

## McrB<sub>s</sub>, a modulator peptide for McrBC activity

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**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, McrB<sub>L</sub> and McrB<sub>s</sub>, are produced from the *mcrB* gene and one, McrC, from the *mcrC* gene. DNA cleavage *in vitro* requires McrB<sub>L</sub>, McrC, GTP and Mg<sup>2+</sup>. We found that DNA cleavage was optimal at a ratio of 3–5 McrB<sub>L</sub> per molecule of McrC, suggesting that formation of a multisubunit complex with several molecules of McrB<sub>L</sub> is required for cleavage. To understand the role of McrB<sub>s</sub>, we have purified the protein and analyzed its role *in vitro*. At the optimal ratio of 3–5 McrB<sub>L</sub> per molecule of McrC, McrB<sub>s</sub> 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 sub-optimal DNA cleavage, addition of McrB<sub>s</sub> enhanced DNA cleavage, presumably due to sequestration of excess McrC. We suggest that the role of McrB<sub>s</sub> is to modulate McrBC activity by binding to McrC.**

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

### Introduction

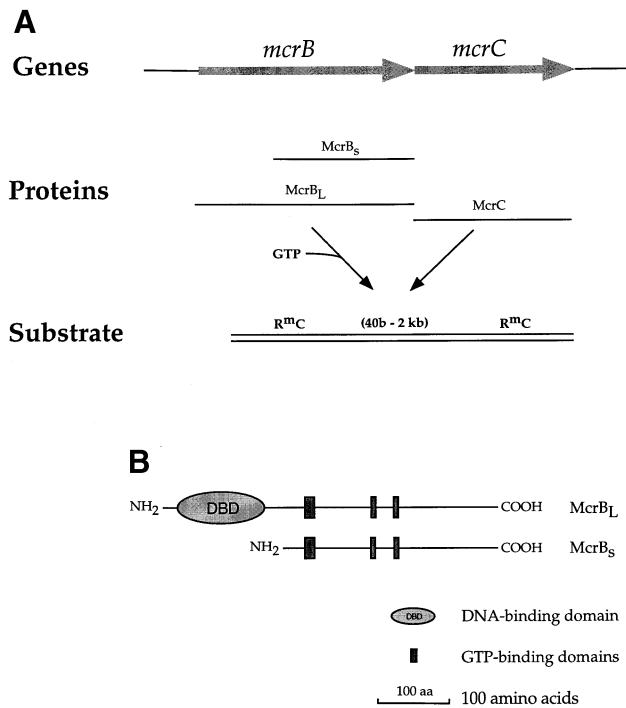
*Escherichia coli* K-12 contains at least four restriction systems to monitor the origin of invading DNA and determine its fate (Bickle and Krüger, 1993). In addition to the type I *EcoKI* system, which restricts unmodified DNA, there are three systems, McrA, Mrr and McrBC, which specifically recognize 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 McrB<sub>L</sub> of 53 kDa and a small McrB<sub>s</sub> protein of 34 kDa (Ross *et al.*, 1987, 1989a; Dila *et al.*, 1990; Krüger *et al.*, 1992). McrB<sub>s</sub> 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). McrB<sub>s</sub> alone or in the presence of McrC cannot support restriction *in vivo* (Beary *et al.*, 1997; D.Dila and E.A.Raleigh, unpublished results). The *mcrC* gene directs the synthesis of a 39 kDa McrC gene product (Ross *et al.*, 1989b).

DNA cleavage *in vitro* requires McrB<sub>L</sub>, McrC, GTP and Mg<sup>2+</sup> (Sutherland *et al.*, 1992). McrB<sub>s</sub> is not required for this reaction. DNA binding abilities have been attributed to McrB<sub>L</sub> (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 McrB<sub>L</sub> 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 McrB<sub>L</sub> and McrC. A maximal rate was obtained at 3–5 McrB<sub>L</sub> per molecule of McrC, showing that DNA cleavage occurs by a multisubunit complex. Excess of either protein decreased the DNA cleavage rate. Because McrB<sub>s</sub> may retain domains for protein–protein interaction which are also found in the full-length protein, it has been proposed that McrB<sub>s</sub> might serve to modulate McrBC restriction activity (Ross *et al.*, 1989a). Strikingly, either over- or underexpression of McrB<sub>s</sub> 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 McrB<sub>s</sub> 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 McrB<sub>s</sub> was more difficult to explain, and the authors suggested that McrB<sub>s</sub> may be required for stabilization of the McrBC restriction complex (Beary *et al.*, 1997).



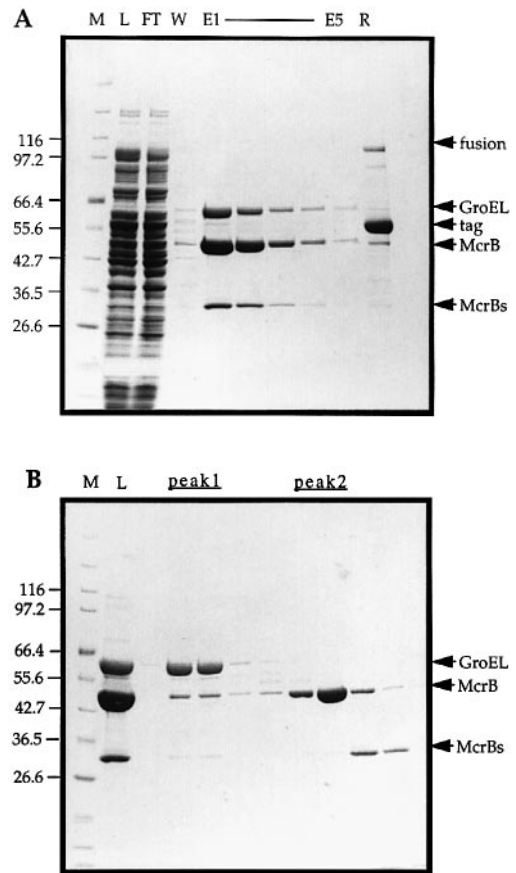
**Fig. 1. (A)** Organization of the McrBC operon. Three proteins, MCrB<sub>L</sub>, MCrB<sub>s</sub> and MCrC, are encoded by the *mcrBC* genes. MCrB<sub>s</sub> lacks the first 161 amino acids of the full-length gene products which comprise the DNA-binding domain. MCrB<sub>L</sub>, MCrC and GTP are required for DNA cleavage. MCrB<sub>s</sub> modulates this activity by binding to MCrC. Two R<sup>MC</sup> 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<sub>L</sub>. This domain is lacking in MCrB<sub>s</sub>. The GTP-binding domains are shown as proposed by Dila *et al.* (1990).

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

## Results

### Overexpression and purification of MCrB proteins

Three major gene products are encoded by the *mcrBC* locus. Two polypeptides, MCrB<sub>L</sub> (53 kDa) and MCrB<sub>s</sub> (34 kDa), are produced from the *mcrB* gene, and one, MCrC (39 kDa), from the *mcrC* gene. The MCrB<sub>L</sub> and



**Fig. 2. (A)** Purification of MCrB<sub>L</sub> using the IMPACT I system. From each fraction, a sample was taken and analyzed by SDS–PAGE followed by staining with Coomassie Blue. Lanes are: M, broad range protein marker (NEB); L, load of clarified crude extract from induced cells; FT, flow through; W, wash; E1–E5, fractions eluted from the column after overnight incubation; and R, proteins remaining bound to the column. **(B)** Purification of MCrB<sub>L</sub> using the gel filtration column Sephacryl S-200. All samples are analyzed by SDS–PAGE and stained with Coomassie Blue. Lanes are: M, broad range protein marker (NEB); L, the pooled and concentrated load on the sizing column; peak 1, samples taken from the first peak; and peak 2, samples taken from the second peak from the column.

McrcB<sub>s</sub> proteins were purified using the IMPACT protein purification system [New England Biolabs Inc., Beverly, MA (NEB)]. Briefly, the tripartite fusion protein contains the MCrB<sub>L</sub> 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<sub>L</sub> 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<sub>L</sub> 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<sub>L</sub> at 53 kDa, MCrB<sub>s</sub> at 34 kDa and a protein migrating with an apparent *M<sub>r</sub>* of 60 kDa (Figure 2A). The 60 and 34 kDa proteins were analyzed by N-terminal peptide

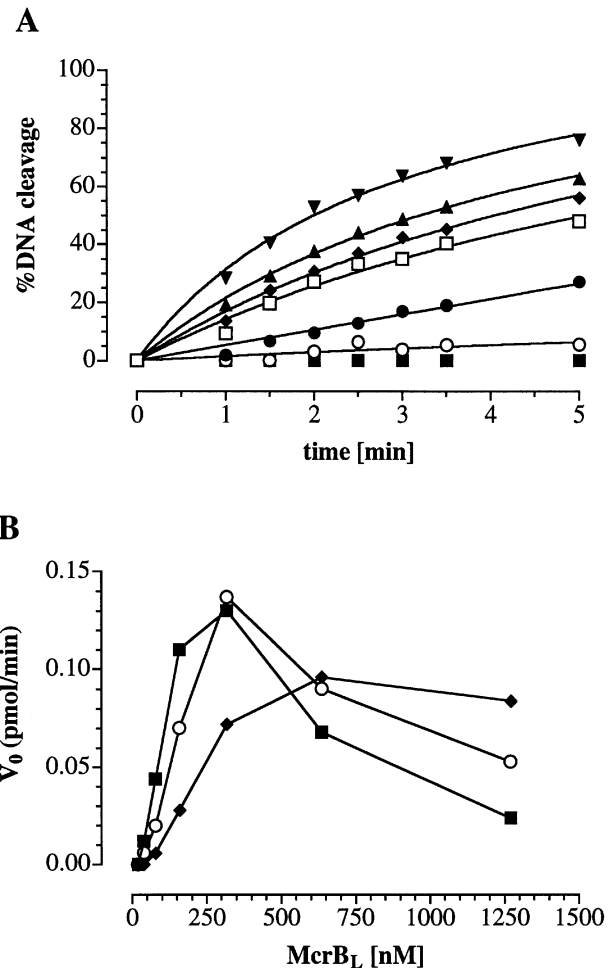
sequencing. The 60 kDa protein was found to be the molecular chaperon GroEL. The 34 kDa McrB<sub>s</sub> protein was confirmed to start 161 amino acids downstream from the N-terminus of McrB<sub>L</sub>, at the same position as shown previously (Zheng *et al.*, 1992).

To remove GroEL and McrB<sub>s</sub> from McrB<sub>L</sub>, the eluate from the chitin column was purified further on a gel filtration column. The result of this separation is shown in Figure 2B. The protein preparation obtained was nearly pure as judged from the Coomassie Blue-stained gel, but contained trace amounts of McrB<sub>s</sub>. We usually obtained 5–6 mg protein/l of culture using this two-column purification method. To verify that the IMPACT purification method did not alter the properties of the enzyme, the DNA cleavage efficiency of our preparation was compared with an McrB<sub>L</sub> preparation purified using conventional methods (gift from F.J.Stewart, NEB) and found to be identical.

To analyze the role of the McrB<sub>s</sub> 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 McrB<sub>L</sub>. As with the full-length protein construct, co-purification of GroEL was observed. After separation on the gel filtration column, the McrB<sub>s</sub> 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<sub>L</sub> 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<sub>L</sub> 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<sub>L</sub> 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<sub>L</sub> concentration on the course of the cleavage reaction. DNA cleavage was slow if McrC (100 nM) was in a molar excess over McrB<sub>L</sub> (curves from 19–79 nM McrB<sub>L</sub>). At a molar ratio of ~3 McrB<sub>L</sub> molecules to one McrC molecule, the reaction rate reached a maximum. At higher McrB<sub>L</sub> concentrations (635 and 1270 nM), the reaction rate decreased, showing the inhibitory effect of excess McrB<sub>L</sub>. 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<sub>L</sub>. Also, at high McrB<sub>L</sub> concentrations, the efficiency decreased. Inhibition by McrB<sub>L</sub> 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

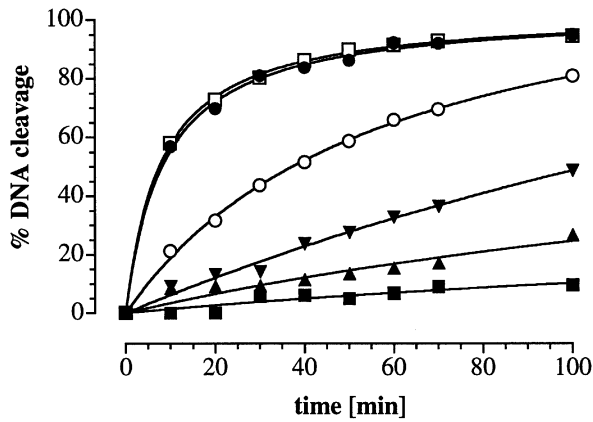


**Fig. 3.** (A) DNA cleavage at different McrB<sub>L</sub> concentrations. Samples containing (□) 1270 nM, (▲) 635 nM, (▼) 317 nM, (◆) 158 nM, (●) 79 nM, (○) 39 nM or (■) 19 nM McrB<sub>L</sub>, 100 nM McrC and 4 nM pMC63/M.*FnuDII*/PvuII were pre-incubated at 37°C for 15 s and the reaction started by addition of 1 mM GTP. For each concentration, 10 µl aliquots were removed at 0, 1, 1.5, 2, 2.5, 3, 3.5 and 5 min time points, and cleavage products were analyzed on a 1% (w/v) agarose gel. The percentage of substrate DNA converted to product is plotted as a function of time. (B) The initial velocities of the DNA cleavage reaction (V<sub>0</sub>) were determined at (◆) 200 nM, (○) 100 nM and (■) 60 nM McrC and using the same McrB<sub>L</sub> concentrations as in (A). Initial velocities were measured and plotted as a function of McrB<sub>L</sub> concentration.

this was due to inhibition by McrB<sub>L</sub> as discussed below. In summary, a ratio of ~3–5 McrB<sub>L</sub> 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<sub>s</sub>

It has been suggested that McrB<sub>s</sub> 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<sub>s</sub> alone or in the presence of McrC was not able to cleave DNA, confirming that full-length McrB<sub>L</sub> is required for this reaction (data not shown). To understand further the role of McrB<sub>s</sub>, the DNA cleavage activity of McrBC (using 125 nM McrB<sub>L</sub> and 60 nM McrC) was measured in the presence of increasing amounts of McrB<sub>s</sub>. Figure 4 shows the effect of McrB<sub>s</sub> on the



**Fig. 4.** Inhibition of the McrBC-mediated DNA cleavage by McrB<sub>s</sub>. McrB<sub>L</sub> (125 nM) was pre-mixed with (□) 31.2 nM, (●) 62.5 nM, (○) 125 nM, (▼) 250 nM, (▲) 500 nM or (■) 1 μM of McrB<sub>s</sub> in the presence of 4 nM pMC63/M.FnuDII/PvuII and 1 mM GTP. The reaction was started by including 60 nM McrC in the reaction. For each concentration of McrB<sub>s</sub>, 10 μl aliquots were removed at 0, 10, 20, 30, 40, 50, 60, 70 and 100 min, and cleavage products were analyzed on a 1% (w/v) agarose gel. The percentage of DNA substrate converted to product is plotted as a function of time.

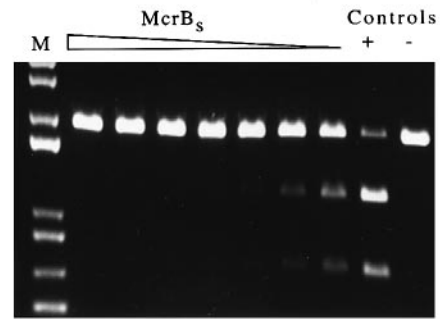
course of the DNA cleavage reaction. The reaction was inhibited in a concentration-dependent manner and was almost completely abolished at 1 μM McrB<sub>s</sub>.

There are several possible ways in which McrB<sub>s</sub> could inhibit the McrBC cleavage reaction. McrB<sub>s</sub> could interfere by competing with McrB<sub>L</sub> for DNA-binding sites, by sequestering McrC or by forming non-functional complexes with McrB<sub>L</sub> (Ross *et al.*, 1989a; Beary *et al.*, 1997). We have addressed these possibilities by pre-incubating McrB<sub>s</sub> with McrB<sub>L</sub> or McrC and measuring the effect on the DNA cleavage reaction. As shown in Figure 5, there was a stronger inhibitory effect when McrB<sub>s</sub> was pre-incubated with McrC than with McrB<sub>L</sub>. These data are consistent with the model whereby McrB<sub>s</sub> inhibits the reaction by binding to and sequestering McrC. In addition, this inhibitory effect was only observed when McrB<sub>s</sub> 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 McrB<sub>s</sub> in 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 McrB<sub>s</sub> in the absence of GTP (data not shown).

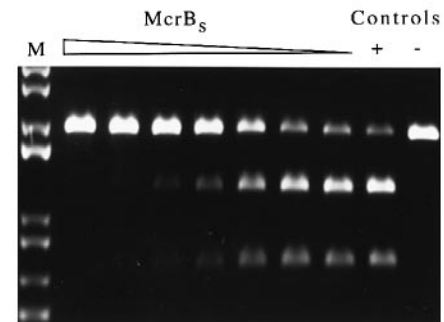
#### Activation of DNA cleavage by McrB<sub>s</sub>

Inhibition of the production of McrB<sub>s</sub> *in vivo* leads to a decrease in McrBC activity (Beary *et al.*, 1997). The authors suggested that McrB<sub>s</sub> 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 McrB<sub>L</sub> to McrC in the cell. Our results (Figure 3B) demonstrated that if either McrB<sub>L</sub> or McrC are in excess, the rate of the cleavage reaction is suboptimal. Under such conditions, the addition of McrB<sub>s</sub> might increase the efficiency of the reaction by sequestering a fraction of McrC. This would result in a more favorable ratio of McrB<sub>L</sub> and McrC for the formation of cleavage-

#### A Preincubation: McrC+McrB<sub>s</sub>



#### B Preincubation: McrB<sub>L</sub>+McrB<sub>s</sub>



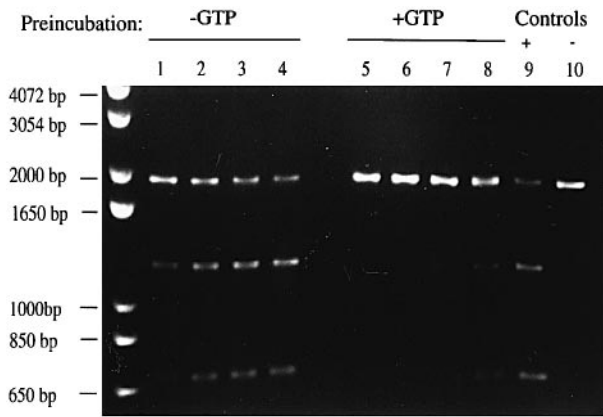
**Fig. 5.** (A) McrB<sub>s</sub> inhibits the reaction by sequestering McrC.

Increasing amounts of McrB<sub>s</sub> were pre-incubated for 15 min at 21°C with 60 nM McrC in the presence of 4 nM pMC63/M.FnuDII/PvuII and 1 mM GTP. The reaction was started by adding 125 nM McrB. The concentrations of McrB<sub>s</sub> from left to right were 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.0312 μM. (B) The same amounts of McrB<sub>s</sub> as used in (A) were pre-incubated for 15 min at 21°C with 125 nM McrB<sub>L</sub>, 1 mM GTP and 4 nM pMC63/M.FnuDII/PvuII. The reaction was started by adding 60 nM McrC. After 15 min at 37°C, the reaction products were analyzed on a 1% (w/v) agarose gel. The positive control (+) was performed by pre-incubating 60 nM McrC under the same conditions as above and starting the reaction with 125 nM McrB<sub>L</sub>. The negative control (-) contains 125 nM McrB<sub>L</sub> but no McrC. The DNA size standard is a 1 kb plus ladder with sizes of 4, 3, 2, 1.6, 1, 0.85, 0.65 and 0.5 kb (Gibco-BRL).

competent complexes. To test this hypothesis, 158 nM McrB<sub>L</sub> was mixed with 200 nM McrC, a ratio that yielded suboptimal cleavage efficiencies in previous experiments (Figure 3B). The reaction velocity was measured in the absence and presence of 125 nM McrB<sub>s</sub>. As shown in Figure 7, it was found under these conditions that McrB<sub>s</sub> increased the rate of DNA cleavage by ~50%.

#### Discussion

Recent investigations in a number of laboratories have begun to elucidate the biochemical mechanism of *mcrBC* restriction. McrB<sub>L</sub>, McrC, GTP and Mg<sup>2+</sup> 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<sub>L</sub> and McrC. The optimal ratio of 3–5 McrB<sub>L</sub> per McrC suggests that DNA cleavage occurs by a multisubunit complex. The assembly of this complex is modulated by McrB<sub>s</sub>. If either of the three proteins was in excess, the reaction was inhibited. Inhibition by excess



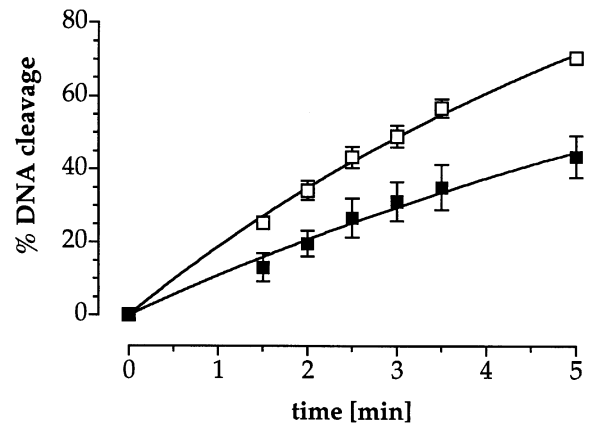
**Fig. 6.** GTP dependence of the McrB<sub>s</sub> interaction with MrcC. Concentrations of 1250, 125, 62.5 and 31.25 nM McrB<sub>s</sub> were pre-incubated for 15 min at 21°C with 60 nM MrcC in the absence (lanes 1–4) or presence (lanes 5–8) of 1 mM GTP and 4 nM pMC63/*M.FnuDII/PvuII*. The DNA cleavage reaction was started by addition of 125 nM McrB<sub>L</sub> and in the left side of the figure with 1 mM GTP. After incubation for 15 min at 37°C, the reactions were stopped by adding stop buffer and then analyzed on a 1% (w/v) agarose gel. The positive control (lane 9) was performed by pre-incubating 60 nM MrcC under the same conditions as above and starting the reaction with 125 nM McrB<sub>L</sub>. The negative control (lane 10) contains only 125 nM McrB<sub>L</sub>. The DNA size standard is a 1 kb plus ladder (Gibco-BRL).

McrB<sub>L</sub> could be due to DNA substrate sequestration since McrB<sub>L</sub> alone can bind to the methylated target sites (Krüger *et al.*, 1995; Gast *et al.*, 1997; F.J.Stewart and E.A.Raleigh, in preparation).

Increased amounts of MrcC, above the optimal ratio, inhibited DNA cleavage. Inhibition of restriction was also observed *in vivo* upon overexpression of MrcC (Beary *et al.*, 1997). This occurs presumably by formation of complexes with McrB<sub>L</sub> which lack the correct stoichiometry and are therefore non-functional for DNA cleavage. Previous studies had shown that GTP hydrolysis by McrB<sub>L</sub> is stimulated by MrcC (Pieper *et al.*, 1997). In these experiments, a 1:1 ratio of McrB<sub>L</sub> and MrcC 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 MrcB–MrcC interaction and thus is maximal at a 1:1 ratio.

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

The optimal ratio of 3–5 McrB<sub>L</sub> for each MrcC molecule in the reaction suggests that inhibition by McrB<sub>s</sub> might occur through subunit poisoning of an oligomeric complex. Several models could explain how McrB<sub>s</sub> interferes with the assembly of that complex. Inhibition by McrB<sub>s</sub> could



**Fig. 7.** Activation of the MrcBC reaction by McrB<sub>s</sub>. The experiment was performed as described in the legend of Figure 3A, except that the protein concentrations were 158 nM McrB<sub>L</sub> and 200 nM MrcC. The DNA cleavage rate was measured in the absence of McrB<sub>s</sub> (■) and after including 125 nM McrB<sub>s</sub> (□). The order of addition of the proteins was McrB<sub>L</sub>, MrcB<sub>s</sub> and then MrcC. The reaction was started by addition of 1 mM GTP.

occur by (i) sequestering McrB<sub>L</sub> in non-functional complexes, (ii) sequestering the MrcC subunit or (iii) binding to and blocking the DNA-binding sites. Since the cleavage-competent complex requires 3–5 McrB<sub>L</sub> molecules, it is conceivable that inhibition by McrB<sub>s</sub> occurs by poisoning this complex as proposed in model 1. This would be expected if McrB<sub>s</sub> binds to McrB<sub>L</sub>, so that assembly of a functional McrB<sub>L</sub> oligomer is impaired. Pre-incubation of McrB<sub>s</sub> with McrB<sub>L</sub> or MrcC demonstrated that inhibition occurs by binding to MrcC (Figure 5) as proposed in model 2. Since McrB<sub>L</sub> has the N-terminal DNA-binding domain, it presumably assembles preferentially on the DNA. Even though McrB<sub>s</sub> might form complexes with McrB<sub>L</sub> 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<sub>L</sub> which is largely missing in McrB<sub>s</sub> (Gast *et al.*, 1997). However, the truncation in McrB<sub>s</sub> (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<sub>s</sub> retains some ability to bind and block the sites on the DNA. However, preliminary DNA-binding experiments with McrB<sub>s</sub> did not detect binding activity (data not shown).

Since the molar ratios of the three proteins are crucial for MrcBC 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<sub>L</sub> and McrB<sub>s</sub> (Ross and Braymer, 1987; Ross *et al.*, 1989a; Dila *et al.*, 1990). Maxicell analysis detected a relative ratio of 3:3:1 for McrB<sub>L</sub>, McrB<sub>s</sub> and MrcC, respectively (Ross *et al.*, 1989a). This ratio suggests that under native conditions McrB<sub>s</sub> is required to maintain an optimal ratio between McrB<sub>L</sub> and MrcC. This view is supported by *in vivo* experiments, showing that reducing the level of McrB<sub>s</sub> leads to a decrease of MrcBC activity (Beary *et al.*, 1997).

Several host factors, mainly proteases and chaperones,

have been implicated in post-translational gene regulation. Recent *in vivo* experiments 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 McrB<sub>L</sub> (Figure 2) and McrB<sub>S</sub>. Treatment with UV light, a stress condition which induces GroEL expression (Krueger and Walker, 1984), leads to McrBC restriction alleviation (Dharmalingam 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 McrB<sub>L</sub> or McrB<sub>S</sub> *in vivo* could be a means to regulate McrBC restriction by transiently sequestering one of the subunits. However, the biological significance of GroEL–McrB<sub>L</sub> or GroEL–McrB<sub>S</sub> interaction can be questioned since its co-purification is a common problem of overexpressed proteins.

Regulation by a McrB<sub>S</sub> 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 translation 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, McrB<sub>S</sub> 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'-TAATACGACTCACTATAGGGG-3' (NEB 1248), is complementary to the T7 RNA polymerase promoter on pER273. Alternatively, for the construction of the McrB<sub>S</sub> purification vector, the forward primer was 5' CGGCCACATATGTCAAAAATGATC 3' containing an *NdeI* site (underlined). The reverse primer, 5'-CGGGGCTCTTCGACCCGTAGTCCCCTAATAAATTTGTTGG-3' contains a *SapI* site (underlined) and the sequence of 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 MgSO<sub>4</sub>, 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 McrB–intein fusion protein, and the vector pSBGYB, expressing the McrB<sub>S</sub>–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 McrB proteins on the chitin column

Expression and purification procedures were the same for all fusion constructs. For McrB<sub>L</sub>, the vector pBGYB was transformed into the *E. coli* strain ER 2267, 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<sub>600</sub> of ~0.5. The culture was then transferred to a 21°C air shaker and induced with 0.4 mM isopropyl-β-D-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 McrB<sub>L</sub> 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 McrB<sub>L</sub> 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 McrB<sub>L</sub> were pooled and dialyzed against 10 mM Tris–HCl, pH 7.5 (21°C), 200 mM NaCl, 0.1 mM Na<sub>2</sub>EDTA, 1 mM DTT and 50% glycerol. Relative protein concentrations were determined using the Bradford method with a bovine serum albumin (BSA) standard (Bio-Rad Inc., CA) or for McrB<sub>L</sub> and McrB<sub>S</sub> 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, pMC63, was that used previously by Stewart and Raleigh (1998). Briefly, this 1935 bp plasmid contains two *BsrUI* sites (CGCG) which can be methylated by *M.FnuDII* to generate <sup>m5</sup>CGCG. The methylated cytosine is preceded by a guanine residue to generate the McrBC-susceptible GmC. The two GmC sites are separated by 63 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 *BsrUI*, which cannot cleave the methylated sequence. When protection against *BsrUI* was complete, pMC63/*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 McrB<sub>L</sub>, McrB<sub>S</sub> and McrC 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 NIH Image 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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