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Citation for published version:

Keatch, SA, Su, TJ & Dryden, DTF 2004, 'Alleviation of restriction by DNA condensation and non-specific DNA binding ligands' *Nucleic Acids Research*, vol 32, no. 19, pp. 5841-5850. DOI: 10.1093/nar/gkh918

Digital Object Identifier (DOI):

[10.1093/nar/gkh918](https://doi.org/10.1093/nar/gkh918)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher's PDF, also known as Version of record

Published In:

Nucleic Acids Research

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Alleviation of restriction by DNA condensation and non-specific DNA binding ligands

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Received September 17, 2004; Revised and Accepted October 13, 2004

ABSTRACT

During conditions of cell stress, the type I restriction and modification enzymes of bacteria show reduced, but not zero, levels of restriction of unmethylated foreign DNA. In such conditions, chemically identical unmethylated recognition sequences also occur on the chromosome of the host but restriction alleviation prevents the enzymes from destroying the host DNA. How is this distinction between chemically identical DNA molecules achieved? For some, but not all, type I restriction enzymes, alleviation is partially due to proteolytic degradation of a subunit of the enzyme. We identify that the additional alleviation factor is attributable to the structural difference between foreign DNA entering the cell as a random coil and host DNA, which exists in a condensed nucleoid structure coated with many non-specific ligands. The type I restriction enzyme is able to destroy the 'naked' DNA using a complex reaction linked to DNA translocation, but this essential translocation process is inhibited by DNA condensation and the presence of non-specific ligands bound along the DNA.

INTRODUCTION

In every cell, it is important to be able to distinguish between different DNA molecules, for example between replicated DNA versus non-replicated DNA or host DNA versus foreign DNA. In both these situations, the cell contrives to ensure that the DNA molecules are chemically different. In both eukaryotes and prokaryotes, DNA methylation, where cytosine is modified to C5-methyl-C or N4-methyl-C and adenine is modified to N6-methyl-A, is of particular importance for this chemical distinction. These different chemical modifications allow various enzyme systems to distinguish between the methylation states of the DNA and carry out appropriate reactions. However, there are situations such as cell stress and DNA damage in which chemically identical DNA molecules have to be differentiated by an enzyme. The normally impressive chemical ability of an enzyme to discriminate between different substrates is of no use in this situation, so how is this discrimination achieved?

This question is raised most clearly by the phenomenon of restriction alleviation (RA) in bacteria, the existence of which

has been known for many years (1,2) but which is only partially understood at a molecular level. In the RA process, the DNA restriction/modification (R/M) system of the host bacterium is switched off or reduced in effectiveness for a period of time to allow, for example, repair of DNA damage induced by UV irradiation (1,3,4) or by chemical agents (5–8) or, when introducing an R/M system into a naïve host, the establishment of modification upon the chromosome (9–13). It can also be induced by the loss of the modification function by mutation (3,14). R/M systems either modify (methylate) newly replicated, hemimethylated host DNA, which contains the methylated parental strand and the unmethylated daughter strand, or destroy unmethylated foreign DNA. In situations of incomplete modification of the host DNA, R/M systems can be lethal. It is known that type I R/M systems have RA control to prevent lethality (9,11) whilst type II R/M systems function as selfish genetic elements (15) and kill their hosts.

RA raises two important questions about the nature of the DNA in the cell. First, when the chromosomal DNA sequences recognized by the R/M system exist in a partly methylated state during DNA repair or a fully unmethylated state when a new R/M system is acquired by a naïve host, how does the host cell control the potentially lethal activity of the host type I R/M system on its chromosome (7,16)? Second, during RA a reduced level of restriction activity is still present and forms a barrier to phage infection (1–3,5,6) indicating that the unmodified target sequences on the phage DNA can be distinguished from the unmodified target sequences on the host DNA by the resident type I R/M system. How is this accomplished when both DNA molecules are chemically identical (7,16)? We wish to address these two questions in this paper.

The RA phenomenon appears to apply only to type I R/M systems, which combine both restriction endonuclease and modification methyltransferase activities within a single multifunctional enzyme complex [reviewed in (16–19)]. These systems, despite their complexity, are very widespread in prokaryotes occurring in at least 50% of bacteria (20). Type I R/M systems are grouped into four families, IA to ID (21). Type I R/M enzymes contain two restriction subunits, two modification subunits and a single sequence specificity subunit; they usually prefer to modify host DNA when it exists in a hemimethylated state following DNA replication and to destroy foreign DNA, which usually contains unmodified target sequences. The complex restriction reaction commences with an irreversible attachment to the unmodified target sequence followed by reeling in of the DNA on either side of the target sequence towards the enzyme (often referred to as

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DNA translocation driven by ATP hydrolysis) with concomitant formation of DNA loops and eventual double-strand cleavage of the DNA when translocation is halted by some obstruction to further translocation. These obstructions are usually another type I R/M enzyme (22), a Holliday junction (23,24) or complete translocation of circular DNA containing only a single target sequence [e.g. (25)]. The restriction subunits are responsible for both the ATP-hydrolysis dependent DNA translocation and for DNA cleavage as they contain essential amino acid motifs for DNA helicase motor activity and for Mg²⁺-dependent endonuclease activity (26–30). At the collision site, each endonuclease domain, one in each restriction subunit, introduces a single-strand break. Since the breaks are in close proximity to each other, the overlapping DNA ends melt and the DNA is cleaved. The nature of the cleaved ends is still not clear (31–33). Furthermore, once cleavage has occurred, the enzyme apparently remains bound to the DNA (34) and continues to hydrolyse ATP without turnover in the complete restriction process (32,35,36).

There appear to be (at least) two forms of RA. For type IA and IB R/M systems, RA has been linked to a genotypic effect (11–13) involving the products of the *clpXP* genes (10). We will refer to this as ‘family-specific RA’, which so far is known for only the type IA and IB families. ClpXP is a proteolytic enzyme complex, which degrades the restriction subunits of the type I R/M enzyme but only if the R/M enzyme has commenced the complex restriction process (3). It is the translocation process during restriction, which signals the clpXP protease to attack the type I R/M enzyme and degrade the restriction subunits. In this manner, the potentially lethal effects of the type I R/M enzyme on the chromosome are prevented at least for the IA and IB families. However, not all type I R/M systems are subject to RA via the clpXP protease but RA is still observed and no specific genotype has so far been associated with this second form of RA (7). We will refer to this as ‘general RA’ and, on the basis of our results, we propose that it applies to all type I R/M systems including those which also have family-specific RA. The two forms of RA, general and family-specific are effective in limiting damage to the host chromosome. The physical basis of general RA is not known, but it has been suggested that it may be related to the difference between the higher order structure of the chromosomal DNA present in the nucleoid and foreign DNA, which will adopt a random coil conformation as it enters the cytoplasm of the cell (7,16). Thus, the type I R/M enzyme will see two different DNA structures, and it effectively exists in two different ‘compartments’ in the cell, either associated with the nucleoid where its main function is to maintain methylation or in the cytoplasm where its role is to intercept foreign unmethylated DNA. There is some evidence that when in the cytoplasmic fraction the enzyme can associate with the inner membrane (3,37,38).

The structure of the nucleoid has been the focus of much attention for many years (39,40). In the absence of cell stress, the nucleoid is always partially coated with a range of ‘histone-like’ proteins (40,41) and their concentration varies depending on growth conditions (42). Such an extensive array of non-sequence-specific ligands bound along the DNA is likely to hinder a translocating type I R/M enzyme. Although it has been demonstrated that a single repressor protein bound to DNA does not hinder translocation (43),

it is noteworthy that the presence of saturating concentrations of intercalating dye molecules does substantially hinder restriction (44,45) implying that a barrier of non-specifically bound ligands would pose a problem for translocation by type I R/M enzymes. Whilst the nucleoid DNA is typically only partially compacted by bound protein thereby allowing DNA replication to proceed, under conditions of stress which induce RA, it has recently been observed that the nucleoid can slowly convert to a semicrystalline form and undergoes a well-known process termed ‘condensation’ in which the DNA double strands become aligned with each other in compact arrays (46–48). This condensed DNA is not available for replication or transcription as these processes are shut down during conditions of DNA damage to allow time for DNA repair. Hence, it is therefore possible that the condensed nucleoid is also generally resistant to restriction. It has been shown that a variety of simple type II restriction enzymes are inhibited *in vitro* by DNA condensation (49,50) but for these enzymes there is no indication of any association with the RA phenomenon (4,5,7). Condensation is likely to be an even more acute problem for a type I R/M system dependent upon DNA translocation.

In this paper, we compare the activity of EcoKI on ‘naked’ DNA with that on DNA coated with non-sequence specific ligands and with DNA condensed by the addition of a polyamine to address the questions about general RA posed by Murray (16) concerning the difference between self and foreign DNA when both are unmethylated. DNA condensation is achieved by the addition of the polyamine spermidine (Sp³⁺), a well-known physiological condensing agent and as non-specific DNA binding ligands, we use several dyes including a dye which binds in the minor groove of DNA, Hoechst 33258, and two dyes which intercalate between base pairs, ethidium bromide (EtBr) and the cyanine dye YOYO.

MATERIALS AND METHODS

EcoKI purification

EcoKI was prepared from 8 l of NK311 cells (6) containing the plasmid pBE3 (11) as previously described (51). Concentration of enzyme was determined by UV absorption at 280 nm. 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.

DNA plasmid preparation

Plasmid DNA was purified from *E.coli* strain ER2426 as described in (52). This strain lacks the EcoKI R/M system hence the DNA contains unmodified EcoKI sites. Plasmid pBR322 and its derivative, pBRsk1 (27) were used in our experiments. pBR322 contains two target sites for EcoKI, whereas pBRsk1 contains only one EcoKI target site. Both plasmids contain one EcoRI site. When linear DNA was required, plasmid DNA was cut with EcoRI in high-salt EcoKI buffer. DNA concentration was determined by UV spectroscopy and purity by agarose gel electrophoresis

using 1× TBE (10× TBE: 0.89 M Tris base, 0.89 M boric acid, 20 mM EDTA) and DNA loading buffer (10× DNA loading buffer: 20% Ficoll 400, 0.1 M Na₂EDTA, 1% SDS, 0.25% (w/v) bromophenol blue, pH 8).

DNA condensation by spermidine and binding of dyes to DNA

Spermidine (Sigma Aldrich) was dissolved in low-salt EcoKI buffer and the pH adjusted to pH 7.9 if required. This stock solution was added to DNA solutions and left for 30 min before adding EcoKI and its reaction ingredients. The dyes ethidium bromide (EtBr), Hoechst 33258 and YOYO (Molecular Probes) were added to DNA at various dye molecule to DNA base-pair ratios and incubated for 30 min at 45°C before adding EcoKI and its reaction ingredients.

DNase digestion

Assays were carried out in low-salt EcoKI buffer with 1 mM CaCl₂ and the ionic strength adjusted using NaCl. Plasmid pBRsk1 DNA (50 nM) was incubated with 0.1 µg/ml DNase I at 37°C. The reaction was stopped by the addition of EDTA to a final concentration of 77 mM. Samples were then run on 0.8% agarose gels, in 1× TBE with EtBr staining.

Restriction assays

Assays were typically carried out in low-salt EcoKI buffer with the ionic strength adjusted with NaCl. Reactions were carried out with 50 nM plasmid DNA, 0.1 mM S-adenosyl-methionine (SAM, New England Biolabs), 50 µg/ml BSA (New England Biolabs) and 2 mM ATP (Roche). EcoKI (67 nM) was used for digestion of DNA containing one EcoKI target site and 134 nM EcoKI for DNA with two sites. Reactions were typically carried out in 180 µl total volume, with 22 µl being removed at specific time points and added to EDTA (77 mM final concentration) in order to stop the reaction. EcoKI was denatured by heating the sample to 68°C for 30 min. This treatment also served to displace Hoechst and YOYO from the DNA and allow their replacement with EtBr. It is known that YOYO makes the DNA migrate differently from DNA stained with EtBr (53) and this treatment allowed all densitometry to be performed in the same manner with EtBr. Loading buffer was then added to samples before being run on 0.8% agarose gels in 1× TBE buffer. EtBr was used to stain the DNA, which was visualized using a UV transilluminator (UVP, TFM-30) and images acquired using a digital camera (Fujifilm FinePix S602Zoom) with a 3 mm thick, 570 nm cut-on filter (Schott, Germany) and Adobe Photoshop software. Densitometry was carried out on gel images using Scion Image software (Scion Corporation). In order to analyse the data thoroughly, a conversion factor was determined to allow direct comparison between supercoiled, nicked or linear DNA as these different topological forms bind varying amounts of dye.

Translocation assays

EcoKI translocation was measured by a coupled enzyme ATPase assay (54). In this reaction, the rate of decrease in NADH is proportional to the rate of ATP hydrolysis by EcoKI and is measured via absorbance at 340 nm. For this assay, 5 nM DNA with one EcoKI target site or 2.5 nM DNA with two

EcoKI target sites was incubated with 10 nM EcoKI, 50 µg/ml BSA, 0.1 mM SAM, 1 mM phosphoenol pyruvate (Sigma), 250 µM NADH (Sigma), in low-salt EcoKI buffer, pH 7.9, with a final reaction volume of 300 µl. Pyruvate kinase/lactate dehydrogenase (Roche) was dialysed against low-salt EcoKI buffer and used fresh on the day of experiment at a concentration of 13 µg/ml. A baseline was recorded at 340 nm before starting the reaction by adding ATP to 2 mM. The rate of decrease of NADH was recorded and the corresponding rate of ATP hydrolysis calculated using an extinction coefficient for NADH of 6.22 for a 1 mM solution, 1 cm pathlength at 340 nm.

Data analysis was performed using Grafit (Erithacus Software, UK) and Dynafit (55).

RESULTS

Determination of conditions for condensation of plasmid DNA

DNA condensation has been shown to be highly dependent not only on the nature of the condensing agent but also on the ionic strength and pH of the solution (56). Given that spermidine carries a charge of 3+ and is strongly basic, these two parameters must be carefully controlled when attempting to assay the EcoKI activity. An assay for condensation using the non-specific nuclease DNaseI was successfully used under the solution conditions appropriate for assaying EcoKI. DNaseI was only able to digest both linear and circular DNA at Sp³⁺ concentrations of <6 mM as shown by the presence of undigested DNA on agarose gel electrophoresis, Figure 1. Slight protection was observed at 5 mM Sp³⁺ but full protection of linear DNA and maintenance of circular DNA, albeit with

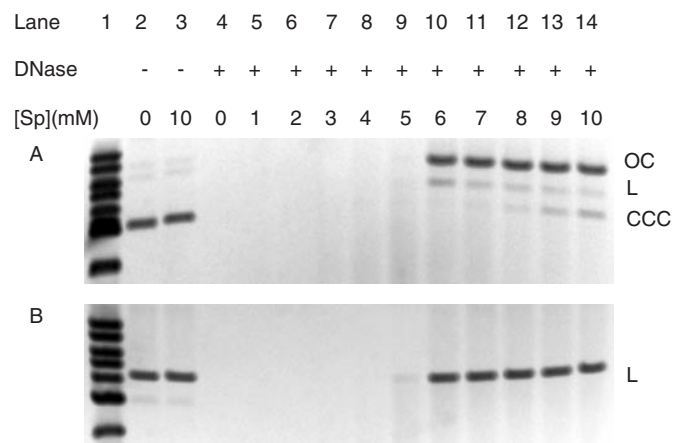


Figure 1. Effect of spermidine-induced condensation on digestion of DNA by DNase I. DNase I (1 µg/ml) was used to digest 50 nM supercoiled (A) and linear (B) pBRsk1 at 37°C in low-salt EcoKI buffer with the ionic strength made up to 100 mM with NaCl. Lane 1 contains a DNA size marker (10, 8, 6, 5, 4, 3 and 2 kb from top to bottom). Lanes 2–14 contain DNA in the presence (plus sign) or absence (minus sign) of DNaseI and varying concentrations of spermidine ranging from 0 to 10 mM as indicated. The different conformations of DNA are as follows: CCC, covalently closed circular (supercoiled); OC, open circular (nicked) and L, linear DNA. Both CCC and L pBRsk1 were condensed by 6 mM spermidine. It can be seen that although the L DNA appears completely condensed at 6 mM spermidine, the CCC DNA is mainly in an OC conformation with some L DNA.

some single-strand nicking, was only observed when the spermidine concentration equalled or exceeded 6 mM. These results are consistent with spermidine-induced compaction of the DNA under these experimental conditions (56).

EcoKI restriction activity on plasmid pBRsk1 as a function of condensation and intercalation

Having defined the conditions which lead to the onset of DNA condensation in the EcoKI assay buffer, we analysed the DNA cleavage rates of EcoKI as either spermidine or intercalating dyes were added whilst maintaining pH and ionic strength constant. Figure 2 shows the progressive digestion of pBRsk1 supercoiled plasmid by EcoKI. It is clear that the

closed circular form of the plasmid is converted to a nicked open circular form and then to a linear form but the rates at which these steps occur decreases significantly as both $[Sp^{3+}]$ and $[dye]$ increase (EtBr results not shown). After lengthy incubations, the linear product is gradually digested further. This secondary cleavage of DNA is often observed with type I enzymes. In the uninhibited assay, Figure 2A, the level of nicked DNA rapidly accumulates and then is further degraded by the acquisition of a second nick which must be in close proximity to the first. Of greatest interest is the accumulation of nicked plasmid once the DNA has condensed or the dye to base pair ratio reaches 1:32, Figure 2B, C and D. In the presence of condensation or dyes, the level of nicked DNA builds up ~ 5 – 10 times slower compared with the uninhibited

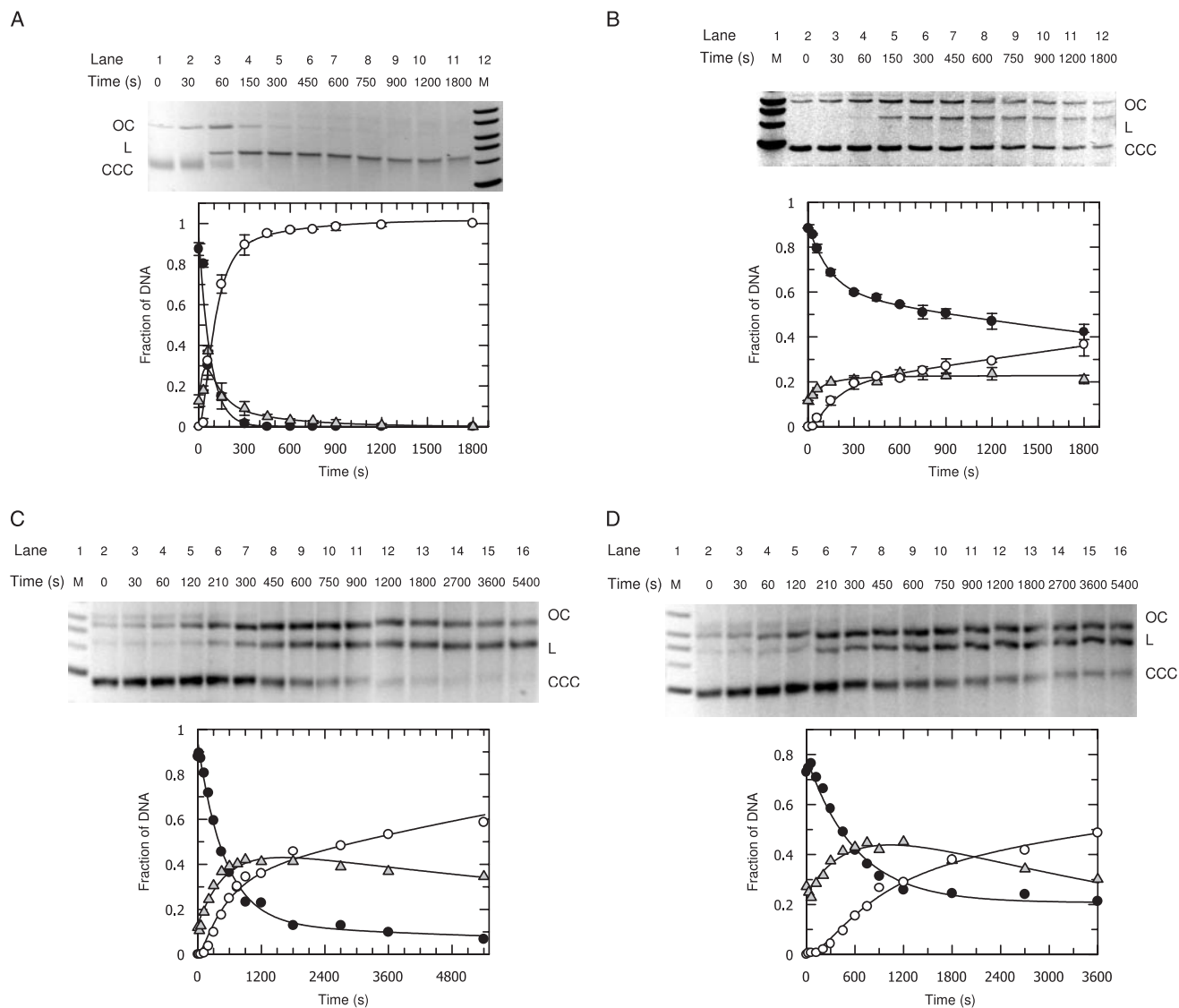
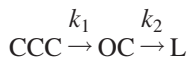


Figure 2. Degradation of 50 nM CCC pBRsk1 by 67 nM EcoKI in low-salt EcoKI buffer with the ionic strength made up to 100 mM with NaCl. The gels show the time course of DNA digestion with CCC DNA being converted to OC then to L DNA. DNA markers of the sizes indicated are shown in lanes marked with an M. The results of densitometry of these gels are shown below each gel with the proportion of CCC DNA shown as filled circles, OC DNA as shaded triangles and L DNA as open circles. The fitted lines are derived from the kinetic model described in the text. All experiments were repeated at least in duplicate (Hoechst 33258) or in triplicate (other data sets) and representative error bars for SD are shown in sections A and B. (A) pBRsk1 being digested by EcoKI. Markers 10, 8, 6, 5, 4 and 3 kb from top to bottom. (B) Digestion of the DNA preincubated and condensed with 6 mM spermidine. Markers 6, 5, 4 and 3 kb from top to bottom. (C) Digestion of the DNA preincubated with Hoechst 33258 at a dye to base pair ratio of 1:32. Markers 6, 5, 4 and 3 kb from top to bottom. (D) Digestion of DNA preincubated with YOYO at a dye to base pair ratio of 1:32. Markers 8, 6, 5, 4 and 3 kb from top to bottom.

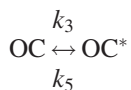
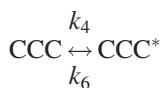
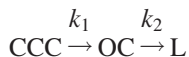
assay, but then breaks down even more slowly to linear DNA. The level of nicked plasmid reaches 20% or more of the total DNA when the spermidine concentration reaches 6 mM and 40% when the dye to base-pair ratio reaches 1:32. It appears that at least a proportion of the nicked DNA is either completely or very nearly completely resistant to cleavage to a linear form. Perhaps in these situations, further nicking does occur but the nicks are too far away from the initial nick to allow denaturation of the DNA to a linear form.

Kinetic modelling of the rate of DNA cleavage

In the absence of spermidine or dye, the restriction reaction pathway proceeds from closed circular (CCC) to open circular (OC) to linear (L) DNA and the restriction data obtained can be described by the model below.



However, the presence of even small amounts of spermidine or dye slows the progression through this reaction pathway, and the persistence of large amounts of uncleaved or nicked DNA after long periods of incubation suggests that these additives can sequester the DNA in uncleavable forms, CCC* for closed circular DNA and OC* for open circular DNA. As the binding of both spermidine and the dyes to DNA are not irreversible processes, we assumed that the formation of CCC* and OC* was reversible. Therefore, we added two additional steps to describe the reaction in the presence of spermidine or dye. The densitometric data, some of which is shown in Figure 2, has been fitted to the following model.



This more complex model [applied equally well to both the spermidine data and the dye data, and the derived rate constants for spermidine are given in Table 1. It is apparent from the rate constants and the derived equilibrium constants that the two kinetic steps involving CCC* and OC* are of minor importance, and therefore difficult to measure accurately, at spermidine concentrations <6 mM where the DNA is uncondensed but becomes very significant upon condensation. Similarly, as the dye concentration increases they become more important (data not shown). Figure 3 shows the rate constants k_1 and k_2 as a function of $[\text{Sp}^{3+}]$ or $[\text{dye}]$. It is apparent that both these rate constants are significantly slowed by condensation and by dye binding as expected from a qualitative inspection of Figure 2. Prior to DNA condensation, however, the rate constants increase significantly indicating that at low concentrations, spermidine actually enhances the restriction reaction. This effect was noted many years ago for the EcoAI type I R/M enzyme (57) and for some type II restriction enzymes (49,50).

Either condensation or the presence of intercalating dyes strongly inhibits the restriction process of EcoKI. The collapse of DNA due to Sp^{3+} into a tightly packed structure would be expected to restrict access of EcoKI to its target site, but we can see that perhaps surprisingly this is not too severe as the rate of nicking is still appreciable indicating that the enzyme can compete effectively with the Sp^{3+} to find its target site. Perhaps more surprising is the dramatic effect of the dyes. Even very small amounts of dye, where on an average one dye molecule is bound for every few hundred base pairs, reduces the rate of conversion of closed circular DNA to open circular DNA and then to linear DNA. These small dyes are clearly a severe block to either protein-DNA binding or EcoKI activity or both.

ATP hydrolysis

The ATP hydrolysis assay measures ATP consumption throughout the restriction process and does not distinguish between any particular binding, translocation or cutting event. However, it is observed that the rate of hydrolysis remains constant for any particular set of experimental conditions. Figure 3A shows the variation in rate as a function of spermidine. The rate is slightly decreased by low concentrations of spermidine but drops by 2/3 to 3/4 of its uninhibited value at 6 mM spermidine and still further at higher concentrations as condensation is completed. Even at the highest concentrations of spermidine where all of the DNA will be

Table 1. Rate constants for the degradation of closed circular plasmid pBRsk1 by EcoKI determined using the model described in the text

[Sp ³⁺] mM	Rate constants (s ⁻¹)						Equilibrium constants	
	k ₁	k ₂	k ₃	k ₄	k ₅	k ₆	k ₃ /k ₅	k ₄ /k ₆
0	0.0150 ± 0.0030	0.0262 ± 0.0100	1.93 × 10 ⁻⁸ ± 0.0030	1.13 × 10 ⁻⁹ ± 0.0110	0.0020 ± 0.0010	0.1563 ± 1.8	9.65 × 10 ⁻⁶	7.22 × 10 ⁻⁹
2	0.0187 ± 0.0003	0.0276 ± 0.0075	1.93 × 10 ⁻⁸ ± 0.0019	1.13 × 10 ⁻⁹ ± 0.0011	0.0041 ± 0.0016	0.0051 ± 0.0037	4.71 × 10 ⁻⁶	2.21 × 10 ⁻⁷
4	0.0197 ± 0.0029	0.0331 ± 0.0087	1.93 × 10 ⁻⁸ ± 0.0016	1.13 × 10 ⁻⁹ ± 0.0008	0.0019 ± 0.0006	0.0044 ± 0.0027	1.01 × 10 ⁻⁵	2.57 × 10 ⁻⁷
6	0.0037 ± 0.0002	0.0083 ± 0.0020	0.0152 ± 0.0006	0.0033 ± 0.0001	0.0003 ± 0.0001	0.0004 ± 0.0001	50.67	8.25
8	0.0020 ± 0.0001	0.0020 ± 0.0006	0.0079 ± 0.0004	0.0025 ± 0.0001	0.0002 ± 0.0001	0.0004 ± 0.0001	39.50	6.25
10	0.0002 ± 0.0001	0.0002 ± 0.0001	0.0093 ± 0.0004	0.0022 ± 0.0001	0.0029 ± 0.0005	0.0017 ± 0.0001	3.20	1.29

It is clear from the equilibrium constants that the side reactions involving the formation of DNA structures resistant to cleavage by EcoKI become significant only once DNA condensation has occurred.

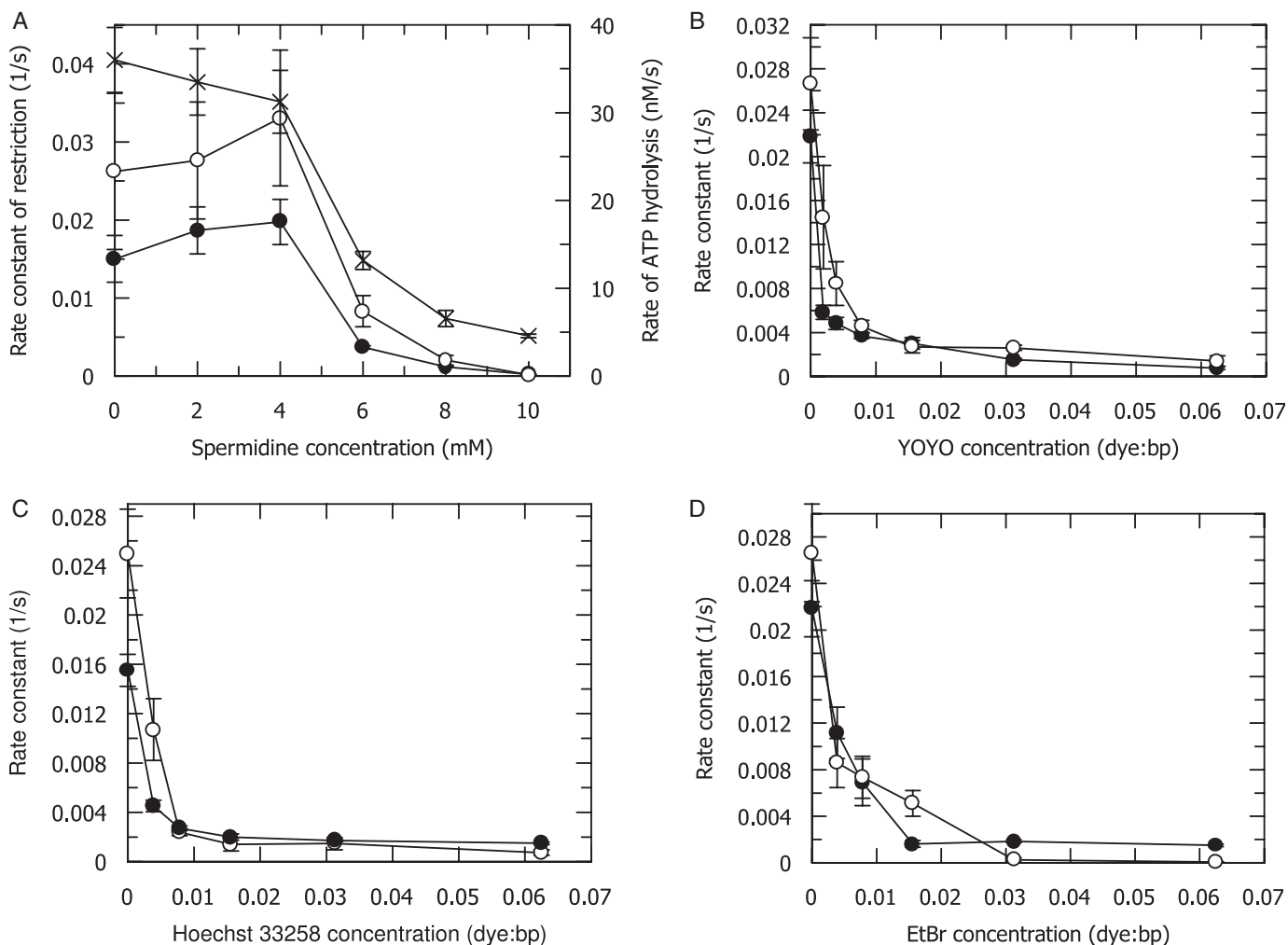


Figure 3. The effect of spermidine and dye molecules on the rates of the endonuclease and ATP hydrolysis reactions of EcoKI on CCC pBRsk1. The rate constants for the cleavage of CCC DNA to OC form (filled circles), OC DNA into L form (open circles) derived by fitting data, such as those shown in Figure 2 collected at different concentrations of spermidine or dye, to the full kinetic model given in the text are shown. The rate of ATP hydrolysis (\times) derived from the ATPase assay in the presence of varying amounts of spermidine is also shown. (A) Effect of spermidine concentration on EcoKI activity. It can be seen that at low concentrations of spermidine the rate of cleavage is enhanced but once condensation occurs at 6 mM spermidine or greater, the activity is greatly reduced. The rate of ATP hydrolysis decreases as spermidine is added, particularly at high concentrations when the DNA has condensed. (B) Effect of YOYO concentration on DNA cleavage by EcoKI. (C) Effect of Hoechst 33258 concentration on DNA cleavage by EcoKI. (D) Effect of EtBr concentration on DNA cleavage by EcoKI.

condensed, ATP hydrolysis is observed indicating that the enzyme can still locate its target site on the condensed DNA and at least attempt to translocate DNA. The presence of YOYO also has a strong inhibitory effect on ATP hydrolysis (data not shown) with 50% inhibition achieved with 1 dye molecule per 128 bp of DNA.

Other DNA substrates

The above experiments were also repeated for closed circular pBR322 and pBR322 linearized with EcoRI, both of which contain two target sites for EcoKI (data not shown), and for linear pBRsk1 which has one target site. The presence of two target sites allows the enzyme to continue degrading the DNA after conversion to the linear form. This results in a smear of

small fragments on agarose gels and requires the addition of a further kinetic step for linear DNA degrading to these fragments. This further complicates the kinetic analysis without revealing any significant new features. ATP hydrolysis occurs at similar rates for all DNA substrates tested including the linear DNA molecule, which contains only a single EcoKI site (data not shown) even though this molecule is virtually resistant to cleavage.

DISCUSSION

Our experiments show that the restriction activity of EcoKI is strongly compromised by the condensation of DNA by spermidine and by the presence of multiple ligands bound non-specifically along the DNA lattice. Condensation or the

presence of dye molecules inhibits the rates of hydrolysis of ATP required for DNA translocation and double-strand cleavage of the DNA. As a consequence of the slowed kinetics of ATP hydrolysis, DNA translocation and double-strand cleavage, a substantial amount of the DNA substrate is only subject to single-strand nicking. Although obstruction of EcoKI binding must occur to some extent, the presence of substantial quantities of nicked DNA rules out the simple idea that condensation or intercalating dyes achieve their inhibitory effect purely by blocking access of EcoKI to its binding site.

It has been shown that type I R/M enzymes make a double-strand break once translocation has stalled either by collision with another translocating type I R/M enzyme (22) or by a strong blockage to translocation such as a junction in the DNA (23,24) or complete translocation of a circular plasmid containing a single target sequence [e.g. (25)]. It appears from our results that compromising the translocation by DNA condensation leads most often to single-strand breaks. Our results do not indicate if multiple single-strand breaks are introduced. If multiple nicks are introduced, then they are either too far apart for strand separation to easily occur or the breaks introduced by a particular restriction subunit are all on the same strand of DNA. Occasionally a double-strand break is made on the condensed DNA, but the frequency is much reduced compared to uncondensed DNA. Previously, it was shown for the EcoR124II type I R/M enzyme that nicks by themselves are not able to induce a second cleavage to produce a double-strand break (43), so it appears that the endonuclease domain in the restriction subunit is only able to make single-strand breaks on one strand of the DNA. During translocation on uncondensed pBRsk1, the two restriction subunits can cooperate with each other to make a double-strand break when the translocation stalls after the entire plasmid has been reeled in. However, stalling translocation of these subunits before they collide means that the two nicks are uncoordinated and fail to lead to linearization of the DNA.

The restriction process is also strongly inhibited by the coating of the DNA with non-specific binding ligands such as dyes. Previously, it has been found that saturating amounts of the intercalator EtBr block restriction activity (44,45). Such inhibition has also been demonstrated for the type II restriction enzyme EcoRI (58). Our results show that substantial inhibition of both translocation and cleavage is achieved by even one dye molecule being present on an average for every 256 bp along the DNA. The presence of dye molecules on the DNA will undoubtedly hinder EcoKI in locating and binding to its target site as the three dye molecules, Hoechst 33258 (59,60), EtBr (61) and YOYO (53) have high nanomolar to micromolar binding affinity for DNA comparable to the binding affinity of EcoKI. However, competition for DNA binding between dye and EcoKI cannot be the entire cause of the observed reduction in activity since at least a proportion of the enzyme must be able to bind to its site as all the binding processes are equilibrium processes. Once EcoKI has bound to the DNA, it is committed to hydrolyse ATP and to introduce single-strand breaks in the DNA. The appearance of single-strand breaks and hydrolysis of ATP indicates that the translocation and cleavage processes are being hindered not only indirectly by competition between the enzyme and the dye for the EcoKI target sequence but also directly in the same manner as found when spermidine is present. It has previously been

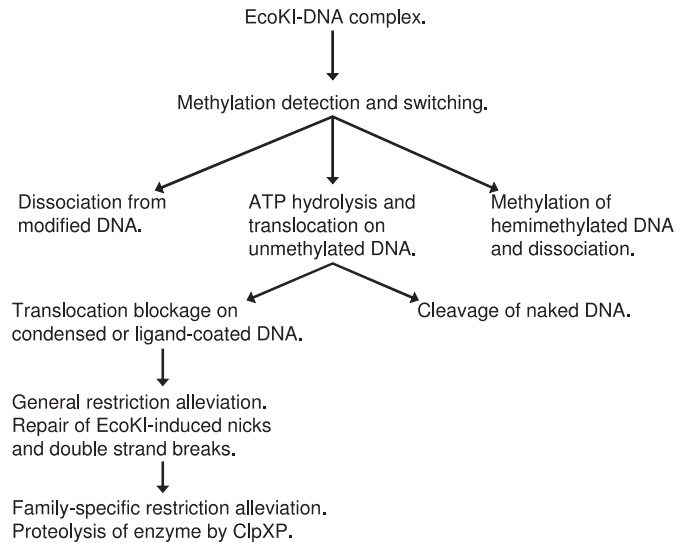


Figure 4. Diagrammatic representation of the pathways followed by the EcoKI type I R/M enzyme during restriction and restriction alleviation.

shown that a single protein bound to DNA is insufficient to halt a translocating type I R/M enzyme (43), but in our experiments it appears that multiple ligands bound in a non-specific manner to the DNA are a very effective block to the type I R/M enzyme. Given that EcoKI can translocate DNA bi-directionally, it appears that the motors are both moving DNA but are stalling before they can translocate the entire DNA. Instead of stalling by colliding with each other, they are stalling prematurely against the barriers formed by bound dye molecules. Each restriction subunit may then introduce a single-strand break in the DNA but since these two nicks are now unlikely to be adjacent to each other, the DNA is not linearized.

We wish to relate our observations, summarized in Figure 4, to those previously made on restriction alleviation and on the structure of the nucleoid. It is apparent that the type I R/M enzyme can find itself in three different situations: (i) diffusing in the cytoplasm looking for protein-free 'naked' foreign DNA; (ii) bound to the nucleoid during DNA replication when it is partially coated with histone-like proteins; or (iii) bound to a fully condensed nucleoid formed under conditions of cell stress.

The nucleoid and RA

Cell stress. It has been found that there are two sorts of RA, family-specific and general, initiated under conditions of cell stress caused by 2-aminopurine, nalidixic acid or UV irradiation, or by the creation of potentially lethal $r^{+}m^{-}$ mutant type I R/M enzymes or introduction of a new R/M system (7). The family-specific RA, as explained earlier, is due to a genotypic effect but no genotype has yet been found for general RA so it was suggested that this mechanism could be due to some feature of the structure of the nucleoid (16).

We believe that the experimental conditions used here, namely coating the DNA with non-specific dye molecules and condensing it with spermidine, mimic the nucleoid structure encountered by EcoKI and other type I R/M enzymes *in vivo*. Therefore, the *in vivo* implication of our results is that the EcoKI DNA translocation process on the nucleoid

is drastically slowed by both condensation and non-specific protein binding and the enzyme stalls. Furthermore, the production of single and double-strand breaks in the host DNA by the R/M enzyme is strongly inhibited by nucleoid structure, particularly the structures induced by conditions of stress where the nucleoid becomes highly ordered and condensed (47,48). Double-strand breaks, which have been observed by pulsed field gel electrophoresis of *E.coli* chromosomal DNA (62), will still occur but very slowly, infrequently and in an uncoordinated manner. These cleavage events can be repaired by DNA ligase and recombination processes.

Acquisition of new type I R/M systems. A similar argument can be made to explain the observation that bacterial cells can acquire new type I R/M systems easily by transformation, transduction and conjugation, but the restriction activity, in contrast to modification, takes a long time to become manifest and RA is activated (9–13). Upon introduction of the new R/M system, protein is slowly synthesized and assembled into the R/M enzyme. Some of this newly synthesized enzyme will clearly be able to attack the foreign DNA should it be unmodified; however, the bulk of the enzyme will be sequestered by binding to the nucleoid where it can either slowly carry out its methylation reaction or trigger the restriction reaction in response to the unmodified targets on the nucleoid. Our *in vitro* data suggest that should the restriction reaction be triggered *in vivo*, the translocation and cleavage functions will be inhibited by the nucleoid structure allowing the normal DNA repair processes to handle the occasional damage produced by the type I R/M enzyme.

By extrapolating from our *in vitro* results to the situation prevailing *in vivo*, we conclude that the restriction activity of the type I R/M enzymes, which is completely dependent upon extensive translocation, would be so inefficient on nucleoid DNA that restriction will not be a major problem for the cell and one observes the general RA phenomenon, Figure 4. In addition to general RA, family-specific RA is an extra feature in which some of the type I R/M enzymes are subjected to clpXP-dependent proteolysis whilst they are attempting to move along the nucleoid DNA.

Nucleoid structure versus uncondensed foreign DNA

When phage attacks a type I R/M proficient cell, which is already under stress and showing RA, it is observed that restriction of the phage is not entirely abolished (1–3,5,6). This indicates that the unmodified, chemically identical, foreign and host DNA molecules can be distinguished by the restriction enzyme. This can be explained by the existence of two pools of type I R/M enzyme (3,37,38). The pool of enzyme bound to the nucleoid will be dealt with by general and family-specific RA as described above but the cytoplasmic pool of the enzyme will bind to the incoming phage DNA. This foreign DNA will be in an uncondensed, random coil form. This form of DNA is the perfect substrate for the translocation process prior to double-strand cleavage and this substrate will not induce general RA. It would appear reasonable to suggest that in this situation, the type I R/M enzyme can complete its restriction reaction so rapidly that if it were susceptible to family-specific RA, then the clpXP enzyme would be physically unable to locate, bind and destroy the restriction enzyme before the restriction enzyme had destroyed the

incoming DNA. Translocation rates for type I R/M enzymes have been measured to be between 100 and 550 bp/s in each direction (22,63–65) so even if two target sequences were 10000 bp apart, the clpXP would only have between 12 and 50 s to act. It is also perhaps noteworthy that EcoKI can form a large dimer species on unmethylated, random coil DNA if the DNA contains two target sequences (66,67) even though this extra complexity is not essential for enzyme activity and therefore seemingly superfluous. Such a dimer is unlikely to be able to form on chromosomal DNA packaged in the nucleoid as the two target sequences will rarely be in close physical proximity. Perhaps this dimer species is not susceptible to clpXP family-specific RA, whereas the monomer species bound on the nucleoid would still be susceptible? One final point of interest arises by comparing the maximum forces generated by the EcoR124I type I R/M enzyme and those generated by polymerases and by the forces required to disrupt nucleosomes. Single molecule experiments reveal that the EcoR124I R/M enzyme cannot move against forces >5 pN (65), whereas the polymerases can move against forces of 20 pN or more and nucleosomes require 20–40 pN to be disrupted [reviewed in (68)]. It would appear that the type I R/M enzymes do not generate enough force to drive their way past obstructions or through condensed DNA.

CONCLUSION

We believe our results show the answer to the question ‘how are unmodified sequences present in the resident bacterial chromosome either because of DNA damage or because the host has no R/M system, distinguished from those in foreign DNA that has recently entered the bacterial cell?’ (7,16) to be that DNA in the nucleoid is condensed and coated with non-sequence-specific ligands whereas foreign DNA is relatively naked and in a random coil conformation. These differences in the higher order structure have an enormous effect on the activity of the type I R/M enzymes.

ACKNOWLEDGEMENTS

We thank Professor Noreen Murray and Dr Garry Blakeley, Institute of Cell and Molecular Biology, University of Edinburgh for many useful discussions about restriction alleviation. This work was supported by a studentship to S.A.K. awarded by the MRC and a grant from the EPSRC.

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