*9638–9649* *Nucleic Acids Research, 2016, Vol. 44, No. 20* *Published online 15 July 2016*
*doi: 10.1093/nar/gkw639*

# Inter-sigmulon communication through topological promoter coupling

**Teresa del Peso Santos and Victoria Shingler***

Department of Molecular Biology, Umeå University, Umeå SE 90187, Sweden

Received June 02, 2016; Revised July 01, 2016; Accepted July 06, 2016

## ABSTRACT

**Divergent transcription from within bacterial intergenic regions frequently involves promoters dependent on alternative σ-factors. This is the case for the non-overlapping $\sigma^{70}$- and $\sigma^{54}$-dependent promoters that control production of the substrate-responsive regulator and enzymes for (methyl)phenol catabolism. Here, using an array of *in vivo* and *in vitro* assays, we identify transcription-driven supercoiling arising from the $\sigma^{54}$-promoter as the mechanism underlying inter-promoter communication that results in stimulation of the activity of the $\sigma^{70}$-promoter. The non-overlapping 'back-to-back' configuration of a powerful $\sigma^{54}$-promoter and weak $\sigma^{70}$-promoter within this system offers a previously unknown means of inter-sigmulon communication that renders the $\sigma^{70}$-promoter subservient to signals that elicit $\sigma^{54}$-dependent transcription without it possessing a cognate binding site for the $\sigma^{54}$-RNA polymerase holoenzyme. This mode of control has the potential to be a prevalent, but hitherto unappreciated, mechanism by which bacteria adjust promoter activity to gain appropriate transcriptional control.**

## INTRODUCTION

Signal-responsive control of promoter activity is critical for the ability of bacteria to rapidly adapt their gene expression to prevailing conditions. Responses to diverse environmental cues are built into control of promoter activity by a number of means, including control of the availability of multiple dissociable alternative σ-factors of the RNA polymerase holoenzyme (σ-RNAP) that direct the transcriptional machinery to the different classes of promoters in the genome (reviewed in 1). These dynamic changes in the composition of the pool of σ-RNAP underscore promoter occupancy—the first step of transcriptional initiation, which results in the formation of the initial closed promoter DNA–RNAP complex. This initial step, and the subsequent steps of transcriptional initiation leading to DNA melting and strand separation to form the transcriptionally competent open-complex, can all be played upon by classical DNA-binding regulators and global regulatory factors like the nucleotide alarmone ppGpp and the DksA transcription factor, which independently and synergistically directly target RNAP to alter its performance (2).

In addition to the above, DNA supercoiling is a major regulator of gene expression (3,4), with individual promoters being activated, repressed or essentially unaffected by changes in supercoiling status (5). Such changes can affect promoter activity either directly, by altering the DNA structure and melting energy, or indirectly by affecting the binding of RNAP and transcriptional regulators (6). Species specific DNA topoisomerases, nucleoid-associated proteins (NAPs, such as HU, H-NS, IHF, FIS and Dps), and RNA polymerase itself all play a part in the network of interactions that constrain DNA in macro- and micro-domains within which supercoiling fluxes occur (7–9 and references therein).

As originally proposed by Liu and Wang (3) in their 'twin-domain model', RNA polymerase plays a pivotal role both as a topological barrier and as the driver of local fluxes in DNA supercoiling. As it transcribes along the DNA and the nascent transcript size increases, rotation of RNAP around the DNA double helix is increasingly constrained so that this powerful machine generates positive supercoils (over-wound DNA) in the direction of transcription and negative supercoils (under-wound DNA) behind it (3,10–15). The topological distortions created within these domains can be neutralized by type I (topoisomerase I) and type II (DNA gyrase) topoisomerases, which remove negative and positive supercoils, respectively (16), or can be dissipated by diffusion through the DNA to exert its regulatory effects on promoter activity (17 and references therein).

Most works on the repercussions of DNA topology effects through supercoiling have involved artificial mutant promoters or mutations of regulatory regions [e.g. $P_{leu\text{-}500}$ (18–21) and CtrA binding sites (22)] or overlapping promoters dependent on the house hold sigma-factor, $\sigma^{70}$ [e.g. $P_{ilvY}/P_{ilvC}$ (5,23,24), $P_{fepA}/P_{fes}$ (25), and promoters of the *foo* operon (26)]. However, divergently firing intergenic promoters can be dependent on different σ-factors. In addition to an overlapping configuration, divergently firing promot-

*To whom correspondence should be addressed. Tel: +46 90 7852534; Fax: +46 90 772 630; Email: victoria.shingler@molbiol.umu.se

ers can be non-overlapping but orientated 'face-to-face', producing partially overlapping transcripts, or orientated 'back-to-back', with an intervening sequence in between. Among these, the latter appears the most common (27,28) and is the promoter configuration found in the plasmid encoded *dmp*-system for (methyl)phenol catabolism by *Pseudomonas putida* CF600 (see scheme in Figure 1A).

Within the *dmp*-system, the two divergently firing promoters are dependent on $\sigma^{70}$ and the alternative $\sigma^{54}$ factor. In contrast to $\sigma^{70}$- and other $\sigma^{70}$-like RNAP holoenzymes that can spontaneously initiate transcription, $\sigma^{54}$-RNAP forms thermodynamically stable closed promoter complexes and so strictly requires activation by a member of a specialized family of mechano-transcriptional activators (reviewed in 29). The $\sigma^{70}$-Pr promoter controls the levels of DmpR—the obligate (methyl)phenol-responsive mechano-transcriptional activator of the powerful $\sigma^{54}$-Po promoter, which drives transcription of the genes for the specialised catabolic enzymes (30–33). In contrast to the $\sigma^{54}$-Po promoter, the $\sigma^{70}$-Pr promoter is intrinsically weak and requires the co-action of ppGpp and DksA to overcome constraints imposed by poor binding of $\sigma^{70}$-RNAP and a slow rate of open-complex formation (34–36).

The $\sigma^{70}$-Pr promoter is also stimulated by factors required for activity at the $\sigma^{54}$-Po promoter, creating an auto-regulatory feed-forward loop (34). Here, we specifically address the role of DNA topology in this inter-promoter communication. The data pin-point transcription-driven changes in DNA supercoiling that overcome two Pr promoter constraints as the underlying mechanism. These results provide the first example of topological coupling between promoters dependent on different σ-factors. As discussed herein, because transcribing RNAP can act as a driver of DNA supercoiling, this non-overlapping 'back-to-back' configuration of a powerful and weak promoter offers a previously unknown means of inter-sigmulon communication that renders the $\sigma^{70}$-dependent promoter subservient to signals that elicit $\sigma^{54}$-dependent transcription without it possessing a cognate binding site for the $\sigma^{54}$-RNAP holoenzyme. This mode of control has the potential to be a prevalent, but previously unappreciated, mechanism by which bacteria can adjust promoter activity to integrate diverse signals for transcriptional control.

## MATERIALS AND METHODS

### Bacterial strains, plasmids and culture conditions

Bacterial strains (Supplementary Table S1) were cultured in Luria-Bertani/Lennox (LB) medium (AppliChem GmbH) at 37°C for *Escherichia coli* and 30°C for *P. putida*. Cultures were supplemented with carbenicillin (Cb, 100 μg ml$^{-1}$ for *E. coli*; 1000 μg ml$^{-1}$ for *P. putida*), tetracycline (Tc, 5 μg ml$^{-1}$ for *E. coli*; 50 μg ml$^{-1}$ for *P. putida*), or tellurite (Tel, 20 μg ml$^{-1}$ for *P. putida*), when appropriate for strain or resident plasmid selection. Plasmids were constructed by standard DNA techniques as detailed in supplementary material. The fidelity of the DNA regions generated by PCR amplification or by insertion of synthetic double stranded linkers was confirmed by DNA sequencing.

### Luciferase assays

Quantitative luciferase assays were performed on cultures grown and assayed at 30°C as described by Sze and Shingler (37). To ensure balanced growth, overnight cultures were diluted and grown into exponential phase before a second dilution to an $OD_{600}$ of 0.05–0.08 and initiation of the experiment with or without the addition of the DmpR effector 2-methyphenol. Light emission from 100 μl 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.

### Purified proteins for *in vitro* assays

Native *P. putida* KT2440 core RNAP, $\sigma^{70}$-RNAP holoenzyme, $\sigma^{54}$, His-DksA and the constitutively active ΔA2-His-DmpR protein were purified as previously described (31,33,34,38).

### Topoisomerase I-treatment of plasmids

Ten microgram of supercoiled transcription templates were treated during 1 h at 37°C with 2 μl of calf thymus topoisomerase I (6 U/μl, Invitrogen) in buffer containing 50 mM Tris–HCl pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM EDTA, 30 μg/ml bovine serun albumin (BSA). After a phenol:chloroform:IAA (25:24:1) extraction, the DNA was precipitated with isopropanol and 0.3 M sodium acetate, washed with 70% ethanol, dried and resuspended in 25 μl RNase free-$H_2O$. Topoisomerase I-treated plasmids were resolved, as described below, to observe the integrity of the DNA and the distribution of the different topoisomers.

### Topoisomer resolution

Topoisomers of plasmid DNA isolated from bacteria cultured to different growth phases were resolved on a 0.8% agarose gel containing 1.5 μg/ml chloroquine. Gels were run at 15 V for 20 h in 45 mM Tris–borate/1 mM EDTA buffer, stained with SYBR green (Life technologies) and documented using a LAS 4000 ImageQuant system (GE Healthcare).

### *In vitro* transcription assays

Standard single-round *in vitro* transcription assays (final volume 20 μl) were performed with 10 nM template DNA and the indicated concentration of *P. putida* $\sigma^{70}$-RNAP and/or $\sigma^{54}$-RNAP at 30°C in a buffer containing 35 mM Tris–Ac pH 7.9, 70 mM KAc, 5 mM $MgAc_2$, 20 mM $NH_4Ac$, 1 mM DTT, and 0.275 mg/ml bovine serum albumin, as previously described (34). Reactions were incubated for 10 min to allow open-complex formation, followed by initiation of transcription by addition of nucleotides (final concentrations: 500 μM ATP, 200 μM GTP, 200 μM CTP, 80 μM UTP and [$\alpha^{32}$P]UTP (5 μCi at >3000 Ci/mmol, Perkin Elmer)). Simultaneous addition of heparin (0.125 mg/ml) was used to prevent re-initiation. Reactions were terminated after 10 min incubation by addition of 5 μl stop/loading buffer (150 mM EDTA, 1.05 M NaCl, 14

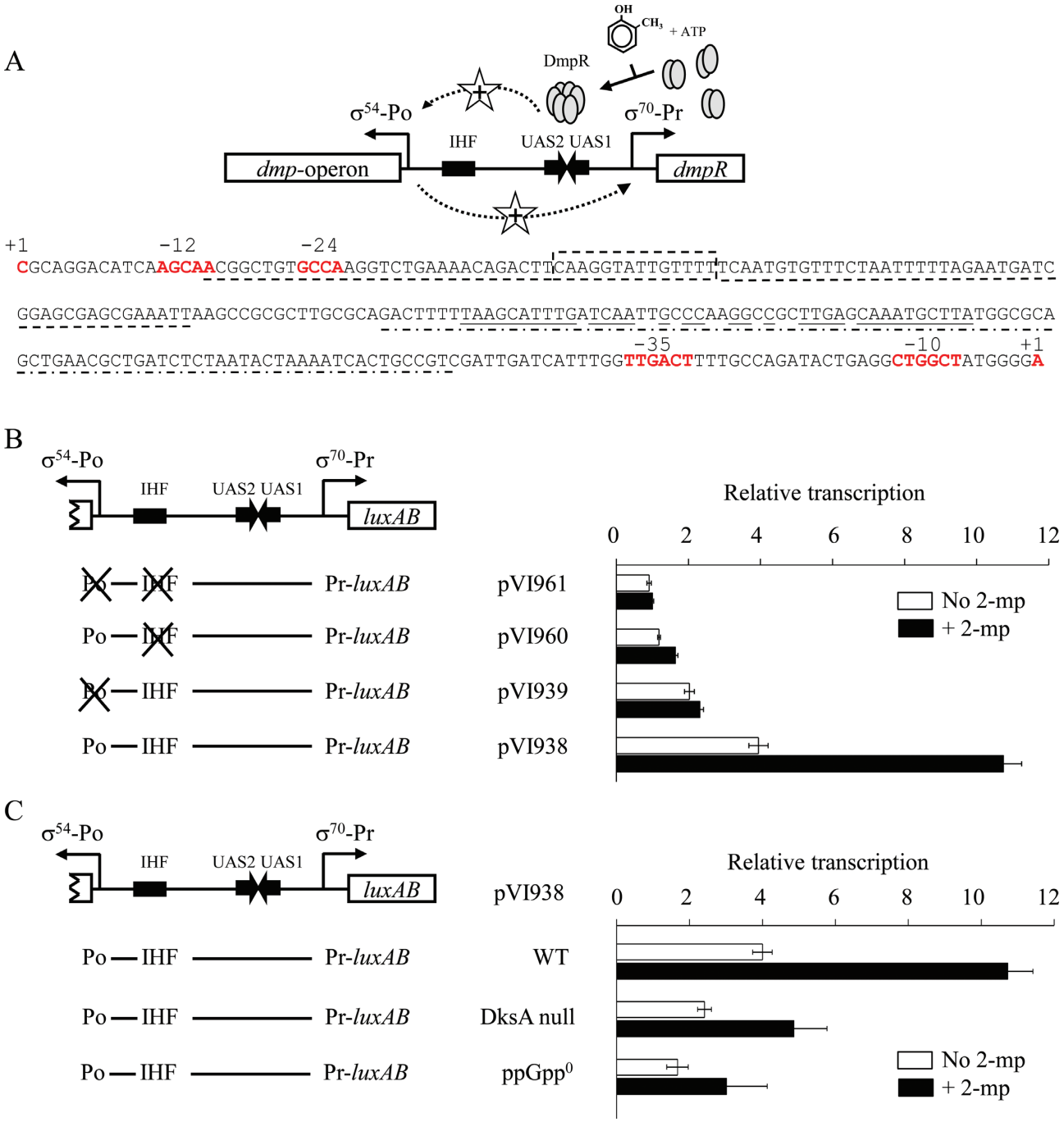


**Figure 1.** Divergent promoter activity stimulates transcription from $\sigma^{70}$-Pr. (**A**) Upper: schematic illustration of the *dmp*-system with the coding regions of *dmpR* and the *dmp*-operon shown as open boxes (not to scale). The relative locations of the binding sites for IHF (black box) and DmpR (inverted black arrows labelled UAS2 and UAS1) within the 406 bp intergenic region are shown relative to the $\sigma^{54}$-Po *dmp*-operon promoter and the $\sigma^{70}$-Pr promoter of *dmpR* that drive divergent but non-overlapping transcription. Stimulatory effects are indicated by dotted arrows with stars. Inactive DmpR dimers (ovals) require pre-binding of an aromatic effector before ATP-binding triggers oligomerisation into the transcriptional promoting form. Lower: nucleotide sequence of the 266 bp $\sigma^{54}$-Po to $\sigma^{70}$-Pr region with the promoter elements and the +1 transcriptional start sites highlighted in red (34–36,57). The IHF binding site (dashed box) and the extent of its DNase I protection (dashed line), the UASs for DmpR (underlined) and the extent of its DNase I protection (dot-dashed line) are taken from (58,59). (**B**) Stationary phase activities of LB-cultured *P. putida* KT2440::*dmpR*-Tel harbouring the indicated Pr-*luxAB* luciferase transcriptional reporter plasmids cultured in LB in the absence (white bars) or presence (black bars) of 2 mM of the potent DmpR effector 2-methylphenol. Pre-defined mutations (34) that abolish binding of $\sigma^{54}$-RNAP to Po (TGGC $N_7$ TTGCT to GTCG $N_7$ TTGTC) or IHF to its target site (AAACAAT $N_3$ CTTG to AAAGTT $N_3$ CAAC) are indicated by crosses. Values for relative transcription are from triplicate determinations from two independent experiments ± SE, normalized by setting the value of pVI961 in the absence of 2-methylphenol as 1. (**C**) Stationary phase activities of *P. putida* KT2440::*dmpR*-Tel and its DksA null or ppGpp° counterparts carrying pVI938, grown and normalised as described under panel (B).

M urea, 10% glycerol, 0.037% xylene cyanol, 0.037% bromophenol blue). Transcripts (282 nt for Pr from supercoiled and topoisomerase I-treated plasmids, 143 nt from linearized templates; 310 nt for Po from supercoiled templates) were resolved on a 5% or 6% polyacrylamide gel containing 7 M urea, and quantified using phosphor-imaging.

### Electro-mobility shift assays (EMSA)

Mixtures (final volume 15 μl) contained 2 nM radio-labeled DNA probe and the indicated amounts of *P. putida* His-DksA and/or $\sigma^{70}$-RNAP. Binding reactions were incubated for 60 min at 4°C, in buffer (35 mM Tris–Ac pH 7.9, 70 mM KAc, 5 mM $MgAc_2$, 20 mM $NH_4Ac$, 1 mM DTT). Where indicated, heparin was added to the binding reaction to a final concentration of 0.15 mg/ml, and the mix was further incubated for 5 min. The resulting complexes were resolved using 4.5% native polyacrylamide gels buffered with 45 mM Tris–borate/1 mM EDTA. Probes were radio-labeled as detailed in supplementary material.

### DNase I and $KMnO_4$ footprinting assays

Assays (final volume 15 μl) were performed as previously described (35). In both assays, binary complexes were formed using 40 ng of DNA (10 or 17 nM depending on size, radio-labeled as detailed in supplementary material). DNA fragments were incubated with different concentrations of *P. putida* $\sigma^{70}$-RNAP for 30 min at 30°C or for 60 min at 4°C in buffer containing 35 mM Tris–Ac pH 7.9, 70 mM KAc, 5 mM $MgAc_2$, 20 mM $NH_4Ac$, 1 mM DTT, and 1 mg/ml bovine serum albumin. After complex formation, heparin-sensitivity was determined by exposure to heparin (final concentration 0.15 mg/ml) for 5 min. In $KMnO_4$ footprinting assays, ternary complexes were generated by addition of NTPs (final concentration 200 μM of each) and further incubation for 20 s at 30°C. Binary complexes (but not the ternary complexes) were then disrupted by exposure to 350 mM NaCl for 20 s.

## RESULTS AND DISCUSSION

### Divergent promoter stimulation within the *dmp*-system is independent of ppGpp and DksA and is non-reciprocal

The $\sigma^{54}$-Po and $\sigma^{70}$-Pr promoters drive divergent transcription from within a 406 bp intergenic region between the ATG initiation codons of *dmpR* and the first gene of the *dmp*-operon (see schematic Figure 1A). Removal and/or mutations of features required for activity of the $\sigma^{54}$-Po promoter, e.g. its promoter elements, $\sigma^{54}$, and IHF or the binding site for IHF, but not DmpR *per se*, results in defective transcription from the Pr promoter upon entry into stationary phase (Figure 1B and 34). The combinatorial effects of IHF and $\sigma^{54}$-RNAP binding in *P. putida* result in ∼4-fold elevation of Pr output, with superimposed DmpR-mediated activation of Po resulting in a net ∼10-fold elevation of Pr activity, as judged using *in vivo* transcriptional reporter plasmids in cultures grown in rich medium in the presence or absence of 2-methylphenol, the most potent effector of DmpR activity.

Stationary phase activity of the Pr promoter is stimulated by binding of ppGpp and DksA to $\sigma^{70}$-RNAP (34,35). Therefore, we next determined if Po-mediated stimulation of Pr activity also occurs in the absence of these regulatory molecules. Consistent with previous data, lack of either ppGpp or DksA resulted in lower Pr output. Nevertheless, stimulation of Pr output by activity at the Po promoter was still observed (compare open and black bars, Figure 1C), demonstrating that the mechanism underlying this level of regulation can act independently of these two global regulators.

Control of Pr output by the Po promoter places a $\sigma^{70}$-dependent promoter under control of the $\sigma^{54}$-sigmulon without possessing a cognate $\sigma^{54}$-RNAP binding site. To determine if inter-sigmulon communication functions in both directions within this regulatory region, we similarly monitored Po-output using otherwise identical transcriptional reporters that differed only by possession of the Pr promoter. However, lack of Pr activity had no discernible effect on Po output *in vivo* or *in vitro* (Supplementary Figure S1A and S1B). This contrasts the stimulatory effect of Po activity on Pr output, at which *in vivo* stimulation can be recapitulated *in vitro* (Figure 1B, Supplementary Figure S1C and 34). Hence, in this case, inter-sigmulon communication only functions in one direction.

### Inter-sigmulon communication from Po to Pr is independent of relative phasing

The 264 bp region between the +1 start sites for Po and Pr has to accommodate two different RNAP holoenzymes, dimeric IHF and the multimeric active form of DmpR. Given the occupation by IHF and DmpR that extend close to, or into, regions predicted to be bound by the RNAPs (∼−50 to +20, see Figure 1A), this region appears crowded. Therefore, we first considered the possibility that DNA bending caused by binding of proteins formed a stimulatory nucleoprotein complex. Within such a complex, upstream DNA and/or $\sigma^{54}$-RNAP could specially interact with $\sigma^{70}$-RNAP to stimulate its activity at Pr – a process which might further be facilitated by open-complex formation and/or transcription from Po.

Since formation of a three dimensional nucleoprotein complex would be disrupted by changes in phasing between the two promoters, we generated derivatives with +5 or +15 bp (half helical turn; off-set phasing), or reconstituted wild-type or +10 bp (on-set phasing) within the region between the UASs for DmpR and the Pr promoter (Supplementary Figure S2). As shown in Supplementary Figure S2, these manipulations had little effect on the ability of Po to mediate stimulation of Pr activity either *in vitro* or *in vivo*. These data, together with our previous finding that transcription from Pr can likewise be stimulated by activity of an unrelated constitutively active divergent promoter—the $\sigma^{70}$-dependent $\lambda P_L$ promoter (34)—refute the idea that a nucleoprotein complex underlies this transcriptional stimulatory mechanism.

### Inter-promoter communication through DNA supercoiling and topological promoter coupling

As outlined in the introduction, active transcription from a promoter induces tension within the DNA through driving positive supercoiling in the forward direction of RNAP and negative supercoiling in the opposite direction. Since dissipation of negative supercoiling through the DNA could affect the kinetics of transcriptional initiation at a divergent promoter, we next addressed supercoiling as a potential mechanism underlying Pr stimulation via Po activity.

As an initial step, we monitored the consequences of divergent transcription on Pr activity in *P. putida* (as in Figure 1 and Supplementary Figure S2) and in *E. coli*. In contrast to *P. putida*, no stimulatory effect could be detected in *E. coli* during any growth phase (compare Figure 2A and B) despite similar levels of DmpR to promote activity from Po (Figure 2C). Note that the enhanced Pr output seen at the exponential-to-stationary phase transition, which occurs irrespective of divergent transcription, is due to nutrition depletion, with consequent ppGpp production and concomitant direct stimulation of the performance of $\sigma^{70}$-RNAP at the Pr promoter. Given the different behaviour in the two organisms, we monitored the topoisomer distribution of a small plasmid (~4 kb pSEVA541, 39) to assess the DNA supercoiling status over growth. Examination of pSEVA541 topoisomers revealed that the supercoiling homeostasis differs, with DNA of *E. coli* remaining more negatively supercoiled than that of *P. putida*, Figure 2D. These findings are consistent with the idea that an increase in negative supercoiling, induced by divergent transcription from Po, underlies stimulation of Pr output in *P. putida*, while in *E. coli* the more negatively supercoiled status of the DNA by-passes this level of regulation.

To directly examine if activity of Po generated sufficient alterations in supercoiling to affect the activity of other divergent promoters, we compared the repercussions of transcription from Po on the activity of two $\sigma^{70}$-dependent promoters appropriately placed within the context of the Po to Pr intergenic region. The two promoters chosen for this analysis were the weak $P_{leu\text{-}500}$ promoter—an extensively studied mutant derivative of the $P_{leu}$ promoter that is highly dependent of negative supercoiling for its activity (19,40–43)—and the powerful $\lambda P_L$ promoter. Activity from the $P_{leu\text{-}500}$ promoter was stimulated to an even higher extent (~3.5-fold) than Pr (~2.75-fold) by induction of activity of the divergent Po promoter, while $\lambda P_L$ promoter activity remained unaffected (Figure 3A). These results clarify that transcription from the $\sigma^{54}$-Po promoter is sufficiently powerful to stimulate activity of a known supercoiling-sensitive $\sigma^{70}$-promoter.

Since transcription from $P_{leu\text{-}500}$ is known to be highly dependent on topological promoter coupling with a divergent promoter, the results above support the notion that, upon activation, transcription from the Po promoter generates a domain of increased negative supercoiling that stimulates Pr activity. Because pharmacological agents such as the gyrase inhibitor novobiocin used to manipulate supercoiling in *E. coli* (23) were ineffective in experiments with *P. putida*, we next generated a series of transcriptional reporters that allowed an artificial increase in negative supercoiling within the intergenic region to test this issue.

To manipulate *in vivo* supercoiling, we took advantage of our previous finding that a strong divergent $\sigma^{70}$-promoter can also stimulate transcription from Pr (34), by generating derivatives where the $\sigma^{54}$-Po promoter is replaced by the powerful $\sigma^{70}$-$P_{trc}$ promoter or a mutant variant. In addition, we also included a derivative where the $P_{trc}$ promoter controls expression of *tetA*. Because transcription, translation and anchorage of membrane proteins occurs concurrently in bacteria, expression of membrane proteins such as TetA further hinders rotation of the RNAP around the DNA and leads to the accumulation of higher levels of transcription-driven supercoiling in plasmids (41,43–45). The data in Figure 3B shows that the $P_{trc}$ promoter stimulates Pr activity 5-fold, as compared to its mutant counterpart, while $P_{trc}$-driven expression of TetA results in a remarkable 43-fold increase in Pr activity. Based on this data, we conclude that Po mediated stimulation of Pr activity occurs through topological promoter coupling that results in a local negative supercoiling domain within the intervening DNA of the two promoters.

### Promoter derivatives that alleviate rate-limiting steps of Pr activity

Having established that negative DNA supercoiling underlies Pr stimulation by divergent transcription, we next addressed which step(s) of transcriptional initiation from Pr is stimulated by this mechanism. Changes in superhelicity can affect transcription initiation in several ways, depending on the promoter characteristics. If binding is a rate-limiting step for promoter output, the effect of negative supercoiling on the helical twist can change the structure of the promoter DNA to a form that can be recognized by the RNAP to allow binding (5,46). If open complex formation is a rate-limiting step, negative supercoiling can provide the energy of activation required to destabilize local regions of the DNA duplex in order to favour DNA melting and the formation of the open-complex (5).

Both Pr ($^{-35}$TTGACT$^{-30}$ -$N_{17}$- $^{-12}$CTGGCT$^{-7}$, consensus residues underlined) and $P_{leu\text{-}500}$ ($^{-35}$TTGACA$^{-30}$ -$N_{17}$- $^{-12}$TGCCAC$^{-7}$) are intrinsically weak promoters. Pr naturally lacks the highly conserved -11A, while the -11A→G substitution is the mutation that renders the $P_{leu\text{-}500}$ supercoiling sensitive. The -11A and the -7T of the -10 element are particularly important for single stranded DNA binding by region 2.3 of $\sigma^{70}$, with lesser and varying contributions from the bases at positions −10 to −8 (47–49). In addition, the −11A plays a crucial role in the nucleation of promoter DNA melting, with substitution to other bases resulting in a slow rate of open-complex formation (35,50,51).

Previous analysis of the Pr promoter showed that introduction of a consensus A at the −11 position resulted in hyperactivity of Pr through enhancing binding of $\sigma^{70}$-RNAP to form the initial closed-complex and stimulation of the kinetics of open-complex formation—two steps that are both facilitated by the net co-action of ppGpp and DksA (35). We reasoned that one or both of these steps likely contributed to the sensitivity of Pr to divergent transcription by $\sigma^{54}$-Po and other promoters ($\sigma^{70}$-$\lambda P_L$ and $\sigma^{70}$-$P_{trc}$). To

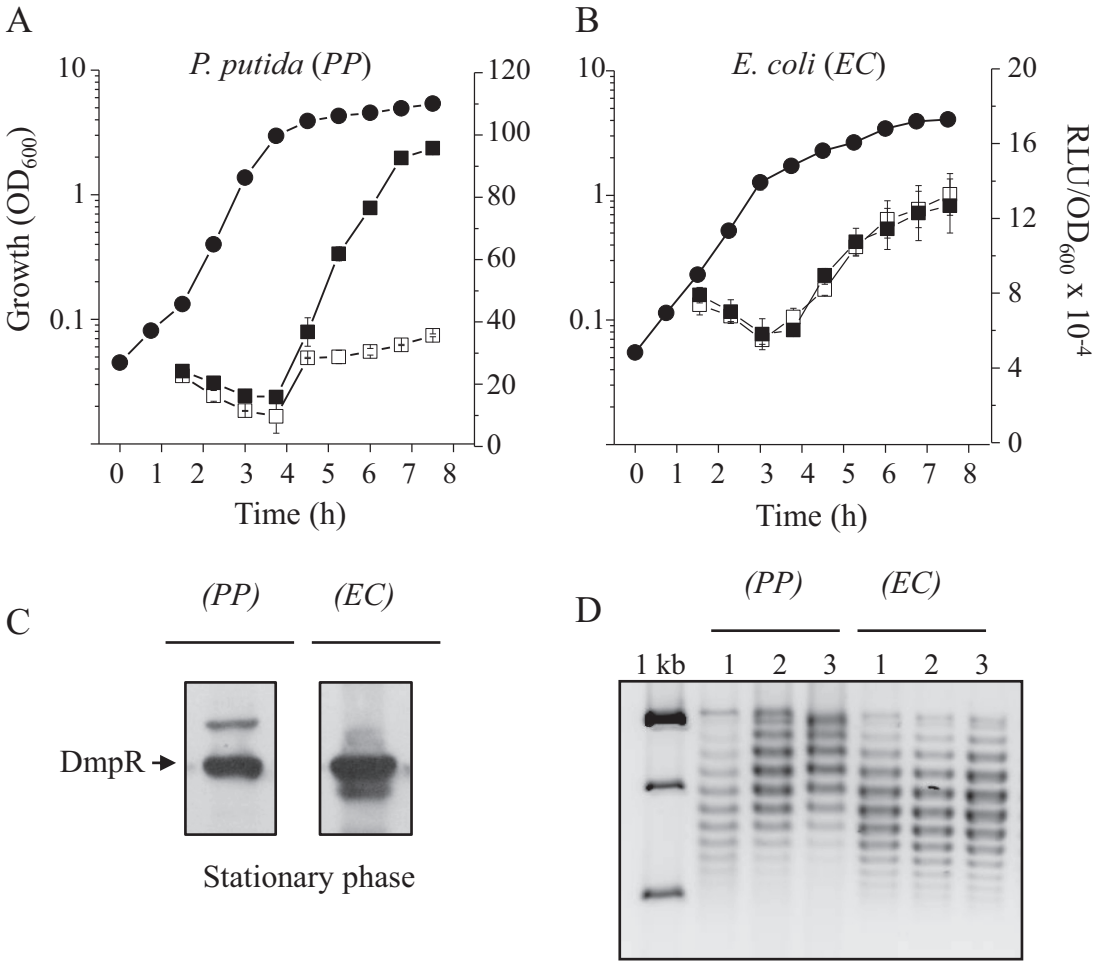


**Figure 2.** Pr promoter stimulation by Po activity coincides with changes in DNA supercoiling. (**A**) Luciferase reporter gene assay of *P. putida* KT2440::*dmpR*-Tel harbouring the Pr-*luxAB* transcriptional reporter plasmid pVI938 (−265 to +215 relative to Pr +1). Growth (circles) and luciferase activity profiles (squares) are of LB-cultured cells grown at 30°C in the absence (open symbols) or presence (closed symbols) of 2 mM of the DmpR effector 2-methylphenol. Values are the average of triplicate determinations ± SE. Where not discernible, SE are within the size of the symbol. (**B**) Luciferase reporter gene assay of *E. coli* MG1655 harbouring the Pr-*luxAB* transcriptional reporter plasmid pVI938 and pVI2404, which constitutively produces DmpR-His. Cells were grown and treated as under panel A. Note the change of scale compared to panel A, and that the two growth curves essentially superimpose so that the open circles (no effector) growth curve are not shown. (**C**) Western analysis of DmpR levels in 30 μg of soluble protein from *P. putida* (*PP*) or *E. coli* (*EC*) cells harvested at the seven hour time point in the presence of 2-methylphenol as in panels A and B. Images are from the same exposure of proteins co-resolved on the same 10% polyacrylamide gel. (**D**) Topoisomer distribution of pSEVA541 extracted from cells at different growth phases: 1, mid exponential ($OD_{600}$ ~ 0.5); 2, exponential-to-stationary phase transition ($OD_{600}$ ~3 for *PP*, ~2 for *EC*); 3, stationary phase ($OD_{600}$ ~ 4.0, 7 h time point). Note that topoisomers with higher superhelical density migrate faster.

test this idea, in addition to the T-11A substitution ($Pr_{T\text{-}11A}$), we generated a promoter variant designed to be altered in only one of these two steps, namely binding of $\sigma^{70}$-RNAP to form the initial closed-complex. For this, we generated $Pr_{UP}$, a derivative of Pr that possesses an AT-rich UP element between positions −60 and −40 relative to the +1 start site of *dmpR* (Figure 4A)—to facilitate formation of the closed-complex step by providing additional double stranded DNA interactions through the α-subunits of RNAP. We then subjected $Pr_{UP}$ to the same series of *in vitro* assays previously used to delineate the properties of $Pr_{T\text{-}11A}$ (35).

As anticipated, electro-mobility shift assays (EMSA) performed at 4°C (which maintains closed $\sigma^{70}$-RNAP-promoter complexes) revealed that $Pr_{UP}$, like $Pr_{T\text{-}11A}$, results in increased binding of $\sigma^{70}$-RNAP as compared to $Pr_{WT}$, and that binding of $\sigma^{70}$-RNAP is further stimulated by the presence of DksA at all three promoters (Figure 4B and C). DNase I footprinting experiments performed at 4°C verified that the UP element of the $Pr_{UP}$ derivative resulted in an expected promoter-upstream extension of protection by $\sigma^{70}$-RNAP (Table 1).

To assess the potential of these promoter variants to form open-complexes, we performed DNase I (Figure 5A and Supplementary Figure S3) and $KMnO_4$ footprinting at 30°C (Supplementary Figure S4). As summarised in Table 1, both $Pr_{WT}$ and $Pr_{UP}$ exhibited heparin-sensitive protection patterns typical of closed or unstable intermediate complexes, i.e. extending to the +1 position. Again, $Pr_{UP}$ resulted in an expected promoter-upstream protection extension. The complexes formed by $Pr_{WT}$ and $Pr_{UP}$ contrast those formed with $Pr_{T\text{-}11A}$, which spontaneously formed

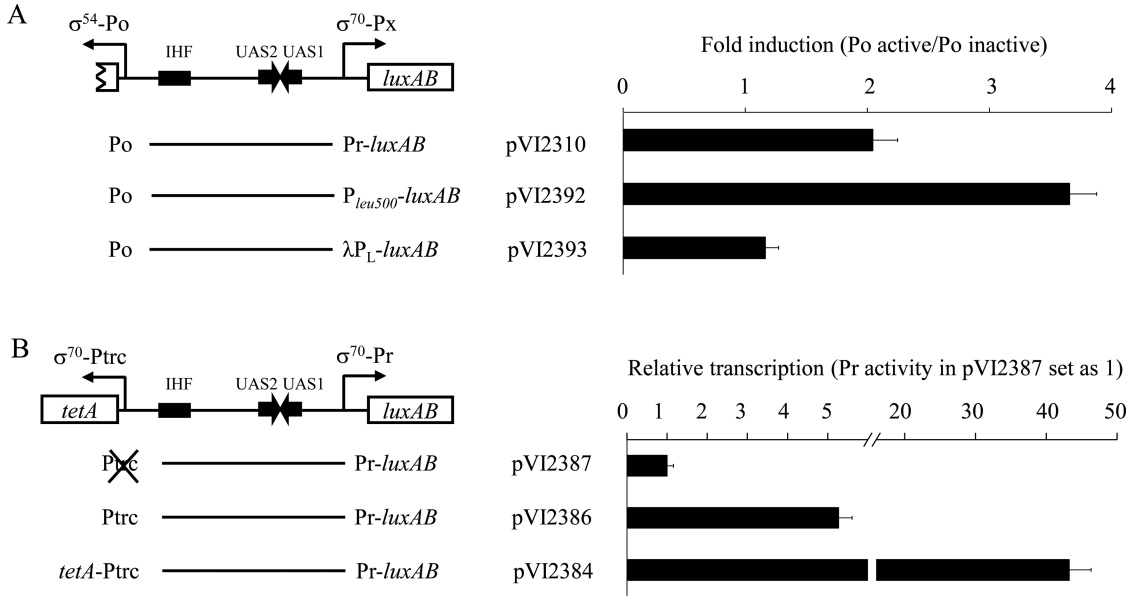


**Figure 3.** Topological promoter coupling underscores Pr promoter stimulation. (**A**) Left, schematic of the luciferase transcriptional reporter plasmids used to test the effect of activity at the $\sigma^{54}$-Po promoter on divergent transcription from a supercoiling-dependent $\sigma^{70}$-promoter (P*leu-500*) and a strong $\sigma^{70}$-promoter ($\lambda P_L$). Right, graphed values represent the fold induction in stationary phase promoter activity in response to Po activity in LB-cultured *P. putida* KT2440::*dmpR*-Tel. Values are that average of triplicate determinations from two independent experiments ± SE and were determined by dividing the activity found when cultured in the presence of 2-mM 2-methylphenol (Po active) by those cultured in the absence of the DmpR effector (Po inactive). (**B**) Effect of negative supercoiling on Pr activity. The graphed values correspond to stationary phase luciferase activities of LB-cultured *P. putida* KT2440 carrying the Pr-*luxAB* transcriptional reporter plasmids illustrated on the left, in which the Po promoter has been replaced by either $\sigma^{70}$-$P_{trc}$, $\sigma^{70}$-$P_{trc}$ controlling expression of *tetA*, or a mutant variant of $\sigma^{70}$-$P_{trc}$ (indicated by a cross). Values are the average of triplicate determinations from two independent experiments ± SE normalized by setting the value of pVI2387 as 1.

**Table 1.** Summary of promoter complexes formed with $Pr_{WT}$, $Pr_{UP}$ and $Pr_{T\text{-}11A}$

| Footprinting[a] | $Pr_{WT}$ | $Pr_{UP}$ | $Pr_{T\text{-}11A}$ |
|---|---|---|---|
| DNase I 4°C NC | −57 to +2 | −60 to +2 | −57 to +2 |
| DNase I 30°C NC | −37 to +1 | −60 to +1 | −37 to +17 |
| C | −50 to +1 | −56 to +1 | −50 to +22 |
| KMnO4 30°C NC | – | – | −11, −6, +1 |
| C | – | – | −7, −5 |

[a]NC, non-coding strand; C, coding strand. For DNase I footprinting, the extent of the DNA protected by binding of $\sigma^{70}$-RNAP is given relative to the +1 transcriptional start of *dmpR*. In the case of KMnO4 footprinting, numbers refer to the positions of reactive thymines in binary complexes.

heparin-stable open-complexes, to result in (i) footprints extending to +17 (on the non-coding strand) and +22 (on the coding strand), and (ii) ready detection of reactive thymines within the open-complex (Table 1, Figure 5A, Supplementary Figures S3 and S4; 35).

The inability of $Pr_{WT}$ and $Pr_{UP}$ to spontaneously form open-complexes suggested that the $Pr_{UP}$ variant, as expected, is unaltered in its kinetics of open-complex formation. To verify that this was the case, we determined the activities of the three Pr promoter variants over time in single-round *in vitro* transcription assays using linear DNA templates. In these assays, the measurement of full-length transcripts represents the number of heparin-stable (transcriptionally-competent) complexes present at each time point. The kinetics for $Pr_{UP}$, like $Pr_{WT}$, were slow and did not reach saturation within the time course of the experiment (Figure 5B). The $Pr_{T\text{-}11A}$ variant, on the other hand, exhibited markedly accelerated kinetics as previously shown (35). Taken together, these data verify that $Pr_{UP}$ has increased affinity for binding to $\sigma^{70}$-RNAP (as does $Pr_{T\text{-}11A}$) but exhibits slow kinetics for formation of transcriptionally competent open-complexes that are indistinguishable from those of $Pr_{WT}$.

### Both binding affinity for $\sigma^{70}$-RNAP and open-complex formation kinetics contribute to supercoiling sensitivity and Pr stimulation by divergent transcription

To determine the consequences of the defined alterations in $\sigma^{70}$-RNAP binding and open-complex kinetics of the three Pr promoter variants, we first assayed their sensitivity to divergent transcription *in vivo* using transcriptional reporters. As shown in Figure 6A, increased binding of $\sigma^{70}$-RNAP by $Pr_{UP}$ resulted in ∼6- to 10-fold increased Pr output; however, stimulation by activity of the divergent Po promoter was curtailed (∼1.5-fold as compared to ∼2.75-fold for $Pr_{WT}$ in the stationary phase). In the case of $Pr_{T\text{-}11A}$, the combined increased affinity for $\sigma^{70}$-RNAP and accelerated open-complex formation kinetics, results in ∼10- to 25-fold increased promoter activity (depending on the growth

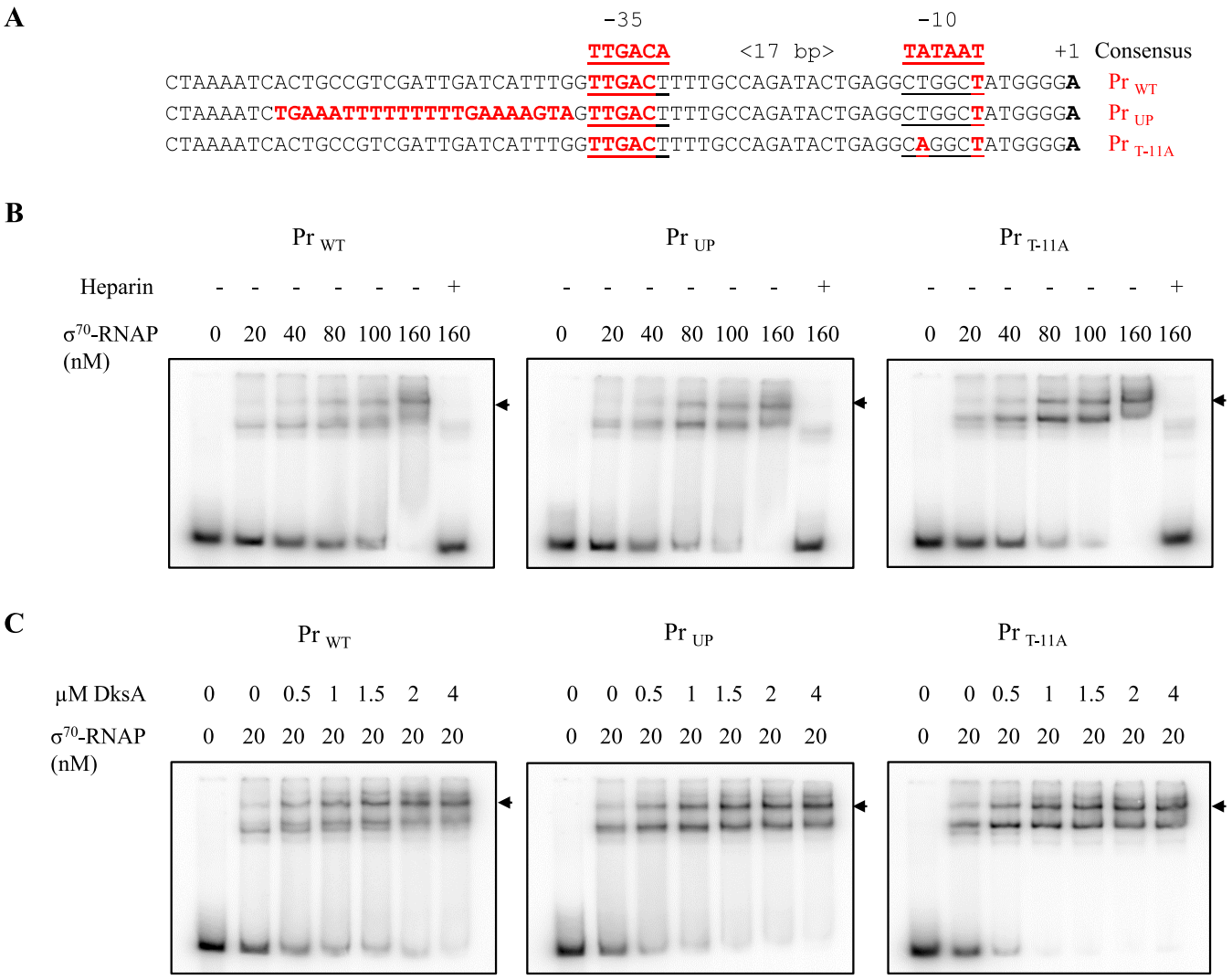


**Figure 4.** $Pr_{UP}$ bypasses the $\sigma^{70}$-RNAP binding defect of $Pr_{WT}$. (**A**) Sequences of Pr wild-type ($Pr_{WT}$) and its UP ($Pr_{UP}$) and T-11A ($Pr_{T\text{-}11A}$) variants. Promoter −10 and −35 elements are underlined with bases matching the consensus indicated in red. The UP element introduced in Pr $_{UP}$ variant is likewise shown in red. (**B**) EMSA assays with linear DNA probes (2 nM) encompassing the −100 to +123 $Pr_{WT}$ region or its $Pr_{UP}$ or $Pr_{T\text{-}11A}$ variants and increasing amounts of $\sigma^{70}$-RNAP (0, 20, 40, 80 or 160 nM) at 4°C. Where indicated (+), heparin was added during the last 5 min of a 60 min binding. Results are representative of at least three independent experiments. The major Pr promoter complexes are indicated by arrows. The additional, faster migrating, complex is as previously observed (35) and is presumed to be $\sigma^{70}$-RNAP binding to a cryptic but non-functional promoter site or different conformations of the double stranded DNA in these native gels. (**C**) EMSA assays as in panel B, but with 20 nM $\sigma^{70}$-RNAP and increasing amounts of DksA (0, 0.5, 1, 1.5, 2 or 4 μM) at 4°C. Results are representative of 3 independent experiments.

phase) and total insensitivity to the activity of the divergent Po promoter. These results demonstrate that compromised $\sigma^{70}$-RNAP binding and slow open-complex formation kinetics are both targeted to contribute to the net stimulation of wild-type Pr activity by divergent transcription.

To further analyse the repercussions of divergent transcription and sensitivity to the supercoiled status of the DNA, we also analysed the performance of the three promoter variants *in vitro* using supercoiled and relaxed (topoisomerase I-treated) plasmid DNA as templates (Figure 6B). *In vitro*, activity from the divergent Po promoter resulted in readily detectable increased transcription from $Pr_{WT}$, but not for $Pr_{UP}$, which is curtailed in this property *in vivo*. However, transcript levels from both $Pr_{WT}$ and $Pr_{UP}$ are decreased when using relaxed topoisomerase I-treated templates, showing the importance of negative supercoiling for efficient transcription from both these promoters. In contrast, and consistent with the *in vivo* insensitivity to divergent transcription in any growth phase, the strong $Pr_{T\text{-}11A}$ promoter variant was also insensitive to the supercoiled status of the DNA *in vitro*.

The above results suggest that while both compromised $\sigma^{70}$-RNAP binding and open-complex formation kinetics contribute to sensitivity to divergent transcription, it is primarily the slow kinetics of open-complex formation—i.e. DNA melting—which renders the Pr promoter sensitive to the superhelicity of the DNA. Taken together with the analysis of the repercussions of natural and artificial changes in the *in vivo* supercoiling status of plasmid DNA (Figures 1–3 and 5A), this analysis of the performance of different

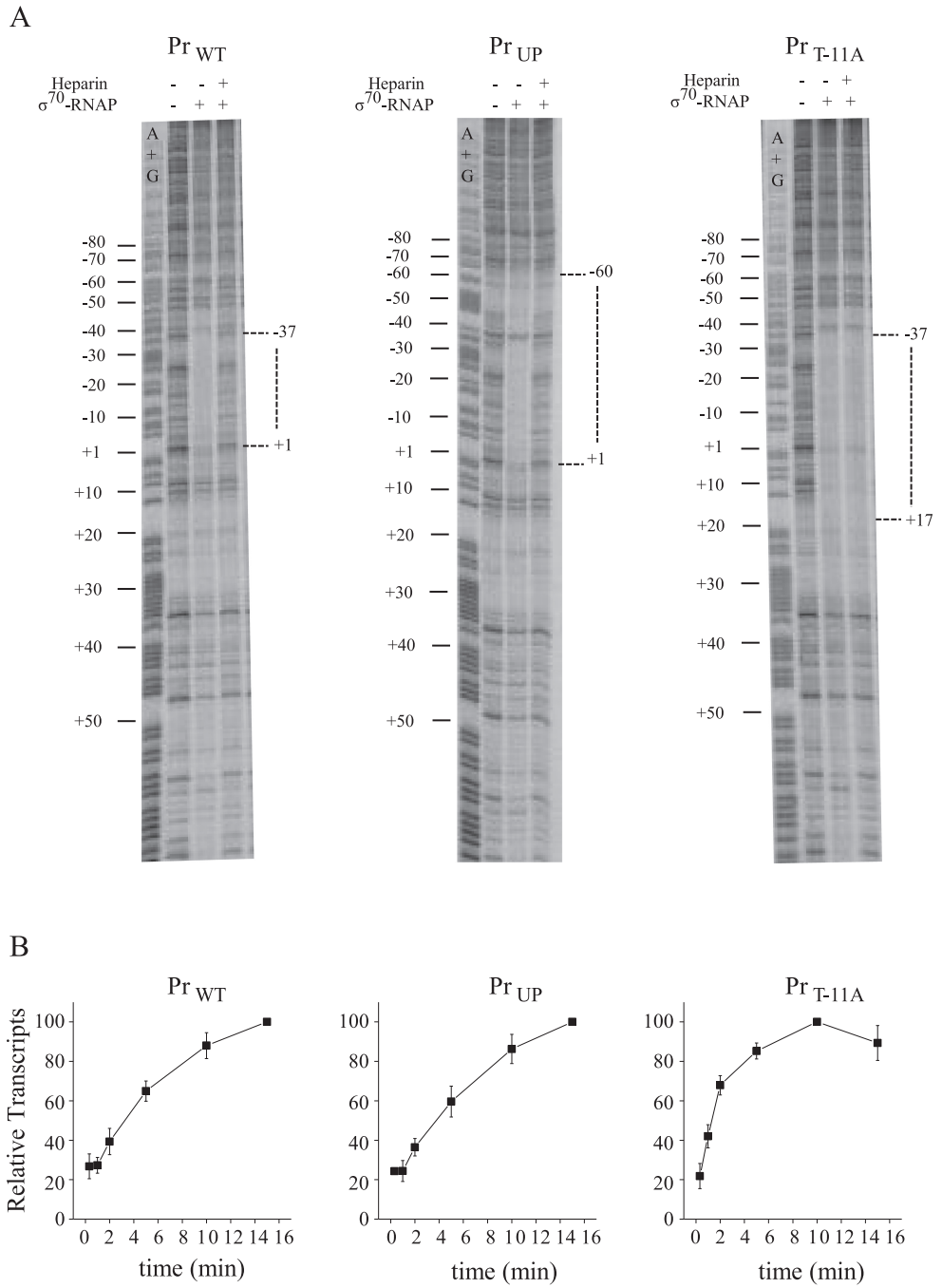


**Figure 5.** $Pr_{UP}$ maintains the slow open-complex formation kinetics of $Pr_{WT}$. (**A**) DNase I footprinting of $\sigma^{70}$-RNAP (100 nM) binding to the non-coding strand (−112 to +126; 17 nM) of $Pr_{WT}$, $Pr_{UP}$ or $Pr_{T\text{-}11A}$ at 30°C for 30 min. The regions protected from DNase I cleavage are indicated between dashed lines. A + G indicates Maxam and Gilbert sequencing reaction. (**B**) Single round *in vitro* transcription assays on linear DNA templates encompassing the (−265 to +8) $Pr_{WT}$ region, or corresponding $Pr_{UP}$ or $Pr_{T\text{-}11A}$ variants. Assays were performed at 30°C with 10 nM template and 25 nM $\sigma^{70}$-RNAP. Relative transcripts after different incubation times (from 20 s to 15 min) for heparin-stable complex formation are shown with the maximum transcript levels obtained in each case set as 100. Data are the average of two or more independent experiments ± SE.

Pr promoter variants lends strong support for a key role of DNA supercoiling in topological coupling from the $\sigma^{54}$-Po promoter to result in stimulation of the intrinsically weak $\sigma^{70}$-Pr promoter. In contrast to the case of two promoters dependent on the same σ-factor, because Po is dependent on the levels of $\sigma^{54}$-RNAP, this mechanism constitutes a new mode of inter-sigmulon communication whereby performance of a $\sigma^{70}$-promoter is controlled in response to signals that elicit transcription dependent on an alternative form of RNAP.

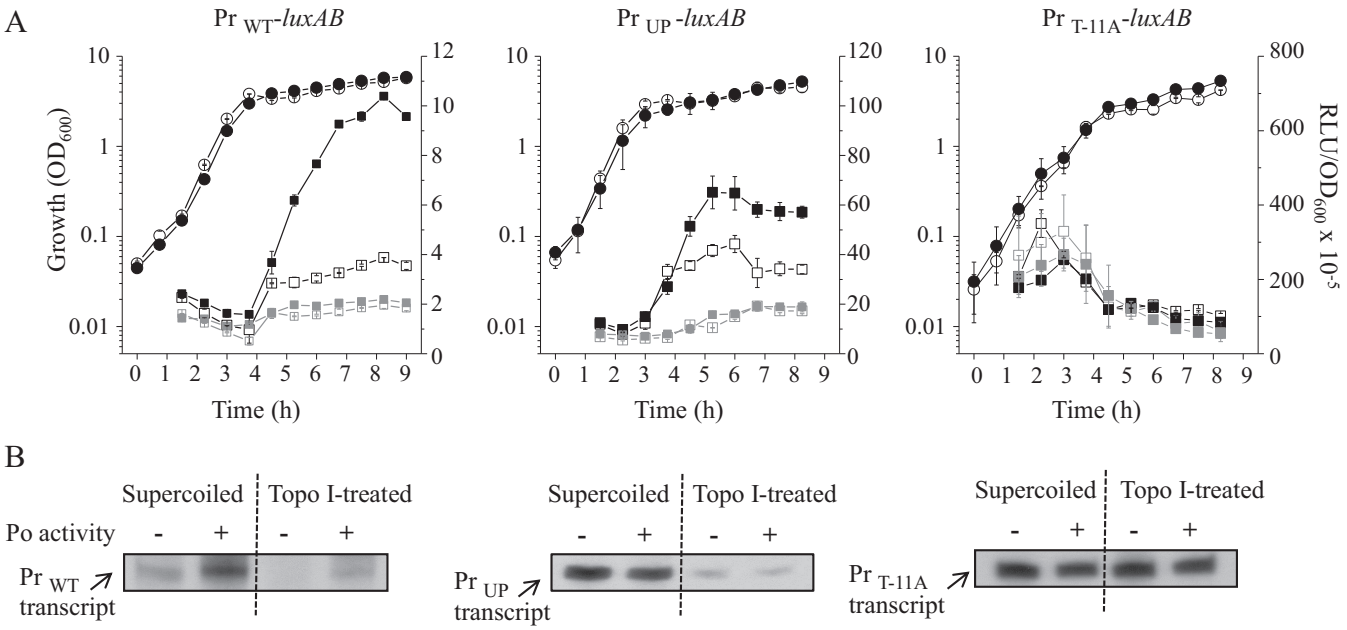


**Figure 6.** Topological promoter stimulation is reduced by enhanced binding of $\sigma^{70}$-RNAP and accelerated open-complex kinetics. (**A**) Luciferase reporter gene assay of *P. putida* KT2440::*dmpR*-Tel harbouring different Pr-*luxAB* (−265 to +215) transcriptional reporter plasmids bearing the wild-type (black) or a mutated (grey, as under Figure 1B) divergent Po promoter. Growth (circles) and luciferase activity profiles (squares) are of LB-cultured cells grown in the absence (open symbols) or presence (closed symbols) of 2 mM of 2-methylphenol. Values are the average of triplicate determinations ± SE. Where not discernible, SE are within the size of the symbol. (**B**) Single-round *in vitro* transcription assays with 10 nM supercoiled or topoisomerase I-treated plasmid template DNA carrying different (−265 to +8) Pr derivatives. Reactions contained 25 nM $\sigma^{70}$-RNAP, 100 nM ΔA2-His-DmpR in the presence (+) or absence (−) of 25 nM $\sigma^{54}$-RNAP. The autoradiographs show differential exposures of the transcripts from the three promoters to accommodate different promoter strengths and in each case are representative of three or more independent experiments.

### Inter-sigmulon communication as a potentially versatile regulatory device

Recognition of different promoter sequences by alternative σ-factors lies at the top of the hierarchy of events that allow bacteria to adapt to changing conditions—they determine when and under what circumstances the distinct promoter classes within the genome are active. The production and/or activities of σ-factors are themselves governed in response to environmental signals by sophisticated and dedicated mechanisms that operate at all known levels of control (1). Because the consequent global reorganization of the composition of the σ-RNAP holoenzyme pool is associated with concomitant alterations in DNA superhelicity (7), structural coupling to changes in DNA topology provides a mechanism for amplifying (or quenching) promoter activities in response to a similar set of signals. As shown here using the $\sigma^{54}$- and $\sigma^{70}$-dependent promoters of the *dmp*-system, integrating signal-responsive control through transcription-driven topological coupling between 'back-to-back' divergent promoters dependent on different σ-factors presents a simple and effective strategy to allow interplay between promoters of different sigmulons, in this case, resulting in stimulation of the activity of a $\sigma^{70}$-promoter in response to signals that elicit $\sigma^{54}$-dependent transcription from the $\sigma^{54}$-promoter.

Divergent transcription of a regulatory gene and at least one cognate promoter under its control is a common theme in bacterial regulatory circuits, with different circuits employing promoters dependent on either the same or different σ-factors. The newly identified means of inter-sigmulon regulation found here does not need to be limited to the case of $\sigma^{70}$ and $\sigma^{54}$—since conceptually it could operate with promoters dependent on any σ-factor. We suggest that analogous interplay between promoters dependent on different σ-factors has the potential to be a widely utilised regulatory device for signal-integration. However, to be effective, it would require one strong promoter to drive alterations in the activity of a weak promoter because transcription-coupled hypernegative supercoiling is dependent on promoter strength (52)—only stronger promoters, where many repeated cycles of transcription initiation takes place, would be anticipated to generate sufficiently high levels of negative supercoiling in an intergenic region to affect transcription from a divergent promoter. Consistent with this notion, transcription from the strong $\sigma^{54}$-Po promoter, or either of the strong $\sigma^{70}$-dependent $\lambda P_L$ or $P_{trc}$ promoters, can stimulate transcription from the weak $\sigma^{70}$-Pr promoter, but transcription from $\sigma^{70}$-Pr does not affect transcription from the divergent $\sigma^{54}$-Po promoter. Conversely, there is much evidence that improving promoter strength, through mutations in conserved (e.g. -10, -35 and discriminator sequences) and non-conserved (e.g. the −35 to −10 spacer region) promoter elements, can counteract sensitivity to DNA supercoiling (4,6,46,53,54). Again, consistent with these findings, pre-defined mutations that accelerate the rate of open-complex formation and $\sigma^{70}$-RNAP binding to $\sigma^{70}$-Pr contribute to its sensitivity to supercoiling and stimulation by the activity of a divergent promoter.

In addition to growth, DNA superhelicity can be affected by many environmental parameters such as temperature, pH, osmotic stress, anaerobiosis and nutrient availability (16,55,56). These signals overlap with those that elicit synthesis of the stringent response alarmone ppGpp that, along with DksA, plays a key role in orchestrating the composition of the RNAP holoenzyme pool and promoter activity (reviewed in 1). While divergent transcription causes stimulation of $\sigma^{70}$-Pr activity even in the absence of ppGpp and DksA, both topological promoter coupling and the direct action of ppGpp and DksA on the performance of $\sigma^{70}$-RNAP at Pr target the same steps of transcriptional initiation and so work hand-in-hand to amplify its activity under metabolic stress conditions.

## CONCLUDING REMARKS

Inter-sigmulon communication as described here provides both a mechanism to overcome regulatory paradoxes that can arise due to the composition of the σ-RNAP holoenzyme pool, and offers an effective alternative regulatory tactic to having two promoters dependent on alternative σ-factors driving transcription of the same gene. As such, it may well be a prevalent mechanism by which promoter activity is tuned during genome-wide adjustments to prevailing conditions.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

## ACKNOWLEDGEMENTS

The authors are indebted to Eleonore Skärfstad for expert technical assistance.

## FUNDING

Swedish Research Council [621-2011-4791 to VS]; Carl Trygger´s Foundation for Scientific Research [CTS-11-420, to V.S.]; European Molecular Biology Organization through a Long-Term Research Fellowship [540-2009 to T. d. P.-S.]. Funding for open access charge: The Swedish Research Council.
*Conflict of interest statement.* None declared.

## REFERENCES

1. Osterberg,S., del Peso-Santos,T. and Shingler,V. (2011) Regulation of alternative σ-factor use. *Annu. Rev. Microbiol.*, **65**, 37–55.
2. Ross,W., Sanchez-Vazquez,P., Chen,A.Y., Lee,J.H., Burgos,H.L. and Gourse,R.L. (2016) ppGpp binding to a site at the RNAP-DksA interface accounts for Its dramatic effects on transcription initiation during the stringent response. *Mol. Cell*. **62**, 1–13.
3. Liu,L.F. and Wang,J.C. (1987) Supercoiling of the DNA template during transcription. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 7024–7027.
4. Travers,A. and Muskhelishvili,G. (2005) DNA supercoiling - a global transcriptional regulator for enterobacterial growth? *Nat. Rev. Microbiol.*, **3**, 157–169.
5. Opel,M.L., Arfin,S.M. and Hatfield,G.W. (2001) The effects of DNA supercoiling on the expression of operons of the *ilv* regulon of Escherichia coli suggest a physiological rationale for divergently transcribed operons. *Mol. Microbiol.*, **39**, 1109–1115.
6. Niehus,E., Cheng,E. and Tan,M. (2008) DNA supercoiling-dependent gene regulation in *Chlamydia*. *J. Bacteriol.*, **190**, 6419–6427.
7. Geertz,M., Travers,A., Mehandziska,S., Sobetzko,P., Chandra-Janga,S., Shimamoto,N. and Muskhelishvili,G. (2011) Structural coupling between RNA polymerase composition and DNA supercoiling in coordinating transcription: a global role for the omega subunit? *mBio*, **2**, doi:10.1128/mBio.00034-11.
8. Higgins,N.P. (2014) RNA polymerase: chromosome domain boundary maker and regulator of supercoil density. *Curr. Opin. Microbiol.*, **22**, 138–143.
9. Dorman,C.J. (2014) Function of nucleoid-associated proteins in chromosome structuring and transcriptional regulation. *J. Mol. Microbiol. Biotechnol.*, **24**, 316–331.
10. Wu,H.Y., Shyy,S.H., Wang,J.C. and Liu,L.F. (1988) Transcription generates positively and negatively supercoiled domains in the template. *Cell*, **53**, 433–440.
11. Tsao,Y.P., Wu,H.Y. and Liu,L.F. (1989) Transcription-driven supercoiling of DNA: direct biochemical evidence from *in vitro* studies. *Cell*, **56**, 111–118.
12. Deng,S., Stein,R.A. and Higgins,N.P. (2004) Transcription-induced barriers to supercoil diffusion in the *Salmonella typhimurium* chromosome. *Proc. Natl. Acad. Sci. U.S.A.*, **101**, 3398–3403.
13. Samul,R. and Leng,F. (2007) Transcription-coupled hypernegative supercoiling of plasmid DNA by T7 RNA polymerase in *Escherichia coli* topoisomerase I-deficient strains. *Jo. Mol. Biol.*, **374**, 925–935.
14. Ma,J., Bai,L. and Wang,M.D. (2013) Transcription under torsion. *Science*, **340**, 1580–1583.
15. Chong,S., Chen,C., Ge,H. and Xie,X.S. (2014) Mechanism of transcriptional bursting in bacteria. *Cell*, **158**, 314–326.
16. Drlica,K. (1992) Control of bacterial DNA supercoiling. *Mol. Microbiol.*, **6**, 425–433.
17. Rovinskiy,N., Agbleke,A.A., Chesnokova,O., Pang,Z. and Higgins,N.P. (2012) Rates of gyrase supercoiling and transcription elongation control supercoil density in a bacterial chromosome. *PLoS Genet.*, **8**, e1002845.
18. Tan,J., Shu,L. and Wu,H.Y. (1994) Activation of the *leu-500* promoter by adjacent transcription. *J. Bacteriol.*, **176**, 1077–1086.
19. Chen,D., Bowater,R. and Lilley,D.M. (1994) Topological promoter coupling in *Escherichia coli*: Δ*topA*-dependent activation of the *leu-500* promoter on a plasmid. *J. Bacteriol.*, **176**, 3757–3764.
20. Fang,M. and Wu,H.Y. (1998) A promoter relay mechanism for sequential gene activation. *J. Bacteriol.*, **180**, 626–633.
21. Chen,C.C. and Wu,H.Y. (2003) Transcription-driven DNA supercoiling and gene expression control. *Front. Biosci.*, **8**, d430–d439.
22. Ouimet,M.C. and Marczynski,G.T. (2000) Transcription reporters that shuttle cloned DNA between high-copy *Escherichia coli* plasmids and low-copy broad-host-range plasmids. *Plasmid*, **44**, 152–162.
23. Rhee,K.Y., Opel,M., Ito,E., Hung,S., Arfin,S.M. and Hatfield,G.W. (1999) Transcriptional coupling between the divergent promoters of a prototypic LysR-type regulatory system, the *ilvYC* operon of *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.*, **96**, 14294–14299.
24. Opel,M.L. and Hatfield,G.W. (2001) DNA supercoiling-dependent transcriptional coupling between the divergently transcribed promoters of the *ilvYC* operon of *Escherichi coli* is proportional to promoter strengths and transcript lengths. *Mol. Microbiol.*, **39**, 191–198.
25. Masulis,I.S., Babaeva,Z., Chernyshov,S.V. and Ozoline,O.N. (2015) Visualizing the activity of *Escherichia coli* divergent promoters and probing their dependence on superhelical density using dual-colour fluorescent reporter vector. *Sci. Rep.*, **5**, 11449.
26. Tessier,M.C., Graveline,R., Crost,C., Desabrais,J.A., Martin,C., Drolet,M. and Harel,J. (2007) Effects of DNA supercoiling and topoisomerases on the expression of genes coding for F165(1), a P-like fimbriae. *FEMS Microbiol. Lett.*, **277**, 28–36.
27. Beck,C.F. and Warren,R.A. (1988) Divergent promoters, a common form of gene organization. *Microbiol. Rev.*, **52**, 318–326.
28. Yamada,M., Kabir,M.S. and Tsunedomi,R. (2003) Divergent promoter organization may be a preferred structure for gene control in *Escherichia coli*. *J. Mol. Microbiol. Biotechnol.*, **6**, 206–210.
29. Shingler,V. (2011) Signal sensory systems that impact $\sigma^{54}$-dependent transcription. *FEMS Microbiol. Rev.*, **35**, 425–440.

30. O'Neill,E., Wikstrom,P. and Shingler,V. (2001) An active role for a structured B-linker in effector control of the $\sigma^{54}$-dependent regulator DmpR. *EMBO J.*, **20**, 819–827.
31. Wikstrom,P., O'Neill,E., Ng,L.C. and Shingler,V. (2001) The regulatory N-terminal region of the aromatic-responsive transcriptional activator DmpR constrains nucleotide-triggered multimerisation. *J. Mol. Biol.*, **314**, 971–984.
32. Bernardo,L.M., Johansson,L.U., Solera,D., Skarfstad,E. and Shingler,V. (2006) The guanosine tetraphosphate (ppGpp) alarmone, DksA and promoter affinity for RNA polymerase in regulation of $\sigma^{54}$-dependent transcription. *Mol. Microbiol.*, **60**, 749–764.
33. Bernardo,L.M., Johansson,L.U., Skarfstad,E. and Shingler,V. (2009) $\sigma^{54}$-promoter discrimination and regulation by ppGpp and DksA. *J. Biol. Chem.*, **284**, 828–838.
34. Johansson,L.U., Solera,D., Bernardo,L.M., Moscoso,J.A. and Shingler,V. (2008) $\sigma^{54}$-RNA polymerase controls $\sigma^{70}$-dependent transcription from a non-overlapping divergent promoter. *Mol. Microbiol.*, **70**, 709–723.
35. del Peso-Santos,T., Bernardo,L.M., Skarfstad,E., Holmfeldt,L., Togneri,P. and Shingler,V. (2011) A hyper-mutant of the unusual $\sigma^{70}$-Pr promoter bypasses synergistic ppGpp/DksA co-stimulation. *Nucleic Acids Res.*, **39**, 5853–5865.
36. del Peso-Santos,T., Landfors,M., Skarfstad,E., Ryden,P. and Shingler,V. (2012) Pr is a member of a restricted class of $\sigma^{70}$-dependent promoters that lack a recognizable -10 element. *Nucleic Acids Res.*, **40**, 11308–11320.
37. Sze,C.C. and Shingler,V. (1999) The alarmone (p)ppGpp mediates physiological-responsive control at the $\sigma^{54}$-dependent Po promoter. *Mol. Microbiol.*, **31**, 1217–1228.
38. Hager,D.A., Jin,D.J. and Burgess,R.R. (1990) Use of Mono Q high-resolution ion-exchange chromatography to obtain highly pure and active *Escherichia coli* RNA polymerase. *Biochemistry*, **29**, 7890–7894.
39. Martinez-Garcia,E., Aparicio,T., Goni-Moreno,A., Fraile,S. and de Lorenzo,V. (2015) SEVA 2.0: an update of the Standard European Vector Architecture for de-/re-construction of bacterial functionalities. *Nucleic Acids Res.*, **43**, D1183–D1189.
40. Lilley,D.M. and Higgins,C.F. (1991) Local DNA topology and gene expression: the case of the *leu-500* promoter. *Mol. Microbiol.*, **5**, 779–783.
41. Chen,D., Bowater,R., Dorman,C.J. and Lilley,D.M. (1992) Activity of a plasmid-borne *leu-500* promoter depends on the transcription and translation of an adjacent gene. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 8784–8788.
42. Chen,D., Bowater,R.P. and Lilley,D.M. (1993) Activation of the *leu-500* promoter: a topological domain generated by divergent transcription in a plasmid. *Biochemistry*, **32**, 13162–13170.
43. Mojica,F.J. and Higgins,C.F. (1996) Localized domains of DNA supercoiling: topological coupling between promoters. *Mol. Microbiol.*, **22**, 919–928.
44. Lodge,J.K., Kazic,T. and Berg,D.E. (1989) Formation of supercoiling domains in plasmid pBR322. *J. Bacteriol.*, **171**, 2181–2187.
45. Lynch,A.S. and Wang,J.C. (1993) Anchoring of DNA to the bacterial cytoplasmic membrane through cotranscriptional synthesis of polypeptides encoding membrane proteins or proteins for export: a mechanism of plasmid hypernegative supercoiling in mutants deficient in DNA topoisomerase I. *J. Bacteriol.*, **175**, 1645–1655.
46. Borowiec,J.A. and Gralla,J.D. (1987) All three elements of the *lac* $p^S$ promoter mediate its transcriptional response to DNA supercoiling. *J. Mol. Biol.*, **195**, 89–97.
47. Matlock,D.L. and Heyduk,T. (2000) Sequence determinants for the recognition of the fork junction DNA containing the -10 region of promoter DNA by *E. coli* RNA polymerase. *Biochemistry*, **39**, 12274–12283.
48. Fenton,M.S. and Gralla,J.D. (2001) Function of the bacterial TATAAT -10 element as single-stranded DNA during RNA polymerase isomerization. *Proc. Natl. Acad. Sci. U.S.A.*, **98**, 9020–9025.
49. Feklistov,A. and Darst,S.A. (2011) Structural basis for promoter-10 element recognition by the bacterial RNA polymerase sigma subunit. *Cell*, **147**, 1257–1269.
50. Heyduk,E., Kuznedelov,K., Severinov,K. and Heyduk,T. (2006) A consensus adenine at position -11 of the nontemplate strand of bacterial promoter is important for nucleation of promoter melting. *J. Biol. Chem.*, **281**, 12362–12369.
51. Schroeder,L.A., Gries,T.J., Saecker,R.M., Record,M.T. Jr, Harris,M.E. and DeHaseth,P.L. (2009) Evidence for a tyrosine-adenine stacking interaction and for a short-lived open intermediate subsequent to initial binding of *Escherichia coli* RNA polymerase to promoter DNA. *J. Mol. Biol.*, **385**, 339–349.
52. Zhi,X. and Leng,F. (2013) Dependence of transcription-coupled DNA supercoiling on promoter strength in *Escherichia coli* topoisomerase I deficient strains. *Gene*, **514**, 82–90.
53. Hatfield,G.W. and Benham,C.J. (2002) DNA topology-mediated control of global gene expression in *Escherichia coli*. *Annu. Rev. Genet.*, **36**, 175–203.
54. Dorman,C.J. (2006) DNA supercoiling and bacterial gene expression. *Sci. Progr.*, **89**, 151–166.
55. Dorman,C.J. (1996) Flexible response: DNA supercoiling, transcription and bacterial adaptation to environmental stress. *Trends Microbiol.*, **4**, 214–216.
56. Cameron,A.D., Stoebel,D.M. and Dorman,C.J. (2011) DNA supercoiling is differentially regulated by environmental factors and FIS in *Escherichia coli* and *Salmonella enterica*. *Mol. Microbiol.*, **80**, 85–101.
57. Shingler,V., Bartilson,M. and Moore,T. (1993) Cloning and nucleotide sequence of the gene encoding the positive regulator (DmpR) of the phenol catabolic pathway encoded by pVI150 and identification of DmpR as a member of the NtrC family of transcriptional activators. *J. Bacteriol.*, **175**, 1596–1604.
58. Sze,C.C., Laurie,A.D. and Shingler,V. (2001) *In vivo* and *in vitro* effects of integration host factor at the DmpR-regulated $\sigma^{54}$-dependent Po promoter. *J. Bacteriol.*, **183**, 2842–2851.
59. Sze,C.C., Moore,T. and Shingler,V. (1996) Growth phase-dependent transcription of the $\sigma^{54}$-dependent Po promoter controlling the *Pseudomonas*-derived (methyl)phenol *dmp* operon of pVI150. *J. Bacteriol.*, **178**, 3727–3735.