A Mutational Analysis of the PD...D/EXK Motif Suggests That McrC Harbors the Catalytic Center for DNA Cleavage by the GTP-Dependent Restriction Enzyme McrBC from *Escherichia coli*†

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ABSTRACT: McrBC is a unique restriction enzyme which binds specifically to the bipartite recognition sequence $R^mCN_{\sim 30-\sim 2000}R^mC$ and in the presence of GTP translocates the DNA and cleaves both strands at multiple positions within the two R^mC "half-sites". It is known that McrBC is composed of two subunits: McrB which binds and hydrolyzes GTP and specifically interacts with DNA and McrC whose function is not clear but which has been suspected to harbor the catalytic center for DNA cleavage. A multiple-sequence alignment of the amino acid sequence of *Escherichia coli* McrC and of six presumably homologous open reading frames from various bacterial species shows that a sequence motif found in many restriction enzymes, but also in other nucleases, the PD....D/EXK motif, is conserved among these sequences. A mutational analysis, in which the carboxylates (aspartic acid in McrC) of this motif were substituted with alanine or asparagine and lysine was substituted with alanine or arginine, strongly suggests that Asp244, Asp257, and Lys259 represent the catalytic center of *E. coli* McrC. Whereas the variants D244A (or -N), D257A (or -N), and K259A are inactive in DNA cleavage (K259R has residual DNA cleavage activity), they interact with McrB like wild-type McrC, as can be deduced from the finding that they stimulate the McrB-catalyzed GTP hydrolysis to the same extent as wild-type McrC. Thus, whereas McrC variants defective in DNA cleavage can stimulate the GTPase activity of McrB, the DNase activity of McrC is not supported by McrB variants defective in GTP hydrolysis.

Restriction enzymes occur ubiquitously among prokaryotic organisms and serve to protect bacterial cells against bacteriophage infection, because invading foreign DNA is highly specifically cleaved by the endonuclease activity of these enzymes (for reviews, see refs *1* and *2*). Different types of restriction enzymes, which differ in subunit composition, cofactor requirement, and mode of action, have been found and classified, among them some that only restrict methylated DNA. Three different methylation-dependent restriction enzymes encoded by the *mcrA*, *mrr*, and *mcrBC* genes were found in *Escherichia coli* (reviews in refs *3* and *4*).

The McrBC system, identified in *E. coli* by Raleigh and Wilson (*5*) and Noyer-Weidner et al. (*6*), is composed of two subunits, McrB and McrC, and requires GTP for its function. It was known through the work mainly of Raleigh and co-workers (*5*, *7*−*10*) and Noyer-Weidner and co-workers (*6*, *11*−*13*) that the products of two genes, McrB (M_r = 54 kDa) and McrC (M_r = 41 kDa), cooperate in recognizing and cleaving hemimethylated or fully methylated DNA with the consensus sequence 5′-$R^mCN_{\sim 30-\sim 2000}R^mC$-3′, where ^{m}C can be 5-methylcytosine, 5-(hydroxymethyl)-cytosine, or N4-methylcytosine (*9*, *14*). It was shown that DNA cleavage occurs at multiple positions between the two R^mC "half-sites" and is strictly dependent on GTP hydrolysis. In accordance with this finding, McrB harbors the three consensus sequences for G proteins: GxxxxGK, DxxG, and NKxD (*15*, *16*), the latter being not quite canonical in McrB (*8*, *17*). It was shown, furthermore, that McrB binds to methylated DNA, while McrC does not, and that GTP and McrC stimulate DNA binding by McrB (*13*, *18*). It was subsequently demonstrated experimentally that the specific DNA binding activity resides in the N-terminal domain of McrB (*19*, *20*), whereas the C-terminal domain is responsible for GTP binding and hydrolysis (*18*, *20*). The *mcr*B gene encodes not only the full-length McrB protein but also a shorter version, $McrB_s$, which lacks the 161 N-terminal amino acids (*12*, *21*). This truncation is produced by internal in-frame translational initiation. $McrB_s$, alone or in the presence of McrC, cannot support restriction in vivo (*22*). In vitro studies had demonstrated that $McrB_s$ in the presence of GTP binds McrC and, depending on the relative ratios of full-length McrB and McrC, can promote or inhibit DNA cleavage (*23*). It was suggested that the role of $McrB_s$ is to modulate McrBC activity by binding to McrC.

The precise stoichiometry of McrB and McrC in the active restriction enzyme is not known. It is clear, however, that oligomeric complexes are formed in the presence of DNA (*19*, *23*), which may be the prerequisite for translocation of DNA by McrBC. It was demonstrated recently that after

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Table 1: Results of BLAST Searches for Apparent McrB and McrC Homologues[a]

species	data source	McrB homologue *E* value[b]	McrC homologue *E* value[b]
Staphylococcus aureus MRSA	Sanger unfinished	2×10^{-73}	1×10^{-35}
Clostridium difficile	Sanger unfinished	4×10^{-31}	6×10^{-25} (with *Y. pestis*[c])
Pseudomonas syringae pv. tomato	TIGR unfinished	7×10^{-25}	0.002
Streptococcus mutans	UOKNOR unfinished	1×10^{-23}	–
Campylobacter jejuni	TrEMBL	6×10^{-23}	4.5
Yersinia pestis	Sanger unfinished	2×10^{-21}	0.034
Streptococcus pneumoniae	TIGR unfinished	1×10^{-20}	–
Porphyromonas gingivalis W83	TIGR unfinished	2×10^{-20}	–
Helicobacter pylori J99	TrEMBL	2×10^{-19}	–
Deinococcus radiodurans (plasmid MP1)	TrEMBL	1×10^{-16}	8×10^{-4}
Bacillus subtilis	TrEMBL	1×10^{-13}	–
Clostridium perfringens (fragment)	TrEMBL	2×10^{-11}	–
Bacillus stearothermophilus	UOKNOR unfinished	1×10^{-10}	–
Bacillus anthracis	TIGR unfinished	3×10^{-10}	–
Lactococcus lactis (plasmid pTR2030)	TrEMBL	2×10^{-9}	-
D. radiodurans	TrEMBL	1×10^{-7}	–
Synechocystis PCC6803	TrEMBL	1×10^{-28} (with *L. lactis*[c])	–

[a] As of April 2001. [b] Expected value. This parameter describes the number of hits expected to be seen by chance when searching a database of a particular size. [c] With the respective *E. coli* query sequences, the *E* values were above the threshold value of 10.

binding to R^mC sites, McrBC translocates DNA in a reaction dependent on GTP hydrolysis. It was concluded that cleavage occurs in response to the encounter of two translocating McrBC complexes (*24*), a process similar to that proposed for other nucleoside triphosphate-dependent restriction enzymes (reviews in refs *1*, *25*, and *26*). It was suggested that McrBC hydrolyzes GTP not only for translocation of DNA but also for the assembly and disassembly of oligomeric complexes (*18*); this suggestion has been supported by the identification of the sequence homology of McrB with the AAA^+ family of ATPases, a class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes (*27*). This class includes proteins involved in DNA replication [e.g., DnaA (*28*)], recombination [e.g., RuvB (*29*)], and repair [e.g., MutS (*30*)]. Very recent experimental evidence indeed shows that in the presence of GTP, McrB forms heptameric or tetradecameric ringlike structures (*31*).

The catalytic center for DNA cleavage has not yet been identified in McrBC. It could be located in McrB, possibly in proximity to, or overlapping, the specific DNA binding site in the N-terminal part, but it could also be associated with the McrC subunit, for which no function has been established as yet, other than a stimulatory effect on the GTPase activity of McrB (*18*) and on the formation of high-molecular mass complexes involving McrB, McrC, and DNA (*13*, *19*, *32*). For both possibilities, examples are known in other restriction enzymes. Type IIs restriction endonucleases are two-domain proteins. One domain is responsible for specific DNA binding, and the other domain harbors the catalytic center [e.g., *Fok*I (*33*)]. Type I and III restriction enzymes, in contrast, have separate subunits for specific DNA binding and DNA cleavage (reviews in refs *25*, *27*, and *34*). Whereas an inspection of the amino acid sequence of McrB did not reveal any motifs characteristic for nucleases, viz., the PD....D/EXK motif typical of many restriction enzymes (*35*), the LAGLIDADG motif characteristic of many homing endonucleases (*36*), or the DRGH motif found in nucleases of different functions [e.g., NucA (*37*)], in McrC a PD....D/EXK motif is present (Table 1) in which the partially conserved proline is replaced with a threonine. Furthermore, this motif is present in all sequences of McrC-like proteins, making it a very good candidate for representing the catalytic center of McrC, as independently suggested previously (*89*). In the paper presented here, we report the results of a mutational analysis of the presumptive nucleolytic center of McrC, which allow us to conclude that McrC is responsible for DNA cleavage by McrBC and that the sequence TD^{244}....$D^{257}AK$ represents the catalytic center of McrC.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media. For the preparation of the methylated substrate plasmid pBW201, the methylation-tolerant *E. coli* strain TC410 [*mcrA*$^-$, Δ(*mrr-hsdRMS-McrBC*)201, *minA*, *minB*, *rpsL*, *sup*$^+$] (*6*, *12*) was used.

The vector pBBIMcrC for overexpression of His_6-tagged McrC was constructed as follows. pBBINucA (*38*), a descendant of pET-3d (*39*), was cleaved with *Bam*HI and *Nde*I. Into the resulting large fragment was inserted the McrC gene, which had been amplified by PCR using the GST-McrC expression vector pBN213 (*13*, *40*) as a template, and primers introducing the appropriate restriction sites (*Nde*I at the 5′-end of the gene with the primer 5′-GATACATATGGACCAACAAATTATTAGGG, *Bam*HI at the 3′-end of the gene with the primer 5′-ATATGGATCCTATTTGAGATATTCATCGAAAATG). After ligation, the resulting plasmid was used to transform *E. coli* LK111(λ) cells. For protein expression, *E. coli* BL21(DE3)pLysS cells (*39*) were transformed with pBBIMcrC. *E. coli* TC410 and LK111(λ) cells were grown in LB medium with 75 μg/mL ampicillin. In the case of BL21(DE3)pLysS cells, 20 μg/mL chloramphenicol was added.

Site-Directed Mutagenesis. Site-directed mutagenesis of McrC was performed using a protocol described by Kirsch and Joly (*41*), starting from the His_6-McrC expression vector pBBIMcrC. In a first PCR with a mutagenic reverse primer and an appropriate forward primer, products of 218 or 255 base pairs were generated, which were used in a second PCR as megaprimers for inverse PCR. The products were then

used to directly transform *E. coli* LK111(λ) cells.

forward primer	5′-GTCAAAACAAAGGACACTACCG
mutagenic reverse primers	
D244A	5′-TTCTGACGATCGAATGGTGATGGCAGTTTCC
D244N	5′-TTCTGACGATCGAATGGTGATATTAGTTTCC
D257A	5′-TGCTCTTATAGTATTTGGCGGCAACGAT
D257N	5′-TGCTCTTATAGTATTTGGCATTAACGAT
K259A	5′-TGCTCTTATAGTATGCGGCATCAACGAT
K259R	5′-TGCTCTTATAGTATCTGGCATCAACGAT

With the first two mutagenic primers was introduced an additional *Pvu*I site (CGATCG), whereas the other four primers led to elimination of a *Hinc*II site (GTYRAC). Transformants were screened by digestion of plasmid minipreps (Qiagen) with the appropriate restriction enzymes. The *mcrC* gene of marker-positive clones was sequenced on both strands using the *Taq* cycle sequencing protocol (Perkin-Elmer) and an Applied Biosystems 373A sequencer.

Protein Purification. The His_6-tagged McrC (wild type and variants) was purified by a small-scale Ni−NTA−agarose batch procedure. Cultures (500 mL) of *E. coli* BL21(DE3)-pLysS cells containing pBBIMcrC (wild type and variants) were grown at 37 °C in LB broth, 50 μg/mL ampicillin, and 20 μg/mL chloramphenicol to an OD_{600} of 1.0. The temperature was reduced to room temperature, and expression was induced by 0.5 mM IPTG. After 20 h, cells were harvested by centrifugation (Beckman JS 4.2 rotor, 4200 rpm, 15 min), washed with buffer A [20 mM Hepes-KOH (pH 7.6), 1 mM DTT, 50 mM KCl, and 10% (v/v) glycerol], resuspended in 25 mL of buffer A containing 20 mM imidazole, and sonicated (Branson sonifier, position 5, 50%, 2 × 2 min on ice). After centrifugation (Beckman JA 20 rotor, 20 000 rpm, 1 h), 1 mL of a Ni−NTA−agarose suspension (Qiagen) equilibrated in buffer A was mixed with the clear supernatant and incubated for 1 h at 4 °C with gentle shaking. After centrifugation (10 min, 2000 rpm, Beckman JS 4.2 rotor) and washing, the His_6-tagged proteins were eluted with 1 mL of 200 mM imidazole in buffer A and stored at −20 °C. A wild-type His_6-McrC preparation of approximately 20−60% purity was obtained by Ni−NTA−agarose (Qiagen) column chromatography. For experiments requiring homogeneous protein preparations (circular dichroism), a cation exchange chromatographic step with phosphocellulose (Whatman) was included. Protein concentrations were determined by the method of Bradford (*42*) with bovine serum albumin as the standard or by absorbance at 280 nm with a molar extinction coefficient ϵ of 51 160 M^{-1} cm^{-1}, calculated by the method of Pace (*43*). Molar concentrations of the partially purified McrC preparations were estimated on the basis of the relative intensity of the bands in the SDS−PAGE analysis and the known amount of protein loaded onto the gel, as determined by the Bradford assay.

GTP Hydrolysis Assay. GTP hydrolysis by McrB in the absence and presence of McrC was assayed by measuring the amount of radioactive inorganic phosphate liberated after incubation with [γ-^{32}P]GTP (DuPont-NEN) in buffer B (buffer A with 10 mM $MgCl_2$ and 100 μg/mL bovine serum albumin) at 25 °C. Standard conditions were 0.5 μM McrB and 10 μM GTP/[γ-^{32}P]GTP in a reaction volume of 20 μL. Nonhydrolyzed GTP was removed by vortexing the reaction mixture with 1 mL of a suspension of 70 mg/mL charcoal in 20% ethanol, 0.2 M HCl, and 0.1 M KH_2PO_4. After centrifugation, the Cerenkov radiation of the supernatant was measured.

DNA Cleavage Assay. DNA cleavage activity of McrBC was assayed with *Eco*RI-linearized pBW201, a 4842 bp plasmid harboring the *Bsu*FI methylase gene leading to in vivo methylation at the first cytosine of CCGG and thus creating multiple McrBC recognition sites (*13*). The reaction mixture typically contained 10 nM plasmid, 0.5 μM McrB and McrC, and 1 mM GTP in buffer B. After incubation at 25 °C, 10 μL aliquots (containing 320 ng of plasmid DNA) were withdrawn and the reaction was stopped by addition of EDTA. Samples were analyzed by electrophoresis in 1% agarose gels.

Circular Dichroism Spectroscopy. Circular dichroism spectra of McrC and McrC variants were recorded on a Jasco J-710 dichrograph at 20 °C in a cylindrical cuvette with a path length of 0.05 cm.

Database Sequence Search and Alignment. Sequence similarity searches were performed with the BLAST program (*44*). For nucleotide sequence databases, TBLASTN, and for protein sequences, BLASTP was used with standard parameters. Searches were done on the NCBI web site (http://www.ncbi.nlm.nih.gov) and the Expasy server (http://www.expasy.ch) of the Swiss Institute of Bioinformatics (SIB). Preliminary sequence data were obtained from The Institute for Genomic Research (TIGR; http://www.tigr.org) and The Sanger Centre (http://www.sanger.ac.uk). In the case of nucleic acid databases, the full-length ORFs containing the McrC-homologous part found by a TBLASTN search were generated by translation of the respective genomic DNA sequences or contigs. Multiple alignment of *E. coli* McrC and similar sequences was performed with the program ClustalX version 1.8 (*45*) with default parameters.

RESULTS

Sequence Database Search for Putative McrC Homologues. No restriction system similar to that of *E. coli* McrBC has been characterized in biochemical terms yet. The sequences of members of the type I and type III systems, which functionally resemble McrBC, are not significantly similar to that of McrB or -C, not to mention the different nucleotide cofactor requirement (ATP instead of GTP). Searches in the SWISSPROT protein sequence database did not reveal any protein sequence that was significantly similar to that of either McrB or McrC, but some sequences (listed in Table 1) showed up in the TrEMBL database and in some unfinished microbial genome sequencing projects. The updated list contains 17 putative McrB homologues with highly significant expectation values (<0.001). The similarities are most pronounced in the GTP binding domain of McrB, as was already shown in a sequence alignment of *E.coli* McrB and the five putative homologues present in the databases two years ago (*17*). A similar search for McrC in genomic databases reveals only six putative McrC homologues. Their degree of similarity to *E. coli* McrC is considerably lower than for McrB and in some cases is at a level which could be considered statistically insignificant. Nevertheless, there is strong evidence that even these sequences represent true McrC homologues, because for each McrC-like sequence a corresponding McrB homologue exists in the respective organism. Furthermore, the putative McrB

and McrC genes are organized in the same manner as in the *E. coli* genome. The McrC coding sequence is always found immediately downstream of the McrB ORF, and in all but one organism, the genes even slightly overlap, as is the case in the *E. coli mcrBC* operon (data not shown).

A multiple alignment of the seven McrC-like sequences (shown in Figure 1) reveals several narrow regions which are highly conserved. Two of these contain a sequence motif which is already well-known from several type II restriction endonucleases to represent the catalytic center (*35*). This motif with the canonical sequence PD...D/EXK has been confirmed to be involved in catalysis of DNA cleavage by crystallographic as well as mutational analyses for several type II restriction endonucleases and other nucleases (see Table 2). Thus, the assumption that this motif in the *E. coli* McrC sequence (here $TD^{244}...D^{257}AK$) is part of the catalytic center for DNA cleavage by the McrBC restriction system as well seemed justified. We tested this hypothesis via a mutational analysis of McrC.

Site-Directed Mutagenesis, Protein Expression, and Purification. Six *E. coli* McrC variants with single amino acid substitutions were produced. We changed aspartic acid residues 244 and 257 and lysine 259 which constitute the presumptive catalytic motif either to alanine or more conservatively to asparagine (positions 244 and 257) and arginine (position 259). All six variants, carrying an N-terminal His_6 tag, were overexpressed using a T7 expression vector (see Materials and Methods). The proteins, which were all produced in amounts similar to the amount of wild-type His_6-McrC, were purified by a single-step small-scale batch procedure with Ni−NTA−agarose, which yielded a protein preparation enriched in McrC, and containing between 20 and 60% McrC (McrC concentrations given in this paper are estimates based on the protein concentration and the purity of the preparation as determined by the gel electrophoretic analysis; cf. Materials and Methods). These preparations proved to be free of nonspecific endonuclease activity and contained only little background GTPase activity and were therefore considered suitable for the subsequent functional assays. Only for the experiment shown in Figure 4 was wild-type McrC purified further by a second chromatographic step utilizing a phosphocellulose column, which resulted in a preparation that was approximately 90% pure. To demonstrate the structural integrity of the McrC variants, small amounts of the McrC variants were also purified to near homogeneity; circular dichroism spectra of wild-type McrC and of the variants were recorded and shown to be identical within the limits of error (Figure 2).

DNA Cleavage. The six McrC variants were tested for their ability to complement McrB to give a fully active McrBC restriction system which is able to cleave methylated substrate DNA in the presence of GTP and Mg^{2+}. In the cleavage assay shown in Figure 3, linearized pBW201, a 4.8 kb plasmid which contains 18 McrBC recognition sites due to in vivo methylation by the plasmid-encoded methyltransferase M.*Bsu*FI, was used. As expected, no DNA cleavage occurs in the absence of McrC. Addition of 0.5 μM wild-type McrC (equimolar to McrB) leads to complete digestion of the 4.8 kbp substrate DNA, resulting in a 1.9 kbp fragment (corresponding to the large nonmethylated region containing the M.*Bsu*FI gene; cf. Figure 3A) and a multitude of smaller fragments which are not resolved in this gel. Two partial digestion products of 3.6 and 2.9 kbp, most likely resulting from single cleavage at the "best" McrBC sites (positions 1232−1299 and 2923−2999 with a spacing of 67 and 76 bp, respectively), can be observed at lower McrC concentrations (0.1 μM). Five of the six McrC variants are completely inactive in supporting DNA cleavage, namely, all three alanine mutants (D244A, D257A, and K259A) and the two asparagine mutants (D244N and D257N). Only the conservative substitution of Lys259 with arginine results in a variant with residual activity. The partial digestion observed with 0.5 μM K259R after 60 min is slightly less advanced than that with 0.1 μM wild-type McrC after 10 min. Thus, a residual activity of ca. 2% of wild-type McrC can be estimated for this variant.

GTP Hydrolysis. The only function that had been associated with McrC previously, other than its involvement in high-molecular mass complexes with McrB and DNA, was its stimulatory effect on the GTP hydrolysis catalyzed by McrB. This stimulation must be caused by direct interaction of two subunits. We investigated whether this McrB−McrC interaction is disturbed by the amino acid exchanges introduced into McrC. If this was the case, then one could not exclude the possibility that the variants are inactive in DNA cleavage because of global structural alterations. The results of the GTPase stimulation assays shown in Figure 4 clearly demonstrate that this is not the case. In Figure 4A, the concentration dependence of the stimulatory effect of a pure wild-type McrC preparation on the GTPase activity of 50 nM McrB is shown. It is represented by a saturation curve which approaches a plateau value at an approximately 1:1 stoichiometric McrB:McrC ratio. Figure 4B shows results of a similar experiment with the six variants and a wild-type McrC preparation which had been purified in the same manner (only passed over the affinity column) as the variants to allow for a direct comparison, each with a 2:1 and 6:1 excess of McrC (100 and 300 nM, respectively) over McrB (50 nM). Each of the six variants exhibits a stimulatory effect which is indistinguishable in its maximal amount from that of wild-type McrC measured in parallel. Furthermore, with each variant, as with the wild-type enzyme, the maximum is already reached at a 2:1 McrC:McrB ratio. This means that neither the mechanistic details of GTPase stimulation by McrC nor the strength of the McrB−McrC interaction is noticeably affected by the mutations that have been introduced. This finding provides strong evidence that the exchanged amino acid residues indeed are directly involved in phosphodiester bond cleavage, because the respective McrC variants are nearly or totally unable to support DNA cleavage.

DISCUSSION

It had not been unequivocally established which subunit of McrBC harbors the active site for DNA cleavage. As DNA cleavage is only observed in the presence of McrC and no other functions had been associated with this subunit so far, McrC is a good candidate to be responsible for DNA cleavage, in particular as a sequence comparison with several obviously homologous proteins whose DNA sequences recently appeared in the database revealed a conserved sequence motif (Figure 1) characteristic of the active site of many restriction endonucleases (*35*) and other nucleases (*27*, *46*−*49*), the PD...D/EXK motif (*89*). We have carried out a

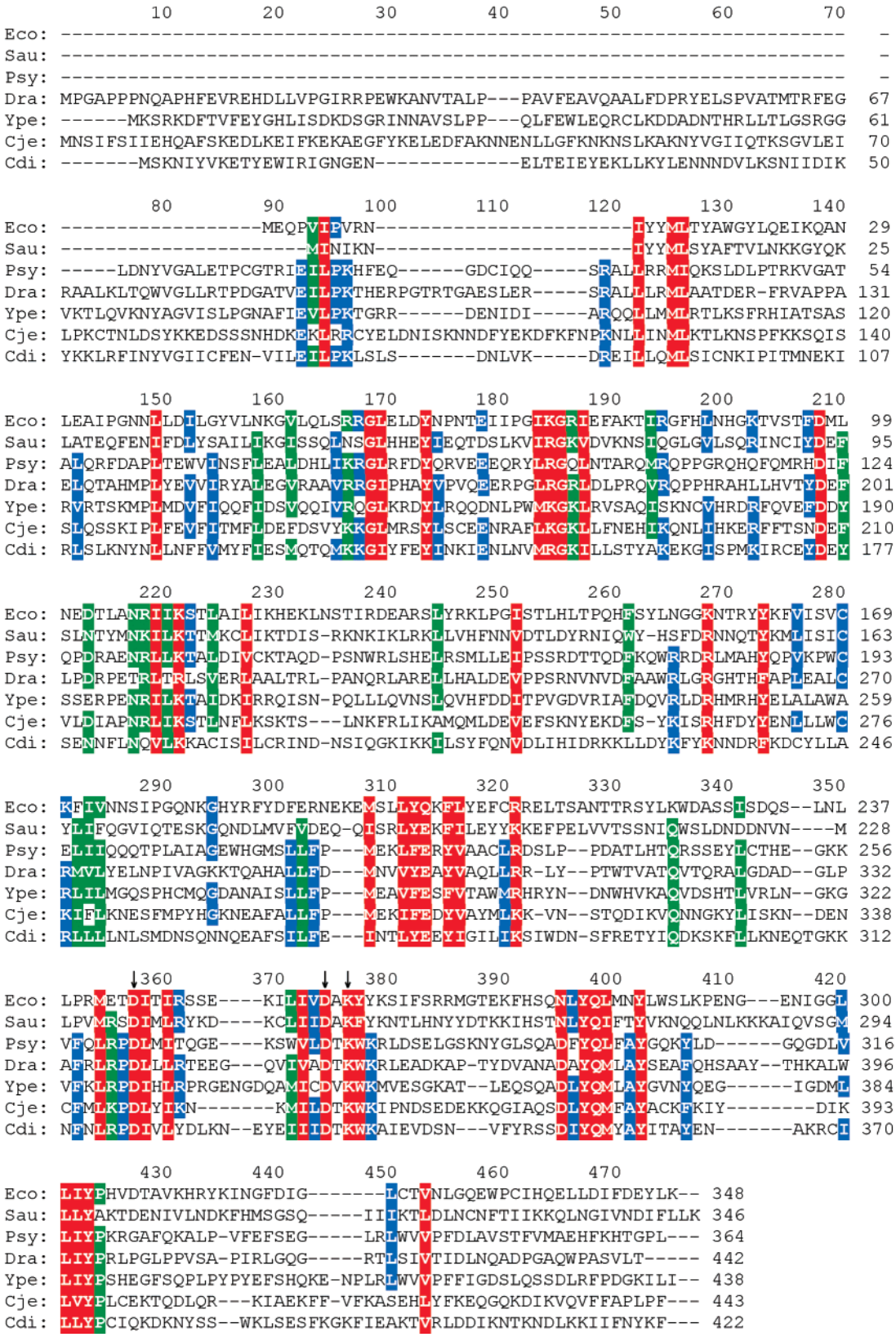

FIGURE 1: Multiple alignment of the *E. coli* McrC amino acid sequence and six homologous ORFs: Psy, *Pseudomonas syringae* pv tomato; Dra, *Deinococcus radiodurans*; Ype, *Yersinia pestis* CO-92; Cje, *Campylobacter jejuni*; Cdi, *Clostridium difficile*; Eco, *E. coli* K-12; and Sau, *Staphylococcus aureus* MRSA. The sequences were found in BLAST searches of finished and unfinished microbial genomes on the NCBI web site, or in the case of the *D. radiodurans* sequence (Q9RZI4; encoded on plasmid MP1) in the TrEMBL database. The alignment was done with the program ClustalX (version 1.8) with default parameters. Levels of conservation are indicated in red (conserved in all seven sequences), green (in six sequences), or blue (in five sequences). Note that *E. coli* McrC positions D244, D257, and K259 (marked by arrows) are absolutely invariant in all sequences.

mutational analysis of the PD...D/EXK motif in McrC, i.e., TD[244]....D[257]AK, by generating and characterizing the variants D244A, D244N, D257A, D257N, K259A, and K259R. These variants were produced as His_6-tagged proteins and assayed

Table 2: Bona Fide Catalytic Sequence Motifs in Type II Restriction Endonucleases and Other Nucleases, As Identified by Crystal Structure Analyses

enzyme	PD motif	D/EXK motif	ref
type II restriction endonucleases			
*Eco*RI	**PD**^{91}	**E**^{111}A**K**	*72*
*Eco*RV	**PD**^{74}	**D**^{90}I**K**	*73*
*Bam*HI	I**D**^{74}	**E**^{111}FE	*74*
*Pvu*II	N**D**^{58}	**E**^{68}L**K**	*75, 76*
*Crf*10I[a]	**PD**^{134}	(**E**^{204})S^{188}V**K**	*77*
*Fok*I	**PD**^{450}	**D**^{467}T**K**	*33*
*Bgl*I	**PD**^{116}	**D**^{142}I**K**	*78*
*Mun*I	**PD**^{83}	**E**^{98}I**K**	*79*
*Nae*I	T**D**^{86}	**D**^{95}C**K**	*80*
*Bgl*II	I**D**^{84}	**E**^{93}VQ	*81*
*Ngo*MIV[a]	**PD**^{140}	(**E**^{201})S^{185}C**K**	*82*
*Bso*BI	V**D**^{212}	**E**^{240}L**K**	*83*
other nucleases			
λ-exonuclease	**PD**^{119}	**E**^{129}L**K**	*84*
E. coli Mut H	Q**D**^{70}	**E**^{77}L**K**	*85*
E. coli Tns A[a]	T**D**^{114}	(**E**^{149})Q^{130}V**K**	*31*
S. solfataricus Hjc	**PD**^{42}	**E**^{55}M**K**	*86*
P. furiosus Hjc	V**D**^{36}	**E**^{46}V**K**	*87*
T7 Endo I	**PD**^{55}	**E**^{65}T**K**	*88*
for comparison			
E. coli McrC[b]	T**D**^{244}	D^{257}**AK**	*89*
*Eco*KI[b]	A**D**^{316}	**E**^{330}A**K**	*65*
*Eco*P1I[b]	**PD**^{898}	**E**^{916}T**K**	*66*

[a] The crystal structure analysis shows that the second acidic residue of the PD....D/EXK motif is recruited for the catalytic center from a distal part of the structure. [b] The identification of the catalytic residues is based on sequence alignments and, in part, mutational analyses.

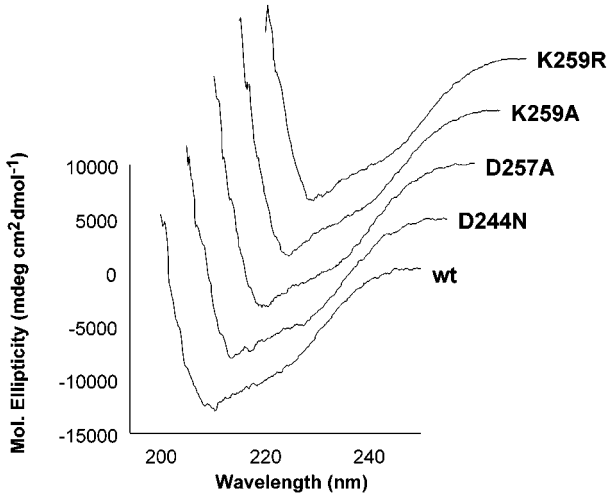

FIGURE 2: Circular dichroism spectra of wild-type McrC and McrC variants. The circular dichroism spectra of wild-type McrC (1.6 μM, left) and the McrC variants D244N (1.7 μM), D257A (0.57 μM), K259A (7.7 μM). and K259R (6.4 μM) were recorded in 20 mM Hepes-KOH (pH 7.4), 1 mM DTT, 10% glycerol, and 1.5 M KCl. Individual spectra are offset by 5 nm (abscissa) and 5000 mdeg cm^2 dmol^{-1} (ordinate).

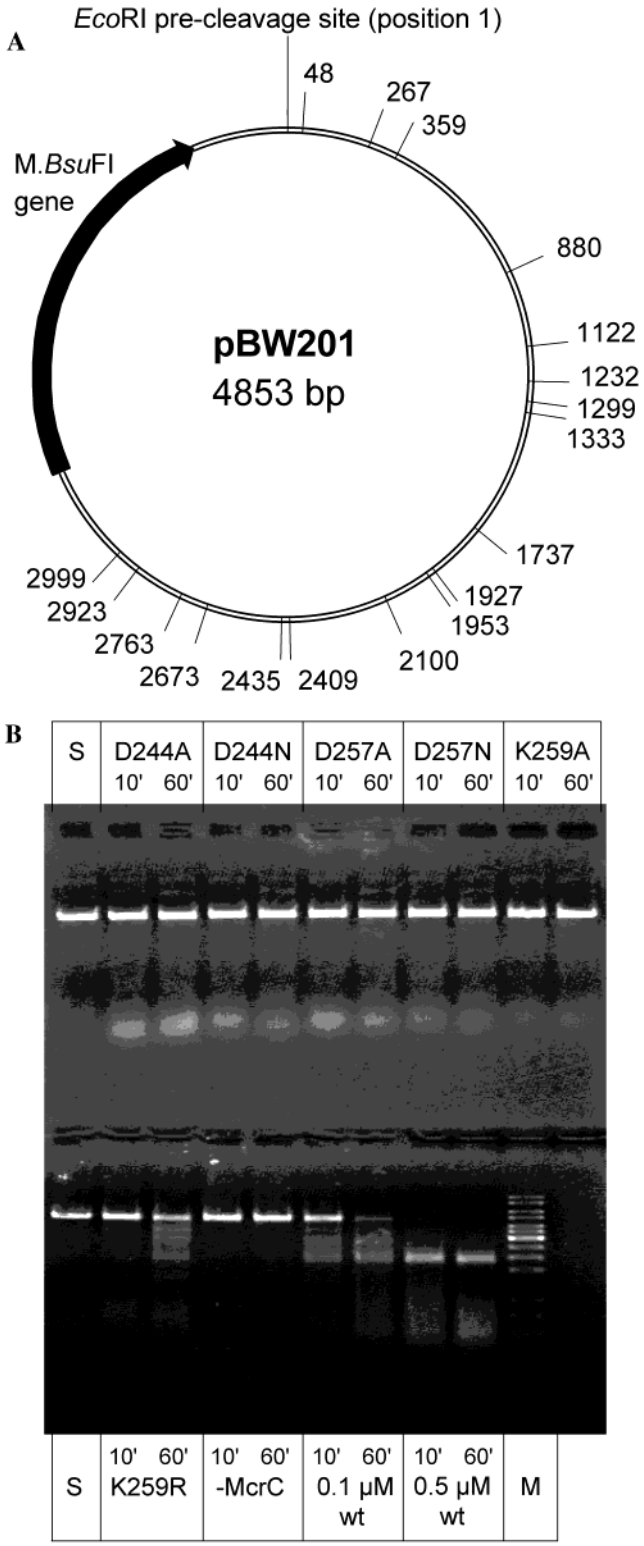

FIGURE 3: DNA cleavage assay. (A) Map of the methylated substrate plasmid pBW201 with all R^{m}C sites and the single *Eco*RI site used for linearization indicated. (B) Agarose gel electrophoretic analysis of cleavage reactions with 0.5 μM McrB and 0.5 μM McrC variants as well as without McrC, 0.1 and 0.5 μM wild-type McrC, carried out for 10 and 60 min each: S, substrate DNA; M, size marker (1 kbp ladder).

for their DNA cleavage activity in the presence of McrB. Our results show that all variants are inactive in plasmid DNA cleavage, with the exception of K259R which exhibits an approximately 50-fold decrease in activity compared to wild-type McrC. This finding suggests that the TD^{244}....D^{257}-AK motif is a bona fide PD...D/EXK motif.

The PD...D/EXK motif has been unequivocally identified by crystallographic analyses in 12 type II restriction endonucleases, as well as in λ-exonuclease, *E. coli* MutH, *E. coli* TnsA, T7 endonuclease I, *Sulfolobus solfataricus* and *Pyrococcus furiosus* Holliday junction resolvase (Table 2), and, with modifications, also in *E. coli* Vsr (*50*). In addition, in several other type II restriction endonucleases, the relevance of this motif for catalysis has been demonstrated by a mutational analysis (*51*). In this context, it must be pointed

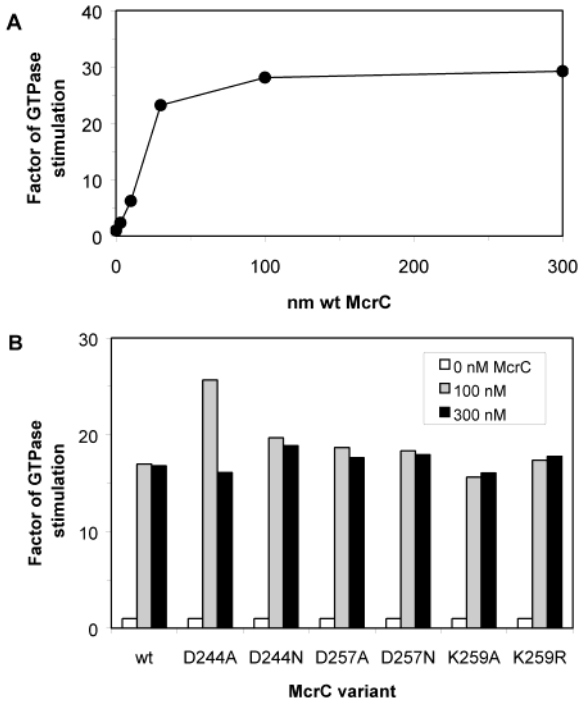

FIGURE 4: Stimulation of the McrB-catalyzed GTP hydrolysis by McrC variants. (A) Wild-type McrC concentration dependence of McrB GTPase activity. (B) Comparison of the GTPase stimulatory effect exerted by wild-type McrC and McrC variants. Concentrations were 10 μM [γ-^{32}P]GTP, 50 nM McrB, and 0, 3, 10, 30, 100, and 300 nM wild-type McrC in panel A and 0, 100, and 300 nM wt or mutant McrC in panel B. GTP hydrolysis was monitored by measuring the amount of radioactive phosphate released. Note that in panel A a near-homogeneous preparation of wild-type McrC was used, whereas in panel B only enriched preparations of wild-type McrC and the McrC variants were used. Furthermore, the relative stimulation of the McrB GTPase activity by McrC may vary depending on the background GTPase activity of the McrB and McrC preparation. This may explain why in panel A a 30-fold stimulation was observed whereas in panel B an only 17−26-fold stimulation could be detected for wild-type McrC and the McrC variants. The experiments shown were carried out only once; on the basis of numerous GTPase experiments carried out with McrBC, we estimate that the values that are given are reliable within ±30%.

out that the "final" proof for a PD...D/EXK motif to be involved in catalysis requires a crystal structure analysis of an enzyme−substrate complex in conjunction with a mutational analysis and mechanistic studies. For example, for the homing endonuclease I-*Ppo*I, it was assumed on the basis of a mutational analysis that the PD^{109}....D^{140}NK motif represents the Mg^{2+} binding site of this enzyme (*52*), an assignment which could not be confirmed by the cocrystal structure analysis of the I-*Ppo*I−DNA complex (*53*, *54*), homology considerations (*55*), and mechanistic studies (*56*, *57*).

As shown in Table 2, the PD...D/EXK sequence motif identified in *E. coli* McrC is quite typical with respect to the composition and distance of the two parts of the bipartite sequence motif. The detailed functions of the individual amino acid residues within this motif are still being discussed for all members of the PD...D/EXK family of nucleases. There is consensus, however, that the two carboxylates (D and D/E) and the main chain carbonyl of the hydrophobic residue X are involved in binding the divalent metal ion cofactor(s), typically, Mg^{2+} or Mn^{2+}. The lysine residue could be involved in positioning and/or activating the attacking nucleophile as well as in transition state stabilization. The mutational analysis of the carboxylates of the PD...D/EXK motif, for example, in *Eco*RI and *Eco*RV, had demonstrated that the substitution of these residues with neutral amino acid residues led to variants with a more than 1000-fold decrease in activity or no measurable activity at all (*58*−*62*). It was shown for *Eco*RI that the substitution of Glu111 with aspartic acid and Lys113 with arginine is better tolerated (*61*, *63*), demonstrating that the conservation of charge is essential for activity, similar to the results shown here for Lys259 of McrC.

The inactivity of McrC D244A or -N, D257A or -N, and K259A variants could, in principle, be due to an impairment of the McrB−McrC interaction. To exclude this possibility, we have measured the stimulatory effect of McrC on the McrB-catalyzed GTPase activity. Within experimental error, all variants exhibit the same relative stimulation of the GTPase activity of McrB as wild-type McrC. This means that the McrB−McrC interaction is not affected by the mutations that had been introduced but rather that the amino acid substitutions directly interfere with the nucleolytic function of McrC by removing functional groups essential for catalysis, viz., the negative charge of Asp244 as well as Asp257 and the positive charge of Lys259. It is interesting to note that McrC subunits defective in DNA cleavage fully support the GTPase activity of McrB, whereas McrB subunits defective in GTP cleavage fully suppress DNA cleavage by McrC (*17*), demonstrating that GTP hydrolysis is the obligatory prerequisite for DNA cleavage.

It could be argued that the effects of the mutations are not due to defects in the catalytic center but due to defects in DNA binding. This appears unlikely as we have no evidence for any alteration in DNA binding of McrB in the presence of McrC as compared to the McrC mutants. Another, though remote, possibility is that the mutations interfere with DNA translocation. As DNA translocation so far can only be measured by its effect, namely, DNA cleavage, we cannot exclude the possibility that the extent of DNA translocation is reduced when McrB is incubated with mutant instead of wild-type McrC.

The results presented here suggest that McrC indeed harbors the catalytic center for DNA cleavage. It had been shown previously that MrcB is mainly responsible for GTP binding and cleavage (*8*, *18*, *24*), but also for specific DNA binding (*13*, *19*). This means that specific DNA binding and DNA cleavage are activities of two different subunits that have to cooperate to achieve specific DNA cleavage. This is also the case for other nucleoside triphosphate-dependent restriction endonucleases, namely, type I and type III restriction enzymes (*25*, *34*, *64*). Type I restriction endonucleases are composed of three different subunits (HsdS, HsdM, and HsdR), one of which is responsible for specific DNA binding (HsdS) and one of which harbors the nucleolytic center (HsdR). Similarly, type III restriction enzymes are composed of two different subunits (Mod and Res), one of which is responsible for specific DNA binding and modification (Mod) and the other of which is involved in DNA translocation and cleavage (Res). It is intriguing to note that members of both the families of type I (e.g., *Eco*KI) and type III restriction enzymes (e.g., *Eco*P1I) share with McrBC the presence of a nucleolytic subunit with a PD...D/EXK motif at the presumptive active center (Table 3) (*65*,

Table 3: Subunit Composition and Domain Organization of Nucleoside Triphosphate-Dependent Restriction Enzymes

	McrBC	type I (*Eco*KI)	type III (*Eco*P1I)
subunit composition	B_nC_m[a]	R_2M_2S	Res_2Mod_2
subunit function			
NTP hydrolysis	B (N-terminal domain)	R (C-terminal domain)	Res (N-terminal domain)
DNA recognition	B (C-terminal domain)	S	Mod
DNA cleavage	C	R (N-terminal domain)	Res (C-terminal domain)
DNA methylation	−	M	Mod

[a] The subunit composition has not yet been determined; *n* and *m* are greater than 1 and may differ in the presence of GTP and/or DNA.

66). The catalytic subunits of these three different types of nucleoside triphosphate-dependent restriction endonucleases have only one catalytic motif per subunit. This means that they have at least to dimerize to afford double-strand cleavage. Indeed, McrBC (*19*, *23*) and type I (*26*, *67*) and type III restriction enzymes (*66*) form multimeric complexes, which bind to two recognition sites (or half-sites, depending on the definition) and, accompanied ("fueled") by nucleoside triphosphate hydrolysis, translocate DNA which is cleaved in both strands, triggered by collision between two restriction enzyme molecules (*24*, *68*−*71*). The similarity in the basic reaction catalyzed by these types of restriction enzymes should not conceal the fact that there are great differences in the mechanistic details which are also reflected in the different architectures of these enzymes (Table 3).

In conclusion, our results suggest that that the PD...D/EXK motif in McrC is part of the catalytic center of the McrBC restriction enzyme, a suggestion that could not have been made if the results presented in this paper were judged on their own but rather relies heavily on structural and biochemical observations made for other nucleases.

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