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Localization of the Type I Restriction–Modification Enzyme *Eco*KI in the Bacterial Cell

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To localise the type I restriction–modification (R-M) enzyme *Eco*KI within the bacterial cell, the Hsd subunits present in subcellular fractions were analysed using immunoblotting techniques. The endonuclease (ENase) as well as the methylase (MTase) were found to be associated with the cytoplasmic membrane. HsdR and HsdM subunits produced individually were soluble, cytoplasmic polypeptides and only became membrane-associated when coproduced with the insoluble HsdS subunit. The release of enzyme from the membrane fraction following benzonase treatment indicated a role for DNA in this interaction. Trypsinization of spheroplasts revealed that the HsdR subunit in the assembled ENase was accessible to protease, while HsdM and HsdS, in both ENase and MTase complexes, were fully protected against digestion. We postulate that the R-M enzyme *Eco*KI is associated with the cytoplasmic membrane in a manner that allows access of HsdR to the periplasmic space, while the MTase components are localised on the inner side of the plasma membrane. © 2000 Academic Press

Key Words: **protein localisation; restriction–modification; spheroplast; subcellular fraction; membrane-associated proteins.**

Restriction and modification (R-M) systems provide the host bacteria with immunity to infection by foreign DNA and protect cellular DNA from restriction by methylation of adenosyl or cytosyl residues within the sequence recognised by the restriction enzymes. The type I R-M systems are the most complex so far discovered and are classified into four distinct families: IA, IB, IC, and ID (1, 2).

Type I enzymes are encoded by three genes, *hsdR*, *hsdM*, and *hsdS*. The products of all three genes are required for restriction, while for modification (methylation) only the products of the *hsdS* and *hsdM* genes are absolutely required (3, 4). The subunit stoichiometry of the endonuclease (ENase) is $R_2M_2S_1$ (5). However, the HsdS and HsdM subunits can also form an independent DNA methyltransferase (MTase) with a stoichiometry of M_2S_1, which is an intermediate in assembly of the ENase (6, 7). The *hsdR* gene is transcribed from its own promoter (P_{RES}), while the *hsdM* and *hsdS* genes are transcribed from a separate promoter (P_{MOD}) (2). The subunit HsdS (product of the gene *hsd*S) plays the key role of recognition of the DNA target sequence, and the ENase cuts DNA at random sites distant to the recognition sequence as a consequence of ATP-dependent DNA translocation past the enzyme, which remains bound to the recognition site (8).

Since DNA cleavage by type I restriction enzymes occurs by a very unusual and highly energy-dependent mechanism, these enzymes are believed to be involved not only as a defence mechanism for bacterial cells against foreign DNA, but also in some type of specialised recombination system controlling the flow of genes between bacterial strains. Restriction may facilitate recombination by generating recombinogenic double-stranded breaks in homologous incoming DNA and it has been suggested that type I R-M system would be particularly effective in this role (2, 9). Considering the fact that the bacterial cell is highly a organised system comprised of functional entities characterised by their structural components and enzymes, the question arises, how this multimeric, multifunctional enzyme is localised in the bacterial cell to ensure all these functions are controlled in an appropriate manner. A periplasmic localisation would be well adapted for restriction, but recombination as well as DNA methylation requires a cytoplasmic localisation. However, the cellular localisation of restriction enzymes has been less than thoroughly analysed. Results referring to the cellular localisation of the more simple

Abbreviations used: R-M, restriction–modification; ENase, endonuclease; MTase, methyltransferase.

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type II restriction enzymes are contradictory. Initial studies have shown that at least a fraction of *Eco*RI and *Pst*I activities could be recovered from the periplasm (10, 11). Application of electron microscopic immuno-gold technique recovered the *Eco*RI MTase from the cytoplasm; however, the *Eco*RI ENase was found in the periplasmic space (12, 13). These results were not supported by a study in which cellular compartmentation of *Eco*RI ENase was analysed using either parental, or *tolA* excretory strains, of *Escherichia coli*. The *Eco*RI activity was almost entirely recovered in the cytoplasmic fractions and consequently was not released into the extracellular medium by a *tolA* mutant (14).

An even more complicated situation can be expected for the cellular localisation of the type I restriction enzymes because of their multimeric structure and functional complexity. First results, indicating a possible periplasmic location of the *Eco*KI ENase, were published by Schell and Glover (15). It was shown that the type I R-M system of *E. coli* K-12 (*Eco*KI) did not restrict DNA of phage lambda, which had already passed through the bacterial membrane, but modification of this DNA did occur. That observation implied that the restriction enzyme component of this system might be localised either in the periplasm, or at the surface of the bacterial membrane, and that the MTase is cytoplasmic. We have used immunoblotting of subcellular fractions to test this assumption. In this paper, we show that the ENase and MTase of *Eco*KI are both membrane-associated, probably through interaction with chromosomal DNA. However, the HsdR subunit crosses the cytoplasmic membrane allowing it access to the periplasmic space. These experiments demonstrate a novel mechanism for control of restriction versus modification for *Eco*KI ENase and provide further insight into the mechanisms by which the bacterial cell can control opposing functions of an enzyme.

MATERIALS AND METHODS

Microbiological techniques, plasmids, and bacterial strains. Bacterial strains and plasmids used are listed in Table 1. Strain *E. coli* QR47-3, constructed in our laboratory, is a derivative of *E. coli* QR47 (16) with the *hsd* gene deletion transduced from *E. coli* C into *E. coli* K (QR47) using a two-step P1-mediated transduction (17).

Recombinant plasmids used in this work are derivatives of the vector pBR322 (18) or pACYC184 (19). *In vivo* R-M assays were carried out as described previously (4).

Phage buffer, complex LB medium, vitamin supplements were as described (4). The solid medium was LB or M9 with agar added at 1.5%. Soft agar overlay was LB with agar added at 0.6%. Antibiotics were used in the following concentrations: ampicillin, 100 mg ml^{-1}; chloramphenicol, 50 mg ml^{-1}. The strain QR47-3 was transformed with the plasmids pVMC3 producing ENase (*hsdR, hsdM,* and *hsdS* genes), pVMΔC23 producing MTase (*hsdM* and *hsdS* genes) and plasmids pMS_K64, pVM27, and pACWR93 coding for individual subunits (Table 1) using the method described in (20).

TABLE 1

Bacterial Strains and Plasmids

	Characteristics	Reference
Strains		
E. coli K-C600	*thr, leu, thi,* R^+M^+	(44)
E. coli K-QR47-3	*lac,* Δ (*hsd R-S*) R^-M^-	(17)
Plasmids		
pMS_K64	Ap^R, *hsdS*	(45)
pVM27	Cm^R, *hsdM*	(17)
pACWR93	Cm^R, *hsdR*	(17)
pVMC3	Ap^R, *hsdRMS*	(5)
pVMΔC23	Ap^R, *hsdMS*	(46)

Isolation of subcellular fractions. Cells were converted to spheroplasts by incubation at 4°C for about 50 min, with lysozyme and EDTA as previously described (21). The conversion was monitored by phase contrast microscopy. Centrifugation at 13,000*g* for 20 min separated a periplasmic fraction (supernatant) from spheroplasts (pellet). Spheroplasts were subjected to osmotic lysis and, in some experiments disrupted also by sonication. Lysates were separated by centrifugation at 50,000*g* for 1 h into the cytoplasmic fraction (supernatant) and the membrane fraction (pellet). Washed pellet was resuspended in SDS sample buffer and boiled for 5 min to solubilise membrane proteins, or extracted with Triton X-100 as described in the following section. To prepare total cell proteins bacteria were resuspended in SDS sample buffer and boiled for 5 min.

Analysis of proteins. Polypeptides from the subcellular fractions and from the total cell extract were separated by SDS-PAGE (22) using purified enzyme *Eco*KI as a standard marker (5). The antigens were transferred to 0.45 μm PVDF membrane using a Semi-Dry Blotter (Sigma). Individual subunits of ENase *Eco*KI were identified by polyclonal antibodies raised in rabbit against purified enzyme. The immune complexes were detected by the chemiluminescence following the protocol of the ECL Western blotting system (Amersham).

Triton and Triton–EDTA extraction of the envelope preparation. Extraction of spheroplast-envelope preparations with Triton X-100, to solubilise cytoplasmic membrane proteins, and with Triton X-100 plus EDTA, to solubilise outer membrane proteins, was carried out as described (23).

The envelope fraction was extracted with 2.5% Triton X-100 in 10 mM Tris-HCl, pH 7.5, and 10 mM $MgCl_2$ at room temperature for 20 min followed by centrifugation for 60 min at 50,000*g*. The extraction was repeated with 2.5% Triton X-100 without $MgCl_2$.

Triton-insoluble pellet was then extracted with 2.5% Triton X-100 containing 7.5 mM EDTA. Both Triton-soluble and Triton–EDTA-soluble supernatants were stored at −20°C. Triton–EDTA insoluble pellet was solubilised by boiling in SDS sample buffer (22).

Trypsin accessibility. Spheroplasts, resuspended in the isotonic buffer (21), were incubated with trypsin from bovine pancreas (Fluka) at 30°C for 2 h, where upon trypsin inhibitor from soybeans (Fluka) was added. Spheroplasts were then collected, lysed and separated into cytoplasmic and membrane fractions as described above. The control samples were prepared by the same procedure with the omission of either trypsin or trypsin inhibitor.

Benzonase treatment. Spheroplasts were subjected to osmotic lysis. $MgCl_2$ was added (up to 10 mM), followed by treatment with benzonase (Merck) (1 unit/1 ml of spheroplast suspension) on ice for 20 min. Lysed spheroplast were then separated by centrifugation into the cytoplasmic and the envelope fractions and the presence of *Eco*KI subunits was checked in both fractions.

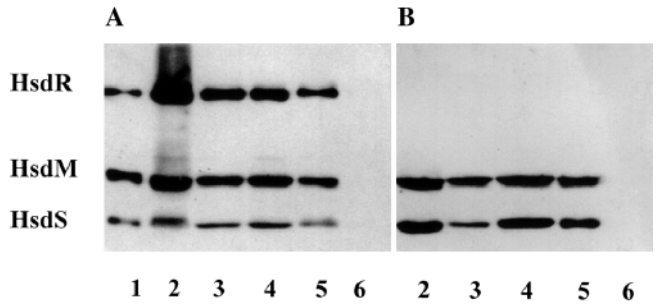

FIG. 1. Immunoblotting of subcellular fractions isolated from *E. coli* QR47-3 harbouring the plasmids with complete endonuclease operon (A) or methylase operon (B). Lane 1, standard (purified R.*Eco*KI); Lanes 2, total cell extracts; Lanes 3, cytoplasmic fractions (from spheroplasts after osmotic lysis); Lanes 4, cytoplasmic fractions (from spheroplasts after sonication); Lanes 5, membrane fractions (from spheroplasts after osmotic lysis); Lanes 6, membrane fractions (from spheroplasts after sonication).

RESULTS AND DISCUSSION

Analysis of Subcellular Fractions

Following the analysis of spheroplasts from *E. coli* QR47-3[pVMC3] cells producing ENase, or QR47-3[pVMΔC23] producing MTase, surprisingly all three Hsd subunits were detected in both cytoplasmic and membrane fractions when spheroplasts were lysed by osmotic shock. However, sonication of the spheroplasts, which disturbs the membrane integrity, released the subunits into the cytoplasm (Fig. 1).

Analysis of subcellular fractions from cells in which only one of each Hsd subunit was produced revealed that HsdR and HsdM were both present as soluble protein in the cytoplasm, while HsdS was insoluble, and sedimented with the membrane fraction (Fig. 2). Insolubility of HsdS subunits has been already demonstrated (7, 24); although the HsdS subunit of the *Eco*AI system is soluble unless the order of the domains is altered (25).

HsdR and HsdM were shown to be associated with the membrane fraction only when co-produced with HsdS as either ENase or MTase complex (Figs. 1A and 1B). The presence of *Eco*KI subunits in both cytoplasmic and membrane fractions might, therefore, reflect the balance between free subunits, and membrane bound subunits of the assembled R-M enzyme.

To exclude an artifactual distribution of the Hsd subunits due to their overproduction from multicopy plasmids we performed analogous experiments with chromosomally located *hsd* genes. The results obtained with the strain *E. coli* C600 confirmed the same localisation of the *Eco*KI subunits as found for the plasmid system (data not shown).

Since it has been previously postulated that *Eco*KI might be located in the periplasm, this fraction was also analysed for enzyme. Although, traces of all subunits were also found in the periplasm, we believe this to be a false result because of contamination of the same fraction by β-galactosidase (a cytoplasmic protein used as a control). This suggestion was confirmed by the analysis of the *Eco*KI R-M phenotype in a *tolA* strain of *E. coli*, which spontaneously releases periplasmic proteins into the culture medium (26). This strain has been previously used to demonstrate cellular localisation of *Eco*RI (14). From the observation of identical R-M phenotypes for parental (wild-type) and *tolA* strains (data not shown) we could exclude the predominant localisation of free enzyme in the periplasm.

Selective Solubilisation of Envelope Proteins

Schnaitman (23) has shown that cytoplasmic membrane proteins are selectively solubilised from the envelope fraction by extraction with Triton X-100, while Triton X-100 plus EDTA can be used to solubilise the cell wall proteins. (The striking resistance of the outer membrane of *E. coli* to solubilisation by Triton X-100 is probably due to the action of divalent cations in stabilising the structure of the cell wall.)

All three Hsd subunits of the *Eco*KI ENase complex were recovered in the Triton X-100 soluble fraction demonstrating the association of the enzyme with the cytoplasmic membrane rather than with the outer membrane. The HsdS subunit when expressed in the absence of HsdR and HsdM is insoluble and remains in the Triton X-100-EDTA insoluble pellet (data not shown).

Effect of Benzonase on EcoKI-Membrane Association

As the resident DNA is one of the substrates for *Eco*KI we tested whether the membrane association might be mediated through DNA. For this purpose we

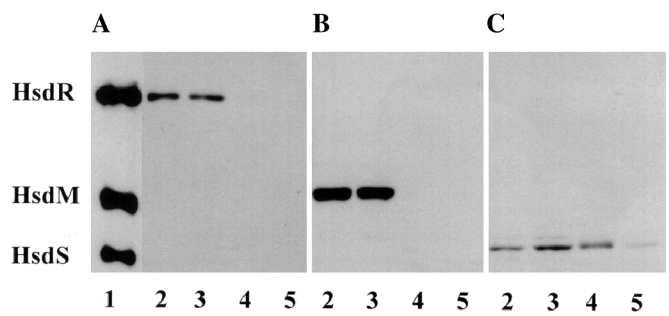

FIG. 2. Immunoblotting of subcellular fractions isolated from *E. coli* QR47-3 harbouring the plasmids with individual *hsd* genes: *hsdR* (A), *hsdM* (B) and *hsdS* (C) Lane 1, standard (purified R.*Eco*KI); Lanes 2, cytoplasmic fractions (from spheroplasts after osmotic lysis); Lanes 3, cytoplasmic fractions (from spheroplasts after sonication); Lanes 4, membrane fractions (from spheroplasts after osmotic lysis); Lanes 5, membrane fractions (from spheroplasts after sonication).

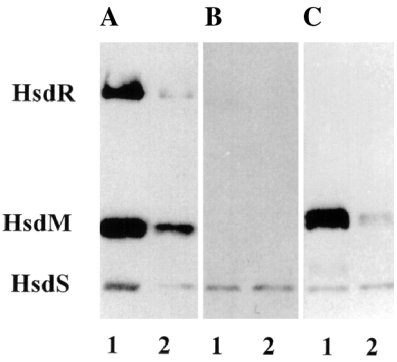

FIG. 3. Effect of benzonase on the association of *Eco*KI endonuclease (A), HsdS subunit (B), and methylase (C) with cytoplasmic membrane. Immunoblotting of membrane proteins. Lane 1, control without benzonase; Lane 2, treatment with benzonase.

used benzonase—a highly active endonuclease that hydrolyses both single and double stranded DNA and RNA to short oligonucleotides (27). Figure 3 demonstrates that, after treatment of spheroplasts with benzonase in the course of osmotic lysis, all three subunits of ENase, or both subunits of MTase were released from the membrane into the soluble fraction. These data lead to the conclusion that the attachment of the enzyme complex to the cytoplasmic membrane is DNA mediated.

Bacterial chromosomal DNA has long been known to be associated with the membrane (28) and this attachment may play a role in the partitioning mechanism of the bacteria (29). Moreover the membrane is directly involved in the control of chromosomal DNA replication (30). Association of replicative plasmid and viral DNA (another potential substrate for ENase) with the bacterial membrane has also been reported (31, 32). The association of *Eco*KI with the bacterial membrane, through DNA binding, may reflect the requirement of the enzyme to act as a maintenance MTase (33) allowing rapid access to newly synthesised (hemimethylated) DNA, which is the natural methylation substrate for both ENase and MTase.

The insoluble HsdS subunit, when produced alone, sedimented with membrane fraction regardless of the presence of benzonase. Obviously only HsdS correctly assembled as either MTase or ENase can mediate the binding of the enzyme to the DNA-membrane complex.

Trypsin Accessibility Experiments

Proteases are reagents commonly employed in the analysis of membrane protein topology. They do not permeate the membranes, because of their size, and the hydrophobic lipid bilayer maintains a reasonably resistant barrier to their action. If intact spheroplasts are exposed to a protease, protein species on the outer face of cytoplasmic membrane, that are accessible to and digestible by the protease, will be degraded, while cytoplasmic proteins, or proteins attached to the inner face of membrane, will be protected (21, 34).

When spheroplasts of the bacterial strain producing ENase (QR47-3[pVMC3]), were treated with trypsin and the proteolysis was stopped by the addition of trypsin inhibitor before osmotic lysis of spheroplasts, only the HsdR subunit of the complex enzyme was digested by the protease as demonstrated by the disappearance of the band corresponding to HsdR polypeptide. In place of HsdR, after proteolysis, two polypeptides T1 and T2 of higher mobilities were detected on Western blot (Fig. 4A). The sum of their molecular masses (82 kDa for T1 and 54 kDa for T2) corresponds to the theoretical molecular mass of HsdR polypeptide (35). Moreover the molecular mass of the proteolytic fragment T2 is identical to fragment T54 produced by limited proteolysis of purified HsdR subunit (36). Under the same conditions HsdM and HsdS polypeptides from either the ENase complex, or from the MTase complex (QR47-3[pVMΔC23]), were fully protected from protease action. However, both HsdM and HsdS subunits were completely digested when the addition of trypsin inhibitor was omitted so that proteolysis continued after lysis of spheroplasts (Fig. 4B), showing that the polypeptides themselves are not structurally resistant to the digestion.

These results imply an association of *Eco*KI ENase with the cytoplasmic membrane in such a way that HsdR (or part of HsdR) is exposed on the outer surface of the cytoplasmic membrane while HsdM and HsdS (MTase component) seem to be hidden at some distance from the actual membrane surface; perhaps on the inner face of cytoplasmic membrane attached through DNA.

Since restriction activity is primarily protective and is meant to prevent phage invasion, one can conclude that it should be localised to the cell periphery. However, the modification activity is that of a maintenance

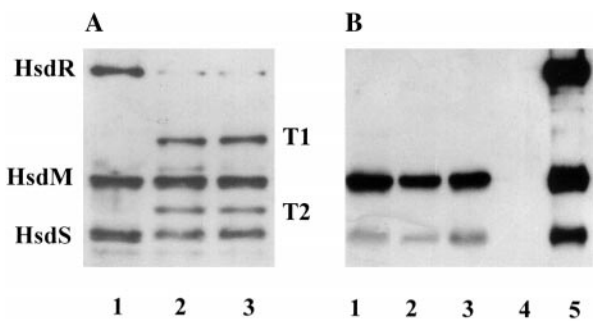

FIG. 4. Analysis of membrane proteins isolated from trypsin-treated spheroplasts of *E. coli* QR47-3 with complete endonuclease operon (A) and methylase operon (B). Lanes 1, membrane proteins—control without trypsin; Lanes 2, membrane proteins prepared from trypsin-treated spheroplasts (trypsin 0.2 mg/ml); Lanes 3, membrane proteins prepared from trypsin-treated spheroplasts (trypsin 0.4 mg/ml); Lane 4, as lane 3 (methylase) without inhibitor; Lane 5, standard (purified R.*Eco*KI).

MTase (33) and would be primarily associated with newly-replicated DNA (which must be methylated on the newly synthesised strand). We have found that the MTase components of the R-M enzyme are associated with the inner side of the cytoplasmic membrane probably via interaction with DNA. However, the HsdR subunit appears to cross the cytoplasmic membrane and is exposed on the periplasmic-side of the membrane possibly allowing easy access to incoming DNA from bacteriophage. Evidence to support such access to incoming DNA is suggested by recent observations involving the translocation of the bacteriophage T7 genome by the *Eco*KI ENase (37). It is also possible that the ENase can initially bind incoming DNA through HsdR-based, non-specific DNA binding (38) even when the ENase is attached to the host chromosome through HsdS.

Recently, Makovets *et al.* (39) have shown that the *Eco*KI R-M enzyme responds differently to incoming phage DNA as a restriction target than it does to chromosomal DNA in cells that have acquired unmodified chromosomal recognition sites. The cellular restriction enzyme, which is capable of destroying the chromosomal unmodified DNA, under the conditions employed in the experiments Makovets *et al.* described, thus leading to cell death, is prevented from such activity by the proteolytic action of ClpXP protease. This protease destroys the HsdR subunit and thus prevents restriction activity by the R-M enzyme in the cell. However, the presence of this protease does not totally alleviate restriction activity *in vivo*. The plating efficiency of bacteriophage lambda is still reduced even when ClpXP protease can destroy the cellular HsdR. We believe that this residual restriction activity reflects the activity of membrane embedded R-M enzyme, where the HsdR subunit is protected from ClpXP proteolysis.

Recently, a number of groups have shown that temporal control of the restriction activity of type I R-M enzymes is accomplished through post-translational mechanisms (7, 39–43). Spatial compartmentalisation as demonstrated in this work, contributes to the control of restriction versus modification activities of these complex R-M enzymes.

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