CBR antimicrobials alter coupling between the bridge helix and the β subunit in RNA polymerase

Anssi M. Malinen1, Monali NandyMazumdar2, Matti Turtola1, Henri Malmi1, Thadee Grocholski1, Irina Artsimovitch2 & Georgiy A. Belogurov1

Bacterial RNA polymerase (RNAP) is a validated target for antibacterial drugs. CBR703 series antimicrobials allosterically inhibit transcription by binding to a conserved α helix (β′ bridge helix, BH) that interconnects the two largest RNAP subunits. Here we show that disruption of the BH-β subunit contacts by amino-acid substitutions invariably results in accelerated catalysis, slowed-down forward translocation and insensitivity to regulatory pauses. CBR703 partially reverses these effects in CBR-resistant RNAPs while inhibiting catalysis and promoting pausing in CBR-sensitive RNAPs. The differential response of variant RNAPs to CBR703 suggests that the inhibitor binds in a cavity walled by the BH, the β′ F-loop and the β fork loop. Collectively, our data are consistent with a model in which the β subunit fine tunes RNAP elongation activities by altering the BH conformation, whereas CBRs deregulate transcription by increasing coupling between the BH and the β subunit.
RNA polymerase (RNAP) mediates synthesis of an RNA copy of the template DNA—the first and often decisive step in gene expression. All RNAPs transcribing cellular genomes are multisubunit enzymes that share homologous catalytic cores\(^1,2\). Bacterial RNAP, a five-subunit complex \(\alpha_2\beta\beta'\omega\), is the simplest model system for studies of fundamental mechanistic properties of all multisubunit RNAPs and a validated target for antibacterial drugs\(^3\).

The cycle of nucleotide incorporation by RNAP is governed by alternate closure and opening of the active site by a \(\beta'\) subunit mobile domain called trigger loop (TL); catalysis of phosphodiester bond formation involves an obligatory closure\(^4,5\), whereas translocation along the DNA requires opening of the active site\(^6\). RNAP also reversibly isomerizes into an off-pathway state that is inhibitory for nucleotide addition. The off-pathway state, aka an elemental pause\(^7\), is the precursor to all regulatory events during transcription elongation, such as longer-lived pauses and termination\(^8\). Long-lived pauses in turn function to synchronize transcription and translation in prokaryotes\(^11,12\) and to recruit regulatory proteins to transcribing RNAP in all domains of life\(^13-15\).

The structural rearrangements accompanying catalysis are relatively well understood. The TL folding into a closed conformation is dependent on the formation of a triple-helical bundle with the \(\beta'\) subunit bridge helix (BH), a long metastable \(\alpha\) helix that spans the active site cleft and moulds into a groove in the \(\beta\) subunit\(^4\) (Fig. 1a). The stability of folded TL is also modulated by its interaction with the \(\beta'\) F-loop, an amino-terminal extension of BH\(^16\). The structural rearrangements associated with isomerization into the elemental pause remain elusive owing to the transient nature of the state. It has been suggested that this isomerization involves fraying of the RNA \(3'\)-end in the active site, kinking of the BH, opening of the \(\beta'\) clamp domain and changes in the template DNA conformation in the vicinity of the active site\(^8,17-20\).

RNAP active site structure evolved to achieve optimal balance between catalytic efficiency, processivity and amenability to regulation\(^21\). First, the stability of folded TL is tuned up to permit both efficient catalysis and rapid translocation that require folding and unfolding of TL, respectively. Second, the propensity to isomerize into the elemental paused state is tuned up to permit both efficient RNA chain elongation and the proper response to regulatory signals. In this work, we present evidence that conformational coupling between the \(\beta\) subunit and BH plays an important role during elongation by RNAP. We also report plausible structural models of CBR703 (N-hydroxy-N'-(3-trifluoromethyl-benzamidine) series inhibitors\(^22,23\) bound in an occluded pocket at the BH-\(\beta\) subunit interface and elucidate mechanistic details of their antibacterial action.

**Results**

RNAPs with amino-acid substitutions at BH-\(\beta\) interface. To gain insights into the mechanism of action of CBR-type antibiotics, we performed the detailed analysis of elongation activities of five RNAPs with substitutions at the BH-\(\beta\) subunit interface, the anticipated binding site of CBRs (Fig. 1b). The BH \(\beta'F773V\) and F-loop \(\beta'P750L\) were first identified in a genetic screen for alleles resistant to CBR-type inhibitors\(^22,23\). \(\beta'F773V\) RNAP was later characterized as pause and terminator resistant\(^24,25\) and has been suggested to have altered translocation and fidelity\(^25\). The D-loop \(\beta'P560S, T563I\) (RpoB5101) RNAP was identified in an in vivo screen as an enzyme with decreased termination\(^26\). The Fork loop \(\beta'V550A\) RNAP was designed to probe interactions of \(\beta'F773\) with the BH Fork loop. The BH \(\beta'H777A\) RNAP was reported to have relatively unaltered \textit{in vitro} activity and mild \textit{in vivo} growth defects in a study by Jovanovic \textit{et al.}\(^27\) Here we reevaluated \(\beta'H777A\) RNAP properties to probe the clash between CBR703 and one of the \(\beta'H777\) conformers revealed by docking experiments (see below).

Most of experiments in this study were performed with wild type and \(\beta'F773V\) RNAPs that also contained a BH \(\beta'N792D\) substitution. The \(\beta'N792D\) substitution increased \textit{Escherichia coli} RNAP sensitivity to streptolydigin (STL)\(^28\) but did not detectably affect the nucleotide addition and translocation rates as well as translocation bias (this work), enabling us to use low concentrations of STL, which do not interfere with fluorescence measurements, to bias RNAP forward in translocation studies.

We assembled variant RNAP transcription elongation complexes (TECs) on chemically synthesized nucleic-acid scaffolds containing fluorescent 6-methyl-isoxanthopterin (6-MI) base in the template strand and used direct time-resolved translocation\(^6\) and nucleotide-addition\(^29,30\) assays to study the effects of substitutions on RNAP translocation equilibrium, translocation rates, catalytic activity and response to CBR703. We also

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**Figure 1 | Binding site of CBR series inhibitors at the BH-\(\beta\) subunit interface.** (a) An overview of the bacterial TEC. \(\beta\) (light blue) and \(\beta'\) (wheat) are depicted as semi-transparent surfaces, \(\alpha\) and \(\alpha'\) (largely obstructed by \(\beta'\)) subunits are depicted as flat grey outlines. BH (orange), F-loop (orange), TL (green—closed conformation, dashed light green—open conformation), RNA (red), template (black) and non-template (grey) DNA strands are depicted as cartoons. Amino-acid residues altered in this study are depicted as spheres. A red arrow indicates the direction of the view in b. (b) CBR703 (sticks with brown carbons and inset) docked at the BH-\(\beta\) subunit interface of \textit{E. coli} RNAP (PDB 4IGC)\(^37\). The native amino-acid residues replaced by CBR703-resistant and-sensitive \(\beta'P560S\) and \(\beta'H777A\) substitutions are depicted as sticks. Cartoons and side chain’s carbons of \(\beta\) and \(\beta'\) are coloured pastel blue and orange, respectively. The outwards\(^37,50,51\) (opaque) and inwards\(^32\) (semi-transparent) facing conformers of \(\beta'\) His777 are shown. Green and black-dashed lines depict polar and \(\pi\)-stacking interactions, respectively. Figure was prepared using PyMOL Molecular Graphics System, Version 1.6.0.0; Schrödinger, LLC. The sources of atomic coordinates are listed in Supplementary Table 3.
compared effects of these substitutions on RNAP response to a regulatory pause site in the presence and absence of CBR703.

**β F773V and β P750L backward bias translocation equilibrium.** All five variant RNAPs translocated forward following the incorporation of the cognate GMP, as judged by increase in 6-MI fluorescence. To assess the completeness of translocation, we compared fluorescence intensities of TECs extended by rNMP and dNMP. The 2’dNMP-extended TECs are anticipated to display higher level of fluorescence than the rNMP-extended TECs unless the latter are fully post-translocated because 2’OH group is essential for stabilizing the pre-translocated state. The 2’dNMP-extended βF773V and β P750L TECs displayed brighter fluorescence than the rNMP-extended TECs (Fig. 2a and Supplementary Fig. 1). In contrast, the fluorescence levels of GMP- and 2’dGMP-extended TECs formed by other RNAPs were the same. Forward-biasing 3’dNMP- and rNMP-extended βF773V and β P750L TECs with the next incoming substrate NTPs and their non-hydrolyzable analogues, respectively, reported fluorescence levels similar to those of the 2’dNMP-extended TECs (Fig. 2a and Supplementary Fig. 1). We concluded that the 2’dNMP-extended βF773V and β P750L TECs are nearly 100% post-translocated, whereas AMP- or GMP-extended TECs contain ~40% and CMP- or UMP-extended TECs contain ~70% of pre-translocated state. Overall, the above experiments revealed that βF773V and β P750L RNAPs displayed a measurable fraction of pre-translocated states, whereas other RNAPs in our set were nearly 100% post-translocated.

**β F773V RNAP translocation is controlled by TL opening.** We employed two antibiotics with established modes of action, tagetitoxin (TGT) and STL, to demonstrate that translocation of βF773V TEC is controlled by opening and closure of the active site by the TL. TGT is a high-affinity pyrophosphate analogue that backward biases RNAP by stabilizing the closed active site. Indeed, addition of saturating amounts of TGT to rNMP-extended TECs reduced their fluorescence to the level of non-extended TECs, which corresponds to the pre-translocated state (Fig. 3). STL binds to the inner face of the BH and stabilizes the open active site conformation, favouring the post-translocated state. Indeed, addition of saturating amounts of STL to rNMP-extended TECs increased their fluorescence to the level of 2’dNMP-extended TECs, which correspond to the post-translocated state (Fig. 2b). CBR703 also forward biased βF773V TECs in a concentration-dependent manner, although it was less potent than STL and failed to quantitatively move the TECs into the post-translocated state (Fig. 2b). Both STL and CBR703 forward biased wild-type RNAP, as evident from their ability to offset the effect of TGT on translocation equilibrium (Fig. 2c). The response of βF773V RNA to STL suggests that the shift of translocation equilibrium towards the pre-translocated state in this RNAP originates, at least in part, from an increased stability of the folded TL. The ability of CBR703 to forward bias βF773V and wild-type RNAPs suggests that the inhibitor destabilizes the folded TL.

Altered RNAPs have decreased forward translocation rate. We performed parallel time-resolved measurements of nucleotide addition and translocation for GMP (and CMP in case of βF773V RNA) addition reactions. The forward and backward translocation rates were inferred from a delay between nucleotide addition and translocation curves using a reversible translocation model, as described in Supplementary Methods. These analyses revealed that RpoB5101 and βV550A substitutions reduced the forward translocation rate to 30–40 s⁻¹ (from 60 s⁻¹ in wild-type RNAP), whereas βF773V and β P750L substitutions reduced the rate to 9 s⁻¹ (Fig. 3 and Supplementary Fig. 2). The backward translocation rate was estimated at 6 s⁻¹ for GMP-extended βF773V and β P750L TECs and at 20 s⁻¹ for CMP-extended βF773V TEC (Fig. 3d), but did not measurably contribute to the kinetics of fluorescence change in other RNAPs and was fixed to zero during analyses. Whereas the 6 s⁻¹ rate could be potentially masked by 60 s⁻¹ forward translocation rate in wild-type RNAP (assuming 10% uncertainty in determination

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**Figure 2 | Effects of CBR703 and substitutions at the BH-β subunit interface on translocation equilibrium.** Top schematic describes the experimental set-up. Best fit curves were simulated using parameters described in Supplementary Table 4. Fluorescence data were averaged over two to three experiments. (a) βF773V TECs display measurable fractions of the pre-translocated state. Fluorescence of rNMP (grey fill) and 3’dNMP (pink fill)-extended TECs normalized to the level of 2’dNMP-extended TECs. White bars depict the effects of the next substrate NTP (pink outline) or its non-hydrolyzable analogue (grey outline). Error bars are s.d. (b) CBR703 and STL forward-biased βF773V RNAP sensitized to STL by the βN792D substitution. Left panel: GMP-extended TEC. Right panel: CMP-extended TEC. TGT (red) and STL (black) quantitatively move the TECs into pre- and post-translocated states, respectively. CBR703 (orange) and cytidine-5’-[(α,β)-methylene]triphosphate (CMPαPP; blue) measurably forward bias the TECs. (c) STL and CBR703 offset TGT effects on wild-type RNA to TST by βN792D substitution. TGT (red) quantitatively converts post-translocated GMP-extended TEC into pre-translocated TEC. TGT is less potent in backward-biasing RNA in the presence of 0.75 μM STL (olive) and 100 μM CBR703 (purple). STL (black) and CBR703 (orange) forward bias RNA in the presence of 0.1 μM TGT.
of equilibrium levels of fluorescence), the 20 s⁻¹ value strongly suggests that β′F773V substitution increases backward translocation rate. The shift of translocation equilibrium towards the pre-translocated state in β′F773V RNAp thus originates from both the decrease in forward and increase in backward translocation rates. The decrease in BH mobility or bendability25 alone is not sufficient to explain such observations, whereas stabilization of the folded TL, in part as a consequence of less bendable BH, satisfactory explains the observed effects.

Altered RNAPs are differentially sensitive to CBR703. We investigated the effect of CBR703 on nucleotide addition and translocation rates of variant RNAPs in the single nucleotide addition assay. Preincubation of TECs with 100 µM CBR703 lengthened nucleotide addition cycle twofold in the wild-type and RpoB5101 RNAPs, had little effect on βV550A and facilitated the completion of the cycle by β′P750L and β′F773V RNAp twofold (Fig. 3c). β′H777A RNAp was inhibited nearly 250-fold and is unaffected in RpoB5101 and stimulated in βV550A, β′P750L and β′F773V RNAp (Fig. 3c). CBR703 also reduced the backward rate of β′P750L TEC below the detection threshold and marginally slowed-down backward translocation of β′F773V TECs (Fig. 3d). The effect of CBR703 on translocation rates of β′P750L and β′F773V RNAp is consistent with its
forward-biasing effect in the equilibrium assay (Fig. 2b) and reinforces the hypothesis that inhibitor promotes the TL unfolding. Consistently, with the results of single nucleotide addition assays, CBR703 permitted rapid processive transcription through the long template by \( \beta'F773V, \beta'P750L \) and \( \beta'V550A \) RNAPs (Fig. 4 and Supplementary Fig. 3). In contrast, transcription by wild-type RNAP was significantly impeded by multiple pauses.

**Altered RNAPs have reduced sensitivity to regulatory pause.** \( \beta'F773V \) and RpoB5101 RNAPs are among the least pause responsive \( E. \ coli \) RNAPs hitherto characterized\(^2^4\). To find out whether pause insensitivity is a general property of RNAPs with substitutions at the BH-\( \beta \) subunit interface, we evaluated their response to the hairpin-stabilized \( hisP \) pause element using a standard single-round in vitro transcription assay\(^3^3\). We used the pIA171 linear transcription template on which the \( hisP \) was positioned downstream from a strong T7A1 promoter (Fig. 4). On this template, radiolabelled transcription complexes can be halted at position A29 when transcription is initiated in the absence of UTP, with ApU dinucleotide, ATP, GTP and \( \alpha-[\text{32P}]CTP \). The halted A29 complexes can then be chased on addition of all four NTP substrates. We found that, similar to \( \beta'F773V \) and RpoB5101 RNAPs characterized earlier\(^2^4\), \( \beta'P750L \) and \( \beta'V550A \) RNAPs were relatively resistant to the \( hisP \) pause (Fig. 4 and Supplementary Fig. 3), suggesting that weakening BH-\( \beta \) subunit contacts universally leads to insensitivity to regulatory pauses. Strikingly, the sensitivity of \( \beta'F773V, \beta'P750L \) and \( \beta'V550A \) RNAPs to \( hisP \) pause was partially restored in the presence of CBR703. The latter result suggests that CBR703 promotes formation of native intermediates in the pausing pathway.

**In silico docking of CBRs at the BH-\( \beta \) interface.** It has been anticipated for some time that CBR-type compounds bind at the BH-\( \beta \) subunit interface of \( E. \ coli \) RNAP near \( \beta'F773 \) based on extensive set of in vivo selected CBR703- and CBR9379-resistant mutations\(^2^2\). Here we used AutoDock Vina\(^3^4\) and GOLD\(^3^5,3^6\) programs, which rely on different algorithms and scoring functions, to identify the binding sites for CBR703, CBR9379 and CBR9393 in \( E. \ coli \) RNAP holoenzyme crystal structure (PDB 4IGC)\(^3^7\). Both Vina and GOLD failed to identify a common binding mode for the three inhibitors to rigid RNAP but robustly recovered overlapping binding sites for CBR9379 and CBR9393 when \( \beta' \text{Leu770} \) and \( \beta' \text{Phe773} \) side chains were set flexible (Supplementary Fig. 4). Moreover, Vina independently recovered the same binding mode for CBR703 (Fig. 1b), a relatively symmetric substructure of CBR9379.

In the resulting models, the structural moieties common for the three CBRs are positioned in a spacious cavity walled by the BH,
translocation traces because of dense temporal sampling. We noted that CBR703 increased the fraction of slow TEC in CBR-sensitive (wild type and RpoB5101) but not CBR-resistant (BV550A and BV773V) RNAPs (Fig. 3 and Supplementary Fig. 2). The increase in fraction of slow TEC is particularly apparent in a set of translocation time curves of the wild-type RNAP recorded at increasing concentration of CBR703 (Fig. 5a) and becomes explicit when the CBR-hypersensitive βH777A RNAP is used (Fig. 5b). We found that the simplest kinetic model consistent with the wild-type and βH777A RNAPs data postulates that the slow TEC is an inactive TEC in slow equilibrium with an active TEC (Fig. 5c). Note that such definition of a slow TEC matches the definition of a paused TEC. The isomerization rate constants were also in the order of those estimated for the elemental pause in single molecule experiments9,38. The model predicts that CBR has a dual effect on transcription: it slows down nucleotide addition twofold and promotes isomerization of active TECs into inactive TECs.

CBR703 promotes TEC isomerization into an inactive state. All assembled TECs that we characterized to date contain 5–25% of a slow reacting TEC (1–2 s⁻¹)⁹. The slow fraction originates at or before the nucleotide addition step, but is only well resolved in translocation traces because of dense temporal sampling. We noted that CBR703 increased the fraction of slow TEC in CBR-sensitive (wild type and RpoB5101) but not CBR-resistant (BV550A and BV773V) RNAPs (Fig. 3 and Supplementary Fig. 2). The increase in fraction of slow TEC is particularly apparent in a set of translocation time curves of the wild-type RNAP recorded at increasing concentration of CBR703 (Fig. 5a) and becomes explicit when the CBR-hypersensitive βH777A RNAP is used (Fig. 5b). We found that the simplest kinetic model consistent with the wild-type and βH777A RNAPs data postulates that the slow TEC is an inactive TEC in slow equilibrium with an active TEC (Fig. 5c). Note that such definition of a slow TEC matches the definition of a paused TEC. The isomerization rate constants were also in the order of those estimated for the elemental pause in single molecule experiments⁹,38. The model predicts that CBR has a dual effect on transcription: it slows down nucleotide addition twofold and promotes isomerization of active TECs into inactive TECs.

A stronger effect of CBR703 on transcription: it slows down nucleotide addition twofold and promotes isomerization of active TECs into inactive TECs. A stronger effect of CBR703 on βH777A RNAP originates from changes in two distinct equilibria: first, the substitution increases CBR703-binding affinity fivefold; second, CBR703 increases bias towards the inactive state 38-fold in βH777A RNAP (Kiso = 0.26, Kbar = 10) but has only 2.5-fold effect in the wild-type RNAP (Kiso = 0.35, Kbar = 0.83). The capacity of CBR703 to promote TEC isomerization into an inactive state inferred from single nucleotide addition experiments in Fig. 5 is entirely consistent with the CBR703 effects on transcription through the long template in Fig. 4 and Supplementary Fig. 3, where CBR703 gradually halts βH777A RNAP at multiple sites and restores pause sensitivity of CBR-resistant RNAPs.

Discussion
Collectively, our data are consistent with the model where disruption of the BH-β subunit contacts relaxes RNAP into a
ground state characterized by the predominantly closed active site, fast and error-prone catalysis, slow translocation and insensitivity to regulatory pauses. βPF773V RNAP is a quintessential example of such RNAP. In contrast, in the wild-type RNAP, motions of the β subunit modulate on-pathway elongation and isomerization into off-pathway states (Fig. 6). Specifically, conformational coupling between the BH and β subunit destabilizes BH–TL interactions in a controllable manner, thereby fine tuning translocation and catalysis. Perhaps independently, the β subunit–BH interactions also control the equilibrium between the active and paused states of the TEC via the BH anchor and switch 1 regions\(^1\). From a structural perspective, decoupling from the β subunit enables the BH to adopt its native helical conformation and to form multiple interactions with the folded TL, thereby promoting the active site closure. The lack of β−induced distortions in the BH structure also diminishes transitions associated with pausing: isomerization of template DNA strand and opening of the clamp domain, which have been structurally linked to the distorted BH\(^2\). In contrast, binding of CBR inhibitors fills the void at the pathway. The latter effect may play the major role in the increasing RNAP propensity to isomerize into inactive state(s). The β subunit also induces larger distortions in the BH conformation, increasing RNAP propensity to isomerize into inactive state(s) that kinetically resembles the natural intermediates of the pausing pathway. The latter effect may play the major role in the antibacterial action of CBR inhibitors. Whereas the overall decrease in elongation rate slows down bacterial growth, a small increase in propensity to isomerize into the inactive state at each sequence position increases the frequency of long-lived pause and arrest events, ultimately leading to premature cessation of transcription that is detrimental for cell viability. Similarly, we hypothesize that deregulated transcription, rather than the slow forward translocation rate, makes βPF773V and βPF750L RNAPs inviable in the absence of CBRs\(^3\). Binding of CBR inhibitors restores the coupling between the BH and β subunit in these RNAPs, thereby restoring transcriptional regulation and supporting viability of the mutant strains.

**Methods**

**Proteins and reagents.** DNA and RNA oligonucleotides were purchased from IDT (Coralville, IA, USA), TdT and GTP from New England BioLabs (Ipswich, MA, USA), and Fidelity Systems (Gaithersburg, MD, USA). TdT was from Epicentre (Madison, WI, USA), CBR703 from Maybridge (Tintagel, UK) and STL from SourcOn-Padena (Tübingen, Germany). Cytidine-5′-[(3′β),-methylene][(3′β)-methylene]triprophosphate and guanine-5′-[(3′β),-methylene]triprophosphate were from Jena Bioscience (Jena, Germany). RNAPs and yeast inorganic pyrophosphatase were expressed and purified as described previously\(^4\). Plasmids are listed in Supplementary Table 1. Template strand oligonucleotides and RNA primers are listed in Supplementary Table 2. Schematics of all nucleic-acid scaffolds used in this study are shown in Supplementary Fig. 5.

**TEC assembly.** TECs (1 μM) were assembled by a procedure developed by Komissarova et al.\(^5\) An RNA primer labelled with Atto680 fluorescent dye at the 5′-end was annealed to template DNA, and incubated with 1.5 μM RNAP for 10 min at 25 °C in TB10D buffer (10 mM MgCl\(_2\), 40 mM HEPES-KOH pH 7.5, 80 mM KCl, 5% glycerol, 2.5% dimethylsulphoxide, 0.1 mM EDTA and 0.1 mM DTT) and with 2 μM of the non-template DNA for 20 min at 25 °C. For TECs used in nucleotide addition measurements, RNA was the limiting component at 1 μM and the template strand was used at 1.4 μM, whereas for TECs used in translocation, the template strand was limiting at 1 μM and RNA was added at 1.4 μM.

**Nucleotide addition measurements.** To determine the incorporation efficiency of NTP, 2′, 3′-dNTP substrates, 1 μM TEC in 20 μl of TB10D buffer was incubated for 10 min with 5 μM substrates at 25 °C and quenched by adding 80 μl of loading buffer (94% formamide, 13 mM Li\(_2\)EDTA and 0.2% Orange G). Time-resolved measurements were performed in an RQF 3 quench-flow instrument (KinTek Corporation, Austin, TX, USA). The reaction was initiated by rapid mixing of 14 μl of 0.4 μM TEC with 14 μl of 400 nM NTP. Both TEC and NTP solutions were prepared in TB10D buffer and, where indicated, supplemented with 100 μM CBR703. The reaction was allowed to proceed for 0.004–10 s at 25 °C, quenched with 86 μl of 0.5 M HCl and immediately neutralized by adding 171 μl of loading buffer (290 mM Tris base, 13 mM EDTA, 0.2% Orange G, 94% formamide). RNAs were separated on 16% denaturing polyacrylamide gels and visualized with Odyssey Infrared Imager (Li-Cor Biosciences, Lincoln, NE, USA); band intensities were quantified using ImageJ software\(^6\).

**Translocation measurements.** RNAP translocation was assayed by monitoring changes in fluorescence of 6-MI base incorporated into template DNA5. Equilibrium levels of fluorescence were determined by recording emission spectra of 6-MI (excitation at 340 nm) with an LS-55 spectrofluorometer (Perkin Elmer, Waltham, MA, USA) at 25 °C. The fluorescence at peak emission wavelength (420 nm) was used for data analysis and representation. Preassembled TECs were diluted at 50–100 μl into 500 μl of TB10D buffer, supplemented with 40 mM pyrophosphatase in a Quartz SUPRASIL Macro/Semi-micro Cell (Perkin Elmer; catalogue number B0631123) and the initial fluorescence was recorded. NTP substrates (5 μM) and RNAP inhibitors were then sequentially added into the cuvette under continuous mixing and incubated for 5 min before taking each reading. Time-resolved measurements were performed in an Applied Photophysics (Leatherhead, UK) SX.188MV stopped-flow instrument at 25 °C. The reaction was initiated by mixing 60 μl of 0.2 μM TEC with 60 μl of 400 nM NTP. Both solutions were prepared in TB10D buffer and, where indicated, supplemented with 0.4–250 μM CBR703. 6-MI fluorophore was excited at 340 nm and emitted light was collected through 400 nm longpass filters. At least three individual traces were averaged for each reported curve.

**Single-round pause assays.** TECs were formed for 15 min at 37 °C with 30 nM linear PCR-generated pIA171 template and 40 nM RNAP holoezyme in 20 mM Tris-acetate, 20 mM Na-acetate, 2 mM Mg-acetate, 14 mM 2-mercaptoethanol, 0.1 mM EDTA and 4% glycerol, pH 7.9. To halt RNAP after the addition of A29, synthesis was initiated in the absence of UTP, with 150 μM ApU, 5 μM ATP and 1 μM CTP supplemented with [α-32P]-CTP. For βPF773V enzyme, ATP and GTP were used at 50 μM and CTP at 10 μM to allow for efficient halted complex formation. Halted complexes were incubated with CBR703.

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**Figure 6 | Antibiotic-binding sites outline an allosteric path mediating regulation of transcription elongation.** (a) The β subunit (light blue) interacts with nucleic-acid determinants implicated in regulation of transcription elongation\(^5\): the nascent RNA\(^7\) (red, blue surfaces) and unpaired non-template DNA\(^5,8\) (black, purple β surface). β′ subunit is depicted as a contour, and α and ω subunits as grey outlines. (b) Allosteric effects (black arrows) travel through rifampicin (RIF, sterically\(^5,9\) and allosterically\(^10\)) inhibits transcription initiation) -binding site (blue surface, amino-acid substitutions alter RNAP pausing propensities\(^2\)) -binding site (cyan surface, amino-acid substitutions lead to pausing defects) outlines the interface where allosteric effects are transferred to the BH (orange cartoon). STL (inhibits transcription elongation by restricting TL movement of the BH–TL interactions) -binding site (yellow surface) marks the region where conformational changes in BH modulate the stability of the folded TL (green cartoon). Myxopyronin (restrains mobility of the β′ clamp\(^1,6\) and alters position of template DNA strand\(^1,6\) binds to the flexible region (pink surface, switch 1 as green cartoon on the background) that controls movement of β′ clamp (green surface) and conformation of the template DNA strand (grey cartoon). β′ subunit is depicted as a contour, and α and ω subunits as grey outlines. The figure was prepared using PyMOL Molecular Graphics System, Version 1.6.0.0; Schrödinger, LLC. The sources of atomic coordinates are listed in Supplementary Table 3.
Docking experiments. The three-dimensional structures of CRB703, CRB9379 and CRB9393 were built in Discovery Studio 3.5 (Accelrys, San Diego, CA, USA) and optimized using Minimize Ligands protocol and CHARMM force field46. In CRB703 and CRB9379 benzene rings interconnected by N- oxhydroxamidine, moiety were modelled in cis-configuration, whereas N-oxhydroxamidine and carbamide (in CRB9379) moieties were modelled planar with non-rotatable C-N and C-O bonds. In CRB9393, the bond between the benzene ring and α-nitrogen of piperezinylmethylnicotin group was set non-rotatable, whereas ternary nitrogen of piperezinyl moiety was protonated and positively charged during docking runs. RNAP fragment comprising amino-acid residues within 20 Å from the putative CBR-binding cavity (Supplementary Data 4) was extracted from E. coli RNAP holoenzyme crystal structure (PDB 4GC1)37 and prepared for docking using Prepare Protein protocol of Discovery Studio (for GOLD runs) and AutoDock tools47 (for AutoDock Vina runs). GOLD 5.2 (ref. 35) docking runs were performed using LIGSITE-binding cavity detection algorithm38 and GoldScore scoring function. AutoDock Vina 1.1.2 docking runs were performed in 25 × 25 × 18 Å search space centred at 130.9, 6.8, −6.7 Å (coordinate space of Supplementary Data 1–4) using default scoring function44.

Data analyses. Time-resolved nucleotide incorporation and translocation data were simultaneously fit to a three-step model using the numerical integration capabilities of KinTek Explorer software49 (KinTek Corporation). The model postulated that the initial TEC16 slowly and reversibly interconverts between inactive and active states and, on the addition of the NTP substrate, undergoes an irreversible transition to TEC17, followed by irreversible translocation except for T/F773V and T/P750L RNAPs where translocation was modelled as a reversible process. Equilibrium titration data were fit to the dissociation equilibrium50,51.

References
1. Werner, F. & Grohmann, D. Evolution of multisubunit RNA polymerases in the three domains of life. Nat. Rev. Microbiol. 9, 85–98 (2011).
2. Svetlov, V. & Nudler, E. Basic mechanism of transcription by RNA polymerase. Biochim. Biophys. Acta 1829, 20–28 (2013).
3. Wang, D., Bushnell, D. A., Westover, K. D., Kaplan, C. D. & Kornberg, R. D. Structural basis of transcriptional pausing. Mol. Cell 27, 409–421 (2007).
4. Sydow, J. F. et al. Structural basis of transcription: mismatch-specific fidelity mechanisms and paused RNA polymerase II with frayed RNA. Mol. Cell 34, 710–721 (2009).
5. Sevostyanova, A., Belogurov, G. A., Mooney, R. A., Landick, R. & Artsimovitch, I. The beta subunit gate is required for RNA polymerase modification by RifA and NusG. Mol. Cell 43, 253–262 (2011).
6. Weixlbauer, A., Leon, K., Landick, R. & Darst, S. A. Structural basis of transcriptional pausing in bacteria. Cell 152, 431–441 (2013).
7. Rein, S. J. & von Hippel, P. H. Thinking quantitatively about transcriptional regulation. Nat. Rev. Mol. Cell Biol. 6, 221–232 (2005).
8. Artsimovitch, I., Chu, C., Lynch, A. S. & Landick, R. A new class of bacterial RNA polymerase inhibitor affects nucleotide addition. Science 302, 650–654 (2003).
9. Villain-Guillot, P., Guaitleri, M., Bastide, L. & Leonetti, J. P. In vitro activities of bacterial inhibitors of bacterial transcription against Staphylococcus epidermidis biofilm. Antimicrob. Agents Chemother. 51, 3117–3121 (2007).
10. Vassylyev, V., Belogurov, G. A., Shabrova, E., Vassylyev, D. G. & Artsimovitch, I. Allosteric control of the RNA polymerase by the elongation factor RifA. Nucleic Acids Res. 35, 5694–5705 (2007).
11. Hedvall, Y. A. et al. The RNA polymerase bridge helix YF1 motif in catalysis, fidelity and translocation. Biochim. Biophys. Acta 1829, 187–98 (2013).
12. Landick, R. & Stewart, J. & Lee, M. G. & Landick, R. Molecular mechanisms of paused RNA polymerase: experimental methods and data analysis. Methods Enzymol. 371, 251–264 (2003).
13. Holmes, S. F., Foster, J. E. & Erle, D. A. Kinetics of multisubunit RNA polymerases: experimental methods and data analysis. Methods Enzymol. 371, 71–81 (2003).
14. Artsimovitch, I. et al. Tagetitoxin inhibits RNA polymerase through trapping of the trigger loop. J. Biol. Chem. 286, 40395–40400 (2011).
15. Takse, S. et al. Inhibition of bacterial RNA polymerase by streptolydigin: stabilization of a stable-bridge-helix active-center conformation. Cell 122, 541–552 (2005).
16. Landick, R., Wang, D. & Chan, C. L. Quantitative analysis of transcriptional pausing by Escherichia coli RNA polymerase: his leader pause site as paradigm. Methods Enzymol. 274, 334–353 (1996).
17. Trott, O. & Olson, A. J. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J. Comput. Chem. 31, 454–461 (2010).
18. Jones, G., Willett, P. & Glen, R. C. Molecular recognition of receptor sites using a genetic algorithm with a description of a desolvation. J. Mol. Biol. 245, 43–53 (1995).
19. Jones, G., Willett, P., Glen, R. C., Leach, A. R. & Taylor, R. Development and validation of a genetic algorithm for flexible docking. J. Mol. Biol. 267, 727–748 (1997).
20. Murakami, K. S. X-ray crystal structure of Escherichia coli RNA polymerase C7070 helix. J. Biol. Chem. 288, 9126–9134 (2013).
21. Herbert, K. M. et al. Sequence-resolved detection of pausing by single RNA polymerase molecules. Cell 125, 1083–1094 (2006).
22. Weinzierl, R. O. The nucleotide addition cycle of RNA polymerase is controlled by two molecular hinges in the Bridge Helix domain. BMC Biol. 8, 134 (2010).
23. Heinkelsheim, P. et al. A site-directed mutagenesis study of Saccharomyces cerevisiae pyrophosphatase. Eur. J. Biochem. 239, 138–143 (1996).
43. Belogurov, G. A. et al. Structural basis for converting a general transcription factor into an operon-specific virulence regulator. Mol. Cell 26, 117–129 (2007).

44. Komissarova, N., Kireeva, M. L., Becker, J., Sidorenkov, I. & Kashlev, M. Engineering of elongation complexes of bacterial and yeast RNA polymerases. Methods Enzymol. 371, 233–251 (2003).

45. Abramoff, M. D., Magalhaes, P. J. & Ram, S. J. Image processing with ImageJ. Biophotonics Int. 11, 36–42 (2004).

46. Brooks, B. R. CHARMM: a program for macromolecular energy, minimization, and dynamics calculations. J. Comput. Chem. 4, 187–217 (1983).

47. Sanner, M. F. Python: a programming language for software integration and development. J. Mol. Graph. Model. 17, 57–61 (1999).

48. Hendlich, M., Rippmann, F. & Barnickel, G. LIGSITE: automatic and efficient detection of potential small molecule-binding sites in proteins. J. Mol. Graph. Model. 15, 389 (1997).

49. Johnson, K. A. Fitting enzyme kinetic data with KinTek Global Kinetic Explorer. Methods Enzymol. 467, 601–626 (2009).

50. Bae, B. et al. Phage T7 Gp2 inhibition of Escherichia coli RNA polymerase involves misappropriation of σ70 domain I.I. Proc. Natl Acad. Sci. USA 110, 19772–19777 (2013).

51. Zuo, Y., Wang, Y. & Steitz, T. A. The mechanism of E. coli RNA polymerase regulation by ppGpp is suggested by the structure of their complex. Mol. Cell 50, 430–446 (2013).

52. Opalka, N. et al. Complete structural model of Escherichia coli RNA polymerase from a hybrid approach. PLoS Biol. 8, e1000483 (2010).

53. Santangelo, T. J. & Artsimovitch, I. Termination and antitermination: RNA polymerase runs a stop sign. Nat. Rev. Microbiol. 9, 319–329 (2011).

54. Touloukhonov, I., Artsimovitch, I. & Landick, R. Allosteric control of RNA polymerase by a site that contacts nascent RNA hairpins. Science 292, 730–733 (2001).

55. Ha, K. S., Touloukhonov, I., Vassylyev, D. G. & Landick, R. The NusA N-terminal domain is necessary and sufficient for enhancement of transcriptional pausing via interaction with the RNA exit channel of RNA polymerase. J. Mol. Biol. 401, 708–723 (2010).

56. Bochkareva, A., Yuzenkova, Y., Tadigotla, V. R. & Zenkin, N. Factor-independent transcription pausing caused by recognition of the RNA-DNA hybrid sequence. EMBO J. 31, 630–639 (2012).

57. Vassylyev, D. G., Vassylyeva, M. N., Perederina, A., Tahirou, T. H. & Artsimovitch, I. Structural basis for transcription elongation by bacterial RNA polymerase. Nature 448, 157–162 (2007).

58. Zhang, Y. et al. Structural basis of transcription initiation. Science 338, 1076–1080 (2012).

59. Campbell, E. A. et al. Structural mechanism for rifampicin inhibition of bacterial RNA polymerase. Cell 104, 901–912 (2001).

60. Artsimovitch, I. et al. Allosteric modulation of the RNA polymerase catalytic reaction is an essential component of transcription control by rifamycins. Cell 122, 351–363 (2005).

61. Mukhopadhyay, J. et al. The RNA polymerase “switch region” is a target for inhibitors. Cell 135, 295–307 (2008).

62. Srivastava, A. et al. New target for inhibition of bacterial RNA polymerase: ‘switch region’. Curr. Opin. Microbiol. 14, 532–543 (2011).

63. Chakraborty, A. et al. Opening and closing of the bacterial RNA polymerase clamp. Science 337, 591–595 (2012).

64. Belogurov, G. A. et al. Transcription inactivation through local refolding of the RNA polymerase structure. Nature 457, 332–335 (2009).