

***EcoR124* and *EcoR124/3*: the first members of a new family of type I restriction and modification systems**

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We have purified the *EcoR124* and *EcoR124/3* restriction enzymes and shown that they are type I enzymes by several criteria: subunit composition, DNA and *S*-adenosylmethionine-dependent ATPase activity, and site-specific DNA methylase activity. By immunochemical criteria these enzymes are related to each other but are unrelated to the two previously investigated families of type I restriction enzymes. They form therefore a new family which we call type IC. The arrangement of the structural genes coding for these enzymes and their transcriptional organisation have been determined. These are different from the common arrangement found for the other two families of type I enzymes.

The type I restriction and modification (R-M) enzymes of *Escherichia coli* and *Salmonella* spp. are complex and multifunctional. The *EcoK*, *EcoB*, *EcoA*, *EcoD*, *EcoDXX1*, *StySP*, *StySB* and *StySQ* restriction enzymes have all been purified and shown to share very similar properties [1–7]. All consist of three different subunits coded by three, contiguous genes, *hsdR*, *hsdM* and *hsdS*, which, with the exception of *EcoDXX1* which is plasmid-coded [5], map to the same chromosomal location [8–11]. Recently it has become apparent that these systems can be grouped into two genetically and antigenically distinct families. One of these, called type IA, comprises *EcoB*, *EcoD*, *EcoK*, *StySB*, *StySP* and *StySQ*, while the type IB systems are *EcoA* and *EcoE* [12–14]. The transcriptional organisation of the type IA *EcoK* genes has been established [15]. One promoter drives the transcription of the *hsdR* gene and a second promoter directs transcription of the *hsdM* and *hsdS* genes. The gene order is *hsdR-hsdM-hsdS* and all genes are transcribed from left to right [15]. The gene order and transcriptional properties of the type IB *EcoA* have been shown to be exactly the same as for *EcoK* [13, 14].

The *IncFIV* plasmids R124 and R124/3 have each been shown to encode a unique R-M specificity [16–18] and the genes for these two R-M systems have both been cloned [19, 20], facilitating the purification of the corresponding enzymes. Under certain circumstances cells may switch expression between *EcoR124* and *EcoR124/3* and this switch appears to involve rearrangements of the plasmid DNA [21]. Genes coding both specificities have been shown to reside on the same plasmid, although only one of them is normally expressed [18], and, at the level of sensitivity of DNA heteroduplex analysis, both sets of structural genes are essentially homologous [19].

In this paper we describe the purification and the characterisation of the *EcoR124* and *EcoR124/3* restriction

enzymes, the transcriptional organisation of the structural genes encoding them, and discuss the relationship of these systems to the types IA and IB R-M systems.

MATERIALS AND METHODS

Bacterial strains and plasmids

E. coli JC7623 carrying the plasmid pCP1005 [19, 20] was used as a source of *EcoR124* and the same strain carrying the plasmid pUNG31 [19, 20] served for the purification of *EcoR124/3*. The complementation assay used to identify the *EcoR124 hsdS* gene was carried out in the strain AB2463(R124/3) using AB2463 and AB2463(R124) as controls. The minicell-producing strain DS410 [22] was used to identify plasmid-coded proteins in the transcriptional organisation experiments. The *lacZ* M15 strain MC1029 [23] was used to screen for recombinant derivatives of pUC18 or pUC19 [24]. The plasmid pUC4K [25] was used for methylation studies with *EcoR124/3*.

Nucleic acid methods

Plasmid DNA was isolated by the method of Birnboim and Doly [26] and, when necessary, was further purified by CsCl gradient centrifugation in the presence of ethidium bromide [27]. All other DNA manipulations were as described in Maniatis et al. [28].

Protein methods and enzyme assays

Sodium dodecyl sulphate/polyacrylamide gel electrophoresis was according to Laemmli [29] and either gels were stained with Coomassie blue or labelled proteins were visualised by fluorography following fixation in 10% acetic acid and immersion in 1 M sodium salicylate [30]. Antibodies were raised against the purified *EcoR124* enzyme in rabbits and these antibodies were used for immune blotting as described [12, 31]. ATPase and methylase assays were carried out as described [15] except that the AdoMet concentration in the methylase assays was reduced to 240 nM.

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Abbreviations. AdoMet, *S*-adenosylmethionine; R-M, restriction and modification.

Enzymes. Type I restriction and modification enzymes (EC 3.1.2.1.3).

Enzyme purification

Both *EcoR124* and *EcoR124/3* were purified using the same protocol. The DNA-dependent and AdoMet-dependent ATPase activity of the enzymes was used to monitor the purifications. *E. coli* JC7623(pCP1005) or JC7623(pUNG31), for *EcoR124* and *EcoR124/3* respectively, were grown in L broth [32] supplemented with 200 µg/ml ampicillin at 37°C to an A_{550} of 1.6 and harvested by centrifugation. The cell paste was divided into 30-g aliquots and frozen at -20°C until used.

A 30-g aliquot of cell paste was suspended in 100 ml buffer A (20 mM Tris/HCl, pH 8.0; 10 mM MgCl₂; 7 mM 2-mercaptoethanol) and the cells were broken by sonication, taking care to maintain the temperature below 8°C. For all subsequent operations the temperature was maintained between 0 and 4°C. The extract was clarified and ribosomes were removed by low and high-speed centrifugation. The supernatant was made 0.2 M in NaCl and nucleic acids were removed by precipitation with 0.5% poly(ethyleneimine), the proteins were concentrated by precipitation with ammonium sulphate (70% saturation) and then dialysed against buffer B (20 mM Tris/HCl, pH 8.0, 50 mM NaCl; 7 mM 2-mercaptoethanol; 0.1 mM EDTA).

Proteins were then loaded on a column of DEAE-Sephacel (Pharmacia, bed volume 60 ml) equilibrated with buffer B which was eluted with a 50–650 mM NaCl gradient in buffer B. In some preparations this step was replaced by a batch adsorption to DEAE-Sephacel followed by elution with 400 mM NaCl. Enzyme-containing fractions were pooled, dialysed against buffer B and loaded on a column of heparin-agarose [33] (40 ml bed volume) in buffer B, proteins were eluted with a 50–550 mM NaCl gradient. Enzyme-containing fractions were pooled and proteins were precipitated in 70% saturated ammonium sulphate. The proteins were dissolved in 2 ml buffer B and fractionated on a column of Sephacryl S-200 (diameter, 2.6 cm; bed height, 100 cm), the enzyme activity eluted in the void volume. The pooled fractions were loaded on a hydroxyapatite column (Ultragel, LKB; bed volume 16 ml). Proteins were eluted with a 20–300 mM sodium phosphate gradient at pH 7.6 containing 20 mM NaCl and 7 mM 2-mercaptoethanol. Active fractions were prepared for storage by dialysis against buffer B supplemented with 10% glycerol.

In earlier preparations chromatography on ATP-agarose was used as a final step and the hydroxyapatite step was omitted. However, fresh supplies of ATP-agarose from two different manufacturers failed to bind either *EcoR124* or *EcoR124/3*.

Because of the large amounts of contaminating ATPases still present during the early stages of the purification, it is not possible to calculate the yield of the purification.

RESULTS AND DISCUSSION

Purification of *EcoR124* and *EcoR124/3*

Both of these enzymes behaved very similarly during purification, as expected from their close genetic relatedness [19, 20]. Fig. 1 shows the proteins present in the enzyme-containing fractions during the course of the purification of both enzymes. The figure demonstrates that, like other type I restriction enzymes, purified *EcoR124* and *EcoR124/3* both contain three different subunits which are the same size for the two enzymes, 116 kDa, 55 kDa and 43 kDa.

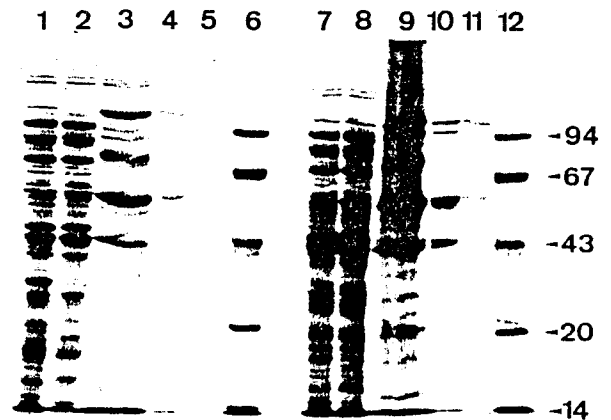


Fig. 1. Enzyme purification. The figure shows a 10% SDS/polyacrylamide gel stained with Coomassie blue of preparations of *EcoR124* (lanes 1–5) and *EcoR124/3* (lanes 7–11). Lanes 6 and 12 contain markers whose molecular masses in kDa are indicated to the right of the figure. Lanes 1 and 7 show whole cell extracts, 2 and 8 pooled fractions after DEAE-Sephacel chromatography, 3 and 9 pooled fractions after heparin sepharose chromatography, 4 and 10 pooled fractions after Sephacryl S-200 chromatography, 5 and 11 the pure enzyme following hydroxyapatite chromatography

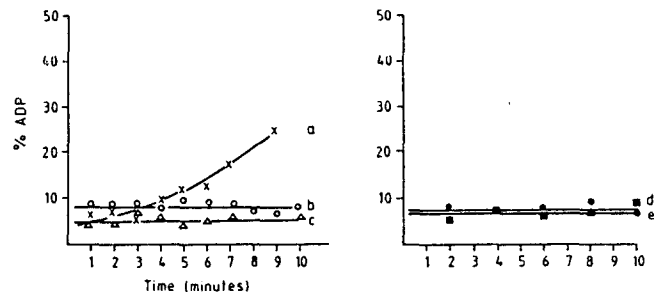


Fig. 2. ATPase activity of *EcoR124/3*. The figure shows the conversion of ATP to ADP when the enzyme is incubated with the following DNA substrates: (a) completely non-modified pUC4K DNA; (b) hemimethylated pUC4K DNA prepared in a *dam*⁺ host; (c) completely methylated DNA prepared in an *EcoR124/3* modifying host; (d) completely unmethylated DNA in the absence of AdoMet; (e) no DNA added. The reactions contained 2.5 µM AdoMet except where stated and were carried out as described [15]

ATPase activity of the enzymes

Fig. 2 demonstrates the effect of methylation of the substrate DNA and of the presence of AdoMet on the ATPase activity of the *EcoR124/3* enzyme. The DNA substrate is the plasmid pUC4K [25] which contains a single site for *EcoR124/3* [34] which happens to overlap a recognition site for the host-coded *dam* methylase. The three DNA samples used in Fig. 2 differ in the state of methylation at the *EcoR124/3* site. One sample was prepared from cells containing the R124/3 plasmid and thus carries methyl groups in both strands of the DNA; another sample was prepared from wild-type *E. coli* and contains a methyl group in one strand of the sequence only, at the *dam* methylation site; the third sample was prepared in *dam*⁻ *E. coli* cells and contains no methyl groups in the *EcoR124/3* recognition site. The figure clearly shows that the ATPase activity has an absolute requirement for AdoMet and for a substrate with completely unmethylated recognition sites. Hemimethylated sites are the normal product of semiconservative DNA replication and thus would

Table 1. *In vivo* restriction by *EcoR124* and *EcoR124/3* of the plasmid pUC4K prepared in different methylating hosts

Host strain	Efficiencies of transformation		
	<i>EcoR124/3</i>	<i>dam</i>	none
AB2463	1	1	10^{-1}
AB2463(R124)	10^{-1}	10^{-1}	10^{-2}
AB2463(R124/3)	1	1	10^{-2}

not be expected to serve as substrates for the ATPase activity, which is associated with DNA cleavage (reviewed in [35]). Nevertheless, this is the first time that this has been demonstrated for any type I restriction enzyme. The *EcoR124* ATPase activity has precisely the same characteristics (not shown) although in this case there was no easy way to prepare a hemimethylated substrate for the enzyme.

Transformation experiments

It is very difficult to demonstrate DNA cleavage reproducibly by either the *EcoR124* or the *EcoR124/3* enzymes *in vitro* for reasons that are not at all clear. Therefore, in order to correlate the results of the ATPase assays described in the last section with DNA cleavage we transformed the three substrate DNAs used for the ATPase assays into cells with different restriction characteristics. The results of this assay are shown in Table 1. The *E. coli* strains AB2463, AB2463(R124/3) and AB2463(R124) were transformed with 1 μ g each plasmid DNA. This last strain served as a control for restriction because all three DNA samples also contain a fully unmethylated *EcoR124* site. It is clear from the data shown in the table that unmethylated sites lead to restriction: transformation frequencies are reduced by an order of magnitude. There is no difference in transformation efficiency between DNA with fully modified sites and DNA with hemimodified sites that can be attributed to restriction. It should be noted that a lack of *dam* methylation leads to a reduction in the efficiency of transformation in all of the hosts tested. This experiment has been repeated three times with no significant differences in the results.

Modification methylation

While most features of the reaction mechanism are similar for the two different families of type I restriction enzymes, the kinetics of the DNA methylation reaction show considerable differences [14]. Further, the methylation of DNA *in vivo* by the type IA enzyme, *EcoK*, is stimulated by the bacteriophage λ *ral* gene product [36, 37] while methylation by the type IB *EcoA* and by *EcoR124* is not affected by the *ral* gene product [38]. The methylation of hemimethylated and unmethylated pUC4K DNA by *EcoR124/3* is shown in Fig. 3. Again, similar data were obtained with *EcoR124* although a hemimethylated DNA sample could not be included in the analysis. The data, while not quantitative, show that methylation in one strand greatly stimulates methylation in the other. Further, the addition of ATP stimulates the methylation of both non-methylated and hemimethylated DNA.

These results are different from those obtained with either *EcoK* or *EcoA* [14]. For *EcoK* the modification of unmethylated DNA is an extremely slow reaction: after 24 h of incubation the reaction has still not gone to completion and

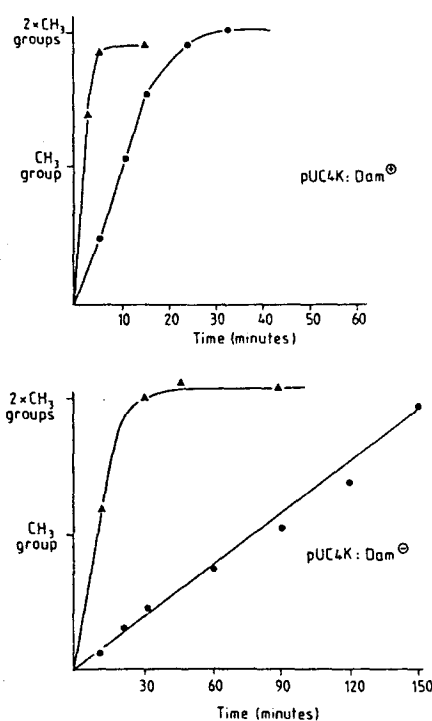


Fig. 3. Methylase activity *EcoR124/3*. The graphs show the influence of ATP (1 mM) on the methylation of hemimethylated DNA (pUC4K:Dam⁺) and non-methylated DNA (Dam⁻). (▲—▲) + ATP; (●—●) no ATP present. The DNA concentrations were 2 μ g/20 μ l reaction mixture for the hemimethylated substrate and 1 μ g/20 μ l for the non-methylated substrate. Reaction conditions were as described [15]

hemimethylated DNA is the only good substrate. Methylation of both kinds of substrates by *EcoK* was reported to be inhibited by ATP. The interpretation of this result is complicated by the fact that the hemimethylated substrate DNA used in these experiments contained 25% unmodified DNA and restriction and methylation may have been competing reactions. *EcoA* methylates both hemimethylated and unmethylated sites equally well but in this case the reaction shows an absolute requirement for ATP.

Antigenic relationships of type I R-M systems

Fig. 4 shows a Coomassie-blue-stained SDS gel of preparations of *EcoK*, *EcoA*, *EcoR124* and *EcoR124/3* together with immune blots of similar gels using antibodies raised against *EcoK*, *EcoA* and *EcoR124*. It is evident that *EcoR124* and *EcoR124/3* are antigenically related to each other and distinct from both *EcoK* and *EcoA*. On the basis of these results the methylation characteristics of the enzymes described in the last section and earlier DNA hybridisation studies [19], we propose that *EcoR124* and *EcoR124/3* are members of a new family of type I R-M systems which we call type IC.

Transcriptional organisation

It has been suggested [20] that the transcriptional organisation of the *EcoR124* and *EcoR124/3* *hsd* genes was similar to that reported for *EcoK* and *EcoA* [13–15]. We have re-examined this question and find a different organisation. The approach that we have employed was to construct plasmids

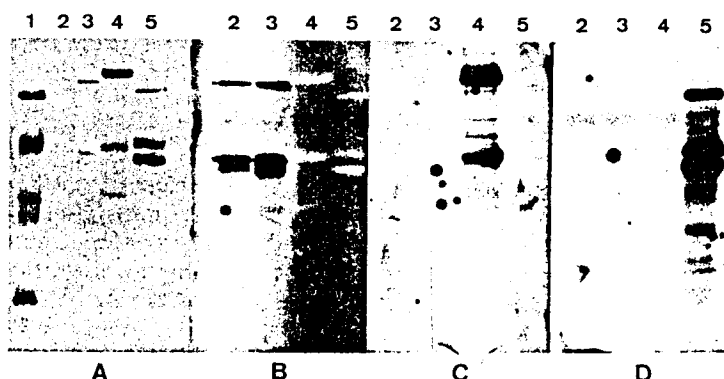


Fig. 4. Antigenic relationships of type I restriction enzymes. (A) Coomassie-blue-stained 10% SDS polyacrylamide gel of the following proteins: lane 1, size markers, as in Fig. 1; lane 2, *EcoR124*; lane 3, *EcoR124/3*; lane 4, *EcoK*; lane 5, *EcoA*. (B, C, D) Immune replicas of gels similar to that shown in (A) probed with antibodies to *EcoR124*, *EcoK* and *EcoA*, respectively, following transfer of the proteins to nitrocellulose filters

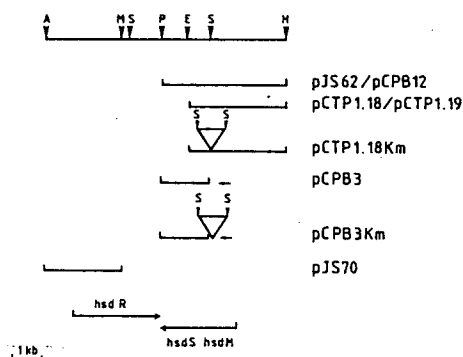


Fig. 5. Plasmid constructions used to investigate the structure of the *EcoR124* *hsd* genes. The top line of the figure is a partial restriction map of the *hsd* region. The symbols used are: (A) *AatII*; (M) *MstII*; (S) *SalI*; (P) *PvuI*; (E) *EcoRI* and (H) *HindIII*. The plasmids pJS62 is derived from R124; the same fragment cloned from R124/3 DNA is pCPB12. The plasmid pCTP1.18Km has a kanamycin-resistance marker inserted into the unique *SalI* site of pCTP1.18. Similarly, pCPB3Km has a kanamycin marker inserted between the *lacUV5* promoter and the *hsdS* gene of pCPB3. The plasmid pJS70 directs the synthesis of a truncated *hsdR* product. At the bottom of the figure is shown the gene order and transcriptional organisation deduced for the *hsd* genes (see text)

containing different parts of the DNA region containing the *EcoR124* or *EcoR124/3* *hsd* genes (Fig. 5). The plasmids were then used to determine which subunits (or parts of subunits) were expressed from them in minicells (Fig. 6). These results, together with the R-M phenotypes of some of the plasmids and the results of complementation analysis confirm the gene order and transcriptional organisation shown at the bottom of Fig. 5.

The cloned *AatII-HindIII* fragment of either R124 or R124/3, shown in Fig. 5, confers a $Res^+ Mod^+$ phenotype on cells and therefore must contain all of the structural genes for the three subunits of the enzyme. Deletion of DNA to left of the *PvuI* site of either R124 or R124/3 DNA (Fig. 5, the plasmids pJS62 and pCPB12 respectively) results in the loss of the ability to restrict DNA but the retention of the ability to modify DNA. Thus, all or part of the *hsdR* gene must lie to the left of the *PvuI* site and all of the *hsdS* and *hsdM* genes must lie to the right of the site. A further deletion of DNA sequences between the *PvuI* site and the *EcoRI* site (Fig. 5, the plasmids pCTP1.18 and pCTP1.19) results in the loss of

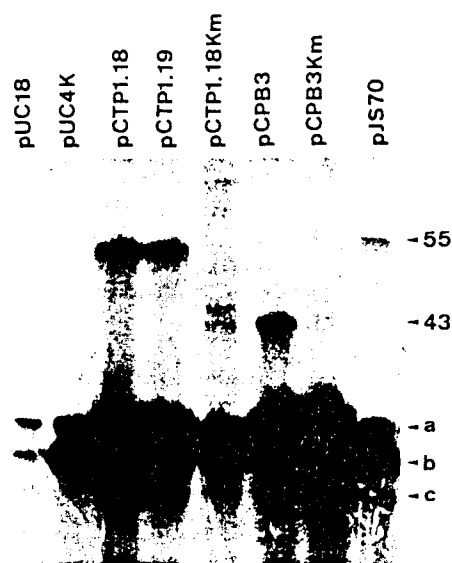


Fig. 6. Plasmid-coded peptides expressed in minicells. Proteins were labelled with [^{35}S]methionine, separated by electrophoresis on a 10% SDS/polyacrylamide gel and the proteins were visualised by fluorography. The structure of the plasmids is shown in Fig. 5. The numbers at the side of the figure are the molecular masses in kDa of the marker proteins, not visible on this fluorogram. The symbols a, b, and c mark the positions of polypeptides coded by the vectors

modification activity. However, minicells containing pUC18 or pUC19 derivatives carrying this *EcoRI-HindIII* fragment express the 55-kDa subunit of the enzyme (Fig. 6). The DNA to the right of the *EcoRI* site thus contains the structural gene for this subunit. The fact that the gene is expressed from both the pUC18 and the pUC19 derivatives, i.e. in both orientations with respect to vector promoters, shows that it is transcribed from its own promoter. The insertion of a kanamycin-resistance determinant into the *SalI* site of the plasmid pCTP1 (Fig. 5) leads to the expression of a truncated protein (Fig. 6) whose size (45 kDa) indicates that the gene for the 55-kDa protein must be transcribed from right to left as shown in Fig. 5.

These results show that the gene for the 43-kDa subunit of the enzyme must lie between the *PvuI* site and the *SalI* site in the gene coding the 55-kDa subunit. This DNA fragment from R124DNA was cloned into the plasmid pUC18 to yield

pCPB3 (Fig. 5). When pCPB3 was transformed into the strain AB2463(R124/3), the result was a strain expressing both *EcoR124* and *EcoR124/3* specificities. The 43-kDa subunit is thus the product of the *hsdS* gene and the 55-kDa subunit must therefore be the product of the *hsdM* gene. Cloning of a kanamycin-resistance determinant into the *SaII* cloning site of the plasmid pCPB3 (Fig. 5) resulted in loss of the expression of the *hsdS* gene, indicating that in pCPB3 it is transcribed from the *lacUV5* promoter in the vector (Fig. 6). Thus *hsdS* and *hsdM* are cotranscribed from a promoter situated to the right of the *hsdM* gene.

A clone containing the *AatII-MstII* fragment of the *hsd* region was constructed (Fig. 5, plasmid pJS70). This plasmid directs the synthesis of a truncated *hsdR* gene product in minicells (Fig. 6, also confirmed by immune blotting). The *hsdR* gene must, therefore, be oriented such that it is transcribed from left to right as drawn in Fig. 5; that is in the opposite direction to the *hsdS-hsdM* operon. This is different from the organisation found for the *EcoK* and *EcoA* *hsd* genes where the gene order is *hsdR-hsdM-hsdS* and all the genes are transcribed from left to right, one promoter being used for *hsdR* and a second promoter for the cotranscription of *hsdM* and *hsdS*.

CONCLUSIONS

We have purified the *EcoR124* and *EcoR124/3* restriction enzymes and have shown that each contains three subunits of 116 kDa, 55 kDa and 43 kDa, the products of the *hsdR*, *hsdM* and *hsdS* genes respectively. The enzymes exhibit DNA-dependent and AdoMet-dependent ATPase activity and are also site-specific DNA methylases [34]. They are thus typical type I restriction enzymes. They are, however, antigenically distinct from the two known families of allelic type I enzymes and we propose that they form a new family, which we call type IC. The genetic organisation of the structural genes of the type IC family is different from that of the other two families in that the operon encoding the *hsdS* and *hsdM* genes is inverted with respect to the *hsdR* gene.

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