JOURNAL OF BACTERIOLOGY, Apr. 1987, p. 1757–1759
0021-9193/87/041757-03$02.00/0

Vol. 169, No. 4

Localization of a Genetic Region Involved in McrB Restriction by *Escherichia coli* K-12

TROY K. ROSS AND H. D. BRAYMER*

Department of Microbiology and the Louisiana Agricultural Experiment Station, Louisiana State University, Baton Rouge, Louisiana 70803

Received 11 November 1986/Accepted 20 January 1987

A 5,500-base-pair *Bgl*II-*Eco*RI fragment proximal to the *hsd* genes of *Escherichia coli* K-12 has been cloned in the plasmid vector pUC9. The resultant hybrid plasmid was shown to complement the *mcrB* mutation of *E. coli* K802. The presence of the hybrid plasmid in strain K802 caused an 18.3-fold drop in transformation efficiency with *Alu*I-methylated pACYC184 relative to unmethylated pACYC184. These results indicate that the cloned DNA is involved in the McrB system of restriction of 5-methylcytosine DNA.

The phenomenon of host-controlled restriction-modification enables bacteria to recognize and degrade foreign DNA molecules that enter the cell. The well-studied systems have been shown to protect their own DNA by specifically methylating (i.e., modifying) certain adenine or cytosine residues within a particular nucleotide sequence. By doing this, the DNA of the host is protected against the action of its own restriction endonuclease (1, 3, 17).

Two restriction systems, RglA and RglB, were reported to degrade the DNA of T-even bacteriophages when the DNA contained 5-hydroxymethylcytosine in a nonglucosylated state. The RglA and RglB systems appear to be identical to the recently described McrA and McrB systems. The two Mcr (for modified cytosine restriction) systems each restrict DNA containing 5-methylcytosine but function independently of each other, as defined by their specificity for different methylated sequences (12). Since it has been reported that the DNA of many organisms contains 5-methylcytosine (5), attempts to clone this DNA in an Mcr^+ strain would result in low transformation efficiencies. The Mcr systems were shown to be functional in many of the common laboratory host strains (12). The present study addresses the genomic location of DNA encoding the McrB system in *Escherichia coli* K-12.

Genetic analysis of *E. coli mcrB* mutants positioned the mutation at 99 min on the chromosome (12), which is near the *hsd* genes (2). The *hsd* region in *E. coli* K-12 encodes the products necessary for the type I restriction-modification activity and consists of three genes, which have been designated *hsdR*, *hsdM*, and *hsdS* (13). Since the position of the McrB system was reported to be within 1 map unit of the *hsd* genes, we chose to analyze the DNA adjacent to the *hsdS* gene for the presence of *mcrB*.

The recombinant phage λ1048, shown to contain nearly the entire *hsd* region in addition to more than 4,000 base pairs (bp) of downstream DNA, was provided by N. Murray (13). The 5,500-bp *Bgl*II-*Eco*RI fragment consisting of DNA adjacent to *hsdS* was purified from λ1048 by endonuclease digestion, agarose gel electrophoresis, and electroelution into a trough (8). DNA digestions were done by using restriction buffer conditions previously described (8). The purified fragment was ligated with *Bam*HI- and *Eco*RI-digested pUC9 (15) DNA by using T4 DNA ligase under the conditions specified by the manufacturer (Bethesda Research Laboratories, Rockville, Md.). The resultant hybrid plasmid, designated pUC9-14 (Fig. 1), was used to transform (10, 14) *E. coli* JM83 (15). Ampicillin-resistant (Ap^r) and Lac^- transformants were selected on MacConkey agar (Difco Laboratories, Detroit, Mich.) with ampicillin (50 μg/ml). Plasmid DNA from several transformants was prepared (7) and subsequently screened by restriction analysis for the presence of the appropriate hybrid plasmid. *E. coli* K802, an $McrB^-$, restriction $(R)_K^-$ and modification $(M)_K^+$ strain (12) provided by New England BioLabs, Inc. (Beverly, Mass.), was transformed to Ap^r with pUC9-14.

The transformed strain, K802(pUC9-14), was then tested for its ability to restrict 5-methylcytosine DNA. Plasmid vector pACYC184 (4) DNA was methylated in vitro by using *Alu*I DNA methylase from New England BioLabs. The methylase reactions were done under the conditions specified by the manufacturer, and successful methylation was verified by attempting to digest the DNA with *Alu*I restriction endonuclease. Agarose gel electrophoresis revealed that predominantly linear and circular molecules of pACYC184 remained after digestion of the methylated DNA with *Alu*I. However, treatment of unmethylated pACYC184 DNA with *Alu*I generated multiple fragments, with the largest being about 800 bp (data not shown). K802(pUC9-14), as well as all of the strains tested, was rendered competent by growing the strain in LB (9) to an optical density at 600 nm of 0.45 to 0.50 and harvesting the cells by centrifugation at 4°C. The cells were then suspended in one-half the original volume of 0.1 M $CaCl_2$ and incubated on ice for 20 min. After recentrifugation, the cells were suspended in 1/10 the initial volume of 0.1 M $CaCl_2$, followed by incubation on ice for 12 to 18 h. A 0.2-ml sample of competent K802(pUC9-14) was mixed with 0.1 μg of either *Alu*I-methylated or unmethylated pACYC184 DNA, and the transformation was done as previously described (14). Dilutions of the transformation mixture were plated on L agar (LB with agar added at 1.5%) with 50 μg of chloramphenicol per ml and 50 μg of ampicillin per ml and incubated overnight at 37°C. The numbers of transformants per 0.1 μg of pACYC184 are shown in Table 1. K802(pUC9-14) showed an 18.3-fold drop in transformation efficiency when the transforming pACYC184 DNA was cytosine methylated compared with the efficiency observed when the DNA was unmethylated.

The restriction assay included testing the R_K^- M_K^+ strain JM107 (16) as the positive control, since it was reported to be $McrB^+$ (12). Under identical conditions, JM107 was shown

* Corresponding author.

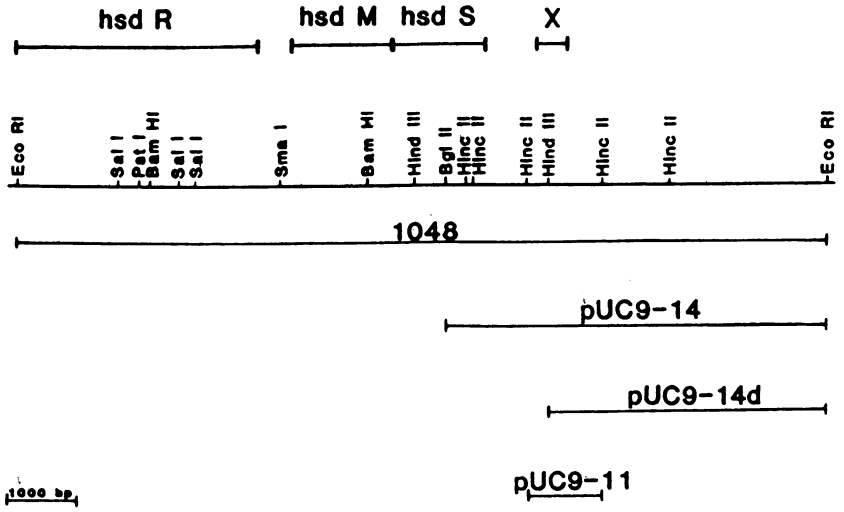

FIG. 1. A restriction map of the *E. coli* K-12 *hsd* region and the more than 4,000 bp of downstream DNA cloned in λ1048. The map shows the deduced location of the gene encoding polypeptide X. The portion of λ1048 DNA inserted in the various hybrid plasmids is shown below the restriction map.

to give an 8.0-fold drop in transformation efficiency when transformed with methylated pACYC184 DNA (Table 1). Selection was made on L agar supplemented with chloramphenicol alone, since JM107 carries no pUC plasmid conferring resistance to ampicillin. Also shown in Table 1 are the results obtained for the two negative controls, K802 and K802(pUC9). Transformants of K802 by pACYC184 were selected on L agar plus chloramphenicol. Transformants of K802(pUC9) by pACYC184 were selected on L agar plus chloramphenicol and ampicillin, to ensure the presence of pUC9 in the host. No significant difference was seen in the transformation efficiencies of either of these strains with unmethylated or methylated pACYC184.

The polypeptides encoded by the *hsd* region in *E. coli* K-12 have molecular weights of 130,000 for *hsdR*, 63,000 for *hsdM*, 50,000 for *hsdS*, and 16,000 for a protein of unknown function. This small polypeptide, designated X, is encoded by a gene downstream of *hsdS* (13). The DNA sequence of the *hsdS* gene and over 900 bp downstream from the termination of *hsdS* indicated that the only open reading frame starts 700 bp beyond *hsdS* (6). A deletion analysis of the region shown in Fig. 1 revealed that upon removal of the 1.8-kilobase *Hin*dIII fragment, both the *hsdS* and X polypeptides are missing (13). The deduced position, then, of the gene encoding protein X would be about the *Hin*dIII site to the right of *hsdS* (Fig. 1; 6, 13). Although protein X is encoded by an adjacent gene, there is no evidence that it is involved with the host specificity phenotype (13).

TABLE 1. Restriction of *Alu*I-methylated pACYC184 DNA

Host strain (plasmid)	No. of transformants[a] obtained with:		Ratio of unmethylated to methylated[b]
	Unmethylated pACYC184	Methylated pACYC184	
K802(pUC9-14)	2.2×10^5	1.2×10^4	18.3
K802(pUC9-14d)	7.2×10^3	1.1×10^4	0.7
K802(pUC9-11)	5.1×10^5	5.9×10^5	0.9
K802(pUC9)	5.0×10^5	4.8×10^5	1.0
K802[c]	1.6×10^6	1.8×10^6	0.9
JM107[d]	4.7×10^4	5.9×10^3	8.0

[a] These data have been normalized to reflect the total number of transformants per 0.1 μg of pACYC184 DNA.
[b] The ratio of transformants obtained with unmethylated pACYC184 DNA relative to transformants obtained with methylated pACYC184 DNA.
[c] Mcr B⁻.
[d] Mcr B⁺.

We investigated the possibility that protein X is involved with McrB function by a deletion analysis of pUC9-14. By taking advantage of the *Hin*dIII site within the pUC9 vector, pUC9-14 was digested with *Hin*dIII, resulting in the generation of two fragments upon analysis by agarose gel electrophoresis. The two *Hin*dIII fragments of 1,450 and 6,750 bp were separated by agarose gel electrophoresis, and the 6,750-bp fragment was purified by electroelution into a trough (8). Upon purification and subsequent ligation of the fragment, it was used to transform K802 to Ap^r. Transformants were screened for the presence of plasmid DNA as previously described (7). Restriction analysis with *Hin*dIII showed that the 1,450-bp fragment of DNA bounded by the *Hin*dIII site in the X region and the *Bgl*II site within *hsdS* had been deleted (Fig. 1).

Since the likely open reading frame for polypeptide X begins nearly 200 bp to the left of the *Hin*dIII site in pUC9-14, this deletion derivative, designated pUC9-14d, should no longer produce the X protein (Fig. 1). pUC9-14d was then tested for its ability to restrict methylated DNA, by using the same approach described above. Results indicated that K802(pUC9-14d) was unable to restrict the *Alu*I-methylated pACYC184 DNA, as is shown by the nearly equal numbers of transformants obtained regardless of the state of the transforming DNA (Table 1). We have not determined the reason for the low numbers of total transformants for the strain containing pUC9-14d.

The DNA sequence just upstream of what is likely to be the 5′ terminus of the gene encoding protein X (6) was analyzed by using a promoter search computer program. The program, called TARGSEARCH, locates potential promoter sites on the basis of their homology with the consensus sequence for *E. coli* promoters (11). The TARGSEARCH program indicated that a potential promoter does exist just upstream from the likely 5′ terminus of the gene encoding protein X (data not shown). This putative promoter gave a homology score of 62.7%, which puts it well over the

45% score reported to be the lower limit for effective promoters (11).

The approach was to then remove much of the DNA downstream of what is likely to be the polypeptide X-coding region but retain a small amount of DNA upstream from the potential 5′ terminus of the region. Mapping the *Hin*cII restriction sites within the X region indicated that this could be accomplished by subcloning an 1,100-bp *Hin*cII fragment containing the unique *Hin*dIII site from the insert DNA of pUC9-14. After the digestion of pUC9-14 with *Hin*cII, the DNA was run on a 6% polyacrylamide gel.The appropriate fragment was cut from the gel, minced, and incubated for 24 h at 37°C in 5 ml of 0.2 M NaCl–20 mM Tris hydrochloride (pH 7.5)–1.0 mM EDTA. The DNA was purified as described in instructions for the use of ELUTIP-d columns (Schleicher & Schuell, Inc., Keene, N.H.). The fragment was ligated with *Hin*cII-digested pUC9 and used to transform JM83. Ap^r and Lac^- transformants were selected on MacConkey agar plus ampicillin. Several colonies were screened for the presence of plasmid DNA (7), and that DNA was further analyzed by restriction analysis and agarose gel electrophoresis. Purified plasmid DNA containing the appropriate insert, designated pUC9-11 (Fig. 1), was used to transform strain K802 to Ap^r. K802(pUC9-11) was subsequently tested for its ability to restrict methylated pACYC184 DNA under the conditions already described. K802(pUC9-11) showed no McrB restriction of the *Alu*I-methylated pACYC184 DNA (Table 1). This is evidence that protein X alone is not capable of McrB restriction, unless the coding region for X extends beyond the boundaries of the 1,100-bp *Hin*cII fragment in pUC9-11.

It is unlikely that the 5′ end of the coding region extends upstream from the left-hand *Hin*cII site of pUC9-11, since the only open reading frame was shown by DNA sequence data to begin 700 bp downstream from *hsdS* (6). This location would put the 5′ terminus well to the right of the left-hand *Hin*cII site of pUC9-11. It is also unlikely that the 3′ end of the coding region for X extends beyond the right-hand *Hin*cII site of pUC9-11, since polypeptide X is reported to have a molecular weight of just 16,000 (13). The open reading frame, then, should not consist of more than 400 to 500 bp of DNA. Therefore, if the 5′ terminus of the protein X-coding region, as well as its potential promoter, is downstream of the left-hand *Hin*cII site and the coding region extends only 400 to 500 bp to the right of that site, then the gene encoding protein X should lie well within the *Hin*cII boundaries (Fig. 1).

In summary, our results demonstrate that the McrB system of restriction in *E. coli* K-12 is encoded by a genetic region adjacent to the *hsdS* gene. Assuming that the deduced location of the gene encoding protein X is accurate, our data suggest that X is involved with McrB restriction. If our assumptions are correct, protein X alone is not responsible for the restriction of 5-methylcytosine DNA. However, we have not experimentally eliminated the possibility that pUC9-11 (Fig. 1) contains only a portion of the coding region for X or that the presumptive promoter located in this region is required for expression of a gene downstream of the gene encoding protein X.

This work was supported in part by the Louisiana State University Agricultural Experiment Station.

We thank Eric Achberger for helpful advice throughout this study and for critical review of the manuscript. We also thank Noreen Murray for generously providing the recombinant phage λ1048 and Alan Biel for reviewing the manuscript.

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