CTP synthetase PP_3207: cyclase, putative PP_4478: succinylglutamic semialdehyde dehydrogenase PP_4476: hypothetical protein PP_2932: amidase PP_1280: alginate O-acetylation protein AlgI PP_0245: S1 RNA-binding domain-containing protein
5/6 matches <17 bp> XXXXXT encoding transcriptional activators		
TTGCCA GTGCAATCGCCTACCAT GGCCCT CCCTTTTGT TTGCCA ACGCGGGTAAGGGG CGGGCT GATTTGATCTG TTGCCA CTGGCGATGGCCA ACCTCGCGT GCTGACCCTGCG TTGAAA TCAGCGAGCGTGACGAC CCCGCT ATCCGAGCGTG TTGACA CCGCGCTGAAC TACGGCGCT TGGCCTGTACCAA TAGACA CCGATGACCGCACT GGGCTTT TCGGCTTCAAT	pVI2243 pVI2244 pVI2245 pVI2246 pVI2255 pVI2256	PP_0513: transcriptional regulator NrdR PP_0583: AraC family transcriptional regulator PP_2833: LysR family transcriptional regulator PP_3544: GntR family transcriptional regulator PP_3717: LuxR family transcriptional regulator PP_2072: AraC family transcriptional regulator
Five out of six matches <17 bp> XXXXXT (–15T and –14G matches to –15 element consensus)		
TTACA TTGTGAATCTTATT TGTGCCCT CCTGCAATCCGG	pVI2257	PP_4551: alpha/beta fold family hydrolase
Five out of six matches <17 bp> XXXXXT (–15T and –13G matches to –15 element consensus)		
TCGACA ATGCCCGATCTTT TCGGCCCT GGAGCCGTCATA	pVI2258	PP_0900: PAP2 family
Five out of six matches <17 bp> XXXXXT (–15T, –14G and –13G matches to –15 element consensus)		
TCGACA TGCTTGTCTATCGAT TGGCACCT GACTTCCCTGC	pVI2259	PP_2826: transcriptional regulator MexT

^aThe potential –35 and –10 elements are underlined; bases matching the consensus sequence of –35, –15 and –10 elements are highlighted in bold.

involvement of bases in the potential –15 element of Pr to its activity. The –15 element consensus—^{–15}**TGGT**^{–12}— is based on a recent bioinformatics analysis of 322 σ^{70} -promoters with verified +1 start sites, which detected a previously unappreciated significant conservation of the –13G base (6). The –15T and –14G of this motif correspond to the ^{–15}TGn^{–13} of the extended –10 motifs. However, unlike the extended –10 ^{–15}TGn^{–13} motif, which is considered to require the binary presence of both the –15T and –14G, the –15 element is degenerate in that possession of even one consensus base can affect promoter activity (6). For example, it has been shown that the presence of a G in the –14 position, even in the absence of the T in position –15, can substantially contribute to transcription activity (e.g. λ P_R) and, vice versa, the presence of a –15T in the absence of the –14G can also enhance promoter activity (e.g. *Plac*, lacUV5, T7A1) (2 and references therein).

The Pr promoter possesses two out of four matches, –14G and –13G, to the consensus –15 element sequence (^{–15}**AGGC**^{–12}, consensus ^{–15}**TGGT**^{–12}). To determine if this potential –15 element plays an important role in Pr activity, we constructed additional transcriptional reporter plasmids carrying derivatives of Pr (–38 to +8) controlling the *luxAB* genes and assayed promoter activity (Figure 5). Simultaneous substitution of both these residues completely abolished detectable Pr activity (G–14A and G–13A in pVI2263, Figure 5). Individual analysis of the contributions of the –14G and –13G bases showed that both residues are involved in

maintaining wild-type Pr activity. Substitution of the G at –13 position for any other base resulted in greatly decreased promoter activity as compared with wild type (25% for G–13C, 7.5% for G–13A and 11.4% for G–13T). Similarly, substitution of the G in the –14 position to either A or C also resulted in greatly reduced promoter activity (to ~11–12% of wild-type Pr), whereas the G–14T substitution retained 62% promoter activity. Examination of the sequence resulting from the G–14T substitution suggests that the promoter activity observed might be due to creation of a new promoter comprising an almost perfect –15 element and a non-existing short –10 (^{–15}**TGCT**^{–12}/^{–11}**GGCTA**^{–7}, consensus ^{–15}**TGGT**¹²/^{–11}**TAAT**^{–7}) spaced 18 bp from the –35 element of Pr. If this was indeed the case, it would imply that a promoter can be functional with only a –35 and –15 element. However, we found that Pr mutant derivatives that improve the –15 element but destroy any match to the short –10 element do not show any promoter activity (compare variants pVI2202 with pVI2207, pVI2254 with pVI2264 and pVI2253 with pVI1020; Figure 5). In addition, a suboptimal 18-bp spacer region between the –35 and –10 elements renders Pr non-functional (Figure 1E). In the light of these results, we conclude that the 62% promoter activity retained by the Pr G–14T variant is because of the tolerance of a T at this position, rather than through creation of a new promoter.

To further characterize the importance of the –15 element, we also generated Pr derivatives with greater matches to the –15 consensus. Individually, a consensus

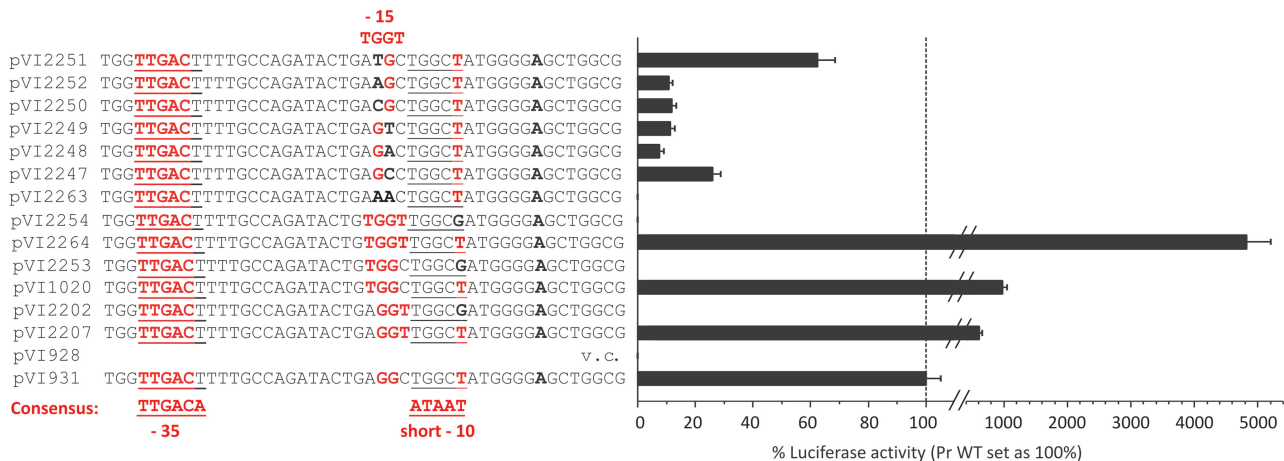


Figure 5. Analysis of the -15 element of Pr. The graph shows the stationary phase luciferase activities of *E. coli* DH5 harbouring -38 to $+8$ Pr luciferase transcriptional reporter plasmid variants encompassing different mutations in the -10 and/or -15 elements. The plasmid names and sequences of these -38 to $+8$ Pr variants are shown to the left. The -35 and short -10 elements are underlined, whereas bases matching the consensus -35 , -15 and -10 elements are shown in red bold-case letters. Substitutions in the sequence that differ from the consensus are indicated in black and bold-case letters as is the $+1$. Values for relative transcription are from duplicate determinations in two to four independent experiments, normalized by setting the value of Pr WT as 100% activity. Error bars are \pm SD.

T at -15 or a consensus T at -12 increases output (~ 10 - and ~ 6 -fold, respectively)—pVI1020 and pVI2207; Figure 5). When combined to give a complete consensus -15 element, the effects are synergistic, resulting in a derivative with an impressive ~ 48 -fold higher activity than wild-type Pr (pVI2264, Figure 5).

The combined data in Figure 5 demonstrate that within native Pr, the -14 G and -13 G together are essential for promoter activity and are thus part of a -15 element. However, the requirement for these specific bases is less strict than those of its -35 and -10 elements, because individual substitution mutants still retain some promoter activity, with G -14 T and G -13 C being the most permissive (62 and 25% activity of wild-type, respectively).

Sequences of the $-35/-15$ spacer and the -6 to $+6$ region are important for productive promoter activity

Given the finding that the -15 element of Pr is essential for activity, re-examination of the non-functional candidate Pr-like promoters (Table 1) shows that all have at least one sequence defect that could potentially account for their lack of activity. To test if all the criteria for Pr-like promoter activity had been defined by our analysis of the -35 , -15 and -10 elements of Pr, we chose the three candidate promoters most closely related in sequence to Pr for correction of their apparent defects, namely, pVI2234 (PP_1129), pVI2239 (PP_4159) and pVI2238 (PP_4476).

As shown in Figure 6, correction for the lack of a -15 element in two of the candidates and the -35 element of the third candidate did not result in promoter activity (pVI2270, pVI2277 and pVI2282; Figure 6), suggesting additional defects within these sequences. In all three cases, the candidate sequences possess different combinations of non-conserved bases within their XXXXT short -10 elements. Therefore, we then additionally substituted the -14 to -7 regions of the candidates with the sequence

of Pr, but again promoter activity was not restored (pVI2272, pVI2279 and pVI2284; Figure 6). The only remaining differences between these sequences and Pr derivatives that are fully functional (Figures 1, 2 and 5) reside in the -29 to -15 spacer region and the DNA downstream of the short -10 element (-6 to $+6$), suggesting that either (or both) of these regions were responsible for inactivity. Therefore, we generated additional derivatives that had either -35 to -7 regions (pVI2274, pVI2281 and pVI2286; Figure 6), or -14 to $+6$ regions (pVI2273, pVI2280 and pVI2285; Figure 6) that are functional in the context of Pr. Because none of these derivatives exhibited any promoter activity, the data reveal that the sequence combination of the -29 to -15 spacer regions and the -6 to $+6$ regions are also essential for promoter activity. Taken together with the results shown in Figures 1, 2 and 5, these results demonstrate the extreme sequence demands for functionality of the Pr promoter in the face of its extremely suboptimal -10 element.

DISCUSSION

Deviation from consensus within promoter elements generally reduces basal promoter activity, thus providing an opportunity for extrinsic factors to stimulate promoter output. Within a list of 599 σ^{70} -promoter sequences with verified $+1$ start sites (14), all have at least two matches to consensus within their -10 elements. In the cases of promoters with only two matches, all possess a combination of either -12 T and -11 A or -11 A and -7 T, i.e. a combination of two of the three most conserved bases within the -10 element (15,16). Here, we perform extensive analysis of the promoter element requirements of Pr—the only experimentally verified σ^{70} -promoter with only one out of six matches to the -10 element consensus sequence ($^{-12}$ CTGGCT $^{-7}$, consensus $^{-12}$ TATAAT $^{-7}$).

<p>A</p> <p style="text-align: center;">-15 TGGT +1</p> <p><u>TTGAC</u>TTTGCCAGATACTGAGGGCTGGCTATGGGGAGCTGG</p> <p><u>TTGACA</u>AGAAAGTAGCTGCCAACAGCCCTGCGAAGACCAAC</p> <p><u>TTGACA</u>AGAAAGTAGCTGCCAGGGAGCCCTGCGAAGACCAAC</p> <p><u>TTGACA</u>AGAAAGTAGCTGCCAGGGCTGGCTGCGAAGACCAAC</p> <p><u>TTGACA</u>AGAAAGTAGCTGCCAGGGCTGGCTATGGGGAGCTGG</p> <p><u>TTGACA</u>TTTGCCAGATACTGAGGGCTGGCTGCGAAGACCAAC</p> <p><u>TTGACA</u>ACGTTTCCATTGCCAACGACGTGGCCAAATACTT</p> <p><u>TTGACA</u>ACGTTTCCATTGCCGGCGACGTGGCCAAATACTT</p> <p><u>TTGACA</u>ACGTTTCCATTGCCGGCTGGCTGGCCAAATACTT</p> <p><u>TTGACA</u>ACGTTTCCATTGCCGGCTGGCTATGGGGAGCTGG</p> <p><u>TTGACA</u>TTTGCCAGATACTGAGGGCTGGCTGGCCAAATACTT</p> <p><u>TTGAC</u>CCAGATCCTGAAGTTGGGCTCGGTGTATCCGTTCCA</p> <p><u>TTGAC</u>TCAGATCCTGAAGTTGGGCTCGGTGTATCCGTTCCA</p> <p><u>TTGAC</u>TCAGATCCTGAAGTTGGGCTCGGTGTATCCGTTCCA</p> <p><u>TTGAC</u>TCAGATCCTGAAGTTGGGCTCGGTATGGGGAGCTGG</p> <p><u>TTGAC</u>TTTGCCAGATACTGAGGGCTGGCTGTATCCGTTCCA</p> <p><u>TTGACA</u> -35</p> <p style="text-align: center;"><u>ATAAT</u> short -10</p> <p style="text-align: center;">Consensus</p>	<p>vector control Pr WT</p> <p>pVI2234 WT</p> <p>pVI2234 A-14G, C-13G</p> <p>pVI2234 -14 to -7 Pr</p> <p>pVI2234 -14 to +6 Pr</p> <p>pVI2234 -29 to -7 Pr</p> <p>pVI2239 WT</p> <p>pVI2239 A-14G, A-13G</p> <p>pVI2239 -14 to -7 Pr</p> <p>pVI2239 -14 to +6 Pr</p> <p>pVI2239 -29 to -7 Pr</p> <p>pVI2238 WT</p> <p>pVI2238 C-30T</p> <p>pVI2238 -14 to -7 Pr and C-30T</p> <p>pVI2238 -14 to +6 Pr and C-30T</p> <p>pVI2238 -29 to -7 Pr and C-30T</p>	<p>pVI928 pVI2230</p> <p>pVI2234 pVI2270 pVI2272 pVI2273 pVI2274</p> <p>pVI2239 pVI2277 pVI2279 pVI2280 pVI2281</p> <p>pVI2238 pVI2282 pVI2284 pVI2285 pVI2286</p>
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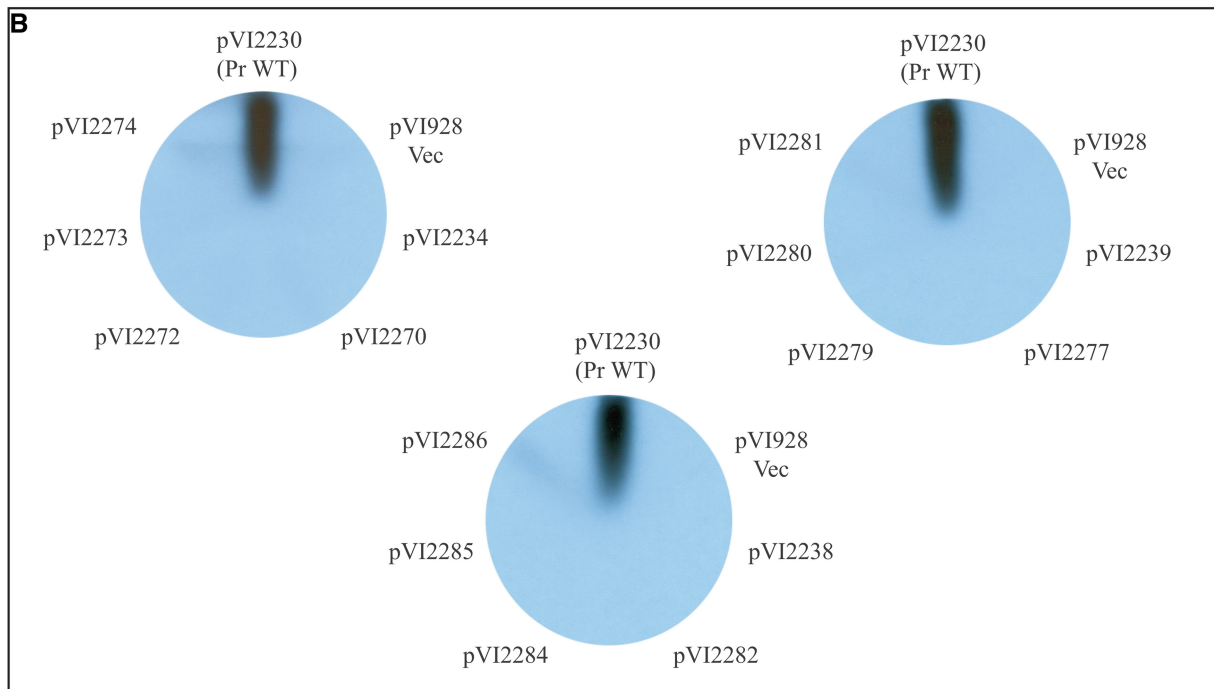


Figure 6. Analysis of the contribution of the -29 to -15 spacer and the -10 downstream region (-6 to +6) to promoter activity. (A) Sequence of the -35 to +6 region of Pr-like sequences present in the indicated reporter plasmids used. The -35, -15 and short -10 elements are underlined with matches to their consensus sequences shown in red bold-case letters. The +1 start corresponding to that of Pr is indicated in bold-case letter. (B) Luciferase plate test assays employing *E. coli* DH5 carrying different transcriptional reporter plasmids exposed for 80 min. The pVI2230 (Pr WT) and pVI928 (the promoter-less vector) are used as positive and negative controls, respectively.

Our analysis of Pr supports the tripartite delineation of σ^{70} -promoter elements into a -35, -15 and short -10 element—consensus $^{-35}\text{TTGACA}^{-30}/12\text{-}16\text{bp}/^{-15}\text{TGGT}^{-12}/^{-11}\text{ATAAT}^{-7}$ (2,6) and demonstrates that the requirements for activity of this promoter are extremely strict (Figures 1, 2, 5 and 6). Promoter activity was abolished by +1/-1 changes of its 17-bp spacer, by substitution

of its single -10 element consensus base (-7T), by substitution of any consensus base within its near-perfect -35 element ($^{-35}\text{TTGACT}^{-30}$, consensus $^{-35}\text{TTGACA}^{-30}$) and by elimination of any match within its -15 element ($^{-15}\text{AGGC}^{-12}$, consensus $^{-15}\text{TGGT}^{-12}$).

Within the -35 element, the -35, -34, -33 and -31 bases that are bound by σ residues through hydrogen

bonds are most highly conserved, whereas conservation is notably lower for the -32 and -30 bases, which interact with σ via weaker van der Waals interactions (6). Unexpectedly, we found a critical role for the non-consensus $-30T$ base of Pr, with substitution to a non-consensus G or C abolishing promoter activity (Figure 2D). Hence, possession of an extremely suboptimal -10 element, as found in Pr, renders its activity sensitive to substitutions of even a non-consensus base of this element.

A similar case is found within the short -10 element of Pr, in which a substitution from the non-consensus $-11T$ to non-consensus C or G also drastically reduced activity (Figure 3A and B). In contrast, a substitution to the consensus A base results in >40 -fold higher activity (8). The short -10 element is involved in ssDNA interactions with region 2.3 of σ^{70} and facilitates open complex formation and maintenance of the transcription bubble surrounding the transcriptional start site. The $-11A$ and the $-7T$ are particularly important for binding, with lesser and varying contributions from the bases at positions -10 to -8 (17,18). Recently, structures of region 2.3 of *Thermus aquaticus* σ^A bound to its target ssDNA have revealed a lock-and-key-type of interaction that accounts for the markedly high conservation of the $-11A$ and $-7T$ bases (19). Within this interaction, the $-11A$ and $-7T$ protrude into hydrophobic pockets, whereas the intervening bases of the $^{-11}ATAAT^{-7}$ DNA remain stacked away from the protein. Region 2.3 of *P. putida* and *E. coli* σ^{70} are identical and highly similar to that of *T. aquaticus* σ^A (Supplementary Figure S5), suggesting that the size and shape constraints of the $-11A$ pocket may tolerate the non-consensus $-11T$ of Pr better than non-consensus C or G. In addition to its contribution to binding with region 2.3 of σ^{70} , the $-11A$ plays a crucial role in the nucleation of promoter DNA melting (20 and references therein), with substitution to other bases resulting in a slow rate of open complex formation (21). We have previously shown that lack of conservation at the -11 position in the Pr promoter compromise both σ^{70} -RNAP binding and the rate of open complex formation—two processes that are stimulated by the action of ppGpp and DksA on σ^{70} -RNAP at this promoter (8).

The Pr promoter possesses two matches to the -15 element consensus ($^{-15}AGGC^{-12}$, consensus $^{-15}TGGT^{-12}$) that, like bases of the -35 element, would be recognized as dsDNA and influence closed promoter complex formation. Substitutions within Pr to generate a variant with a complete consensus -15 element dramatically increases promoter activity (48-fold), whereas simultaneous substitutions of both the $-14G$ and the $-13G$ completely abolishes activity. However, individual substitution of either the $-14G$ or the $-13G$ of Pr reduces, but does not eliminate, its activity (Figure 5). Hence, although this element is important for full promoter activity, substitutions have less dramatic effects than those within its -35 or short -10 elements. A direct interaction between residues E458 and H455 within σ^{70} and the $-14G:C$ base pair is thought to underlie the high conservation of the $-14G$ within the -15 element. Quantitatively, the $-14G$ provides a significantly larger contribution to promoter

activity as compared with a T in position -15 ; on the other hand, the $-13G$ is more conserved than the $-15T$ (2,6 and references therein). Experimentally, the importance of a G at the -13 position had been demonstrated for *T. aquaticus* σ^A promoters (22), but to our knowledge it has not previously been shown for the *E. coli* or *P. putida* σ^{70} -RNAP holoenzymes.

A widely held, but aberrant, belief is that the function of the extended -10 region (which is encompassed within the newly defined -15 element) is only to compensate for a poor or missing -35 element (2). Although this is evident for some promoters, e.g. λ Pre (23) and galP1 (24,25), many promoters possessing this element show reasonable matches to the -35 element and require or benefit from dsDNA interactions through their -35 and/or UP element contacts (2,6). A recent analysis by Djordjevic (6) suggests that -15 elements have a significantly greater propensity to rescue promoters with weak -10 elements as compared with promoters with weak -35 elements, and a much stronger propensity to rescue promoters with a weak overall activity than with weak σ^{70} -dsDNA interactions (i.e. weak -35 element). As outlined above, this is also the case for the -15 element of the Pr promoter, with its intrinsically low activity, near-perfect -35 but extremely suboptimal -10 .

Other well documented examples of promoters with -35 and -15 elements compensating for weak -10 elements include the *gapA*P1 promoter (-10 element AATTTT) (26), Pminor (-10 element GAAAAC) (27,28), and the *ompF* promoter (-10 element AAAGAT) (16,29). However, it is notable that all these weak -10 element promoters still possess three out of six matches to consensus, including a consensus $-11A$. This is in contrast to the extreme case of Pr with its single match at the -7 position (CTGGCT). The T at position -7 is the most conserved of all bases within the short -10 element ($^{-11}ATAAT^{-7}$).

Even with the aid of a -15 element, it is remarkable that Pr is functional given its lack of consensus within its short -10 element. Part of the answer may reside in the sequence of the spacer region and the region downstream of the -7 position, which we also found to be important for Pr activity (Figure 6). In addition to the length of the spacer between the -35 and -10 elements, promoter alignments have revealed that within subsets of σ^{70} -promoters, some sequences in the spacer region are better conserved than parts of the -35 and -10 elements (16). More recently, additional σ^{70} -RNAP/spacer interactions involving the side chain of R451 within σ^{70} (30) and the β' zipper of core RNAP (31) have been found to contribute to closed complex formation at specific promoters, whereas σ^{70} region 1.1 is thought to influence spacer-mediated changes in transcriptional initiation by altering the trajectory of spacer and thus downstream DNA (32). Our current analysis does not allow us to deduce what part of the Pr spacer sequence is required to mediate its effect, however, it appears likely that interaction through one or more of these regions of σ^{70} -RNAP underlies the positive contributions of the spacer DNA to Pr promoter activity.

In its native context, Pr controls transcription of the *dmpR* gene that encodes the master regulator required for phenol catabolism by *P. putida* CF600. We verified that highly similar promoters control analogous regulators from other plasmid- or chromosomally- encoded phenol catabolic systems of Pseudomonads. All but one of these promoters have a single -7T match to the consensus within their -10 regions and can be considered as natural Pr variants (Figure 3), which we collectively refer to as Pr-like or '-10-less' promoters. Because lack of identity with the -10 consensus element leads to mis-predictions or non-detection of these Pr-like promoters using promoter search engines, it seemed likely to us that other previously unrecognized Pr-like '-10-less' promoters might exist. In particular, we anticipated finding such promoters upstream of genes involved in controlling transcriptional regulators or enzymes for secondary metabolism. However, using a purpose-designed program to identify such candidate promoters, we were unable to identify a single functional Pr-like promoter within the entire *P. putida* KT2440 genome (Supplementary Figures S2-S4 and Table 1). Hence, Pr-like promoters form a class of σ^{70} -dependent promoter on their own, which to date are restricted to promoters for regulators of phenol catabolism.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1 and 2 and Supplementary Figures 1-5.

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