The trigger enzyme PepA (aminopeptidase A) of *Escherichia coli*, a transcriptional repressor that generates positive supercoiling

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*Escherichia coli* aminopeptidase A (PepA) is a trigger enzyme endowed with catalytic activity and DNA-binding properties prominent in transcriptional regulation and site-specific DNA recombination. The current work demonstrates that PepA is a repressor in its own right, capable of specifically inhibiting transcription initiation at promoter P1 of the carAB operon, encoding carbamoylphosphate synthase. Furthermore, *in vitro* topology studies performed with DNA minicircles demonstrate that PepA binding constrains a single positive supercoil in the carP1 control region. Such a topological event is understood to constitute an impediment to transcription initiation and may serve as a mechanism to regulate gene expression.

**Keywords**: arginine biosynthesis; carbamoylphosphate synthase; DNA topology; Protein–DNA interactions; pyrimidine biosynthesis; transcription regulation

Trigger enzymes combine metabolic function with regulation of gene expression [1]. Much like bacterial transcription regulators, some of these multifunctional enzymes exert a direct effect on gene expression, having acquired a DNA- or RNA-binding domain. *Escherichia coli* PepA (Aminopeptidase A, alias XerB and CarP) [2–4], belongs to the class of DNA-binding trigger enzymes. Besides peptide degradation [5,6], the protein is involved in two distinct cellular processes: (a) transcriptional control of the carAB operon and possibly of its own gene [4], and (b) site-specific DNA recombination. PepA in conjunction with ArgR or ArcA imposes the directionality of the resolution reaction of ColE1 and pSC101 multimers respectively [7,8]. In both processes the peptidase activity of PepA is not required, and the protein was supposed to play an auxiliary architectural role in the elaboration of higher order structures [4,9–11].

The crystal structure of hexameric PepA is solved [12], but its DNA-binding mode is not well characterized and unusual; it does not involve any classical DNA-binding motif. Structural information and separation-of-function mutants revealed the importance of positively charged residues in the amino-terminal domain that flank a DNA-binding groove running over the C-terminal domain [9,12,13]. DNase I footprinting [4] and atomic force microscopy of PepA-DNA complexes [14] revealed DNA distortions and foreshortening of about 235 bp of the carP1 control region (Fig. 1), likely by wrapping around a single 330 kD PepA hexamer. Previous work indicates that PepA binding is an essential element of the regulatory process, but IHF, PyrH (UMP-kinase), PurR, RutR and Fis are required as well for full range modulation of carP1 activity [11,15–19].

In the current work, we demonstrate that PepA is not only an architectural element, but also a repressor
in its own right, capable of specifically inhibiting the carP1 promoter. Furthermore, we examine how PepA binding affects the topological state of DNA minicircles and demonstrate that PepA binding to the carAB control region constrains a positive supercoil. The introduction of a positive supercoil may constitute a key mechanical element of PepA-mediated repression.

Materials and methods

Construction of plasmids

Plasmids pDD-carP1 [11], and pDD-PartJ [20] are described. Plasmids pDD-carP1 + P2 and pDD-PartJ-long were constructed by amplification of the carAB (−419 to +142 with respect to the carP1 initiation site) and artJ (−535 to +28) control region, with genomic DNA from E. coli MG1655 [F−, F′−, rfa-1, rfb-50, ilyG−] as template and the oligonucleotide pairs AB1Bam (5′-CGGGATCC GTTAGTTGGAAGGAAAGCGG-3′)/AB2Bam (5′-CGG GATCTCGGCTCCGCTTCCAGAAC-3′) and DC1407f (5′-CGGGATCCGCGCCCTGGAATTGACAAAAAG ATACGC-3′)/DC394r (5′-CGGGATCTTTATGATT TTGT GCCGTTG-3′), respectively, as primers. The BamHI-digested amplicons were ligated into vector pDD3 as described [11].

Overexpression and purification of PepA

PepA was purified from an IPTG-induced culture of E. coli strain JM101 transformed with plasmid pKHW1, as described by Devroede et al. [21].

DNA circularization and topology assays

Circularization assays were performed with 567 bp [32P]-labelled DNA fragments, prepared by BamHI digestion of plasmids pDD-carP1 + P2 and pDD-PartJ-long followed by 5′-end labelling. BamHI fragments were dephosphorylated by incubation at 37 °C with two units heat-sensitive alkaline phosphatase (FastAP; Fermentas, Life Technologies, Ghent, Belgium) for 30 min, followed by 30 min inactivation at 75 °C. 5′-end labelling was performed by incubation of ~2 pmol dephosphorylated DNA fragments at 37 °C for 40 min with 10 units T4 polynucleotide kinase (Fermentas, Groningen, the Netherlands) and 50 μCi [γ-32P]-ATP (3000 Ci-mmol−1; Perkin Elmer) in 1× T4 polynucleotide forward reaction buffer, followed by heat inactivation for 10 min at 65 °C. Labelled fragments were purified by gel electrophoresis on 6% polyacylamide. Labelled DNA was recovered by diffusion out of the crushed acrylamide slice submerged in 600 μL elution buffer (0.5 M ammonium acetate, 0.01 M magnesium acetate, 0.1 mM EDTA) and incubated overnight at 45 °C. Acrylamide particles were removed by filtration through a fibreglass plug, and the DNA concentrated by ethanol precipitation in the presence of 15 μg yeast tRNA.

Circularization reaction mixtures were assembled containing 2 μL labelled DNA (about 2 fmol), 3 μL sonicated herring sperm DNA (0.5 mg mL−1) as a nonspecific competitor, increasing concentrations of purified PepA, and 1× T4 DNA ligase buffer (Fermentas) in a total volume of 20 μL, and incubated at 22 °C during 20 min. Linear fragments (bare or PepA-bound) were then ligated by 5 U (1 μL) of T4 DNA ligase (Fermentas) for 60 min at 22 °C. Subsequently, samples were treated with 2 μL proteinase K (Qiagen, Antwerp, Belgium) for 20 min at 37 °C and analysed by gel electrophoresis on 4% polyacrylamide in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2.5 mM EDTA). To reveal the identity of different DNA populations, bands were excised from the gel, DNA was extracted from the gel as described above, and aliquots digested with BamHI (Fermentas FD), HincII (Fermentas), λ-exonuclease (Fermentas) and different topoisomerases: E. coli Topoisomerase I (New England Biolabs, Bioké, Leiden, the
Single-round in vitro transcription

Unless otherwise stated, single-round in vitro transcription assays were performed with supercoiled template DNA (pDD-carP1 or pDD-P<sub>artJ</sub>). Aliquots containing 0.1 pmol template in a total volume of 35 µL transcription buffer (50 mM Tris-HCl (pH 7.8), 50 mM NaCl, 3.0 mM magnesium acetate, 0.1 mM EDTA, 0.1 mM DTT, 25 µg·mL<sup>-1</sup> BSA) were incubated for 20 min at 37 °C with various concentrations of PepA protein, as indicated. Subsequently 1.0 unit of E. coli RNA polymerase holoenzyme saturated with σ<sup>70</sup> subunit (Affymetrix, USB products) was added and the incubation extended for another 25 min. RNA synthesis was initiated with the addition of 15 µL start solution (transcription buffer containing 0.67 mg·mL<sup>-1</sup> heparin, 0.53 mM ATP, GTP and CTP, 0.053 mM UTP, and 3.0 µCi of [α<sup>-32</sup>P]-UTP) (3000 Ci·mmol<sup>-1</sup>; Perkin Elmer). Transcription was allowed to proceed for 5 min and immediately arrested by the addition of 50 µL water-saturated and neutralized phenol. Equal aliquots (10 µL) of the aqueous phase were mixed with 5.0 µL of formamide dye mix, briefly heated (2 min at 90 °C) and the reaction products were analysed by electrophoresis on 6% polyacrylamide gels under denaturing conditions (TBE-urea gels). When indicated, the order of addition of PepA and RNA polymerase was inverted to allow the binding of RNA polymerase to the promoter region prior to the addition of PepA.

Results

PepA is a transcriptional repressor in its own right

To gain further insights into the mode of action of PepA, single-round in vitro transcription assays were performed using supercoiled pDD-carP1 template [11]. The results indicate that PepA specifically inhibits carP1 activity when it is allowed to bind the template prior to the addition of RNA polymerase, whereas no inhibition was observed on internal control transcript RNA1 (Fig. 2A). In contrast, a slight increase in RNA1 synthesis was systematically observed in the presence of increasing concentrations of PepA (Fig. 2A,B), possibly as a consequence of topological modifications of the DNA template (see below and Discussion). PepA had no effect on transcription initiation at P<sub>artJ</sub> [20], included as an additional negative control (Fig. 2C). Therefore, we may conclude that the observed inhibitory effect of PepA at carP1 is specific. When RNA polymerase was allowed to bind prior to the addition of PepA, a strong inhibition was still observed (Fig. 2B), indicating that PepA is able to disrupt a preformed closed RNA polymerase–DNA template complex, or to inhibit its further transition to downstream steps in transcription initiation. A similar in vitro transcription assay performed with topologically unconstrained linearized pDD-carP1 plasmid DNA as template indicates that initiation at carP1 is less efficient but still repressed by PepA. However, in contrast to what was observed with supercoiled template, PepA binding had no effect on RNA1 production with the linear template (Fig. 2D).

Topological characterization of PepA-carP1 operator complexes

To examine how PepA-induced DNA deformations revealed by DNase I footprinting [4] and AFM imaging of 1 : 1 stoichiometric PepA-DNA complexes [14] may...
bring on consequential changes in the DNA helicity state, topological studies were performed using minicircles generated by ligation of linear DNA after or prior to PepA binding. The starting material is purified 5’-[32P]-labelled DNA of 567 bp, covering the carAB control region and bearing BamHI sticky ends (B1B2) (Fig. 1; labelled 4Lin in all figures). Similarly, a BamHI fragment of identical length but corresponding to the artJ control region was included as a control to demonstrate the specificity of PepA action on the topology of the carAB control region (Fig. S2). The results clearly indicate that PepA has an effect on the generation of DNA topoisomers containing the carAB control region, whereas such an effect is not observed with the artJ fragment (Figs 3 and S2). It is worth noticing that the linear carAB and artJ fragments of identical length migrate with remarkably different velocities on polyacrylamide gels (Fig. S2), whereas they comigrate on agarose gel (not shown). This may be explained by intrinsic bending, likely imposed by numerous nucleotide stretches containing at least four consecutive A residues in the carAB fragment (24 stretches, of which several are in phase with the helical repeat), whereas the artJ fragment harbours only four such stretches that are furthermore widely separated. Various topoisomerases (see Fig. 4 and Table S1 for properties and substrate specificities) were subsequently used to analyse the separated and recovered reaction products. The objective of these assays is to determine the nature and number of supercoils generated, or in other words, to estimate the precise change in linking number ΔLk generated upon PepA binding.

Ligation of the carAB fragment in the absence of PepA yielded a preponderant slowly migrating band (Fig. 3, lane 2), which likely corresponds to the relaxed form of the monomeric covalently closed circular DNA (from here on labelled 1Rccc). It is worth mentioning that we used 567 bp fragments, which corresponds to the precise length of 54 helical turns. Due to the fact that the extremities are naturally aligned, the ligation is efficient and consequently nearly no open circular DNA is formed. However, topoisomerons with supercoils can be obtained but generally in small amounts. Indeed, a minor but distinct band that likely corresponds to the −1 topoisomer noted (Cccc − 1) can be observed migrating slower than the 1Rccc population (Fig. 3, lane 2).

In the presence of PepA, added prior to ligation and subsequently removed by proteinase K treatment, two other bands were observed migrating faster than the 1Rccc. Therefore, they most likely correspond to some type of coiled cccDNA (Fig. 3, lanes 3–13; from here on and for reasons developed below, labelled 3Cccc for Coiled covalently closed circular DNA and 2Kccc for Knotted covalently closed circular DNA). The four major DNA populations, 1Rccc, 2Kccc, 3Cccc and 4Lin, were extracted from the gel and, in order to determine their identity, aliquots were submitted to treatment by some or all of the following enzymes: (a) BamHI that was used to generate the labelled fragment, (b) HincII that cuts at 386 bp from the upstream border of the linear fragment (Figs 1 and S3), (c) λ-exonuclease that digests linear (in the 5’-3’ direction) but not circular DNA, (d) topoisomerase I from E. coli (a protein, a type IA enzyme) that transiently cuts one strand of the DNA helix and relaxes exclusively negative supercoils (due to its requirement for a short single-stranded DNA stretch in the substrate; negative supercoiling can be converted into untwisting), (e) eukaryotic calf thymus topoisomerase I (type IB) that also cleaves one strand but is capable of removing both negative and positive supercoils, (f) E. coli gyrase (type IIA) that nick both strands, allowing the introduction of negative supercoiling and
has a weak catenation/decatenation (knotting/unknotting) activity, (g) bacterial topoisomerase IV (type IIA) that is reported to be efficient in removing supercoils of both signs, and in decatenating/catenating cccDNA (see Fig. 4 and Table S1 for enzyme specificities).

Band 4Lin with the fastest migration velocity corresponds to the starting material of linear DNA bearing BamHI sticky ends (Fig. 3, lane 1). Accordingly, cleavage by HincII generated two subfragments (Fig. S3; these migrated out of the gel in conditions used in Fig. 5), and λ-exonuclease nearly completely digested this population.

Band 1Rccc represents the preponderant population of DNA formed upon ligation in the absence of PepA. This band displays the most retarded gel migration and corresponds to the relaxed form of the circularized DNA (Relaxed cccDNA, hence 1Rccc). Indeed, resistance against λ-exonuclease digestion indicates that the corresponding DNA molecules are circular (cccDNA) (Fig. 5C). Moreover, digestion by BamHI and HincII restored the linear DNA fragment, and cleavage by HincII did not generate additional bands that could indicate the presence of linear or circular multimers (Fig. S3). Concordantly, topoisomerase IA (Fig. 5C) or IB (not shown) have no further observable relaxing effect on this population. However, topological constraints may be introduced into initially relaxed cccDNA molecules by DNA gyrase, which generated molecular species with faster migration velocity. A similar effect but to a lesser extent was observed with Topo IV (Fig. 6D), which further reinforces the conclusion that band 1Rccc indeed represents a homogeneous population of relaxed cccDNA molecules.

Band 3Cccc exhibits a similar behaviour similar to that of 1Rccc when treated with λ-exonuclease, BamHI and HincII, which indicates that the corresponding DNA population consists of monomeric cccDNA molecules (Fig. 5D). As formation of this band is PepA dependent and displays a migration velocity faster than that of 1Rccc (Figs 3 and S2), it is likely to have incorporated a topological constraint such as a coil due to PepA binding and wrapping (Coiled cccDNA, hence 3Cccc). Indeed, 3Cccc is insensitive to E. coli topoisomerase I, but serves as a good substrate for calf thymus topoisomerase I, and to a lesser extent for gyrase, indicating that this cccDNA population contains one positive coil (ΔLk = +1) that is constrained upon wrapping of the DNA around PepA prior to ligation (Fig. 5D). The rationale behind this conclusion is based on the following observations. Escherichia coli Topol has no effect on the 3Cccc population, even though we have shown that the enzyme relaxes 567 bp minicircles with a single negative supercoil (Fig. S4). Calf thymus topoisomerase I efficiently converted 3Cccc to two different products: (a) the relaxed form of cccDNA observed as a well-defined band migrating at the same velocity as 1Rccc (ΔLk = 0), and (b) a noticeable band migrating in-between 1Rccc and 3Cccc. This population may as well harbour a trapped negative supercoil (ΔLk = −1) generated by the repetitive activity of type IB topoisomerase. Indeed, type IB topoisomerases relax DNA through a ‘controlled rotation’ mechanism during which multiple rotation events may occur during a cleavage/religation cycle [22–24], conferring upon the enzyme the property of inducing changes in linking number by ± N. This is consistent with the treatment by gyrase, which is capable of introducing negative supercoils or catenate/decatenate by changing the Lk by steps of two. DNA molecules 3Cccc of supposed ΔLk = +1 that undergo a −2 Lk change by gyrase, would culminate in the resultant ΔLk = −1, which is consistent with the position of the −1 topoisomer generated by repeated action of calf thymus gyrase.

\[ \text{Fig. 4. Graphical presentation of the activities of different topoisomerases used on various substrates relevant for this work.} \]

The different DNA forms indicated are covalently closed circular DNA molecules, respectively, negatively (Cccc−) or positively (Cccc+) supercoiled, relaxed (Rccc) or knotted (Kccc). The two types of type II topoisomerases from E. coli (gyrase and Topo IV) are shown in bold. CT Topo I corresponds to calf thymus topoisomerase I, a typical eukaryotic type IB enzyme, a swivelase. Ec Topo I corresponds to the topoisomerase I from E. coli (a protein) that performs a controlled strand passage reaction. The arrows indicate the direction of the reaction catalysed by the enzymes and dashed lines indicate weak activity. Notice that the end product of the unknotting reaction of the Kccc substrate by Topo IV is the relaxed form (Rccc), whereas unknotting of Kccc by gyrase will eventually result in the supercoiled product.
topoisomerase and with the position of the minor product generated upon ligation in the absence of PepA (Fig. 5D). Combined, these observations indicate that band 3Cccc represents cccDNA molecules with one positive supercoil produced by the DNA-remodelling effect of PepA prior to ligation of the ends of the linear fragment.

Band 2Kccc also represents monomeric cccDNA molecules as it exhibits the same behaviour upon treatment by λ-exonuclease, BamHI or HincII as observed for 1Rccc and 3Cccc (Fig. 5E). Moreover, as the apparent migration is clearly faster than 1Rccc and slightly slower than 3Cccc (Figs 3 and 5A), this DNA population most likely contains some kind of topological constraint. Interestingly, 2Kccc is unaffected by treatment with the two topoisomerases of type I, excluding the possibility that this topological constraint would come under the simple form of a supercoil of positive or negative sign. On the other hand, partial sensitivity to gyrase (Fig. 5E) and quasi completed conversion by bacterial topoisomerase IV (Figs 6C and S1) suggest that this band may represent a population of DNA molecules containing a trapped node-coil, as the result of a knot introduced by PepA binding prior to ligation. Indeed, previous studies revealed that an extensive part of the carAB control region, containing the binding sites for various regulatory proteins (Fig. 1), may be wrapped loosely around one PepA hexamer, leaving wobbly loops that may accommodate the direct docking of said regulators [11,14]. It appears thus conceivable that a free moving end of the linear DNA molecule would slip through this interspace, loosely tying a floating knot around the protein. The subsequent ligation of the two ends would create a knotted cccDNA of resultant ΔLk = + 3. Noteworthily, this type of topoisomer was not present when PepA was allowed to bind pre-formed minicircle DNA, a condition in which knot formation is impossible (Fig. S4), further advancing the idea that this band represents a population of knotted cccDNA molecules.

Such knotted cccDNA, resembling the shape of a trefoil (Fig. 4), can only be relieved to a lower degree if both strands are disrupted simultaneously, allowing the passage of the constrained DNA duplex through the gate. Although both E. coli gyrase and topoisomerase IV are of type II topoisomerases, gyrase appears to be less suitable to reveal the possible presence of a knot trapped in the small cccDNA of our experiment due to two of its characteristics: (a) gyrase was reported to be 100-fold less efficient than topoisomerase IV in decatenation in vivo [25], and (b) gyrase is unique among all topoisomerases in its ability to...
To further verify our hypothesis, the four main DNA populations were submitted to treatment by topoisomerase IV (Fig. 6). The results show that band 2Kccc serves as an optimal substrate for the unknotting reaction and was almost entirely converted to cccDNA containing one positive supercoil (3Cccc) and to the relaxed form cccDNA (1Rccc). The reverse reaction, in which topoisomerase IV introduces a knot and converts 3Cccc to 2Kccc, was less efficient (Fig. 6B).

Ligation of the artJ control fragment was similar in the absence and presence of PepA and resulted in the formation of two major products (indicated 1Rccc and 2 Cccc) from gels indicated that both products are monomeric circles (Figs S5 and S6). The major ligation product (indicated Rccc) corresponds to the relaxed covalently closed form, whereas the slightly slower migrating band indicated that Cccc bears a single coil of positive sign. Importantly, formation of this topoisomer of the artJ fragment was not influenced by the presence of PepA, unlike topoisomer formation with the carAB fragment, which is therefore specific at the protein concentrations used in the assays. Although the carAB and artJ DNA fragments have the same length, their structural properties are different as shown by (a) their different electrophoretic mobility as indicated above and (b) their Rccc/Cccc ratio which is also different.

**Discussion**

In the present work we demonstrate for the first time that PepA is a repressor in its own right, capable by itself of inhibiting transcription initiation at carP1, and is not just an architectural element in the elaboration of a higher order nucleoprotein complex, as suggested previously [4,14]. Interestingly, initiation at carP1 was independent of the order of addition of PepA and RNA polymerase. This leads to two possible models: (a) PepA exerts an inhibitory effect on downstream steps of transcription initiation (isomerisation, promoter clearance), or (b) PepA forces the dissociation of closed complexes. The latter hypothesis is favoured taking into account that: (a) RNA polymerase-carP1 complexes generated in the absence of dNTPs are unstable complexes [27], and that (b) this event is in agreement with the presence of a 6 bp GC-rich discriminator box, which constitutes a high-energy barrier for isomerization (see below), as attested to by the reduced promoter strength on linearized versus supercoiled pDD-carP1 template (Fig. 2).

Our present topology study demonstrates that PepA binding constrains a positive supercoil in the carP1 control region, most likely by toroidal wrapping. This effect is specific as it does not occur with the artJ promoter/operator region used as a control. Transcription initiation requires local strand separation and is thus facilitated by negative supercoiling, which can be converted into untwisting [28]. PepA binding and PepA-induced positive supercoiling may therefore constitute an important encumbrance for the upstream steps of transcription initiation, not only because such important remodelling imposes a reorganisation of target sites for the other transcription factors but also by impeding the melting of the DNA duplex. Such an
interrelationship between DNA topology and transcription initiation may also explain the observed increase in the amount of RNA1 control transcript generated in the in vitro assays performed with supercoiled but not with unconstrained linear pDD-carP1 template (Fig. 2A,D). Within the topologically constrained template molecules the generation of a positive supercoil upon PepA binding is automatically compensated by the emergence of a negative supercoil in the rest of the molecule, which in turn could stimulate open complex formation and subsequent RNA1 transcription initiation. It is worth noticing that such an increase was not observed in similar assays performed with PurR or ArgR, two transcription regulators that bend but do not wrap their target operators, including the carAB control region [11,20].

The response to supercoiling differs greatly between different promoters and as such DNA superhelicity can discriminatorily affect the activity of different groups of promoters on a global level. Among the ones with the highest dependency for negative supercoiling are the stringent promoters, which are negatively affected by ppGpp and contain a GC-rich discriminator box [29]. The stringent class of promoters comprises promoters of not only stable RNA genes (rRNA, tRNA) and ribosomal proteins but also genes of de novo UMP synthesis including the pyrBI and carAB operons [30]. PepA-induced positive supercoiling may come into play at this regulation level, amplifying the higher dependency of carAB transcription initiation on negative supercoiling, imposed by the presence of the GC-rich discriminator. In this context it is worth noticing that the synthesis of carbamoylphosphate (CP) is an energy-demanding process (two molecules of ATP consumed per CP molecule produced). The coordination of carP1 activity with the energetic state of the cell via DNA topology-mediated effects is therefore an interesting mechanism. Transcription regulation by mechanisms involving DNA remodelling such as bending, wrapping and looping are observed ubiquitously in nature. In these instances, nucleoprotein complexes are able to efficiently tune the transcription initiation by promoting long-distance interactions, remodelling promoter conformation, and/or by restricting or increasing its accessibility to RNA polymerase [31–36].

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Author contributions
PNLM and DC conceived, designed and performed the experiments; PNLM, DC and MN analysed the data and wrote the paper.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

**Table S1.** Activities, substrate preferences and mode of action of different topoisomerases used.

**Fig. S1.** Circularization assay with the linear 567 bp **carP1** + **P2** fragment analysed on a 1.8% agarose gel.

**Fig. S2.** Formation of DNA minicircles with the **carAB** and **artJ** promoter/operator regions.

**Fig. S3.** Analysis of **carP1** + **P2** ligation products by BamHI and HincII digestion.

**Fig. S4.** Analysis of **carP1** + **P2** minicircles by ligation prior to or after PepA binding.

**Fig. S5.** Circularization assay with the 567 bp **artJ** promoter/operator fragment and characterization of the ligation products with exonucleases and topoisomerases.

**Fig. S6.** Analysis of gel-purified **artJ** ligation products by endo- and exonuclease treatment.