McrB₆, a modulator peptide for McrBC activity

Daniel Panne, Elisabeth A.Raleigh¹ and Thomas A.Bickle²

Department of Microbiology, Biozentrum, Basel University, Klingelbergstrasse 70, CH-4056 Basel, Switzerland and
¹New England Biolabs, 32 Tozer Road, Beverly, MA 01915, USA
²Corresponding author
e-mail: bickle@ubacu.unibas.ch

McrBC is a methylation-dependent endonuclease from Escherichia coli K-12. The enzyme recognizes DNA with modified cytosines preceded by a purine. McrBC restricts DNA that contains at least two methylated recognition sites separated by 40–80 bp. Two gene products, McrB₆ and McrB₇, are produced from the mcrB gene and one, McrC, from the mcrC gene. DNA cleavage in vitro requires McrB₆, McrC, GTP and Mg²⁺. We found that DNA cleavage was optimal at a ratio of 3–5 McrB₆ per molecule of McrC, suggesting that formation of a multisubunit complex with several molecules of McrB₆ is required for cleavage. To understand the role of McrB₆, we have purified the protein and analyzed its role in vitro. At the optimal ratio of 3–5 McrB₆ per molecule of McrC, McrB₆ acted as an inhibitor of DNA cleavage. Inhibition was due to sequestration of McrC and required the presence of GTP, suggesting that the interaction is GTP dependent. If McrC was in excess, a condition resulting in suboptimal DNA cleavage, addition of McrB₆ enhanced DNA cleavage, presumably due to sequestration of excess McrC. We suggest that the role of McrB₆ is to modulate McrBC activity by binding to McrC.

Keywords: GTP/GTPase/McrBC restriction/5-methylcytosine

Introduction

Escherichia coli K-12 contains at least four restriction systems to monitor the origin of invading DNA and determine its fate (Bickle and Krüger, 1993). In addition to the type I EcoKI system, which restricts unmodified DNA, there are three systems, MrcA, Mrr and McrBC, which specifically recognize modified DNA (Noyer-Weidner et al., 1986; Raleigh and Wilson, 1986; Heitman and Model, 1987; Waite-Rees et al., 1991). McrBC is the best characterized of the modification-dependent enzymes. It stands out from the rest of the family of restriction–modification endonucleases in several respects. In contrast to classical restriction–modification systems where methylation of the target sequence provides a means of protection, methylated DNA is an absolute requirement for DNA cleavage to occur. McrBC specifically recognizes DNA containing 5-hydroxymethylcytosine, 5-methylcytosine or 4-methylcytosine preceded by a purine residue (RmC) (Raleigh and Wilson, 1986). Restriction requires at least two RmC sites that are separated optimally by 40–80 bp but can be spaced as far as 2 kb apart (Sutherland et al., 1992; Stewart and Raleigh, 1998).

The mcrBC locus contains two genes, mcrB and mcrC (Figure 1A). Three major polypeptides are encoded by this operon. The mcrB gene encodes a large, full-length gene product termed McrB₆ of 53 kDa and a small McrB₆ protein of 34 kDa (Ross et al., 1987, 1989a; Dila et al., 1990; Krüger et al., 1992). McrB₆ lacks the N-terminal 161 amino acids encoded by the mcrB gene but retains the C-terminal 287 residues (Ross et al., 1989a). This truncation is produced by internal in-frame translational initiation rather than post-translational processing of the full-length product (Ross et al., 1989a; Krüger et al., 1992). McrB₆ alone or in the presence of McrC cannot support restriction in vivo (Ross et al., 1989b). DNA cleavage in vitro requires McrB₆, McrC, GTP and Mg²⁺ (Sutherland et al., 1992). McrB₆ is not required for this reaction. DNA binding abilities have been attributed to McrB₆ (Krüger et al., 1995; F.J.Stewart and E.A. Raleigh, in preparation), and it was shown that the DNA-binding domain resides in a fragment comprising the N-terminal 190 amino acids (Gast et al., 1997). Dila et al. (1990) identified a GTP-binding motif in the central part of the mcrB gene (Figure 1B). This assignment was confirmed by the demonstration that McrB₆ binds and hydrolyzes GTP in an McrC-dependent fashion (Pieper et al., 1997). Thus, McrBC is the only known nuclease which requires GTP for activity.

In this communication, we have shown in vitro that the DNA cleavage rate depends on the relative and the absolute amounts of McrB₆ and McrC. A maximal rate was obtained at 3–5 McrB₆ per molecule of McrC, showing that DNA cleavage occurs by a multisubunit complex. Excess of either protein decreased the DNA cleavage rate. Because McrB₆ may retain domains for protein–protein interaction which are also found in the full-length protein, it has been proposed that McrB₆ might serve to modulate McrBC restriction activity (Ross et al., 1989a). Strikingly, either over- or underexpression of McrB₆ abolished or reduced McrBC restriction in vivo (Beary et al., 1997). The first case can be understood by dominant-negative inhibition in which the truncated variant McrB₆ interferes with the functional assembly of the oligomeric wild-type protein. Similar cases have been reported in the literature (Roman et al., 1991; Treacy et al., 1992; de la Cruz et al., 1993). However, reduction of McrBC restriction by reduced levels of McrB₆ was more difficult to explain, and the authors suggested that McrB₆ may be required for stabilization of the McrBC restriction complex (Beary et al., 1997).
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Fig. 1. (A) Organization of the McrBC operon. Three proteins, McrB_L, McrB_s and McrC, are encoded by the mcrBC genes. McrB_s lacks the first 161 amino acids of the full-length gene products which comprise the DNA-binding domain. McrB_s, McrC and GTP are required for DNA cleavage. McrB_s modulates this activity by binding to McrC. Two R^mC recognition sites, which can be spaced between 40 and 2000 bp, are required for DNA cleavage. (B) Modular organization of the mcrB gene. A DNA-binding domain has been assigned to the N-terminus of McrB_L. This domain is lacking in McrB_s. The GTP-binding domains are shown as proposed by Dila et al. (1990).

The data presented here define the role of McrB_s in restriction by McrBC. We found that McrB_s enhanced or decreased McrBC activity depending on the relative amounts of McrB_L and McrC. At optimal molar ratios of 3–5 McrB_L per molecule of McrC, the addition of McrB_s led to an inhibition of the DNA cleavage reaction. Inhibition was due to binding of McrC, thus decreasing the amount of cleavage-competent McrB_L–McrC complexes. At equimolar ratios of McrB_s and McrC, conditions which gave suboptimal cleavage activity, addition of McrB_s stimulated the DNA cleavage reaction. Stimulation occurred by sequestering excess McrC, leading to more favorable ratios between McrB_s and McrC. Pre-incubation experiments showed that GTP was required for, or stimulated the interaction of, McrB_s with McrC. This suggests that the C-terminal 287 residues of McrB_s and McrB_L contain a domain which interacts with McrC. Inhibition and stimulation of the reaction can be explained by interaction of McrB_s with McrC, which modulates the formation of cleavage-competent McrB_L–McrC complexes.

Results

Overexpression and purification of McrB proteins

Three major gene products are encoded by the mcrBC locus. Two polypeptides, McrB_L (53 kDa) and McrB_s (34 kDa), are produced from the mcrB gene, and one, McrC (39 kDa), from the mcrC gene. The McrB_L and McrB_s proteins were purified using the IMPACT protein purification system [New England Biolabs Inc., Beverly, MA (NEB)]. Briefly, the tripartite fusion protein contains the McrB_L gene fused at the N-terminus of a mutant intein, which is capable of N-terminal junction cleavage in the presence of thiol reagents. A chitin-binding domain is fused to the C-terminus of the intein, which allows affinity purification of the fusion protein on a chitin resin. The intein fusion proteins were constructed so that an additional glycine residue remains at the C-terminus of McrB_s after cleavage from the intein tag. We found that this extra residue increased the cleavage efficiency from the affinity tag as compared with a direct fusion construct.

The McrB_L start site was that used previously (Ross et al., 1989b; Sutherland et al., 1992). After expression at 21°C overnight, 80% of the fusion protein was found to be in the soluble fraction. SDS–PAGE analysis of the fractions from the chitin column showed the full-length McrB_L at 53 kDa, McrB_s at 34 kDa and a protein migrating with an apparent Mr of 60 kDa (Figure 2A). The 60 and 34 kDa proteins were analyzed by N-terminal peptide
maximal stimulation of the GTPase activity of McrB by F.J. Stewart and E. Sutherland, unpublished data). However, inhibitory effect of excess McrBL. Similar experiments showed the effect of McrBL concentration on the course of the reaction by McrBs modulating McrBC activity of DNA cleavage efficiency of our preparation was compared with an McrBL preparation purified using conventional methods (gift from F.J. Stewart, NEB) and found to be identical.

To analyze the role of the McrB protein, a fragment from the mcrB gene coding for the C-terminal amino acids 162–459 was cloned into the IMPACT vector. A soluble protein of the expected size was obtained, expressed and purified as described for McrBL. As with the full-length protein construct, co-purification of GroEL was observed. After separation on the gel filtration column, the McrB preparation was apparently homogeneous as judged from a Coomassie-stained gel. Usually 7–8 mg protein/l of culture was obtained. The McrC protein used in all experiments was a gift from F.J. Stewart (NEB) purified essentially as described by Sutherland et al. (1992).

**DNA cleavage efficiency is dependent on the molar ratios of the mcrBC gene products**

McrB and McrC are the only gene products from the mcrBC genes required for in vitro DNA cleavage activity (Sutherland et al., 1992). Earlier work suggested that a molar ratio of ~5 McrB molecules to one McrC molecule was optimal for DNA cleavage in vitro (E.A. Raleigh, F.J. Stewart, and E. Sutherland, unpublished data). However, maximal stimulation of the GTPase activity of McrB by McrC was obtained with equimolar ratios of the two proteins (Pieper et al., 1997). To examine this issue further, we measured the efficiency of the DNA cleavage reaction at different molar ratios of the two proteins. Figure 3A shows the effect of McrB concentration on the course of the cleavage reaction. DNA cleavage was slow if McrC (100 nM) was in a molar excess over McrB (curves from 19–79 nM McrB). At a molar ratio of ~3 McrB molecules to one McrC molecule, the reaction rate reached a maximum. At higher McrB concentrations (635 and 1270 nM), the reaction rate decreased, showing the inhibitory effect of excess McrB. Similar experiments were performed using a higher (200 nM) and a lower (60 nM) McrC concentration. The initial reaction velocity data from these experiments are summarized in Figure 3B. In all cases, the reaction was less efficient if McrC was in excess over McrB. Also, at high McrB concentrations, the efficiency decreased. Inhibition by McrB was less strong if more McrC was included in the reaction. At the highest McrC concentration used (200 nM), the maximal cleavage rate obtained was lower as compared with reactions with 100 and 60 nM McrC. Presumably this was due to inhibition by McrB as discussed below. In summary, a ratio of ~3–5 McrB per molecule of McrC is required for optimal DNA cleavage efficiency. An excess of either protein inhibited the cleavage reaction.

**Inhibition of the McrBC-mediated DNA cleavage reaction by McrB**

It has been suggested that McrB might play a regulatory role in the McrBC-mediated restriction of DNA (Ross et al., 1989a; Beary et al., 1997; D. Dila and E. A. Raleigh, unpublished data). McrB alone or in the presence of McrC was not able to cleave DNA, confirming that full-length McrB is required for this reaction (data not shown). To understand further the role of McrB, the DNA cleavage activity of McrBC (using 125 nM McrB and 60 nM McrC) was measured in the presence of increasing amounts of McrB. Figure 4 shows the effect of McrB on the
course of the DNA cleavage reaction. The reaction was inhibited in a concentration-dependent manner and was almost completely abolished at 1 μM McrB5.

There are several possible ways in which McrB5 could inhibit the McrBC cleavage reaction. McrB5 could interfere by competing with McrB L for DNA-binding sites, by sequestering McrC or by forming non-functional complexes with McrB L (Ross et al., 1989a; Beary et al., 1997). We have addressed these possibilities by pre-incubating McrB5 with McrB L or McrC and measuring the effect on the DNA cleavage reaction. As shown in Figure 5, there was a stronger inhibitory effect when McrB5 was pre-incubated with McrC than with McrB L. These data are consistent with the model whereby McrB5 inhibits the reaction by binding to and sequestering McrC. In addition, this inhibitory effect was only observed when McrB5 and McrC were pre-incubated in the presence of GTP (Figure 6). This experiment demonstrates that the interaction between the two proteins is GTP dependent.

To verify that this result was not due to instability of McrB5, the absence of GTP, this experiment was repeated using different pre-incubation times. However, a similar extent of inhibition was obtained after short (5 min) and long (30 min) pre-incubation times, excluding instability of McrB5 in the absence of GTP (data not shown).

**Activation of DNA cleavage by McrB5**

Inhibition of the production of McrB5 in vivo leads to a decrease in McrBC activity (Beary et al., 1997). The authors suggested that McrB5 may sequester excess McrC, thus modulating the level of McrBC activity, and that the decrease of restriction may be due to unfavorable molar ratios of McrB5 to McrC in the cell. Our results (Figure 3B) demonstrated that if either McrB L or McrC are in excess, the rate of the cleavage reaction is suboptimal. Under such conditions, the addition of McrB5 might increase the efficiency of the reaction by sequestering a fraction of McrC. This would result in a more favorable ratio of McrB L and McrC for the formation of cleavage-

**Discussion**

Recent investigations in a number of laboratories have begun to elucidate the biochemical mechanism of mcrBC restriction. McrB L, McrC, GTP and Mg2+ are required for in vitro DNA cleavage activity (Sutherland et al., 1992). The data presented here demonstrate that the DNA cleavage rate depends on both the relative and absolute amounts of McrB L and McrC. The optimal ratio of 3–5 McrB L per McrC suggests that DNA cleavage occurs by a multisubunit complex. The assembly of this complex is modulated by McrB5. If either of the three proteins was in excess, the reaction was inhibited. Inhibition by excess
McrBs could be due to DNA substrate sequestration since McrB s alone can bind to the methylated target sites (Krüger et al., 1995; Gast et al., 1997; F.I. Stewart and E.A. Raleigh, in preparation).

Increased amounts of McrC, above the optimal ratio, inhibited DNA cleavage. Inhibition of restriction was also observed in vivo upon overexpression of McrC (Beary et al., 1997). This occurs presumably by formation of complexes with McrB s which lack the correct stoichiometry and are therefore non-functional for DNA cleavage. Previous studies had shown that GTP hydrolysis by McrB s is stimulated by McrC (Pieper et al., 1997). In these experiments, a 1:1 ratio of McrB s and McrC yielded maximal levels of GTP hydrolysis. It is possible that the activities of different functional complexes are monitored by the DNA cleavage and the GTPase assays. It is also possible that GTP binding and/or hydrolysis mediates McrB–McrC interaction and thus is maximal at a 1:1 ratio.

In agreement with previous results in vivo, McrB s can both inhibit or activate the reaction (Figures 4 and 7). A probable interpretation of these results is that when the ratio of McrB s to McrC is optimal, McrB s has an inhibitory effect on restriction due to sequestration of the McrC subunit (Figure 5). This would decrease the concentration of cleavage-competent McrB s–McrC complexes. Alternatively, when the McrC subunit is in excess, McrB s has an activating effect presumably by sequestering excess McrC, that otherwise would inhibit the formation of cleavage-competent complexes. Thus McrB s can be an activator or inhibitor depending on the molar ratios of McrB s and McrC.

The optimal ratio of 3–5 McrB s for each McrC molecule in the reaction suggests that inhibition by McrB s might occur through subunit poisoning of an oligomeric complex. Several models could explain how McrB s interferes with the assembly of that complex. Inhibition by McrB s could occur by (i) sequestering McrB s in non-functional complexes, (ii) sequestering the McrC subunit or (iii) binding to and blocking the DNA-binding sites. Since the cleavage-competent complex requires 3–5 McrB s molecules, it is conceivable that inhibition by McrB s occurs by poisoning this complex as proposed in model 1. This would be expected if McrB s binds to McrB L so that assembly of a functional McrB s oligomer is impaired. Pre-incubation of McrB s with McrB L or McrC demonstrated that inhibition occurs by binding to McrC (Figure 5) as proposed in model 2. Since McrB s has the N-terminal DNA-binding domain, it presumably assembles preferentially on the DNA. Even though McrB s might form complexes with McrB L in solution, those might be deficient in DNA binding and may not interfere with the DNA cleavage reaction. The third model is disfavored by evidence that DNA recognition is mediated by the N-terminal domain of McrB s which is largely missing in McrB s (Gast et al., 1997). However, the truncation in McrB s (missing the N-terminal amino acids 1–161) is shorter than the 1–190 amino acids N-terminal fragment used by Gast et al. (1997) to monitor DNA binding, so it cannot be completely ruled out that McrB s retains some ability to bind and block the sites on the DNA. However, preliminary DNA-binding experiments with McrB s did not detect binding activity (data not shown).

Since the molar ratios of the three proteins are crucial for McrBC activity, an important question is at which ratios they exist in the cell. Expression data from two independent laboratories using different vector constructs, expression and detection methods showed similar amounts of McrB s and McrB L (Ross and Braymer, 1987; Ross et al., 1989a; Dila et al., 1990). Maxicell analysis detected a relative ratio of 3:3:1 for McrB s, McrB L and McrC, respectively (Ross et al., 1989a). This ratio suggests that under native conditions McrB s is required to maintain an optimal ratio between McrB s and McrC. This view is supported by in vivo experiments, showing that reducing the level of McrB s leads to a decrease of McrBC activity (Beary et al., 1997).

Several host factors, mainly proteases and chaperones,
have been implicated in post-translational gene regulation. Recent in vivo studies have shown that establishment of the restriction systems EcoKI and EcoAI in a new host is dependent on the presence of the host genes clpX and clpP (Makovets et al., 1998). It is thought that the ClpXP protease, or one of the components of this complex, can transiently delay the formation of the restriction-competent complex. A similar role in regulation of McrBC restriction could be provided by the molecular chaperone GroEL which we found to co-purify with McrB1 (Figure 2) and McrB2. Treatment with UV light, a stress condition which can be questioned since its co-purification is a recent finding (Krueger and Walker, 1984), leads to McrBC restriction alleviation (Dharmaningam and Goldberg, 1980; Kelleher and Raleigh, 1994). It is known that the association of GroEL with unfolded proteins can prevent unproductive aggregation or that GroEL can enhance assembly of multisubunit complexes. The interaction of GroEL with MrcR1 or MrcR2 in vivo could be a means to regulate MrcRBC restriction by transiently sequestering one of the subunits. However, the biological significance of GroEL–McrR1 or GroEL–McrR2 interaction can be questioned since its co-purification is a common problem of overexpressed proteins.

Regulation by a MrcR2 is reminiscent of the regulation of Tn5 transposition. The transposase (Tnp) is regulated by an N-terminal deletion variant Inh, lacking the first 55 amino acids (de la Cruz et al., 1993). Inhibition by Inh is proposed to occur by formation of mixed oligomers with Tnp. Other examples in which a truncated variant of the protein interferes with the functional assembly of the oligomeric wild-type protein have been reported (Roman et al., 1991; Treacy et al., 1992). The McrBC system offers a striking demonstration of how an internal transduction product may modulate the efficiency of the reaction either by inhibiting or increasing the cleavage rate depending on the ratio of the three mcrBC gene products. Thus, MrcR2 provides the system with a rather sophisticated means for regulation.

**Materials and methods**

**Construction of the purification vectors**

The mcrB gene was amplified by PCR from pER273 (Sutherland et al., 1992). The forward primer, 5'-TATACTGACTCATACTAGGGG-3' (NEB 1248), is complementary to the T7 RNA polymerase promoter on pER273. Alternatively, for the construction of the McrR1 purification vector, the forward primer was 5'-GGCAGGAGACACAAACTGATAACGTCC-3' containing a NdeI site (underlined). The reverse primer, 5'-CGGC(GCT)TTCCGGACCGCTGCTGAGGCTCTTCTATGGG-3' contained a SapI site (underlined) and the sequence of the last the last eight amino acids of the mcrB gene (italics). This primer introduces an additional glycine codon at the end of the mcrB sequence. PCR mixtures contained 1× Vent DNA polymerase buffer (NEB) adjusted to 3 mM MgSO4, 0.25 mM each dNTP, 100 ng of plasmid pER273, 0.4 μM primers and 2 U of Vent DNA polymerase (NEB) in a 100 μl reaction. Amplification was carried out using a Perkin-Elmer Cetus 480 thermal cycler at 95°C for 60 s and then five cycles of 95°C for 60 s, 52°C for 60 s, and 72°C for 120 s. The final step was incubation at 72°C for another 5 min.

Gel purifications of DNA were performed using conventional agarose electrophoresis and GeneClean methods (Bio101 Inc., La Jolla, CA). The vector pBGYB, expressing the McrR–intein fusion protein, and the vector pSBGYB, expressing the MrcR–intein fusion protein, were constructed as follows: the amplified fragments were digested with NdeI and SapI as indicated by the manufacturer (NEB), repurified and ligated overnight at 16°C into NdeI–SapI-digested and gel-purified pMYB140 (NEB). All constructs were verified by sequencing both strands.

**Expression and purification of the MrcR proteins on the chitin column**

Expression and purification procedures were the same for all fusion constructs. For MrcR2, the vector pBGYB was transformed into the E.coli strain ER2267, plated on LB agar plates containing the appropriate antibiotic and grown overnight at 37°C. A freshly transformed colony was transferred into 10 ml of LB broth containing 100 μg/ml ampicillin and grown overnight at 37°C to saturation. This overnight culture was used to inoculate 1 l of LB broth containing 100 μg/ml ampicillin and grown at 37°C to an OD_{600} of ~0.5. The culture was then transferred to a 20°C air shaker and induced with 0.4 mM isopropyl-β-thiogalactopyranoside for 16 h. The cells were harvested and the pellet was resuspended in 50 ml of 4°C cold column buffer [20 mM Tris–HCl, pH 7.5 (21°C), 500 mM NaCl] and broken by sonication. All subsequent steps were carried out at 4°C. After centrifugation at 25 000 g for 30 min, the cleared supernatant was loaded at a flow rate of 0.5 ml/min on a pre-equilibrated 5 ml chitin column. The column was washed with 20 column volumes of column buffer at a flow rate of 2 ml/min. Afterwards, the column was flushed with three column volumes of cleavage buffer [20 mM Tris–HCl, pH 7.5 (21°C), 500 mM NaCl, 30 mM dithiothreitol (DTT)]. The flow was stopped and the column remained at 4°C overnight. Fractions (5 ml) containing MrcR2 were collected by washing the column with column buffer. To assess the efficiency of cleavage from the affinity tag, a sample of the resin was taken and boiled in SDS loading buffer. All fractions were analyzed by SDS–PAGE (Laemmli, 1970).

Fractions containing MrcR2 from the chitin column were pooled and concentrated to 5 ml in a Centriprep-10 concentrator (Amicon Inc., Beverly, MA). The concentrate was loaded on a calibrated HiPrep Sephacryl S-200 (26/60) column (Pharmacia Biotech) equilibrated in 20 mM Tris–HCl, pH 7.5 (21°C), 500 mM NaCl, 1 mM DTT. The column was run at a flow rate of 0.5 ml/min, and 2 ml fractions were collected and monitored by UV absorbance. After elution, the protein-containing fractions were analyzed by SDS–PAGE (Laemmli, 1970). Fractions containing MrcR2 were pooled and dialyzed against 10 mM Tris–HCl, pH 7.5 (21°C), 200 mM NaCl, 0.1 mM Na2EDTA, 1 mM DTT and 50% glycerol. Relative protein concentrations were determined using a Bradford method with bovine serum albumin (BSA) standard (Bio-Rad Inc., CA) or for MrcR2, and MrcR2, using molar extinction coefficients of 73 980 and 39 475/M/cm respectively, calculated according to the method of Pace et al. (1995). Both methods produced similar results. All preparations were stored at –20°C until further use.

**DNA cleavage assays**

The DNA substrate used in all experiments, pCM63, was that used previously by Stewart and Raleigh (1998). Briefly, this 1935 bp plasmid contains two BsuUI sites (CGCC) which can be methylated by M.FnuDII to generate m5CGCG. The methylated cytosine is preceded by a guanine residue to generate the McrBC-susceptible GmC. The two GmC sites are separated by 56 nucleotides. The methylation reactions were performed in M.FnuDII buffer (NEB) containing 10 pMC63, 16 U of M.FnuDII (NEB) and 320 μM S-adenosylmethionine in a 100 μl reaction. The reaction was incubated at 37°C for 3 h. The methylation status was examined by digesting the plasmid with BsuUI, which cannot cleave the methylated sequence. When protection against BsuUI was complete, pCM63/M.FnuDII was ethanol precipitated, washed and resuspended in 50 μl containing 1× buffer 2 (NEB) and 20 U of PvuII. Incubation for 1 h at 37°C to linearize the plasmid was followed by phenol:chloroform extraction and ethanol precipitation. McrBC activity was usually measured in 100 μl of 1× buffer 2 (NEB) supplemented with 100 μg/ml BSA, 1 mM GTP, 500 ng (4 nM) of pMC63/M.FnuDII/PvuII and MrcR2, MrcR2, and MrcR2 concentrations as indicated in the text. At the indicated time points, 10 μl samples were removed and the reactions terminated on ice by addition of 2 μl of stop buffer (10 mM Tris–HCl, 120 mM EDTA, 30% glycerol and 0.25% bromophenol blue). The extent of cleavage was quantified by agarose electrophoresis in 1× TBE containing 0.25 μg/ml ethidium bromide, followed by photography of the UV-illuminated gel. Pictures were saved as TIFF files and the images were analyzed by densitometry using the software ImageJ 1.61. The amounts of uncut substrate and the larger cleavage product were quantified by measuring the areas under the peaks. The extent of relative cleavage was calculated considering that the larger cleavage product contains 63.4% of the full-length substrate (D.Panne, unpublished results). The data were fitted to exponential or linear functions by non-linear regression in PRISM software (GraphPad, San Diego, CA).
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References


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