

EcoA: The First Member of a New Family of Type I Restriction Modification Systems

Gene Organization and Enzymatic Activities

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The characterization of the *EcoA* restriction-modification enzymes from *Escherichia coli* 15T⁻ is described. The reactions catalysed by these enzymes are very similar to those catalysed by the classical type I restriction and modification enzymes, a family of genetically related proteins. The detailed mechanisms, particularly for DNA modification, differ. The genetic and transcriptional organizations are also very similar to those of the classical systems, despite the fact that *EcoA* is not allelic to the others. We demonstrate that the expression of the *EcoA* genes is controlled following conjugative transfer to other strains in such a way that no lethality is observed, probably because the recipient chromosome is completely modified before restriction activity is expressed.

1. Introduction

The type I restriction and modification (R-M) systems of the Enterobacteriaceae are by far the most complex of all the R-M systems discovered to date (for reviews, see Yuan, 1981; Bickle, 1982). The type I restriction enzymes from the *Escherichia coli* strains K12 and B, *EcoK* and *EcoB*, have been purified and extensively characterized. Both contain three non-identical subunits of M_r 135,000, 60,000 and 50,000 (Meselson *et al.*, 1972; Eskin & Linn, 1972), which are coded by three contiguous genes on the *E. coli* chromosome: *hsdR*, *hsdM* and *hsdS* (Boyer & Roulland-Dussoix, 1969; Glover & Colson, 1969; Hubacek & Glover, 1970). Modification methylases have been purified from these strains. They contain the products of the *hsdM* and *hsdS* genes only (Lautenberger & Linn, 1972; Suri *et al.*, 1984a). The genes for the *E. coli* K12 and B systems as well as those encoding the *Salmonella* SB and SP systems map at the same position on the chromosome; near and counter-clockwise to *serB* (Boyer, 1964; Glover & Colson, 1969; Colson & Van Pel, 1974; Bullas & Colson, 1975).

Several lines of evidence indicate that all of the type I R-M systems mentioned so far are closely related; for example, mutations in one system can be complemented by healthy alleles from another (Boyer & Roulland-Dussoix, 1969; Van Pel & Colson, 1974; Bullas *et al.*, 1980). Moreover,

antibodies prepared against *EcoK* cross-reacted with the enzymes from other strains (Murray *et al.*, 1982) and DNA hybridization experiments and comparative nucleotide sequencing studies confirmed these similarities (Sain & Murray, 1980; Murray *et al.*, 1982; Gough & Murray, 1983). The order of the *EcoK* genes has been established as *hsdR*, *hsdM*, *hsdS*. The genes are organized into two transcriptional units; one promoter directs the transcription of the *hsdR* gene, and a separate promoter upstream from *hsdM* serves both *hsdM* and *hsdS*; both promoters read in the same direction (Sain & Murray, 1980).

We have described the purification of the enzymes from a R-M system of *E. coli* 15T⁻, *EcoA* (Suri *et al.*, 1984b). This system was thought to be type I because it maps to the same place on the chromosome as the other type I R-M systems (although linkage relationships are somewhat different from those of the other systems; Arber & Wauters-Willems, 1970), and because it shows the same response to methionine starvation as the better studied systems (Lark & Arber, 1970). Cells containing type I systems survive the starvation because *S*-adenosylmethionine is required for restriction as well as for modification; other R-M systems do not require AdoMet† for restriction and

† Abbreviations used: AdoMet, *S*-adenosylmethionine; kb, 10³ bases or base-pairs.

begin to attack their own chromosomes once DNA replication has generated unmodified recognition sites. Despite these similarities to the other type I R-M systems, we could find no cross-reaction with *EcoK* antisera and no cross-hybridization of the chromosomal DNA with *EcoK*-specific probes was found (Murray *et al.*, 1982). Purification of the *EcoA* activities led to the isolation of two protein species. One of them was a single polypeptide of M_r 98,000 and the other contained two subunits of M_r 64,000 and 55,000. The two-subunit enzyme turned out to be an *EcoA*-specific modification methylase. The other polypeptide alone showed no enzymatic activities but was required, together with the modification methylase, for restriction activity (Suri *et al.*, 1984b; and this paper).

The three-subunit nature of the enzyme, even though the sizes of the subunits are different from those of the other enzymes, points to a relationship with the classical type I enzymes, as does the punctuated nature of the *EcoA* DNA recognition sequence (5'-G-A-G-(N₇)-G-T-C-A-3'; Suri *et al.*, 1984b). In this paper, we describe the genetic and transcriptional organization of the *EcoA* genes and describe some features of the *EcoA* restriction and modification reaction mechanisms. These results are compared, and sometimes contrasted, to the classical type I systems. In addition, we show that when the *EcoA* genes are transferred from one cell to another by conjugation, the expression of the gene for the subunit necessary for restriction is controlled in such a way that the modification methylase can modify the recipient chromosome before active restriction enzyme is made.

2. Materials and Methods

(a) Bacterial and phage strains

E. coli WA2379 (Arber & Wauters-Willems, 1970), a Res_A^+ Mod_A^+ strain, and *E. coli* WA921, a strain lacking a R-M system (Wood, 1966), were used to determine the R-M phenotypes of the deletion mutants. WA921 was also the host used for transformations. WA2677, a Res_A^- Mod_A^+ carrying an F' with a wild-type copy of the *EcoA* genes was obtained from W. Arber and was used as the donor in the conjugation experiments.

The phage λ hsdA Δ 6, an *att⁻ red⁻ gam⁺ cI* vector carrying the *EcoA* genes, was constructed and kindly provided by F. V. Fuller-Pace (Fuller-Pace *et al.*, 1985). λ vir (Jacob & Wollman, 1954) was used for checking R-M phenotypes as described by Colson *et al.* (1965).

(b) Nucleic acid procedures

Phage λ hsdA Δ 6 DNA was extracted with phenol from phage particles that had been purified by CsCl gradient centrifugation (Yamamoto *et al.*, 1970). Plasmid DNA was isolated as described by Birnboim & Doly (1979) and was for some experiments further purified by CsCl/ethidium bromide equilibrium centrifugation (Clewell & Helinski, 1969). Commercially available nucleic acid enzymes were purchased from either New England Biolabs or Boehringer-Mannheim. DNA fragments were separated on agarose gels at concentrations between 0.7% and 1.5% (w/v) or on 3.5% (w/v) polyacrylamide

gels. Fragments were visualized under ultraviolet light illumination after staining with ethidium bromide.

(c) Enzyme and antibody preparations

The purification of both the *EcoK* enzymes and the *EcoA* restriction enzyme and modification methylase have been described (Murray *et al.*, 1982; Suri *et al.*, 1984a,b). Antisera against the purified proteins were raised in rabbits. A total of 150 to 200 μ g of protein in Freund's complete adjuvant was injected intradermally at 8 to 10 sites and the animals were boosted 3 weeks later with half the amount of protein in Freund's incomplete adjuvant.

(d) Immune blotting

EcoA-related proteins in whole cell extracts were analysed by the immune blotting technique described by Towbin *et al.* (1979) with the modifications detailed elsewhere (Murray *et al.*, 1982). Detection of antigen was with ¹²⁵I-labelled protein A followed by autoradiography.

(e) Endonuclease and ATPase assays

All reactions contained 100 mM-HEPES (4-(2'-hydroxyethyl)-1-piperazine-ethanesulphonic acid) (pH 7.4), 6.6 mM-MgCl₂, 14 mM-2-mercaptoethanol, 0.25 mM-EDTA, 1 mM-spermidine, 3.6 μ M-AdoMet and 6% (w/v) polyethylene glycol. ATP, [³H]ATP (Amersham; 26 Ci/mmol), DNA and enzyme were added at the concentrations given in the text and Figure legends. The reaction mixtures were prewarmed at 37°C for 5 min before the reaction was initiated by the addition of enzyme. This consisted of a mixture of the modification methylase and the restriction subunit at an approximate molar ratio of 1:2. Endonuclease activity was monitored by agarose gel electrophoresis. ATPase activity was measured by separating and counting [³H]ATP and [³H]ADP by chromatography on polyethyleneimine (PEI) thin-layer plates as described by Hadi *et al.* (1975).

(f) Modification methylase assay

The reactions contained 100 mM-HEPES (pH 7.4 for *EcoA*, pH 6.7 for *EcoK*), 14 mM-2-mercaptoethanol, 6.6 mM-MgCl₂, 0.25 mM-EDTA and 3.6 μ M of [methyl-³H]AdoMet (Amersham; 70 Ci/mmol). When present, ATP was at a concentration of 1 mM. The concentrations of linearized plasmid DNA and of enzymes are given in the Figure legends. The reactions were preheated to 37°C for 10 min before adding enzyme. Samples of 25 μ l were removed at the indicated times, the reaction was stopped by the addition of phenol and the DNA was separated from low molecular weight radioactive material by chromatography on small columns of Biogel A 0.5 m. The DNA-containing fractions were counted in Instagel (Packard) at an efficiency of 50%.

Heteroduplex DNA was prepared from modified and non-modified plasmids (pBR322 or pSHI44). The plasmids were linearized by cleavage with *SalI* and then equal amounts of modified and non-modified DNA was mixed and sealed into a glass capillary. This was heated to 100°C to melt the DNA and then the temperature was allowed to fall to 55°C over 90 to 120 min to promote reannealing.

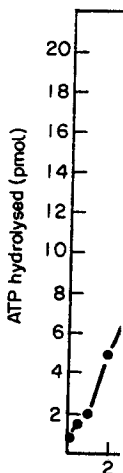
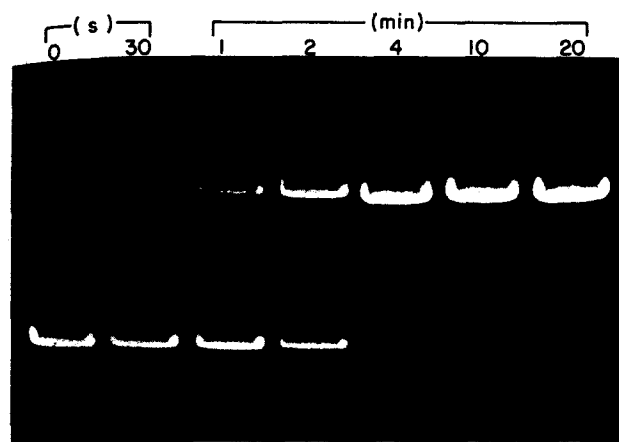
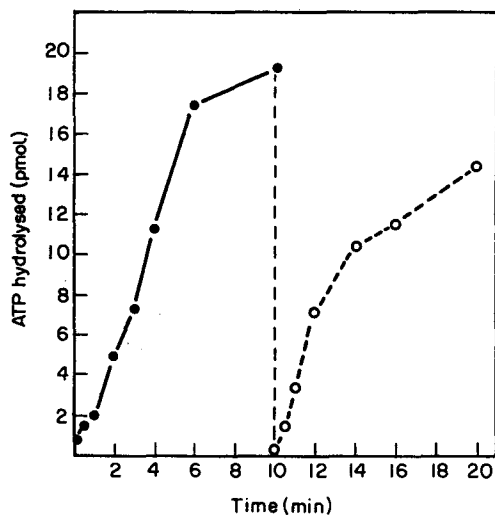


Figure 1. Enzyme activities. (a) Photograph of an agarose gel showing the reactions with 100 mM-HEPES (pH 7.4) and 1 mM-2-mercaptoethanol, 6.6 mM-MgCl₂, 0.25 mM-EDTA and 3.6 μ M of [methyl-³H]AdoMet (Amersham; 70 Ci/mmol) present at 1 mM. The reactions were preheated to 37°C for 10 min before adding enzyme. Samples of 25 μ l were removed at the indicated times, the reaction was stopped by the addition of phenol and the DNA was separated from low molecular weight radioactive material by chromatography on small columns of Biogel A 0.5 m. The DNA-containing fractions were counted in Instagel (Packard) at an efficiency of 50%. (b) ATP hydrolysed (pmol) versus time (s). The reactions were preheated to 37°C for 10 min before adding enzyme. Samples of 25 μ l were removed at the indicated times, the reaction was stopped by the addition of phenol and the rest of the reaction mixture was separated from low molecular weight radioactive material by chromatography on small columns of Biogel A 0.5 m. The DNA-containing fractions were counted in Instagel (Packard) at an efficiency of 50%. (c) ATPase activity was measured by separating and counting [³H]ATP and [³H]ADP by chromatography on polyethyleneimine (PEI) thin-layer plates as described by Hadi *et al.* (1975).



(a)



(b)

Figure 1. Endonuclease and ATPase activities of *EcoA*. The reactions were set up as described in Materials and Methods with unmodified plasmid pSHI44 DNA at a concentration of 30 $\mu\text{g}/\text{ml}$. Non-radioactive ATP was present at 1 mM and ^3H -labelled ATP (36 Ci/mmol) was present at 0.2 μM . The reaction was started by adding the enzyme to a concentration of approx. 15 $\mu\text{g}/\text{ml}$. At the indicated times, 1 μl portions were removed and spotted onto PEI thin-layer chromatography plates to determine the amount of ATP hydrolysed according to Hadi *et al.* (1975), or 20 μl portions were removed and mixed with 5 μl of phenol. Portions (1 μl) of these samples were spotted on PEI plates for the analysis of ATP hydrolysis and the rest of the samples were analysed by agarose gel electrophoresis. After 10 min, non-radioactive and ^3H -labelled ATP in the same ratio as described above were added to increase the ATP concentration to 1 mM and the reaction was monitored for a further 10 min. (a) Photograph of the ethidium bromide-stained agarose gel. The lower band is supercoiled DNA, the intermediate band is linear DNA and the top band is circular nicked DNA. (b) ATP hydrolysed in pmol/20 μl of reaction mix. (●), Initial addition of 20 pmol ATP/20 μl ; (○), second addition of 20 pmol ATP/20 μl .

3. Results

(a) Endonuclease and ATPase activity

We used the plasmid pSHI44 (Marcoli *et al.*, 1980) to investigate the cleavage characteristics of *EcoA* because it contains a single site for the enzyme (Suri *et al.*, 1984b). All three subunits of the enzyme are necessary and the reaction has an absolute requirement for Mg^{2+} , ATP and AdoMet for DNA cleavage. The reaction is greatly stimulated by the presence of spermidine (1 mM) and polyethylene glycol (6%). Figure 1(a) shows a restriction assay with supercoiled pSHI44. The reaction is essentially complete after ten minutes of incubation. All of these features of the reaction are the same as those found for the classical type I enzymes such as *EcoK* (Yuan, 1981; Bickle, 1982). As with these enzymes (Eskin & Linn, 1972; Yuan *et al.*, 1972), cleavage by *EcoA* is accompanied by ATPase activity. Figure 1(b) shows the kinetics of ATP hydrolysis measured during the cleavage reaction described in Figure 1(a); after ten minutes of incubation, almost all of the 20 pmol of ATP present per timepoint (20 μl) were hydrolysed. Although DNA cleavage was completed after ten minutes, fresh ATP added at this time was still hydrolysed, clearly showing that ATP hydrolysis continues after DNA cleavage has terminated. The 18 pmol of ATP hydrolysed during the first six minutes of the reaction per 0.6 μg of DNA (M_r 3.9 $\times 10^6$) correspond to the hydrolysis of approximately 1.2×10^5 ATP molecules per molecule of DNA or per cleavage event.

A unique characteristic of DNA cleavage by the classical type I restriction enzymes is that, while they recognize well-defined sequences in DNA, they cut the DNA randomly, far from the recognition sequences (Horiuchi & Zinder, 1972; Adler & Nathans, 1973; Murray *et al.*, 1973a; Bickle *et al.*, 1978; Rosamond *et al.*, 1979; Yuan *et al.*, 1980). Figure 2, lanes A and B, show that *EcoA* also cuts DNA randomly with respect to its recognition site. The circular DNA was first cleaved with *EcoA*, which cuts it once to generate a full-length linear molecule (see Fig. 1, 10 min timepoint for example) and was then cut with *SalI*, which has a single site on pSHI44. The result is a smear of DNA on the gel, clearly indicating that cleavage by *EcoRI* was random with respect to the *SalI* site. Some linear plasmid DNA is still present in this digest; this is due to cleavage by *SalI* of the supercoiled DNA that was not digested by *EcoA*. We have not been able to attain a complete DNA digest with *EcoA*. The reason for this is probably that, because the reaction requires AdoMet, the methyl donor in the modification reaction, modification and restriction are competing reactions during the incubation and, once the single *EcoA* site in the plasmid is methylated, it is resistant to cleavage. Lanes C and D in Figure 2 show that modified DNA is not a substrate for restriction by *EcoA*.

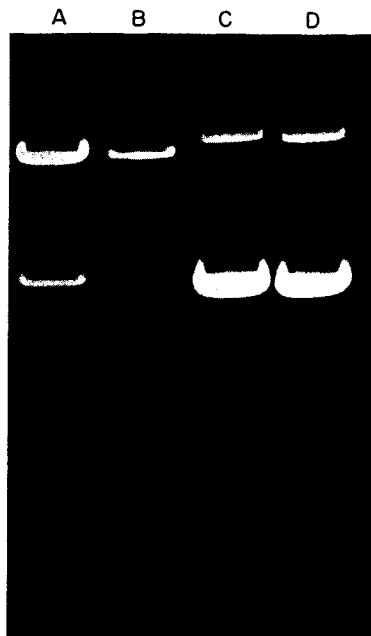


Figure 2. Cleavage by *EcoA* is random and requires non-modified DNA. Reaction mixtures of 20 μ l were set up as described in the legend to Fig. 1, except that ^3H -labelled ATP was omitted. Lane A, non-modified DNA, after 10 min incubation. NaCl was added to 150 mM and the incubation was continued for 1 h at 37°C. Lane B, same as lane A, except that 6 units of *SalI* were present during the 1 h at 37°C. Lane C, modified DNA incubated without enzyme. Lane D, as lane C, except that the incubation was in the presence of *EcoA*. The lower bands in the Figure are supercoiled DNA, the upper bands are linear DNA for lanes A and B, and nicked circular DNA in lanes C and D.

(b) Modification methylase activity

The two subunit enzyme isolated during the *EcoA* purification is a modification methylase, transferring methyl groups from AdoMet to the N⁶ position of specific adenosyl residues within the recognition sequence (Kröger & Hobom, 1984; Suri *et al.*, 1984b). Modification methylase activity is also detected when all three subunits of the enzyme are present in the reaction.

We have compared the reaction kinetics of DNA methylation by *EcoA* with those of the classical type I enzyme, *EcoK*. The plasmid pBR322 linearized with *SalI* was used as a substrate for *EcoK*. This plasmid contains two recognition sites for the enzyme (Kan *et al.*, 1979) and thus can accept a total of four methyl groups. The substrate for *EcoA* was again pSHI44, also linearized with *SalI*. Hemi-methylated DNA (that is, DNA carrying methyl groups in one strand only) was prepared by melting and then hybridizing together equal amounts of modified and non-modified DNA; 50% of such a preparation are hemi-methylated, 25% are non-methylated and 25% are fully methylated.

The time-course of the methylation reaction is shown in Figure 3 for *EcoA*. The *EcoA* methylase

modifies both non-methylated and hemi-methylated DNA at about the same rate. That two methyl groups were incorporated per DNA molecule in the reaction with hemi-methylated DNA reflects the fact that the non-methylated DNA contained in the sample was also a substrate for methylation. There are several notable differences between methylation by the *EcoA* methylase and by the three-subunit restriction enzyme. The restriction enzyme methylates non-methylated DNA somewhat more slowly than it does hemimethylated DNA, and the reaction has an absolute requirement for ATP.

These reaction kinetics are clearly different from those found for the *EcoK* modification methylase and restriction enzyme (Fig. 4). Both of these enzymes methylate non-modified DNA extremely poorly; even after 30 hours of incubation the reaction has not gone to completion. ATP has no effect on the modification enzyme and inhibits the restriction enzyme. The rate of methylation of hemi-methylated DNA by the *EcoK* enzymes is similar to that seen for *EcoA*.

(c) Genetic and transcriptional organization of the *EcoA* structural genes

The *EcoA* structural genes were cloned into a phage λ vector by F. V. Fuller-Pace (*ahsdA*Δ6; Fuller-Pace *et al.*, 1985). We have subcloned a 10.5 kb *HindIII* fragment from this phage DNA into the *HindIII* site of the plasmid pBR322 (Bolivar *et al.*, 1977). Both orientations of the fragment were recovered, giving rise to the plasmids pBS11 and pBS12, respectively. These plasmids were transformed into the strain WA921, which lacks a R-M system, and the transformants were tested for *EcoA*-specific restriction and modification. Both restriction and modification were expressed, independently of the orientation of the insert. The level of restriction of phage λ was increased three- to fivefold as compared to restriction from chromosomally located genes, an effect that is presumably due to the high copy number of the plasmids.

After establishing a restriction map of pBS11 (Fig. 5), a series of deletion derivatives was generated by partial digestion of pBS11 or pBS12 with different type II restriction enzymes followed by religation. Following transformation into WA921, the R-M phenotype of the different derivatives was determined, with the results shown in Figure 5.

The *EcoA*-related proteins produced by these deletion derivatives were analysed by the immune blotting technique of Towbin *et al.* (1979) using antisera raised against *EcoA*. Figure 6 shows several immune blots analysing the proteins produced by the plasmids depicted in Figure 5. A comparison of the *EcoA* proteins produced with the physical maps of the plasmids readily permitted the order of the genes to be established and allowed the transcriptional organization to be worked out.

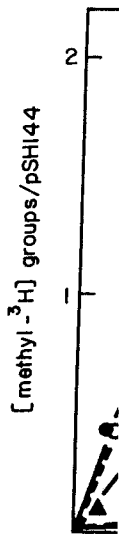


Figure 3. Methylation of DNA by *EcoA* concentration of restriction enzyme (●), methylase + restriction enzyme (■), modified DNA.

Both pBS11 and pBS12 10.5 kb *HindIII* fragments direct the synthesis of polypeptides ar

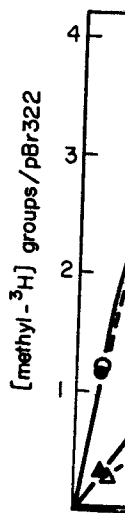


Figure 4. Methylation of DNA by *EcoK* concentration of restriction enzyme (●) was 7.7 μ g/ml for non-modified DNA at about 3 μ g/ml and (▲) is restriction enzyme.

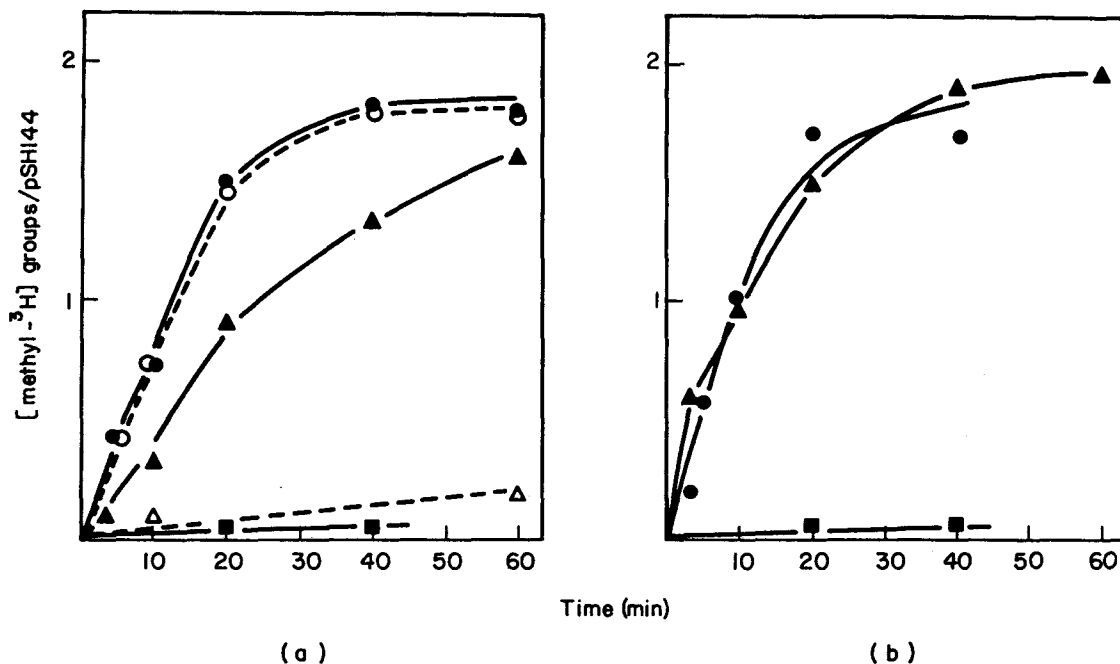


Figure 3. Methylation of (a) non-modified and (b) hemi-methylated DNA by the *EcoA* restriction enzyme and modification enzyme. Reaction conditions and treatment of samples were as described in Materials and Methods. The DNA concentrations were 30 $\mu\text{g/ml}$ for the non-modified DNA and 60 $\mu\text{g/ml}$ for the hemi-methylated DNA. The *EcoA* restriction enzyme was present at about 15 $\mu\text{g/ml}$ and the modification methylase was present at about 5 $\mu\text{g/ml}$. (●), Methylase + ATP; (○), methylase, no ATP; (▲), restriction enzyme + ATP; (△), restriction enzyme, no ATP; (■), modified DNA in (a) with restriction enzyme + ATP and in (b) with methylase + ATP.

Both pBS11 and pBS12, which carry the 10.5 kb *Hind*III fragment in opposite orientations, direct the synthesis of three polypeptides that cross-react with the *EcoA* antisera. The two smaller polypeptides are the modification methylase and

the large polypeptide is the subunit necessary for restriction (Suri *et al.*, 1984b). The deletion of DNA sequences to the right of the *Nru*I site in the plasmid pBS11 Δ NruI (Figs 5 and 6) does not affect the expression of the genes. Deletion of sequences to

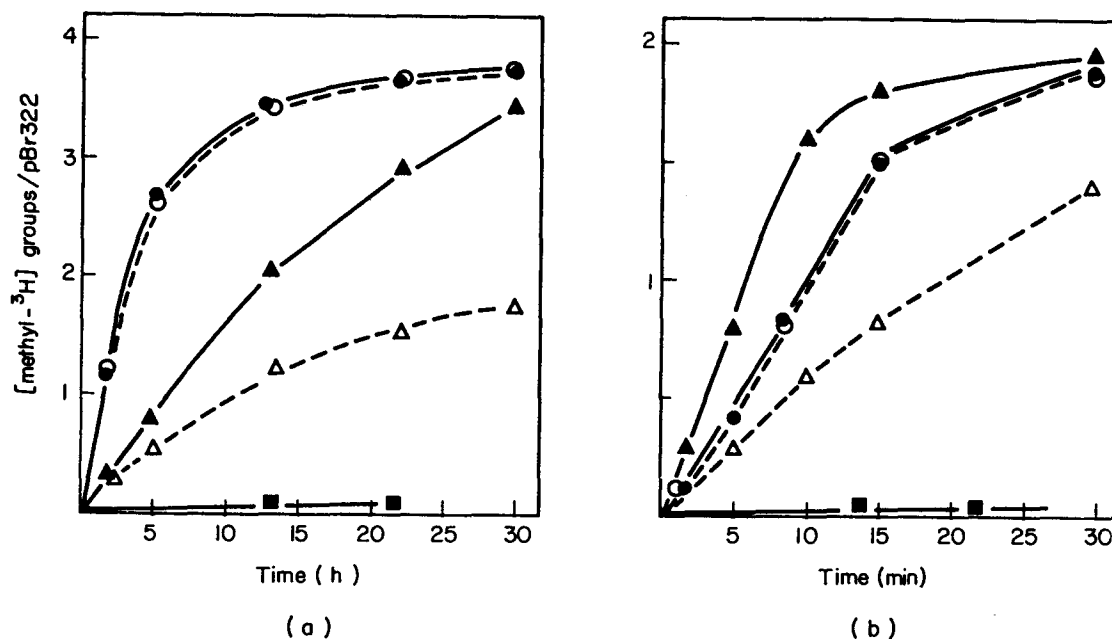


Figure 4. Methylation of (a) non-modified and (b) hemi-methylated DNA by *EcoK*. Reaction conditions were the same as those for Fig. 3, with the following modifications. The pH of the reaction mix was 6.7, the DNA concentration was 7.7 $\mu\text{g/ml}$ for non-modified DNA and 15 $\mu\text{g/ml}$ for hemi-methylated DNA, and *EcoK* restriction enzyme was present at about 3 $\mu\text{g/ml}$ and the modification methylase at about 2.5 $\mu\text{g/ml}$. The symbols are the same as for Fig. 3, except that in (a) (△) is restriction enzyme + ATP and (▲) without ATP.

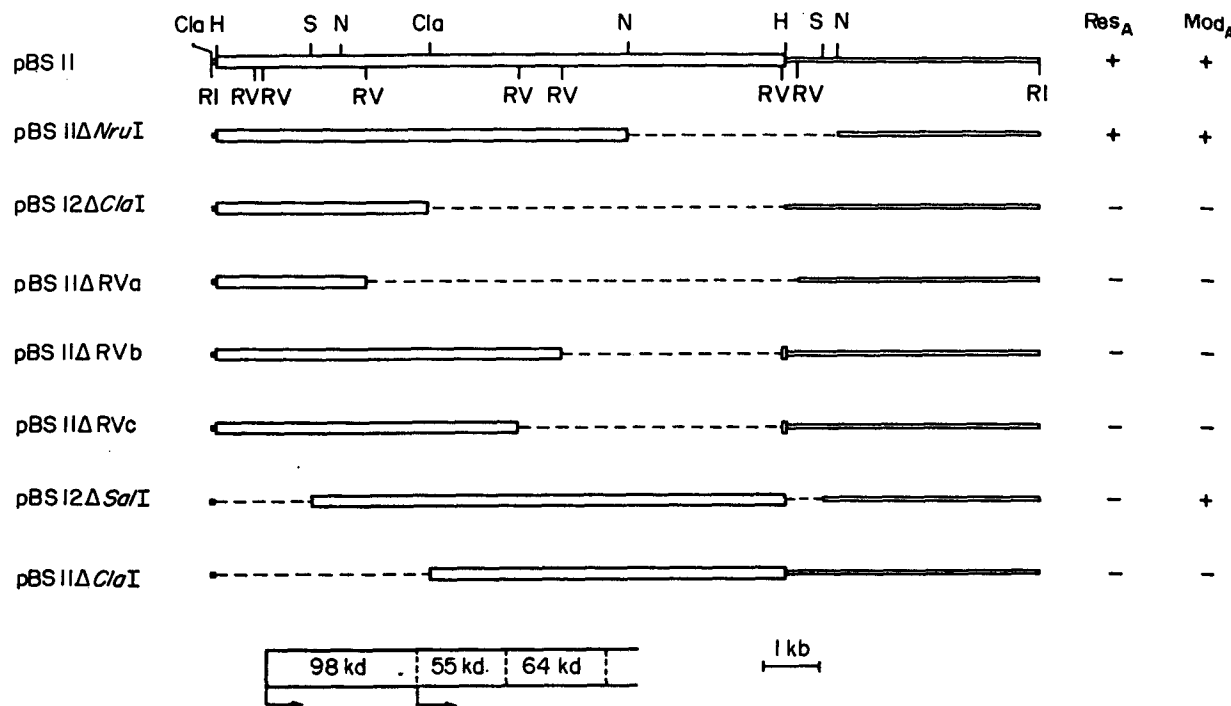


Figure 5. Physical map of the plasmids pBS11, pBS12 and some of their deletion derivatives. The top line shows a restriction map of pBS11; in pBS12 the DNA between the 2 *Hind*III sites is inverted with respect to pBS11. DNA sequences derived from pBR322 are shown as thin lines, those from the *λ*hsdAΔ6 are shown as thick lines. The symbols used for restriction sites are RV, *EcoRV*; RI, *EcoRI*; N, *NruI*; H, *Hind*III; S, *SalI*; Cla, *ClaI*. The plasmids are drawn as though cleaved at the single *EcoRI* site in pBR322. The next lines show the structure of the different deletion derivatives (deleted DNA is indicated by broken lines). The R-M phenotype conferred by the mutants is indicated on the right. At the bottom of the Figure, the approximate location of the 3 *EcoA* structural genes, together with the positions of the 2 promoters and the direction of transcription, are indicated. These positions were deduced from the phenotypes of the plasmids and from the information presented in Fig. 6. The left end of the gene for the 95,000 *M_r* protein has been defined more precisely by *Bal31* deletion analysis and is indicated by a straight line. The boundaries between the genes and the left end of the gene for the 64,000 *M_r* gene are less well-defined and are drawn with broken lines to indicate that fact. kd, $\times 10^3$ *M_r*.

the left of the *NruI* site that leave only 5 kb of the original *Hind*III fragment intact (Figs 5 and 6; pBS12Δ*ClaI*) abolish the expression of both modification methylase polypeptides but do not affect the expression of the restriction subunit. When the deletion extends still further to the left (pBS11ΔRVa), a faint band of *M_r* 70,000 appears and the restriction subunit can no longer be detected. This faint band reacted with antisera raised against the restriction subunit (not shown) and therefore is a truncated restriction subunit, which appears to be unstable. These results show that the restriction subunit gene, which requires approximately 2.7 kb, is the leftmost in the insert of pBS11 and that it is transcribed from left to right. The leftmost boundary of this gene has been mapped more precisely by *Bal31* deletion analysis to about 750 base-pairs from the *SalI* site (Fig. 5; not shown). The deletion mutants pBS11ΔRVb and pBS11ΔRVc (Fig. 5) express the restriction subunit and the smaller of the two methylase subunits. This information allows us to map the gene for the smallest subunit to the right of the restriction subunit gene, and shows that the rightmost gene is

that coding for the subunit of intermediate molecular weight. The deletion in the plasmid pBS12Δ*SalI* (Fig. 5) removes the promoter of the restriction subunit gene. This plasmid expresses both of the methylase subunits (Fig. 6), and the methylase genes are therefore expressed from their own promoter or promoters. The deletion in the plasmid pBS11Δ*ClaI* removes the gene for the restriction subunit and terminates within the gene for the smallest subunit of the enzyme (Fig. 5). This plasmid expresses no *EcoA*-related proteins (Fig. 6) even though the gene for the intermediate subunit is intact. This gene must therefore be transcribed from the same promoter as that for the smallest subunit, from left to right as drawn in Figure 5.

The conclusion from these mapping and transcription studies is that the arrangement of the *EcoA* genes is the same as that of the classical enzyme, *EcoK*. The genes are organized into two transcriptional units, the restriction subunit gene is transcribed from its own promoter, while a separate promoter drives the transcription of both methylase genes. The results are summarized at the bottom of Figure 5.

Figure 6. In vitro synthesis of proteins between 5×10^4 and 10^6 *M_r* in an experiment. The results are summarized at the bottom of the figure.

(d)

It is clear that the DNA is transcribed into two transcripts. The results of a differential centrifugation experiment for the modification methylase subunits. The experiment demonstrates that the production of the restriction subunit is not affected by the transcriptional unit of the restriction subunit gene. The results are summarized at the bottom of the figure. The results are summarized at the bottom of the figure.

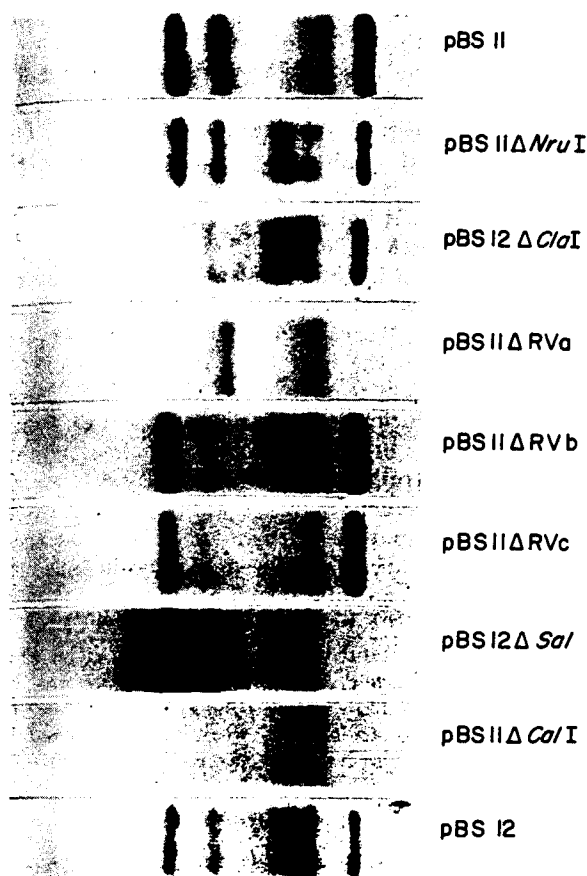


Figure 6. Immune blot analysis of the *EcoA*-related proteins synthesized from the plasmids shown in Fig. 5. Between 5×10^7 and 5×10^8 cells were analysed in each experiment. The Figure is a composite of several experiments. The rightmost band is the restriction subunit.

(d) Control over the expression of *EcoA* restriction

It is clear that the organization of the *EcoA* genes into two transcriptional units potentially allows for differential control over the expression of the genes for the modification methylase and the gene for the restriction subunit.

The experiment shown in Figure 7 is a direct demonstration that some control over the production of the restriction subunit takes place, although the control need not necessarily be at the transcriptional level. Figure 7 shows the results of a conjugation experiment where the *EcoA* genes carried on an F' were transferred into WA921, a restriction-less strain, carrying either the plasmid pBS11ΔC/aI or pBS12ΔC/aI (Fig. 5). The first plasmid expresses no *EcoA* gene products, while the second directs the synthesis of the restriction subunit but not the methylase subunits (Fig. 6). In the 90 minutes that the conjugation was allowed to proceed, the titre of the strain carrying pBS12ΔC/aI dropped about 40-fold, while that of the strain carrying pBS11ΔC/aI increased about fivefold. Twelve exconjugants from both crosses were checked for their R-M phenotype by testing their

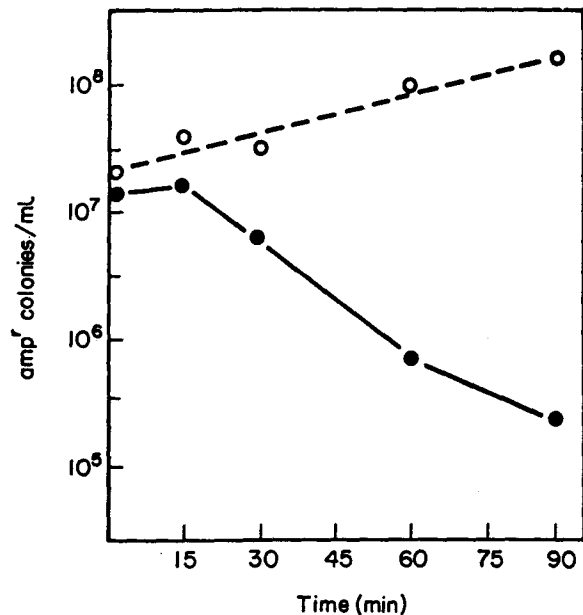


Figure 7. Conjugation experiments with the *EcoA* genes. The donor cells were WA2677(F-A), which were grown in L broth at 37°C with slight aeration to 2×10^8 cells/ml. Recipients were WA921 (pBS11ΔC/aI) or WA921 (pBS12ΔC/aI). They were grown under the same conditions in the presence of 200 μg ampicillin/ml to 1.5×10^8 cells/ml, harvested by centrifugation, resuspended in the same volume of prewarmed L broth and incubated for 20 min. Mating was initiated by mixing 1.8 ml of donor cells with 0.2 ml of recipients and continued at 37°C with slight aeration. At the indicated times, portions were removed and diluted 20-fold into 10 mM-Tris·HCl (pH 8.0), 10 mM-MgCl₂, vortex mixed for 20 s and then further diluted. Portions from the appropriate dilutions were spread on LA plates containing 200 μg ampicillin/ml to select for recipients. Data are presented as the number of ampicillin-resistant (amp^r) colonies/ml of conjugation mix. (O), Recipient is WA921 (pBS11ΔC/aI); (●), recipient is WA921 (pBS12ΔC/aI).

ability to restrict and modify λ phages and for the presence of the F' by susceptibility to phage M13. All of the exconjugants with pBS11ΔC/aI had received and were expressing the *EcoA* genes, and all of them were M13-sensitive. In contrast, none of the surviving WA921 (pBS12ΔC/aI) expressed *EcoA* restriction and none of them was M13-sensitive, indicating that they had not mated. Thus, transfer of the *EcoA* genes into recipients that already contain the restriction subunit is a lethal event, presumably due to restriction of the recipient chromosome. This result implies that the expression of the restriction subunit is regulated during normal gene transfer.

4. Discussion

EcoA has many features in common with the classical type I R-M enzymes such as *EcoK*. Although the molecular weights of all three subunits of the enzyme are different from those of *EcoK*, they are functionally similar in that there is

a large subunit necessary only for restriction, and two smaller subunits that are required for both restriction and modification. In the accompanying paper (Fuller-Pace *et al.*, 1985), it is shown that the smallest subunit is the functional equivalent of the *hsdM* gene product of the classical systems, and that the intermediate subunit corresponds to the *hsdS* product. *EcoA* is a member of a new family of type I R-M systems; Fuller-Pace *et al.* (1985) have found a strain of *E. coli* with a R-M system that is allelic to *EcoA* but which has a different specificity.

The only significant difference in enzymatic activities between the classical type I enzymes and *EcoA* lies in the modification reaction. In both systems, modification methylation is catalysed by both a two-subunit modification enzyme and the three-subunit restriction enzyme. Hemi-methylated DNA is methylated by *EcoA* and *EcoK* at comparable rates. However, only the *EcoA* enzymes are able to methylate non-modified DNA efficiently. ATP is an absolute requirement for this reaction, while the much less efficient *EcoK* is inhibited by ATP. This suggests that, while the spectrum of enzymatic activities is the same for both enzymes, the details of the reaction mechanism are different.

The genetic and transcriptional organization of the *EcoA* genes is very similar to that of the genes for the classical enzyme, *EcoK* (Sain & Murray, 1980). The genes are organized into two transcriptional units, with the genes coding for the subunits of the modification methylase transcribed from one promoter and the restriction subunit gene transcribed from another. This organization potentially allows for differential control of the synthesis of the modification methylase and the restriction enzyme. Although physiological conditions might exist that would make sequential expression of the enzymes desirable, this sort of control would be really advantageous when the structural genes for the enzymes are transferred to a different bacterial strain. Upon transfer of a R-M system from one strain to another, the chromosomal DNA of the recipient must first be modified before restriction activity can be expressed, because any non-modified recognition sites on the recipient chromosome would lead to restriction and consequent cell death.

Our conjugation experiments (Fig. 7) show that the *EcoA* genes can indeed be transferred to other strains efficiently and with little, if any, killing of the recipient. Transfer was a lethal event, however, when the recipients already contained and were expressing the gene for the restriction subunit, presumably because this protein could assemble an active restriction enzyme with the methylase gene products expressed from the incoming DNA. The result implies that the expression of the restriction subunit is regulated during gene transfer. The experiments do not allow us to distinguish between regulation at the transcriptional or some other level.

Crosses performed between strains carrying the classical type I R-M systems have shown that these

systems can be interchanged by conjugation (Boyer, 1964; Boyer & Roulland-Dussoix, 1969). It is clear that the absolute frequency of transfer is reduced relative to a restrictionless female when the recipient expresses an active R-M system. One might ask what might be the advantage for the bacteria to transfer the genes for R-M systems from strain to strain. It has been shown in the laboratory that one response of bacteriophages to the presence of a R-M system in their host is to mutate out the recognition sites in the phage genome (Arber & Kühnlein, 1967; Murray *et al.*, 1973b). It is therefore advantageous to the host to change the specificity of its R-M system from time to time. There might also be an evolutionary advantage to the exchange of R-M systems between different bacterial strains. Once a given strain has acquired a R-M system of a particular specificity from another bacterium, further exchange of genetic material between these two strains would be facilitated greatly, because the restriction barrier would no longer exist.

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