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A novel decatenation assay for DNA topoisomerases using a singly-linked catenated substrate

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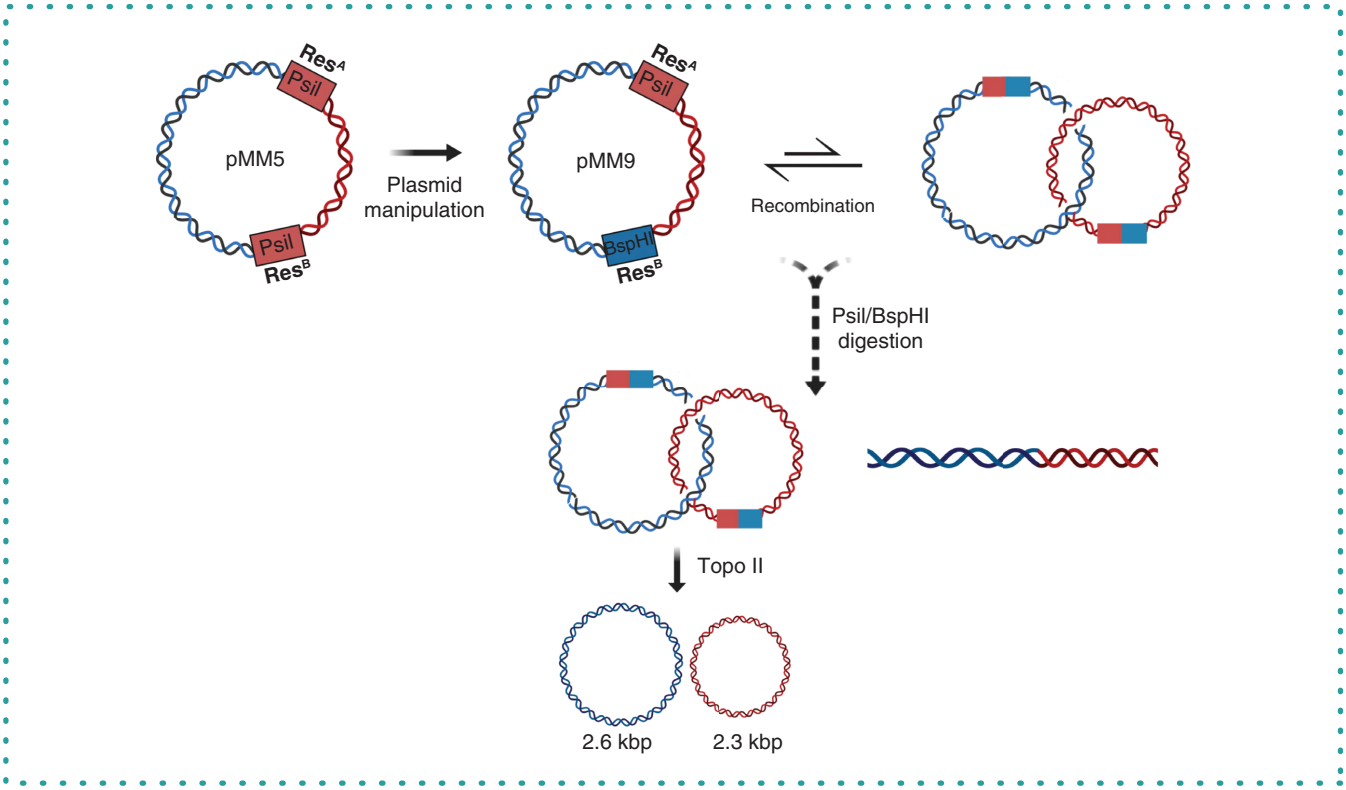
ABSTRACT

Decatenation is a crucial *in vivo* reaction of DNA topoisomerases in DNA replication and is frequently used in *in vitro* drug screening. Usually this reaction is monitored using kinetoplast DNA as a substrate, although this assay has several limitations. Here we have engineered a substrate for Tn*3* resolvase that generates a singly-linked catenane that can readily be purified from the DNA substrate after restriction enzyme digestion and centrifugation. We show that this catenated substrate can be used with high sensitivity in topoisomerase assays and drug-inhibition assays.

METHOD SUMMARY

We engineered a plasmid such that reaction with Tn*3* resolvase generates a singly-linked catenane product. Restriction enzyme digestion and caesium chloride density gradient centrifugation enable the purification of the catenane product substantially free from starting plasmid. This product may be utilized in topoisomerase enzyme assays.

GRAPHICAL ABSTRACT



KEYWORDS:
antibiotics ● anticancer drugs ● decatenation ● gyrase ● topoisomerase

DNA topoisomerases are enzymes that modulate DNA topology [1–3]. These essential enzymes catalyze the interconversion between different topological forms of DNA and are categorized, on evolutionary and mechanistic grounds, into two types: I and II. The two types are distinguished by the number of phosphotyrosyl linkages established in the covalent DNA–protein complex. Type I enzymes form a single linkage, leading to transient single-stranded DNA breaks, whereas type II topoisomerases form transient double-stranded breaks via two phosphotyrosyl linkages. The reactions characteristic of topoisomerases include the relaxation, supercoiling, knotting/unknotting and catenation/decatenation of their DNA substrates.

Topoisomerases are biologically essential enzymes involved in several critical cellular processes including DNA replication [2,4]. The process of replication imposes various topological modifications upon the DNA at different stages. At replication termination, as two replication forks converge, catenation (interlinking) of the daughter chromosomes can result [5] and are removed by the action of type II topoisomerases passing one daughter through a double-stranded break in the other. Naturally occurring DNA catenanes were first observed in mitochondrial DNA isolated from HeLa cell lines [6] and have since been identified to be the predominant method of organizing DNA in the mitochondria of trypanosomes; this mitochondrial DNA is commonly referred to as kinetoplast DNA (kDNA) [7]. Analysis of the kDNA network reveals two types of DNA circles that are interlinked to form an intricate network of maxicircles (20–40 kbp) and mini-circles (~2.5 kbp) [8].

The most common method for assaying the decatenation activity of topoisomerases *in vitro* involves the use of purified kDNA as a substrate [9]. The assay monitors the production of mini-circles released from the kDNA. The inherently complex arrangement of kDNA has several practical implications that can lead to difficulties in interpretation of agarose gel-based assays. One such issue is the significant size of kDNA; its large structure barely enters the pores of an agarose gel, and it is normally retained in the wells. Additionally, the nature of the current assay does not allow for quantitative analysis of decatenation events; this is because the number of links between the maxi- and mini-circles that must be removed to release the circles is variable [10].

Given these issues, we aimed to develop a simpler and more suitable substrate to replace kDNA in decatenation assays. The development of our alternative substrate, a singly-linked DNA catenane, improves the sensitivity and reliability of the assay. The method involves the use of a site-specific recombinase protein, Tn*3* resolvase, which is encoded by the Tn*3* transposon, together with ß-lactamase and Tn*3* transposase genes [11]. Tn*3* resolvase is a DNA site-specific serine recombinase, responsible for the resolution of cointegrate intermediates during the replicative transposition pathway. The specific site of action of resolvase, the 'resolution site' (*res*), encompasses a 114-bp sequence, divided into three subsites: site I (28 bp), site II (34 bp) and site III (25 bp) (Figure 1). DNA cleavage and rejoining reactions occur at site I, whereas sites II and III act as accessory sites and play a role in the regulation of the enzyme's activity. The Tn*3* recombination reaction is initiated by the binding of Tn*3* resolvase subunits to the two *res* sites to form *res*–resolvase complexes. These complexes combine to form the synaptic complex that is composed of two intertwined *res* sites. Subsequently, double-stranded DNA cleavage occurs at each of the res site I sequences, followed by strand exchange and rejoining to re-form two intact *res* sites. When the substrate is a circular DNA molecule containing two directly repeated *res* sites, this process leads to the formation of singly-linked catenanes as products [12].

We have harnessed theTn*3* resolvase reaction to generate a singly-linked catenated product ('bis-cat') comprising two DNA circles of different sizes that can be used in DNA topoisomerase assays, building on recently published work [13]. We modified the Tn*3* res sites so that restriction sites present before recombination are destroyed during the reaction, allowing easy purification of the bis-cat product.

Materials & methods

Tn*3* resolvase overexpression & purification

Overexpression of Tn*3* resolvase was achieved by transforming the expression vector pSA1101 [14] into BL21 (DE3) pLysS competent cells. The *Escherichia coli* culture was grown to late log phase, induced with 0.1 mM of IPTG and grown for a further 3 h. Resolvase purification was carried out as described previously [15].

Plasmids & DNA

Plasmid pMM6 was modified from pMM5 [16] by site-directed mutagenesis at *res* site I. This was achieved using the In-Fusion HD® cloning kit following the manufacturer's guidelines and using the oligonucleotides in Table 1. The conversion of pMM6 into pMM9 involved the removal of three natural BspHI restriction enzyme sites from the pMM6 plasmid, again by site-directed mutagenesis using the In-Fusion HD® cloning kit; primer sequences are given in Table 1. The resultant plasmid, pMM9, was transformed into ER2925 cells (a *dam* methylation-deficient background). Supercoiled pBR322 and kDNA were obtained from Inspiralis Ltd. (Norwich, UK).

Tn*3* resolvase-mediated *in vitro* recombination

The optimum concentration of resolvase determined for an efficient recombination reaction was 20 μM per 1 μM of substrate DNA. Resolvase was diluted in resolvase dilution buffer (20 mM of Tris·HCl [pH 7.5], 1 mM DTT, 0.1 mM EDTA, 1 M NaCl, 50% v/v glycerol) as required. Standard recombination reactions were performed in 50 mM of Tris·HCl (pH 8.2), 10 mM of $MgCl_2$ and 0.1 mM of EDTA.

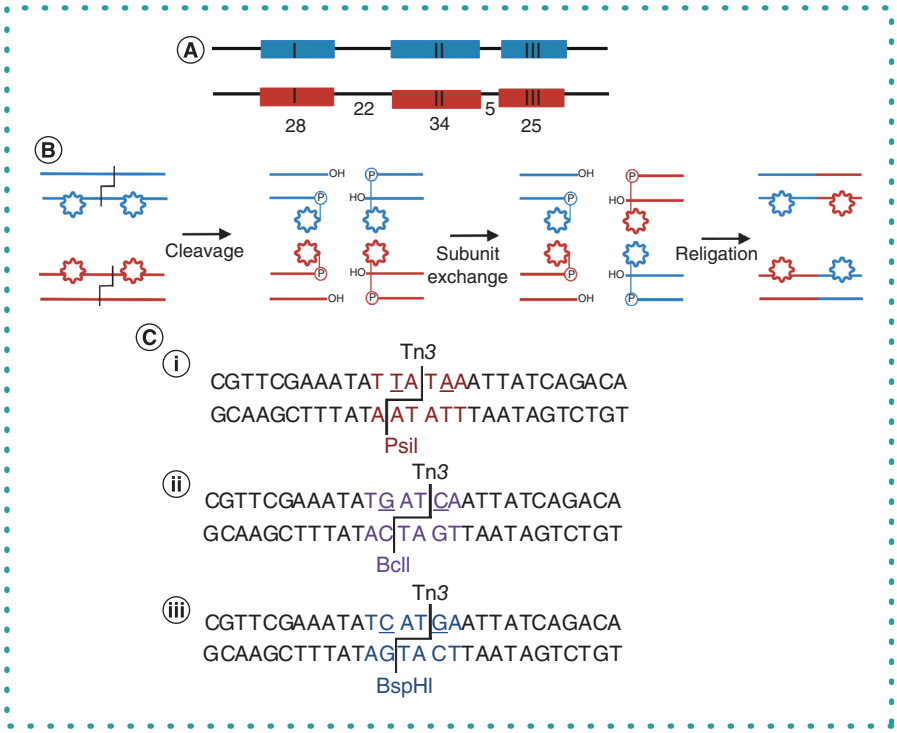


Figure 1. Reaction scheme for Tn*3*-mediated recombination. (A) Representation of the Tn*3* recombination site (*res*); resolvase dimers bind to each of the *res* subsites I, II and III. The numbers indicate the length of each subsite and intermediate regions between subsites in bp. **(B)** The suggested mechanism of action of serine recombinases, focusing on *res* site I. Resolvase dimers (subunits depicted by polygons) are bound to each *res* I site (the *res* I sites are bought into close proximity with one another by the accessory *res* sites II and III to form the synaptic complex, not shown). Resolvase cleaves all four strands at *res* site I, each 5′ end becoming covalently linked to a resolvase subunit via a phosphodiester link. Subsequently, the cleaved DNA strands are exchanged and religated in their new configuration. **(C)** (i) Sequence of *res* site I. The natural PsiI recognition sequence is highlighted in red, and specific Tn*3* sites of cleavage are indicated by black bars. The underlined T and A bases on the top strand of the PsiI recognition sequence indicate nucleotide mutation of which have a minimal effect on Tn*3* resolvase reaction efficiency. (ii) *res* site I PsiI recognition sequence changed to BclI sequence (purple). (iii) *res* site I PsiI recognition sequence changed to BspHI sequence (blue).

Table 1. Plasmids created for use in the Tn*3* recombination reactions.

Plasmid	res^{A} †	res^{B}	Unique RE	Forward primer		Reverse primer	
pMM5	PsiI	PsiI	–				
pMM6	PsiI	BspHI	PsiI	CATGATATTTCGAACGGACTAGTGAGC		CATGAATTATCAGACATAGGAATTCGGCTTCG	
pMM7	PsiI	BclI	BclI PsiI	TGTCTGATAATTGATCATATTTCGAACGGTTGCA		CGTTCGAAATATGATCAATTATCAGACATAGTAAAACG	
pMM9	PsiI	BspHI	BspHI PsiI	1‡	AAGCGCTCATCAGCCCGAAGT	1	GGCTGATGAGCGCTTGTTTCG
				2	GGTAATGAGATTATCAAAAAGGATCTTCAC	2	GATAATCTCATTACCAAAATCCCTTAACGTG
				3	GTCCCATGAGCGGATACATATTTGAATG	3	GTATCCGCTCATGGGACAATAACCCTG

† Res^{A} and Res^{B} denote the restriction enzyme sequence at each site.
‡ For pMM9, the numbers 1, 2 and 3 refer to the forward and reverse primers used to remove all three natural BspHI sites to from the pMM9 plasmid.

Recombination reactions were carried out at 37°C for 1 h, after which the reaction was stopped by incubation at 70°C for 5 min. Recombination reactions were treated with proteinase K (50 μg/ml, 60 min, 50°C) and ethanol precipitated. Approximately 1 mg of Tn*3* resolvase-treated DNA was subjected to BspHI (200 units, NEB) linearization in a final volume of 20 ml for 1 h at 37°C, followed by heat inactivation at 80°C for 20 min. The sample was prepared for separation by caesium chloride density gradient by the addition of 1.019 g of CsCl and 0.11 ml of 10 mg/ml of ethidium bromide solution, per gram of heat-treated solution. Separation was performed using a WX Ultra 1000 centrifuge and a TV860 rotor at 45,000 rpm, at 18°C overnight. The denser supercoiled DNA bands were extracted from the gradient using wide-gauge needles, and ethidium bromide was removed by several washes with water-saturated butanol. The extracted material was then ethanol-precipitated and analyzed on agarose gels.

Topoisomerase assays

DNA topoisomerase enzymes were supplied by Inspiralis Ltd, and assays were carried out following the manufacturer's protocols. Human topo IIα decatenation assays were carried out in the following assay buffer: 50 mM of Tris·HCl (pH 7.5), 125 mM of NaCl, 10 mM of $MgCl_2$, 5 mM of DTT, 1 mM of ATP and 100 μg of ml^{-1} albumin. *E. coli* DNA gyrase assay buffer was 35 mM of Tris·HCl (pH 7.5), 24 mM of KCl, 4 mM of $MgCl_2$, 2 mM of DTT, 1.8 mM of spermidine, 1 mM of ATP and 100 μg ml^{-1} of BSA. Assays were conducted in a volume of 30 μl at 37°C for 30 min with either 200 ng of kDNA (Inspiralis Ltd) or 200 ng of singly-linked catenated substrate; inhibition assays incorporated ciprofloxacin (0–100 μM). Reactions were stopped by addition of 30 μl of STEB (40% w/v sucrose, 100 mM of Tris·HCl pH 8, 10 mM of EDTA and 0.5 mg ml^{-1} of bromophenol blue) and 30 μl of chloroform/isoamyl alcohol (v/v, 24:1), vortexed, centrifuged and the upper aqueous phase analyzed on a 1% (w/v) TAE agarose gel at 85 V for 90 min. Gels were run without intercalator and were post-stained with ethidium bromide before visualization under UV illumination. Gel bands were quantified using GeneTools. Reactions in the presence of inhibitors (etoposide and ciprofloxacin) were carried out as previously with the addition of varying concentrations of inhibitor (Figure 2); in the case of etoposide, reactions were carried at in 1% DMSO with human topo IIα (0.44 nM).

Results & discussion

Construction & purification of singly linked catenated DNA

The decatenation activity of enzymes has conventionally been determined by agarose gel-based assays using kDNA as a substrate. The inherently complex structure of kDNA leads to difficulties in detecting both the substrate and products of the reaction. Here we describe the construction of an alternative substrate, a singly-linked DNA catenane, to replace the use of kDNA in decatenation assays.

Tn*3* resolvase can convert a circular substrate DNA molecule containing two directly repeated *res* sites into a pair of singly-linked catenated DNA rings. However, as a result of imperfect recombination efficiency, the challenge of separating the reaction product from the substrate arises. Substrate and product are identical in terms of DNA sequence and size. Recombination by Tn*3* resolvase involves double-stranded DNA cleavage and strand exchange precisely at *res* site I (Figure 1). We therefore considered modifications of site I that would not be inherited by the product singly-linked catenanes, thus allowing the differentiation of the reaction substrate from the products. Site I comprises a 28-bp sequence containing a natural PsiI restriction enzyme site (TTATAA) at its center; Tn*3* resolvase cleaves each strand symmetrically after the T of the AT in the PsiI site. Therefore, in a plasmid containing two *res* sites, if the PsiI restriction site in one *res* site is changed to a different restriction site (e.g., BclI TGATCA), the product *res* sites will lack both sites. It is thus possible, in theory, to design a substrate containing a restriction site that will be missing in the catenane product. However, sequence modifications at the site of action of Tn*3* resolvase can cause drastic reductions in reaction efficiency [16]. The negative impact can be minimized by retaining the central AT of the PsiI sequence, where the resolvase cleavage occurs, making modifications on either side of this sequence. We aimed to identify a modification that would minimize the negative effect on Tn*3* resolvase efficiency while allowing us to use restriction enzymes to distinguish recombination product from substrate.

The starting substrate, plasmid pMM5, contains two *res* sites (that we term res^A and res^B) each containing a PsiI restriction site. This plasmid was modified to introduce recognition sites for either BspHI (TCATGA) or BclI (TGATCA) into res^B. The resulting plasmids were named *pMM6* (res^A contains a PsiI site [unique in the plasmid], and res^B contains a BspHI site [not unique]) and *pMM7* (res^A contains a PsiI site, and res^B contains a BclI site [both unique]) (Table 1). Both pMM6 and pMM7 can be cleaved by restriction enzymes that will not cleave the catenated recombination product; pMM6 can be cleaved with PsiI, whereas pMM7 can be cleaved with either PsiI or BclI. However, the efficiency of Tn*3* resolvase-mediated recombination of pMM7 was approximately fourfold lower than for pMM6 (results not shown). BspHI cannot be used for pMM6 because it cleaves elsewhere in the plasmid, and using PsiI with pMM6 would be too expensive, due to the high cost of the PsiI enzyme. Therefore, to generate a more economical method, all of the BspHI recognition sequences in pMM6, apart from the one in res^B, were removed by site-directed mutagenesis, creating pMM9. This plasmid can be cleaved either with PsiI (in res^A) or BspHI (in res^B), and neither of these enzymes cleave the catenated product of recombination.

Singly-linked catenane substrates for topoisomerases were produced by treating pMM9 with Tn*3* resolvase. Any unreacted circular pMM9 was cleaved with BspHI, and the supercoiled catenane was purified away from the linearized substrate by ethidium bromide–CsCl density centrifugation as described in the materials and methods section. The resulting singly-linked catenanes purified by this method included a background contamination of uncatenated DNA molecules.

Decatenation reactions with type II topoisomerases

The validation of purified singly-linked catenanes as substrates for topoisomerase-catalyzed decatenation reactions was carried out by analyzing the decatenation activity of enzymes using the new substrate in direct comparison with kDNA. The substrates were tested using two DNA topoisomerases, possessing differing decatenation efficiencies.

Topo IIα is a type II topoisomerase that controls and manipulates the topological state of DNA, principally during the processes of chromosome condensation, chromatid segregation, transcription and translation, by initiating transient double-stranded DNA breaks [4]. Topo IIα has a variety of activities, including the ability to relax both positively and negatively supercoiled DNA and interconvert catenated and decatenated, or knotted and unknotted, DNA forms.

A direct comparison of the singly-linked catenanes and kDNA as substrates for topo IIα-mediated decatenation revealed that the former substrate produced a more discernible and sensitive decatenation assay when compared with kDNA (Figure 2). The singly-linked

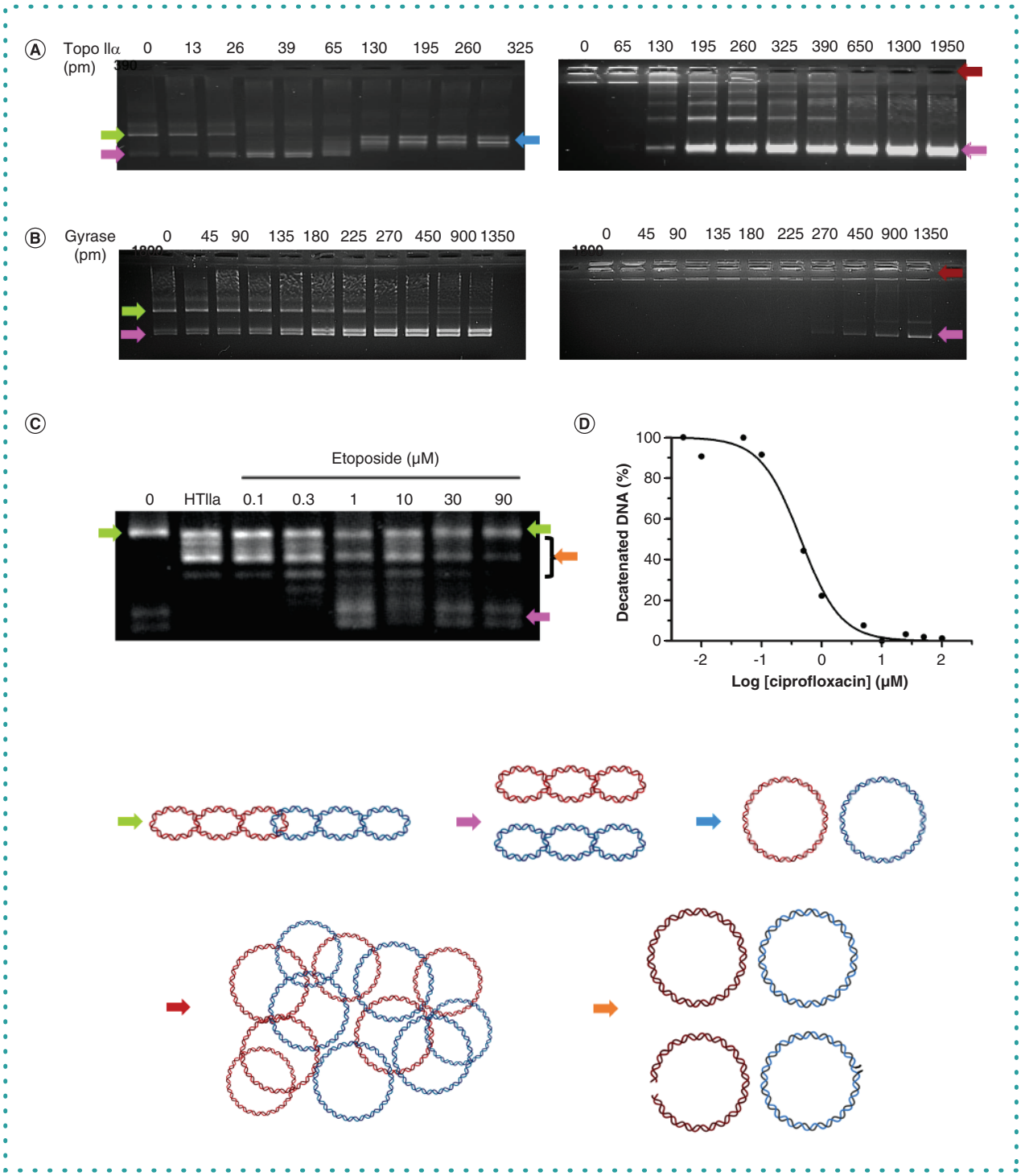


Figure 2. Topoisomerase-mediated decatenation assays. (A) Topo IIα decatenation reaction to compare the use of singly-linked catenanes (bis-cat; left panel) and kinetoplast DNA (kDNA; right panel) as substrates. In addition, 200 ng of singly linked substrate (green arrow) is completely decatenated by 39 pM of topo IIα into supercoiled decatenated DNA molecules (pink arrow); further addition of topo IIα relaxes the supercoiled decatenated DNA molecules (pink arrow) into their relaxed form (blue arrow). An equal amount of kDNA is completely decatenated by 650 pM of topo IIα, almost no decatenation of kDNA is detected at 65 pM of topo IIα, and a large proportion of the substrate can be seen immobile in the wells of the agarose gel (red arrow). **(B)** *Escherichia coli* gyrase-mediated decatenation of singly-linked catenanes (left panel) and kDNA (right panel). The singly linked catenanes are decatenated by 450 pM of gyrase, whereas even at a concentration of 1800 pM of gyrase, it is difficult to ascertain whether kDNA substrate still remains in the wells of the agarose gel. Notably, the singly-linked catenated substrate only (lane 1) also has a background level of supercoiled uncatenated DNA molecules. **(C)** Inhibition of human topo IIα (HTIIa) by etoposide; the IC_{50} can be approximated to be 0.3–1 μM; topo IIα was 0.44 nM. **(D)** Inhibition of *E. coli* gyrase by ciprofloxacin. Plot of gyrase decatenation inhibition assay with singly-linked substrate at increasing levels of ciprofloxacin. The gyrase concentration was 450 pM; the calculated IC_{50} of ciprofloxacin against *E. coli* gyrase was 0.44 μM.

catenanes can be clearly visualized on agarose gels, prior to the addition of enzyme, at which point the kDNA is trapped in the wells of the gel. The addition of topo IIα leads to the decatenation of the singly-linked catenanes in a single step to release two separate circular DNA molecules of visibly distinguishable sizes: 2.6 and 2.3 kbp. In contrast, the reaction of topo IIα with kDNA leads to the appearance of several DNA molecules of varying sizes on the agarose gel; these are likely to be partially decatenated products. Impressively, the use of singly linked DNA catenanes has significantly improved the sensitivity of the DNA decatenation assay by unambiguously demonstrating full decatenation with ~16-fold less enzyme than required to fully decatenate an equal amount of kDNA. In addition, the new substrate described here allows for the visual distinction between two different activities of topo IIα. Under these assay conditions, at topo IIα concentrations of 13–65 pM, the decatenation of the singly-linked dimers into two independent supercoiled DNA molecules is seen. Further addition of topo IIα in excess of 130 pM reveals the DNA relaxation activity of the enzyme, converting the unlinked supercoiled DNA molecules into relaxed forms with a relatively lower mobility in the agarose gel. This illustrates how the bis-cat assay can be used to simultaneously monitor decatenation and relaxation, showing, in this case, that topo IIα is a preferential decatenase; such distinction is not apparent using kDNA. (Note that the gels in Figure 2 are run in the absence of ethidium bromide; in the presence of this intercalator, the relaxed and supercoiled band would run together, allowing easier quantitation of the decatenation reaction.)

E. coli DNA gyrase, a type II topoisomerase, is distinguished by its unique ability to negatively supercoil DNA in an ATP-dependent reaction [17]. The enzyme has also been shown to possess decatenation activity, albeit inefficient in comparison with other type II topoisomerases [18–20]. The singly-linked DNA catenanes and kDNA were subjected to the relatively modest decatenation activity of gyrase to test the effectiveness of each substrate. Evidently, the increased sensitivity provided by the reaction on singly linked DNA catenanes readily allows the detection of gyrase decatenation activity at lower concentrations of enzyme, resulting in two distinct bands on the agarose gel, representing the separate 2.6 and 2.3 kbp supercoiled DNA molecules (Figure 2). The singly-linked DNA catenane shows promise for both the reassessment of previously identified topoisomerases and characterization of novel decatenation activities. No relaxation is seen here, as under these conditions (presence of ATP), gyrase maintains the negative supercoiling of the DNA.

A further application of the singly-linked catenated substrate is for inhibition assays, which are often used to test the efficacy of novel topoisomerase inhibitors. We envisage that the sensitivity of our substrate will enhance the fidelity of the information obtained by gel-based assays. Type II DNA topoisomerases, such as topo IIα and gyrase, are well-known targets of anticancer drugs and antibiotics, respectively [21–23]. We show in Figure 2 an example of this application using topo IIα and etoposide, a well-known anticancer drug targeted to topo II. The approximate IC_{50} from this assay (0.3–1 μM) can be compared with those derived from the literature (50–200 μM IC_{50}) using kDNA as the substrate [24–26], suggesting that the bis-cat assay is more sensitive. Ciprofloxacin, a clinically established gyrase inhibitor, was tested in an exemplar gyrase decatenation inhibition assay using the singly-linked DNA catenanes to assess its half-maximal inhibitory concentration (IC_{50}) (Figure 2). The percentage of decatenation activity was determined by taking the sum of decatenated 2.6 and 2.3 kbp products over a range of ciprofloxacin concentrations, as a fraction of the DNA gyrase decatenation activity in the absence of inhibitor; appropriate background subtractions were made to allow for pre-existing uncatenated DNA molecules in the substrate. The resultant IC_{50} for inhibition by ciprofloxacin was calculated as 0.44 μM, which is similar to values determined previously [27,28].

These examples highlight a potential limitation of the singly-linked substrate – that is, the background level of uncatenated double-stranded DNA molecules. This background contamination with the desired product (<20% of the total DNA) could be a consequence of downstream handling of the DNA but seemingly persists over different resolvase preparations. It is unlikely that the contamination is a direct result of decatenation by a co-purified topoisomerase protein, not only due to the stringent resolvase purification process, which involves denaturation with urea, but also because a topoisomerase capable of decatenating double-stranded DNA catenanes would require an energy investment from the hydrolysis of ATP, which is not present. To investigate whether this background level was attributable to nuclease activity, we exploited the Mg^{2+}-independence of Tn*3* resolvase by conducting the resolvase reaction in an assay buffer lacking the divalent metal. This alteration had minimal effect on the reaction efficiency, but it did not solve the contamination problem; nevertheless, alternative purification methods using hexa-histidine tagged resolvase would be possible. These uncatenated supercoiled product circles could be produced by failure of resolvase to religate one circle in the catenane.

The problem of background-level contamination could theoretically be tackled by manipulation of the pMM9 substrate plasmid to enable the downstream purification of the singly-linked catenanes from their uncatenated counterparts. For example, the molecular mass of the substrate plasmid could be adjusted to increase its mass difference from the largest of the two decatenated circular products to take advantage of downstream gel filtration. An alternative prospect is the introduction of two different DNA triplex-binding sites into pMM9, one in each region between the two *res* sites [29]. Purification could then proceed by the sequential passage of the singly-linked catenane mixture through two columns, each with an attached triplex DNA-forming oligonucleotide complementary to one of the triplex sites in pMM9 [30]. Thus, the catenated substrate would bind to both columns, and the uncatenated DNA molecules (only able to bind to one of the two columns) could be separated.

In conclusion, the singly-linked catenated substrate has been validated using well-characterized topoisomerases. This novel substrate has proved to be a superior alternative to the currently marketed kDNA substrate by significantly enhancing the sensitivity and reliability of gel-based decatenation assays. The potential hindrance of background uncatenated DNA molecules can efficiently be accounted for by making simple background-level subtractions to provide reliable results, as has been demonstrated with the use of an inhibition assay.

Future perspective

The singly-linked catenated substrate (bis-cat) described here proved to be an effective means of characterizing decatenation activities. We envisage that with the focus of future efforts upon the improvement of Tn*3* resolvase-mediated reaction efficiency and downstream purification to remove background uncatenated circles, this would lead to an ideal assay. The additional advantage of the high sensitivity and simplicity provided by the substrate highlights it as a desirable candidate for automated high-throughput assays that may be implemented to test the potency of novel topoisomerase inhibitors on a larger scale.

Author contributions

Experimental work involving the production and optimization of the singly-linked catenated substrate was carried out by NF Waraich. S Jain carried out topoisomerase assays. The study was designed and supervised by A Maxwell and NP Burton, with provision of valuable guidance and materials from WM Stark and SD Colloms. NF Waraich and A Maxwell wrote the manuscript, with input from NP Burton, WM Stark and SD Colloms.

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Financial & competing interests disclosure

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