6540–6546 *Nucleic Acids Research, 2005, Vol. 33, No. 20*
doi:10.1093/nar/gki951

StpA protein from *Escherichia coli* condenses supercoiled DNA in preference to linear DNA and protects it from digestion by DNase I and EcoKI

S. A. Keatch, P. G. Leonard[1], J. E. Ladbury[1,*] and D. T. F. Dryden*

School of Chemistry, The King's Buildings, The University of Edinburgh, Edinburgh EH9 3JJ, UK and [1]Department of Biochemistry and Molecular Biology, University College London, Gower Street, London WC1E 6BT, UK

Received September 12, 2005; Revised and Accepted October 18, 2005

ABSTRACT

The nucleoid-associated protein, StpA, of *Escherichia coli* binds non-specifically to double-stranded DNA (dsDNA) and apparently forms bridges between adjacent segments of the DNA. Such a coating of protein on the DNA would be expected to hinder the action of nucleases. We demonstrate that StpA binding hinders dsDNA cleavage by both the non-specific endonuclease, DNase I, and by the site-specific type I restriction endonuclease, EcoKI. It requires approximately one StpA molecule per 250–300 bp of supercoiled DNA and approximately one StpA molecule per 60–100 bp on linear DNA for strong inhibition of the nucleases. These results support the role of StpA as a nucleoid-structuring protein which binds DNA segments together. The inhibition of EcoKI, which cleaves DNA at a site remote from its initial target sequence after extensive DNA translocation driven by ATP hydrolysis, suggests that these enzymes would be unable to function on chromosomal DNA even during times of DNA damage when potentially lethal, unmodified target sites occur on the chromosome. This supports a role for nucleoid-associated proteins in restriction alleviation during times of cell stress.

INTRODUCTION

The genome of *Escherichia coli* comprises a single circular molecule of DNA, $\sim 4.7 \times 10^6$ bp in length. This large amount of DNA has to be organized into the small volume of the bacterial cell. The condensed state of the genome is, at least in part, owing to its binding to several core nucleoid-associated proteins (1–7). These nucleoid-associated proteins are thought to control the overall DNA conformation and gene regulation (1,6).

Nucleoid-associated proteins can be classified into two groups. The first group are uniformly distributed throughout the nucleoid, and thought to have a major role in DNA structuring (2). These include histone-like nucleoid structuring protein (H-NS), heat-unstable nucleoid protein (HU), integration host factor (IHF), suppressor of td mutant phenotype A (StpA) and DNA-binding protein from starved cells (Dps). The second group bind at specific loci within the nucleoid and are therefore thought to be involved in gene regulation. They comprise curved DNA-binding protein A (CbpA), curved DNA-binding protein B (CbpB), factor for inversion stimulation (Fis), inhibitor of chromosome initiation A (IciA) and sequestration A (SeqA), and are all sequence specific DNA-binding proteins (2). In actively growing cells, the nucleoid is mainly associated with Fis, host factor for phage Qβ (Hfq), HU, StpA and H-NS. However, on entry into stationary phase, the nucleoid content of all these proteins is significantly reduced, with the starvation-induced nucleoid protein Dps becoming the most abundant. This change in nucleoid protein composition during stationary phase accompanies nucleoid condensation and gene silencing (1,8,9).

H-NS is one of the most abundant bacterial nucleoid-associated proteins, and forms concentration-dependent, self-associated complexes (4). It has an important role in the packing of DNA within the nucleoid as well as regulation of many genes dispersed throughout the genome (10). Overproduction of H-NS has been seen to form highly condensed nucleoids (11,12). H-NS dimers are proposed to homo-oligomerize in a head to tail orientation, through their N-terminal domains. The C-terminal domains form the DNA-binding regions, with each H-NS dimer therefore able to bind two separate DNA strands. In this way, H-NS oligomers can bind along the length of a DNA helix and form interstrand links with another DNA helix and therefore bind the DNA helices together in lateral arrays (4). This has been visualized

*To whom correspondence should be addressed. Tel: +44 131 650 4735; Fax: +44 131 650 6453; Email: David.Dryden@ed.ac.uk
Correspondence may also be addressed to J. E. Ladbury. Tel: +44 20 7679 7012; Fax: +44 20 7679 7193; Email: ladbury@biochem.ucl.ac.uk

by AFM *in vitro*, as high concentrations of H-NS were found to collapse plasmid DNA into varying conformations of condensates (13,14). From the images obtained, a model of H-NS condensation was proposed where H-NS binds randomly to the open circle of DNA. When two points on the same DNA molecule come into close proximity, H-NS bound to one can also bind the second, thereby forming intramolecular bridges. The formation of the first bridge would make it easier for further bridging to occur, forming many contacts, with intervening 'bubbles' of unbridged DNA. Further oligomerization of H-NS would cause binding of these bubbles, completely linking the helices together, with a loop at either end. It is thought that the linked helices are possibly interwound to account for the observed loss in DNA length, and the fact that H-NS binding constrains DNA supercoiling. This method of DNA bridging is similar to that of the eukaryotic linker histones H1 and H5, which also contain two DNA-binding domains and form similar tracts of laterally condensed DNA (15).

StpA shares 58% amino acid identity with H-NS, is of similar size and is present in bacterial cells at a similar concentration (1,16). In fact, anti-StpA antibodies bind to both StpA and H-NS (1). It is therefore thought that both proteins will have a similar function and mode of action (1) and DNA bridging by StpA to form condensed DNA structures has been visualized by atomic force microscopy (13,14). In the absence of H-NS, over-expression of StpA can suppress many of the effects of H-NS on gene expression. However, although there is an increase in the production of StpA in hns mutants, it is degraded by the Lon protease (17). This is due to the fact that in wild-type cells, StpA and H-NS form heteromeric complexes which are resistant to Lon. StpA is therefore incapable of compensating for a loss of H-NS and *hns* mutant *E.coli* adopts a pleiotropic phenotype. The heteromeric complexes formed by StpA and H-NS may possess novel properties dependent upon the expression of both proteins (3 and references therein). StpA has been found to bind DNA with four to six times higher affinity than H-NS (3). It has also been shown to interact with RNA, leading to the proposal that StpA may have a role in coupling DNA and RNA processes, such as the coordination of transcription and translation (18). H-NS and StpA have also been shown to have a preference for bent DNA (3). However, Azam and Ishihama (18) used a shorter bent DNA substrate and found that only H-NS favoured bent DNA, with StpA binding with equal affinity to bent and straight substrates. This has been explained by the idea that StpA may recognize a longer curved segment of DNA than H-NS (3).

In this paper we investigate the effects of StpA on the accessibility of DNA to the non-sequence-specific endonuclease, DNase I, and to the sequence-specific type I restriction enzyme, EcoKI, whose complex reaction requires DNA translocation driven by ATP hydrolysis prior to DNA cleavage. Our results support the conclusion that StpA alters the DNA structure sufficiently to prevent access of either nuclease to the DNA, and that this alteration is a coating of the DNA surface and condensation of the DNA into a collapsed state. These effects on EcoKI suggest a possible role for nucleoid condensation in the phenomenon of restriction alleviation whereby the activity of restriction enzymes such as EcoKI is controlled during conditions of cell stress.

METHODS

A cDNA insert corresponding to the stpA gene from *E.coli* (SwissProt primary accession number P30017; kindly provided by J. C. D. Hinton) was cloned previously in the expression vector pET14b (Novagen) between the 5′ Nde1 site and the 3′ BamH1 site. This pET14b vector encoding StpA was kindly provided by A. Petrovic.

A single colony of *E.coli* BL21 (λDE3 pLysS) cells transformed with the StpA plasmid was used to inoculate 100 ml of Luria–Bertani media supplemented with 50 μg/ml chloramphenicol and 200 μg/ml carbenicillin. Overnight culture (10 ml) was used to inoculate 2 l flasks containing fresh media and antibiotics. The cultures were incubated at 37°C until an OD_{600} of 0.7 was reached. StpA protein expression was induced at this point by addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM and the cells were incubated for a further 4 h at 37°C. The cells were collected by centrifugation at 4000 r.p.m. in a Sorvall GS3 rotor and the resulting cell pellet stored at −20°C. The cells were lysed by sonication after resuspension in 20 mM Tris, pH 8.0, 1 M NaCl, 100 μM 4-(2-aminoethyl)benzene sulphonyl fluoride. The cell lysate was centrifuged at 18 000 r.p.m., 4°C in a Sorvall SS34 rotor to remove the insoluble components. The supernatent was then loaded onto a 5 ml Talon metal affinity column equilibrated with 20 mM Tris, pH 8.0 and 1 M NaCl. The column was washed with 20 mM Tris, pH 8.0, 1 M NaCl and 10 mM imidazole before elution with 20 mM Tris, pH 8.0, 1 M NaCl and 150 mM imidazole. Dialysis was used to remove the imidazole from the protein solution.

DNA of plasmid pBR322 (two EcoKI sites) and the virtually identical pBRsk1 [one EcoKI site, (19)] was prepared as described (20). Linear forms of the plasmids were prepared by digestion with EcoRI restriction endonuclease which has a single target site within these plasmids. Given the number of base pairs of DNA in pBR322 (4361 bp) and the molar concentration of the plasmid and of StpA, one can calculate the number of molecules of StpA per base pair and the number of base pairs of DNA per molecule of StpA.

EcoKI enzyme was prepared as described previously (21) and its ATPase and restriction activities were measured as described previously (22). EcoKI hydrolyzes ATP when bound to either circular or linear DNA containing at least one target site but it only cleaves circular DNA with one or more sites and linear DNA with two or more sites. DNA cleavage by EcoKI was dependent upon the presence of its recognition sequences, S-adenosyl methionine and ATP and no non-specific endo- or exonuclease activity was observed.

Buffers used for all experiments were either a 'high salt' EcoKI buffer comprising 33 mM Tris–acetate; 10 mM magnesium acetate; 66 mM potassium acetate; 0.5 mM DTT; pH 7.9 or a 'low salt' EcoKI buffer comprising 10 mM Tris–acetate; 10 mM magnesium acetate; 7 mM 2-mercaptoethanol; pH 7.9. $CaCl_2$ (1 mM) was added to buffers for assays of DNase I activity. The DNA was incubated with the appropriate amount of StpA for 30 min prior to the addition of the nucleases. DNase I and EcoKI restriction reactions were stopped by the addition of EDTA to 77 mM. Samples were analysed by running on a 0.8% agarose gel in Tris–borate–EDTA buffer and staining with ethidium bromide.

RESULTS

Effect of StpA on DNase I activity

StpA-induced condensation of linear DNA. The DNase I assay was used to look at the condensed structure of DNA with varying concentrations of StpA. It was found that all of the linear pBR322 was protected from DNase I digestion by a concentration of one StpA per 64 bp, but at concentrations of one StpA per 96–128 bp only a fraction of the DNA was protected (Figure 1). All other concentrations of StpA were insufficient to protect linear pBR322 from DNase I and it was therefore digested, although we note that some slight smearing could be seen at concentrations of one StpA per 256–128 bp indicating that, for a small fraction of the DNA molecules, the DNA structure had been altered by binding of StpA.

StpA-induced condensation of supercoiled DNA. The effect of StpA concentration on DNase I digestion of supercoiled pBRsk1 was also studied (Figure 2). No concentration of StpA was capable of completely protecting the supercoiled form of pBRsk1, as the DNase I was always able to at least nick the plasmid to form open circular DNA. This nicking activity was not seen with linear pBR322 but this is most probably because nicked linear substrate would be indistinguishable from intact linear substrate on agarose gel electrophoresis. Supercoiled pBRsk1 was nicked by DNase I at concentrations

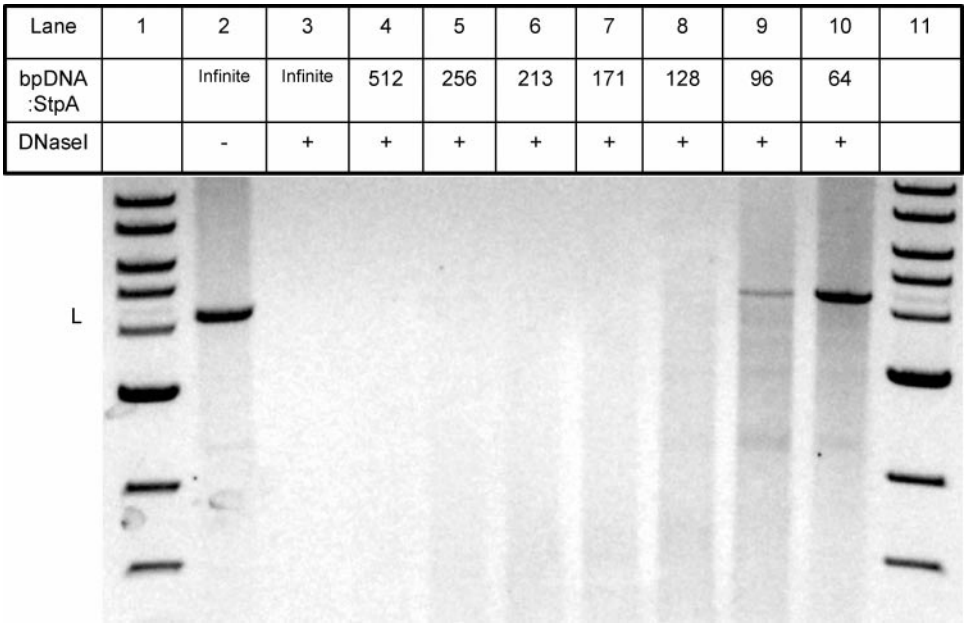

Figure 1. Effect of StpA concentration on DNase I digestion of linear pBR322. Linear pBR322 (L) (50 nM) was digested with 0.1 μg/ml DNase I for 1 h at 25°C in high salt EcoKI buffer, pH 7.9. The gel shows undigested DNA as a band of 4.4 kb (L), with digested DNA being absent from the gel, or present as a faint smear. Lanes 1 and 11 contain linear DNA markers of sizes 1.5, 2, 3, 4, 5, 6, 8 and 10 kb from bottom to top.

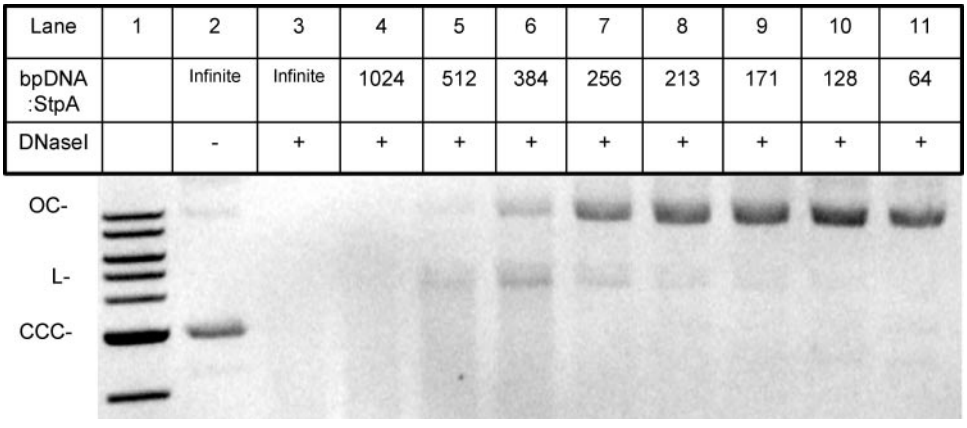

Figure 2. Effect of StpA concentration on DNase I digestion of supercoiled pBRsk1. Supercoiled pBRsk1 (CCC) (50 nM) was digested with 0.1 μg/ml DNase I for 1 h at 25°C in high salt EcoKI buffer, pH 7.9. The gel shows undigested DNA (CCC), with digested DNA being absent from the gel, and DNA protected from digestion present as linear (L) and nicked (OC) species. Lane 1 contains linear DNA markers of sizes 2, 3, 4, 5, 6, 8 and 10 kb from bottom to top.

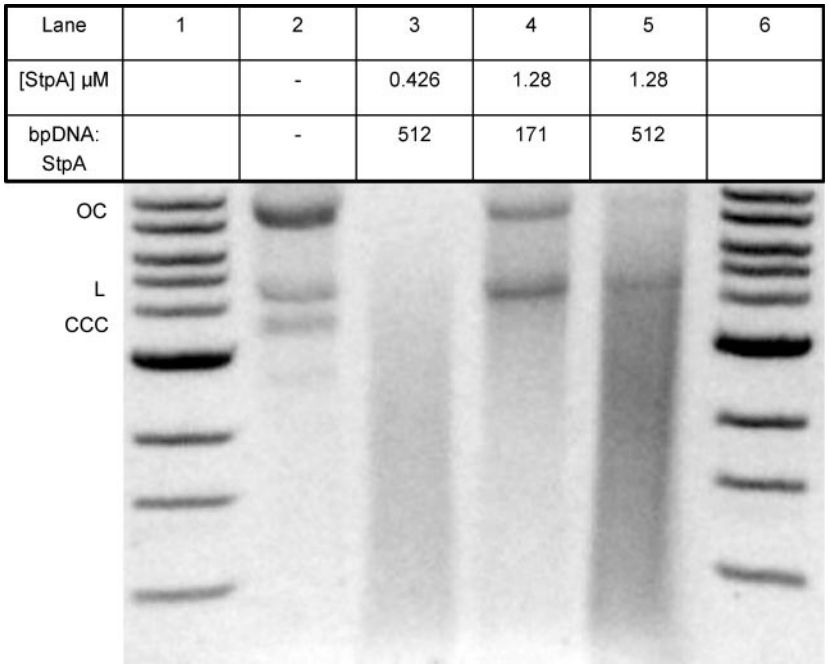

Figure 3. Agarose gel showing effect of StpA concentration on inhibition of DNase I digestion of supercoiled pBRsk1. Supercoiled pBRsk1 was digested by 0.1 μg/ml DNase I in low salt EcoKI buffer, pH 7.9, at 25°C. Lane 3 shows how 50 nM DNA is digested by DNase I with 512 bp DNA per StpA, with a StpA concentration of 0.426 μM. Lane 4 shows DNase I digestion of 50 nM DNA at 171 bp per StpA (1.28 μM StpA). Lane 5 shows how 150 nM DNA is digested by DNase I with 512 bp per StpA, at an StpA concentration of 1.28 μM. Lane 2 is a marker containing supercoiled (CCC), nicked (OC) and linear (L) pBRsk1. Lanes 1 and 6 contain 1 kb linear DNA markers of sizes 1, 1.5, 2, 3, 4, 5, 6, 8 and 10 kb from bottom to top.

of one or more StpA per 256 bp, and converted to a mixture of open circular and linear DNA at a concentration of one StpA per 384 bp. A very faint linear band could also be seen when one StpA per 512 bp was used, although the majority of DNA at this concentration was digested. Lower concentrations of StpA were insufficient to protect supercoiled pBR322 from DNase I. The proportions of the protected DNA changed as the StpA concentration increased. This could be seen as a decrease in the intensity of linear DNA, and an increase in intensity of supercoiled DNA indicating that the DNA was becoming less accessible to DNase I attack with increasing StpA concentration. Although it was possible that StpA was physically blocking the potential cutting sites of DNase I and thereby preventing digestion, this was unlikely owing to the low concentrations of StpA (256 bp: StpA) required to inhibit DNase I. At this low concentration, the relatively small size of StpA would be unable to sufficiently protect the DNA from the indiscriminate cutting of DNase I.

These results suggested that StpA could condense both linear pBR322 and supercoiled pBRsk1. However, it was also possible that StpA inhibits the DNase I enzyme directly. This was shown not to be the case by the control experiment shown in Figure 3. In this experiment, a concentration of StpA was used which caused DNase I inhibition of supercoiled pBRsk1 (171 bp:StpA), but with three times the concentration of DNA, reducing the DNAbp:StpA ratio below the inhibition level (512 bp:StpA). As the total concentration of StpA had not changed, if StpA inhibited DNase I directly, this reaction would have resulted in no digestion of the DNA. However, the DNA was digested and therefore the effect of StpA upon DNA structure appears to be the cause of the reduction in DNase I activity.

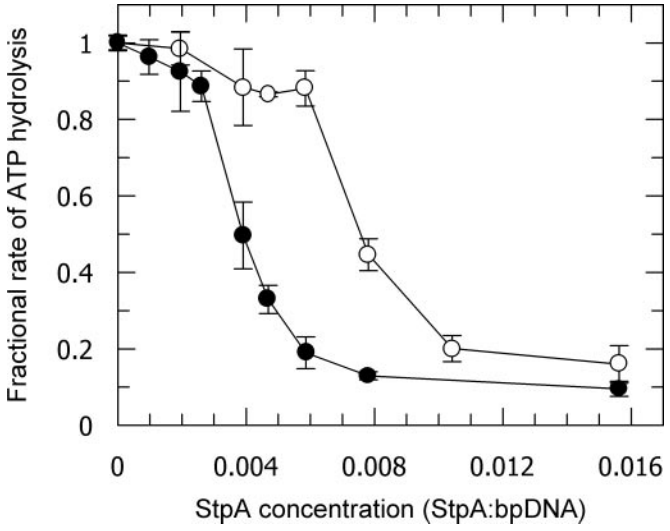

Figure 4. Effect of StpA concentration on the relative rate of ATP hydrolysis of EcoKI with linear and supercoiled substrates. The StpA concentrations are given as the number of StpA molecules per DNA bp. The relative rate of ATP hydrolysis by EcoKI is shown when supercoiled pBRsk1 (closed circles) and linear pBRsk1 (open circles) were used as substrates. Reactions took place in high salt EcoKI buffer, pH 7.9, with 5 nM DNA and 10 nM EcoKI.

Effect of StpA on EcoKI activity

EcoKI ATP hydrolysis. Figure 4 shows the effect of StpA concentration on the ATP hydrolysis activity of EcoKI. The rate of ATP hydrolysis of linear pBRsk1 decreased by up to 10% with addition of StpA to one StpA per 171 bp (0.00585 StpA:bp). A large decrease in ATP hydrolysis rate to around

one-fifth of the uninhibited rate was seen with addition of further StpA to a concentration of one StpA per 96 bp (0.0104 StpA:bp). Upon addition of StpA to one StpA per 64 bp (0.0156 StpA:bp) there was a further decrease in the ATP hydrolysis rate to around one-sixth of its uninhibited rate.

The rate of ATP hydrolysis of supercoiled pBRsk1 decreased by up to 10% with addition of StpA to one StpA per 384 bp (0.0026 StpA:bp). A large decrease in ATP hydrolysis rate to around one-fifth of the uninhibited rate was seen with further addition of StpA to a concentration of one StpA per 171 bp (0.00585 StpA:bp). Upon addition of more StpA to one StpA per 64 bp (0.0156 StpA:bp) there was a further decrease in the ATP hydrolysis rate to around one-tenth of the uninhibited rate.

Linear and supercoiled DNA substrates both followed the same trend in their response to increasing StpA concentrations. However, a higher concentration of StpA was required to inhibit the ATP hydrolysis activity of EcoKI on the linear DNA molecule than on the supercoiled substrate.

EcoKI restriction activity on linear DNA. Figure 5 shows the effect of StpA concentration on the cleavage of linear pBR322 (which contains two recognition sequences for EcoKI) after a 20 min incubation with EcoKI. Owing to the non-specific cleavage reaction of EcoKI on linear DNA, in which the enzyme molecules bound at each recognition sequence have to cooperate to introduce a single double-strand cut at a random location between the two recognition sequences, the DNA migrated as a broad range of fragment sizes rather than as two discrete bands. We observed that EcoKI restriction of linear pBR322 was inhibited by high concentrations of StpA. In the absence of StpA, restriction of linear pBR322 did not go to completion, although most DNA had been restricted after 20 min. However, no EcoKI cleavage of linear pBR322 was visible after 20 min at concentrations of StpA with one or more StpA per 96 bp. EcoKI restriction was also inhibited by one StpA per 128 bp, but a small amount of smearing (indicating cutting) could be seen. Concentrations of one StpA per 256–171 bp appeared to be slightly inhibitory to EcoKI restriction. The usual cutting pattern of EcoKI, as seen in absence of StpA, on pBR322 previously linearized by the EcoRI endonuclease (a band at 3.9 kb and smear from this) was not seen at these concentrations of StpA so although cleavage was occurring, it was not happening at the same locations on the DNA as in the absence of StpA.

EcoKI restriction activity on supercoiled DNA. It can be seen from Figure 6 that EcoKI restriction of supercoiled pBRsk1 (which contains one recognition sequence for EcoKI and which is cut to a linear form only) was also inhibited by high concentrations of StpA. In the absence of StpA, supercoiled pBRsk1 was completely cut into linear DNA. However, no EcoKI cutting of supercoiled pBRsk1 was visible after 20 min at StpA concentrations of one or more StpA per 256 bp. EcoKI restriction was reduced by one StpA per 512–1024 bp. Performing the same control experiment as done for DNase I, it was found that the inhibitory effect of StpA was due to the binding of StpA to DNA rather than a direct interaction of StpA with EcoKI (data not shown).

DISCUSSION

StpA is proposed to act in a way similar to H-NS to form oligomeric arrays that bind DNA helices together (1,13,14). This bridging between helices will be aided by supercoiling, which brings different regions of DNA into close proximity (23–26). StpA has been shown to facilitate bridging between adjacent DNA double-stranded helices with clear evidence that once a bridge has formed, further bridges form next to the bridge and one gets cooperative growth of the bridged region. Dame *et al*. (13,14) show partial collapse of open circular DNA at one StpA per 200 bp. These bridged structures would be expected to restrict access of other proteins to the DNA. Our results show that StpA-induced condensation of DNA inhibits the nuclease activity of DNase I and the ATP hydrolysis and restriction activities of EcoKI. We found that around one StpA per 64–96 bp protected linear DNA whereas supercoiled DNA required only one StpA per 256–384 bp for

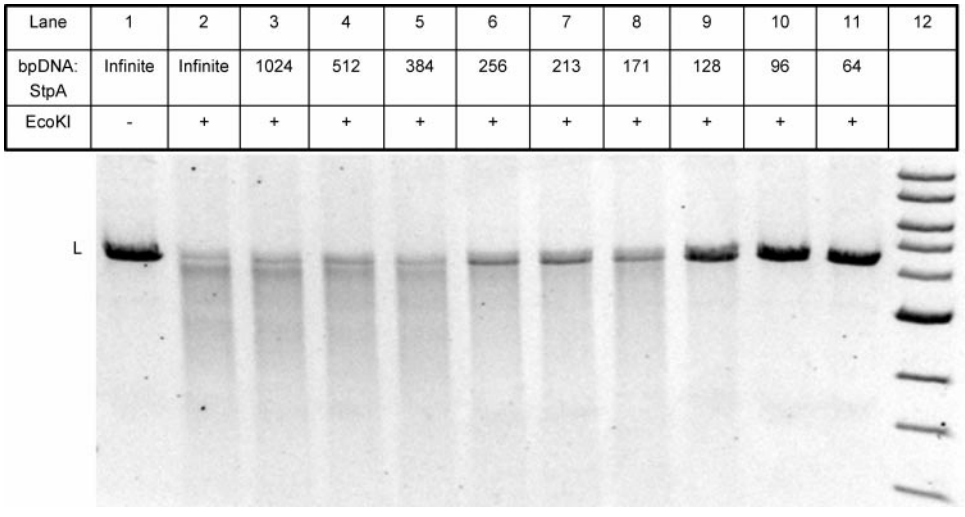

Figure 5. Effect of StpA concentration on the digestion by EcoKI of EcoRI-linearized pBR322. Linear pBR322 (50 nM) was cut in high salt EcoKI buffer, pH 7.9, with varying concentrations of StpA for 20 min. The EcoKI concentration was 67 nM. pBR322 DNA forms a distinct band of 4.4 kb (L) which is cut to form a smear of lower molecular weight. Lane 12 contains a linear 1 kb DNA ladder of sizes 1, 1.5, 2, 3, 4, 5, 6, 8 and 10 kb from bottom to top.

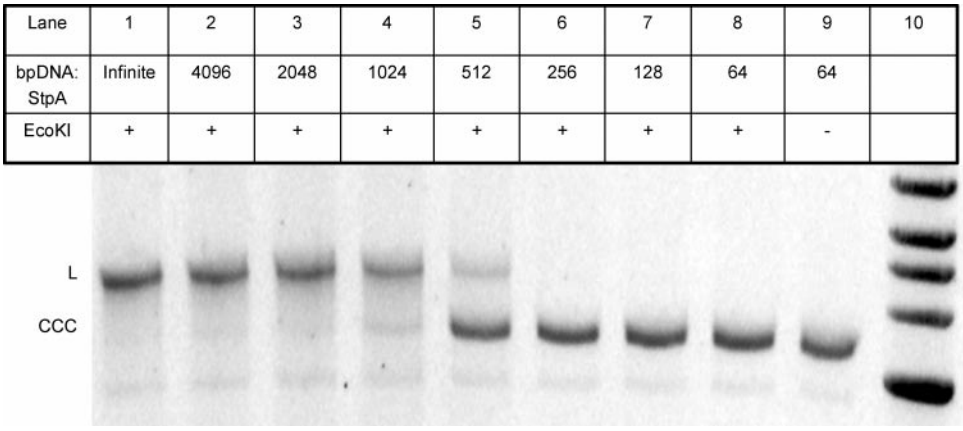

Figure 6. Effect of StpA concentration on the digestion by EcoKI of supercoiled pBRsk1. Supercoiled pBRsk1 (50 nM) was cut in high salt EcoKI buffer, pH 7.9, with varying concentrations of StpA for 20 min. The EcoKI concentration was 67 nM. pBRsk1 is cut from supercoiled DNA (CCC) into a linear band of 4.4 kb (L). Lane 10 contains a linear 1 kb DNA ladder of sizes 3, 4, 5, 6 and 8 kb from bottom to top.

protection. It was also observed that the actual molar concentration of StpA was not responsible for the inhibition of DNase I or EcoKI, but rather it was the number of StpA per DNA bp which was vital for inhibition of these enzymes. StpA bound to the DNA may prevent access of the enzymes to their target sites, non-sequence-specific in the case of DNase I and sequence-specific in the case of EcoKI, or prevent translocation of EcoKI by directly blocking the movement or by bridging DNA helices together. Once a certain critical ratio of StpA to DNA was reached, all of the DNA molecules present in the reaction had been converted to a condensed, nuclease-resistant form. In other words the binding of StpA to DNA, followed by condensation of the DNA, appears to be cooperative in nature.

In supercoiled DNA, regions of DNA helix are in close proximity to each other thus favouring the probability of StpA binding and the cooperative formation of an inter-strand bridge. Linear DNA, however, is best described as an open worm-like coil structure and the average spatial distance between any two segments is related to the number of base pairs between the segments. The closest average distance between segments on linear DNA is found for segments separated by ~1000 bp (26) but the DNA segments are not, on average, in close contact with each other. Hence, an StpA bound to one segment is generally not close to another segment of DNA and the likelihood of bridging will be low until at least the first bridge is formed after which cooperative growth of bridges is again possible. The lower probability of bridge formation on linear DNA than on supercoiled DNA would explain the differences observed in our assays.

It has been calculated by Azam *et al*. (2) that there is one StpA molecule per 190 bp of DNA in *E.coli* cells during the exponential phase, as well as one H-NS per 220 bp. Dame *et al*. (13,14) have shown that this quantity of StpA, along with its homologue H-NS and numerous other nucleoid-associated proteins, would be enough to at least partially condense DNA in the cell. Under conditions of cell stress leading to DNA damage, unmethylated EcoKI target sequences can appear on chromosomal DNA (27). On naked DNA, such sites would lead to the cleavage of the DNA but our results clearly show that physiological quantities of StpA, and by inference proteins such as H-NS, can protect damaged host DNA from unwanted degradation by type I restriction enzymes such as EcoKI. This supports similar conclusions drawn from biophysical studies of the inhibition of EcoKI by DNA-binding dye molecules (22) and genetic studies of the phenomenon of restriction alleviation (27) in which restriction is down-regulated during periods of cell stress. Since a minimal level of ATP hydrolysis continues even once DNA cleavage is abolished, it also appears to be the case that even if EcoKI can bind to DNA in the presence of StpA, the DNA translocation mechanism is insufficiently powerful to displace the StpA molecules from the DNA to expose a DNA cleavage location. Single molecule force measurements on a related type I restriction endonuclease, EcoR124I, indicate that whilst translocation occurs with speeds approaching 1000 bp/s, the force generated by the translocating motors is only of the order of 5 pN (28), significantly less than the force generated by RNA polymerases to transcribe DNA [e.g. see review (29)]. EcoKI translocates rather more slowly at ~100 bp/s (30) and one might assume that the force generated by it is similar or less than that generated by EcoR124I. The weakness of the motors in type I restriction enzymes may, therefore, be more suited to translocation on naked invading phage DNA than on the nucleoid, a feature of advantage to the host cell.

ACKNOWLEDGEMENTS

This work was supported by BBSRC grant 15/17860 to DTFD and a studentship (S.A.K.) from the Medical Research Council. J.E.L. is a Wellcome Trust Senior Research fellow. P.G.L. is funded through a BBSRC studentship. Funding to pay the Open Access publication charges for this article was provided by JISC.

Conflict of interest statement. None declared.

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