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Molecular Cloning of the *mcrBC* (*rglB*) Locus of *Escherichia coli* K12 and Identification of the *mcrBC* Encoded Proteins

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The *mcrBC* (formerly known as *rglB*) locus of *Escherichia coli* K-12 encodes a restriction system which recognizes and cleaves the hmC DNA of T2 and T4 bacteriophages, but hmCT6 is refractory to McrBc action. This locus maps at 98.39 min, on the standard *E. coli* K-12 genetic map, very close to the *hsdS* gene of the *hsdRMS* cluster. In this paper, we report the cloning of the *mcrBC* locus, localisation of the coding region by Tn5 mutagenesis and identification of its protein products. *mcrBC* locus have been localised on a 5.35kb *Bgl*II *Eco*RI fragment and the minimal region needed for RglB activity is a 2.6kb *Hpa*I-*Stu*I fragment. The *mcrBC* is apparently an operon in which a single promoter transcribes all the genes as a single transcriptional unit. Maxicell system and T7 expression system revealed that the *mcrBC* cluster encodes four polypeptides of molecular weights 51kDa, 39kDa, 33kDa and 16kDa and all these four proteins are needed for RglB activity.

Key Words: hmC DNA, Restriction, Cloning *mcrBC* locus, McrBC proteins, rgl locus, RglB activity

Introduction

One of the unique features of the DNAs of bacteriophages T2, T4 and T6 is the hydroxymethylation of their cytosine residues (Wyatt & Cohen 1952). Phage encoded hydroxymethylase converts the cellular dCTP into hydroxy methylcytosine [(hmC) (Flaks & Cohen 1957)] and these modified nucleotides are the sole precursors in phage infected cells since the dCTPase encoded by the phage genome degrades remaining dCTP. In wild type phage infected cells, the hmC residues in the DNA are glucosylated by the phage encoded α and β glucosyl transferases (Lehman & Pratt 1960). Uridine diphosphoglucose (UDPG) is used as substrate in this reaction and consequently absence of either UDPG or glucosyl transferases leads to the production

Dedicated to the memory of S E Luria, who discovered the Rgl restriction systems and has the honour of the first scientist to discover the restriction systems.

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of hmC T-even phages (Hattman & Fukasawa 1963, Revel & Luria 1970).

RglA gene mapping at 26.24min, and *rglB* gene mapping at 98.39min, in the *E. coli* linkage map (Ravi et al. 1985), encode the two restriction systems that cleave specifically hmC DNA (Revel 1967). The cleavage of DNA catalysed by these enzymes occurs at a limited number of sites and the cleaved DNA is usually degraded non specifically by host exonuclease V (Dharmalingam & Goldberg 1976) encoded by the *recBCD* genes. Glucosylated hmC DNA and cytosine DNA are refractory to the cleavage by the Rgl restriction systems, and hence wild type T-even phage and *E. coli* DNA are insensitive to the above enzymes.

Recent experiments showed (Raleigh & Wilson 1986) that the *rglA* and *rglB* encoded polypetides restrict methyl cytosine containing DNA as well (Mcr activity). DNA modified by the methylases, *Pvu*II.M, *Hae* III.M, *Hae*II.M and *Mspl*.M, are restricted by the *rglB* and the DNA modified *Hpa*II.M is restricted by the *rglA*. Based on this additional property, *rglA* and *rglB* loci were also called as *mcr*A and *mcr*B respectively (Raleigh et al. 1989). All the workers in this field recently agreed to the nomenclature *mcrA* and *mcrBC* (Raleigh et al. 1991), replacing the older nomenclature. However, in this paper the Rgl activity will be mentioned whenever restriction of *hmC DNA is described.*

In this connection we report the physical and genetic characterisation of the *mcrBC* locus as well as the identification of proteins encoded by this locus. Our results show that the *mcrBC* locus encodes four proteins. When this work was underway there were two reports describing the characterisation of *mcrBC* locus and its protein products using different approaches. (Ross et al. 1989, Dila et al. 1990). By and large these results support our data and our experiments also bring to light observations not reported earlier. Further we used both maxicell system and T7-promoter expression system to confirm the proteins encoded by the *mcrBC* locus.

Materials and Methods

Bacteria, bacteriophages and plasmids used in this study are listed in table 1. Media and buffers used are described elsewhere (Miller 1978). Routine microbial genetic techniques used were described by Miller (1978).

DNA Manipulations and Plasmid Constructions

Plasmid DNA was prepared using a modified procedure (Dharmalingam 1986) of Kieser's mini preparation method (Kieser 1984). Restriction enzyme digestion and ligation were done as described by the supplier. Transformation, of *E. coli* has been described already (Dagert & Ehrlich 1979). Antibiotics were used at the following concentrations in L-plates, ampicillin 50μg/ml, Kanamycin 50μg/ml and trimethoprim 75μg/ml.

Tn5 Mutagenesis of the Plasmid DNA and Mapping of Tn5 Insertions

Mutagenesis of cloned genes in pDSS17 with Tn5 was done essentially as described by deBruijn and Lupski (1984) using λ421 (λb221, rex::Tn5, *c*1857, O^{am29} p^{am80}). The insertions were mapped by measuring the length of the fragments produced by *Hind*III digestion. There are two *Hind*III sites equidistantly placed; 1.25kb from the ends of the inverted repeats of Tn5. There is an unique *Hind*III site in pDSS17. Three fragments were produced upon digestion of pDSS17::Tn5 with *Hind*III. Of these the 3.42kb fragment is Tn5 specific and is common to all the insertions. The other two fragments are variable in size depending on the site of insertion of the transposon. The result with *Hind*III digestion was confirmed

Table 1 *Bacterial strains, bacteriophages and plasmids*

Strain	Description	Reference or Source
Bactrial strains		
HR112	*rglA2, rglB8*	Revel H R
KD101	Δ *lac-Pro, rglA, rglB*$^+$, *Su*$^-$ *hsdR*$^+$, *hsdM*$^+$	Lab collection
DSS221	KD101, *hsdR*: :MudI 1734, *mcrBC*$^+$	Sozhamannan and Dharmalingam (1988)
DSS2215	DSS221 *hsdR*: :pSG1, *lac*$^+$	" "
DSS101	KD101 *mcrBC*: : Tn10	Ravi et al. (1985)
DS1012	DSS101 Δ*mcrBC*	Sozhamannan and Dharmalingam (1988)
CSR603	*thr*-1, *leuB6*, *proA2*, *phr*-1 *recA*1, *argE*3, *thi*-1, *uvrA6*, *ara*-14, *lacY*1, *galK2*, *xyl*-5, *mtl*-1, *gyrA*98, (*nalA*98) *rpsL*31 *tsk*-33, *SupE*44	Bachmann B. CGSC↑5830
Phage strains		
hmCT4	∝gt57, β gt14	Revel HR
λVir		Lab collection
λpGS1	λpl (209) *lacY*: : Tn9	Weinstock G M
Plasmids		
pPO1734	Mini Mu-MudI 1734	Casadaban M *via* Hughes K T
pUC9		Vieira and Messing (1982)
pUC18		Yanisch-Perron et al (1985)
pBR322		Lab Collection
pBg6		Sain and Murry (1980)
pBGS9		Spratt B G
pT7-5		Tabor and Richardson (1985)
pGP1-2		" "
pMU575		Yang and Pittard (1987)
pRAD778		Raja and Dharmalingam (1991)
pDSS17		
pRAD188		
pRAD191		
pRAD752		This study
pRAD753		
pRAD754		
pRAD755		

by digestion with *Sal*I and double digestion with *Eco*RI and *Hind*III.

Identification of Plasmid Encoded Polypeptides

Plasmid encoded proteins were identified using the maxicell system essentially as described by Sancar et al. (1979) except that glycine (5% final concentration) was used instead of D-cyloserine.

T7 promoter expression system was described already (Tabor & Richardson 1985). In brief, the T7 RNA polymerase encoded by T7 *gene* 1, regulated by lambda P_L promoter was provided by pGP1-2. This plasmid is a P15A replicon and P_L is repressed at 30°C by the temperature sensitive lambda repressor *C*I857. Various constructs were cloned in the multiple cloning site of pT7-5 (a ColE1 replicon) downstream of the T7 *gene* 10 promoter. These two plasmids were introduced into HR112 strain by transformation. Labelling of proteins in T7 expression clones was done as described by Ramalingam et al. (1992). Proteins were separated on 12% SDS-polyacrylamide gels as described already (Laemmli & Favre 1973, Dharmalingam & Goldberg 1976). Labelled proteins were detected by Phosphor Imager (Molecular Dynamics, USA). Dried gels were exposed to the screen for 5hrs and the exposed screens were scanned as described by the manufacturer.

Results

Fine Structure Map Order of mcr BC Gene(s) in the hsd region

McrBC locus was cloned *in vivo* on to lambda pSG1 as follows. Lambda pSG1 was first integrated very close to *mcrBC* utilizing the homology provided by a MudI 1734 inserted previously into *hsdR* gene which is adjacent to *mcrBC* locus. The actual structure of the prophage in the above strain (DSS2215) was deduced by various genetic tests (figure 1 A) and this was described already (Sozhamannan & Dharmalingam 1988a). UV irradiation of DSS2215 led to the production of lambda d*mcrBC*$^+$ transducing particles (figure 1 B2) which also carried *hsdR* and *hsdM* genes along with Kan and Cam resistance genes of lambda pSG. This result indicates that *mcrBC* lies next to *hsdS* and the sequence of genes in this region is *hsdR-hsdM-hsdS-mcrBC*. If *mcrBC* is next to *hsdR* then one would obtain *lac*$^-$ defective transducing particles (figure. 1 B1). However, we could not detect such defective particles. This results clearly proved that *mcrBC* locus lies next to *hsdS* region.

Molecular Cloning of mcrBC Locus

We subscloned the *mcrBC* gene from a recombinant (vector pBR322) plasmid (pBg6) which was shown earlier (Sain & Murray 1980) to carry part of *hsdS* gene but not *hsdM* and *hsdR* genes. Since our results (see above) showed that *mcrBC* is close to *hsdS* and the insert size in pBg6 is 10kb, we presumed the *mcrBC* should be present on pBg6. To test this, pBg6 was transformed into DSS1012 and amp^r transformants were selected. All the 32 amp^R transformants checked showed RglB$^+$ phenotype indicating the presence of *mcrBC* gene in pBg6. The efficiency of plating (e.o.p) of hmCT4 phage on one typical isolate was 4×10^{-5} plaque forming units (pfu)/ml when compared to its e.o.p on permissive strains. This result taken together with the genetic evidence obtained with *mcrBC*$^+$ transducing phages confirmed the order of genes in the hsd region to be *hsdR-hsdM-hsdS-mcrBC*.

Subcloning of the mcrBC Gene

Digestion of pBg6 with *Eco*RI yielded, three fragments, of sizes 9.34kb, 4.07kb and 0.9kb. Of these three, the largest fragment carried the 5.35kb bacterial sequence and most of the pBR322 sequence. In order to see whether this 5.35kb bacterial DNA carries the *mcrBC*

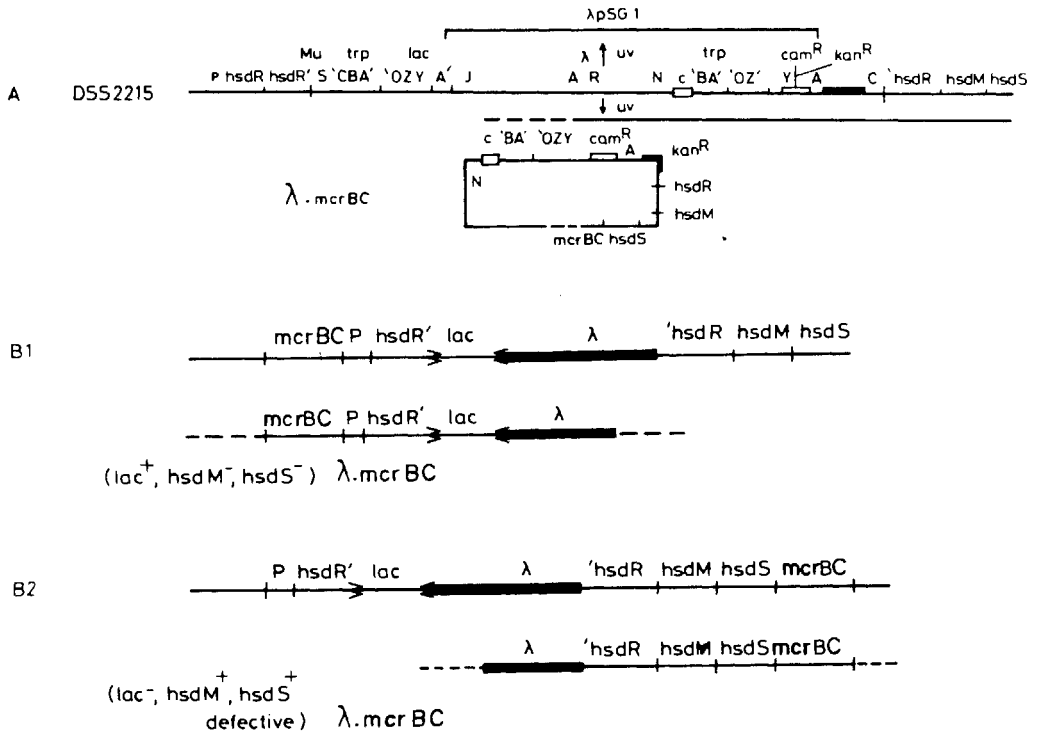

Figure 1 *McrBC* locus is linked to *hsdS* and not hsdR. Prophage structure in DSS2215 was determined earlier (Sozhamannan & Dharmalingam 1988a). The lambda d*mcrBC*$^+$ particles could arise out of the excision event demonstrated schematically in I B2 only if it is linked to *hsdS*. Packaging limit precluded many phage genes and hence these are defective particles. These particles also carry the *hsdM* and *hsdS* genes along with Kan^r and cam^r genes

sequence, pBg6 DNA was cleaved with *Eco*RI and the digested DNA was used for transforming DSS1012 (*mcrBC*$^-$) without *in vitro* ligation. *In vivo* ligation of the fragment containing the pBR322 replicon alone would replicate, and hence amp^R transformants must contain the 5.35kb bacterial sequence. Of the eight amp^R transformants seven harboured the expected size recombinant plasmid and these transformants were also Rgl$^+$ since hmcT4 plated at efficiencies less than 10^{-5} pfu/ml. Plasmid (pDSS17) from one of these transformants was taken for further analysis. This plasmid has a single *Eco*RI site, and is 9.34kb in size. The two similar *Eco*RI fragments (4.07kb & 0.9kb) were missing in pDSS17 in contrast to pBg6 (figure 2).

Further shortening of pDSS17, by subcloning the 3.9kb *Hin*dIII-*Eco*RI fragment in pUC9, (pDSS227) showed that, the 1.45kb fragment extending from the *Bgl*II/*Bam*HI hybrid site to *Hin*dIII is essential for RglB activity (figure 2) since transformants carrying pDSS227 failed to restrict hmCT4.

*Tn*5 *Mutagenesis of pDSS*17

In order to localize the segment encoding the McrBC protein(s), pDSS17 was mutagene-

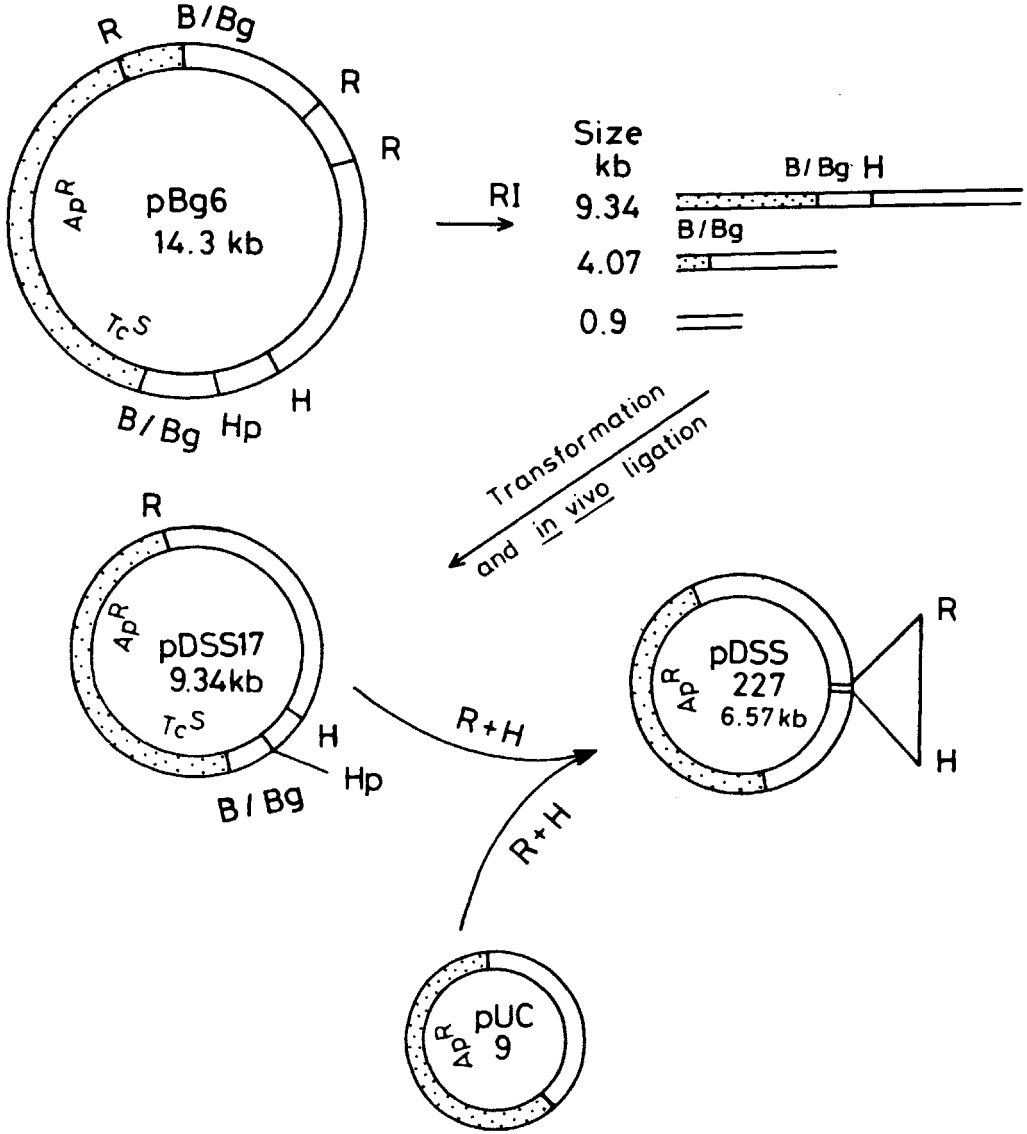

Figure 2 Construction of pDSS17 and pDSS227. The plasmid pBg6 was cleaved with *Eco*RI and the cleaved DNA was used for transformation without ligation. Plasmids from amplicillin resistance colonies were isolated and their restriction map confirmed. pDSS227 was constructed by ligating the 3.9kb *Eco*R1-*Hind*III fragment of pDSS17 into the *Eco*RI-*Hind*III cleaved pUC9 plasmid

nized with Tn5. More than 70% (143/199) of the Tn5 insertions in the insert DNA inactivated the RglB activity (figure 3). If the Tn5 insertions were random, and the frequency of insertions is proportional to the length of the target DNA then this result would imply that a large portion of the insert DNA (about 3.5kb) encodes functions essential for RglB activity. It is likely that McrBC protein is unusually large, or the *mcrBC* locus might encode more than one polypetides. The peptide seen in both tracks is the beta lactamase protein encoded by the vector.

Maxicell experiments clearly showed (Sozhamannan & Dharmalingam 1988b) the production of more than one polypetide by the recombinant plasmids carrying the insert. In order to verify this observation and to confirm the sizes of the proteins encoded by the insert we used T7 promoter expression system. Figure 5A shows the T7 expression

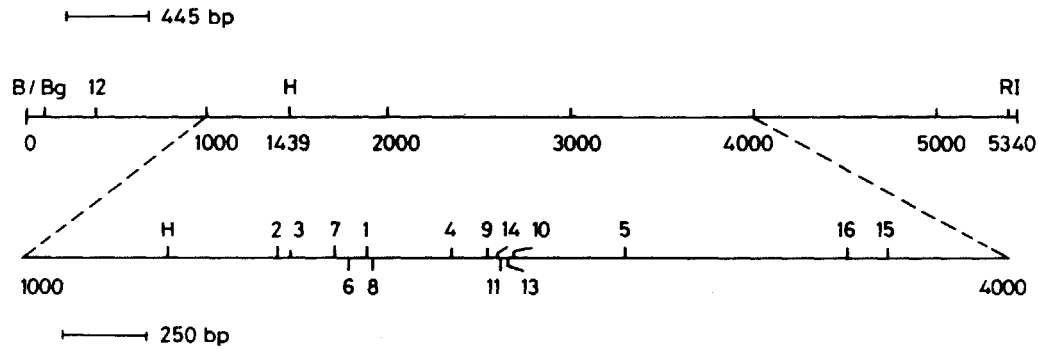

Figure 3 Tn5 mutagenesis of pDSS17. Transposition was performed as described under materials and methods. Details of the map location of each Tn5 insertion is described under materials and methods.

polypetide. Figure 3 also shows the map location of the selected Tn5 insertions which inactivated the RglB activity.

Identification of Proteins Encoded by the mcrBC Locus

Two different systems were used to identify the polypetides encoded by the *mcrBC* locus. Maxicell system was used to identify the proteins encoded by the various plasmid constructs and expressed from the native promoters. The *mcrBC*$^+$ plasmid pBg6 encoded four polypeptides of molecular weights 51kDa, 46kDa, 33kDa and 16kDa (figure 4). pDSS227 which lacks sequences upstream of *Hind*III site did not encode any of these polypetides (lane 2) indicating the presence of a common promoter for all these system we used, figure 5B shows the sequential derivation of pRAD752 from pDSS17 and figure 5C shows the constructs utilized in this study. Transposon insertion experiment described earlier and the experiments of others (Ross et al, 1989, Dila et al. 1990) showed that the region between *Hpa*I and *Stu*I restriction sites is the minimal size needed for encoding the RglB and McrBC functions. Results in figure 6 show that all the RglB$^+$ constructs, including pRAD755 which carries only the *Hpa*I-*Stu*I region, encode four polypetides of sizes 51, 39, 33 and 16KDa. This result confirms the maxicell experiment. However, in T7 expression system a 39 KDa polypetide is detected and the 46kDa protein observed in the maxicell system was not produced.

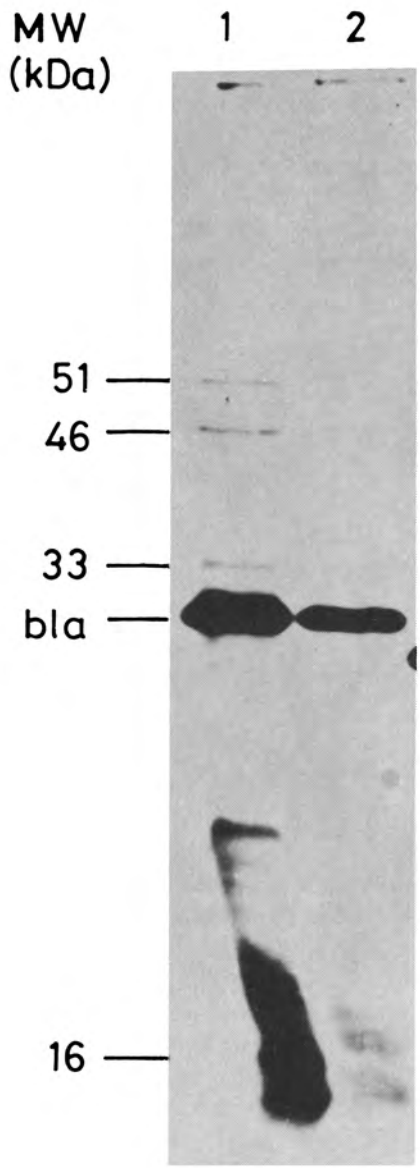

Figure 4 Analysis of proteins encoded by *mcrBC* genes in maxicells. CSR603 carrying pBg6, or pDSS227 were used for the analysis of proteins in 12% SDS-polyacrylamide gels. Track-1, CSR603 (pBg6); track-2, CSR603 (pDSS227)

Additional experiments are needed to clarify this discrepancy. Introduction of a site specific mutation (leading to *mcrBC* minus phenotype) by filling the *Hind*III site (pRAD753) abolished the producion of 51kDa protein indicating that this protein is the first one immediately downstream of the *Hpa*I site. DNA inserted in the opposite orientation failed to encode any polypetide (data not shown).

Discussion

The *mcrBC* locus encoding the polypetides responsible for cleaving hmCT4 DNA has been cloned as a 5.4kb DNA fragment. Fine structure mapping allowed us to locate the *mcrBC* locus next to the *hsdS* gene. Since the *hsdS* region is more than 10kb in length, the above data allowed us to presume that pBg6 which carries 10kb insert but lacking *hsdR* and *hsdM* might carry the *mcrBC* gene. This prediction turned out to be correct.

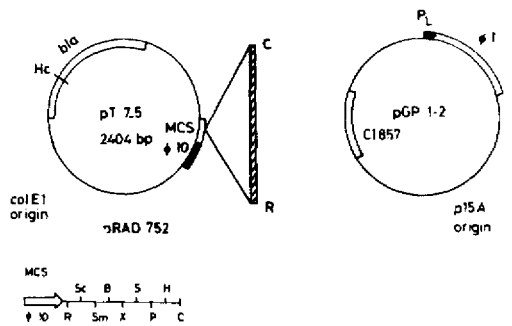

Figure 5A Two plasmid T7 promoter expression system used in this study. pGP1-2 supplied the RNA polymerase of T7 at 42°C since the T7 *gene* 1 is under the control of pL regulated by the temperature sensitive *CI857* lambda repressor. pT7-5 is used in these experiments since in the earlier constructs by Tabor and Richardson the beta lactamase gene was also transcribed by the T7 *gene* 10 promoter. In pT7-5 the beta lactamase gene is in opposite orientation to *gene* 10 promoter and hence only the insert is transcribed at 42°C

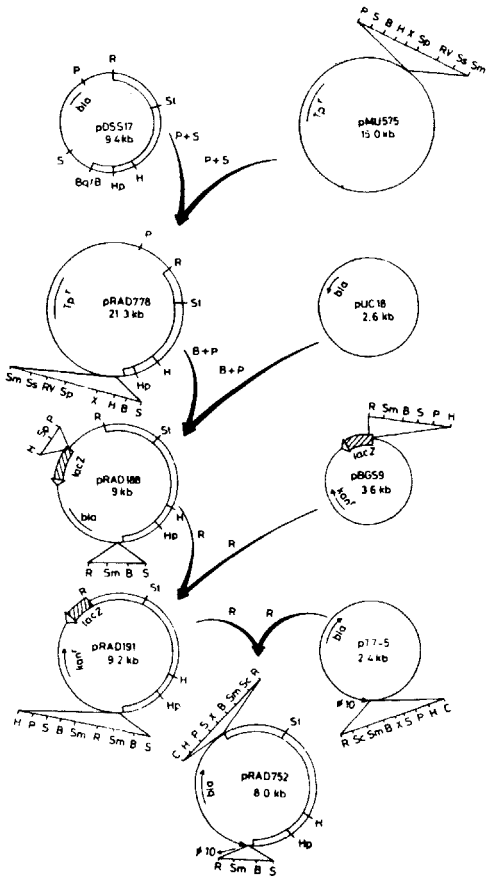

Figure 5B The expression constructs used in this study. pDSS17 (see figure 2) is the starting plasmid and pRAD778 was derived from this as described already (Raja & Dharmalingam 1991). pRAD778 has a copy number of one and its *Pst*I-*Bam*HI fragment was transferred to *Pst*I-*Bam*HI cleaved pUC18. The resulting plasmid pRAD188 was cleaved with *Eco*R1 and this fragment was cloned on to pBGS9, a ColE1 replicon vector, which encodes kanamycin resistance. pRAD191 is the recombinant clone with *Eco*R1 insert. The kan^r property of pRAD191 allowed easy selection of pT7-5 clones carrying the inserts from pRAD191. The *Eco*R1 fragment from pRAD191 was ligated to pT7-5 and the amp^R colonies were selected, and checked for kan^s. These were then tested for RglB$^+$ phenotype. In pRAD753 *mcrBC* is present in the right orientation with T7 RNA polymerase promoter

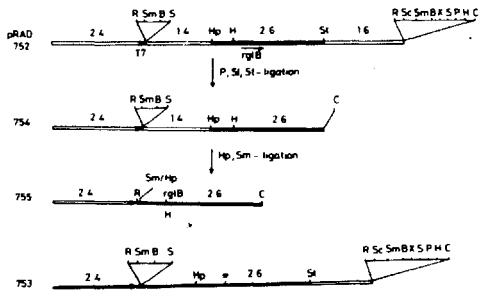

Figure 5C pRAD753 was constructed by filling the *Hind*III site of the partially digested pRAD752, and this blunt end molecule was self ligated. pRAD754 was *constructed by cleaving the pRAD752 with PstI* and the single stranded region was removed by S1 nuclease treatment and then the DNA was cleaved with *Stu*I. The blunt ends were self ligated. pRAD755 was constructed by deleting the *Hpa*I-*Sma*I fragment by restriction enzyme cleavages followed by self ligation. All these clones except pRAD753 are RglB$^+$. Restriction enzyme symbols used are, B-*Bam*HI, C-*cla*I; Hp-*Hpa*I; H-*Hind*III; P-*Pst*I; R-*Eco*RI; S-*Sal*I; X-*Xho*I; RV-*Eco*RV; Ss-*Sst*I; Sp-*Sph*I

Maxicell experiments and T7 promoter expression system revealed that this fragment encodes four polypeptides of molecular weights 51kDa, 39kDa (46kDa in maxicell), 33kDa and 16kDa. Subcloning experiment showed that the deletion of the "region" upstream of the *Hind*III, abolishes the synthesis of all the four proteins in maxicell experiments. This implies that all the four proteins are synthesized from a native promoter localized upstream of the *Hind*III site. This resembles the *hsd* operon in which three polypetides *hsdS*, *hsdM* and *hsdR* are transcribed from a single promoter. T7 promoter expression system confirms the production of four polypetides from the *Hpa*I-*Stu*I fragment. The 39 kDa protein seen in T7 expression system and the 46kDa protein detected in the maxicell system could be the same protein. Perhaps the 46 kDa

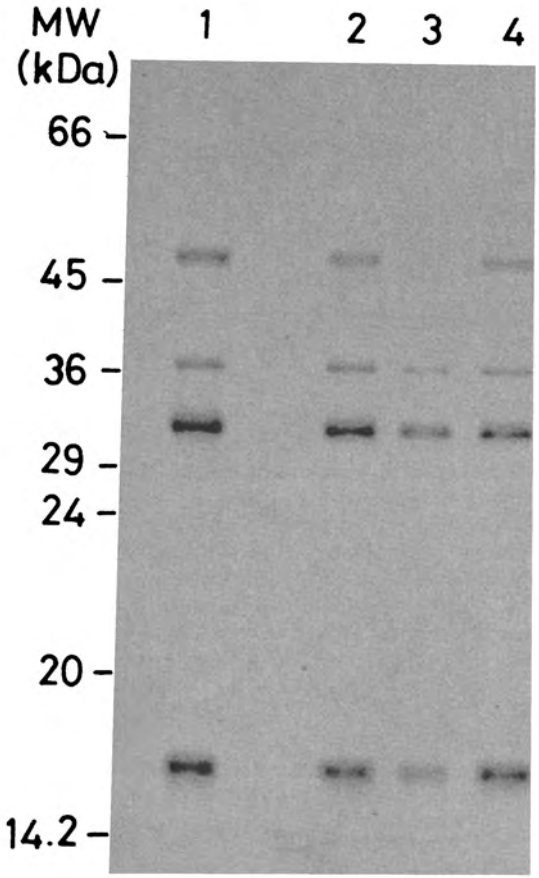

Figure 6 Protein products from T7 expression constructs. Proteins were labelled with ^{35}S-methionine as described under materials and methods. Labelled proteins were separated on a 12% acrylamide gel. Track 1. pRAD752; track 2, pRAD754; track 3, pRAD753 and track 4, pRAD755. Molecular weight of the proteins (calculated from the standard) is marked on the left

peptide observed in maxicells is produced from an upstream start codon. Reports from other laboratories using coupled *in vitro* transcription and translation also showed multiple protein bands (Dila et al. 1990). Our report confirms the production of multiple proteins from *mcrBC* locus. However, the 16kDa protein was not reported by other investigators. Further we clearly demonstrated here that there is only one promoter which is usable *in vivo* since pDSS227 failed to produce any protein in maxicell system. Computer search for reading frames from the published sequence (Dila et al. 1990) revealed two proteins of 53.7kDa and 41kDa. However, the promoter sequence resembling the *E. coli* consensus sequence could not be detected. When we used the translational start sites (SD sequence and ATG) to identify the likely ORFs we could determine from the *mcrBC* sequence two more shorter peptides from the C-terminal regions of the two large ORFs. Absence of a typical *E. coli* promoter structure implies that this segment of DNA may be a recent acquisition from some other source as predicted by Dila et al. (1990) based on the low GC content of this locus. Tn5 insertions at different locations indicated that all the four peptides are required for RglB activity. However some uncertainity arose when these insertions were used for detecting the proteins in maxicell system. This could be due to the complication created by the Tn5 encoded polypetides which comigrated with McrBC proteins. Site specific mutations using either point mutations or by cassetts and analysis of these mutant clones in T7 promoter expression system could solve this problem.

Experiments are underway to analyse the transcripts from the various cloned inserts. This will allow us to study the regulations of production of the multiple proteins. The role of the individual proteins in Rgl and Mcr activities is also being persued.

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