Supplementary Data to:

Recycling of protein subunits during DNA translocation and cleavage by Type I restriction-modification enzymes

Michelle Simons and Mark D. Szczelkun

SUPPLEMENTARY MATERIALS & METHODS

Cloning of EcoKI genes
The EcoKI genes were cloned as follows: The \( hsdR \) gene was amplified from pVMC3 (1) by PCR using oligonucleotides 5\(^{-}\) GCGTAAGTCTCGAGTCAGGCAGCTCGTCCCAGATATA ATCGCTG -3\(^{-}\) and either, 5\(^{-}\) GCGTAAGTCATATGTTATGGGCGCTTAAATATTTGGACAG -3\(^{-}\) (to generate the “full length” clones), 5\(^{-}\)-GCGTAAGTCATATGATGAATAAATCCAATTT TGAAT-3\(^{-}\) (to generate the \( \Lambda 19 \) clones) or 3\(^{-}\)-GCGTAAGTCATATGAAATATTTGGACAGGC -5\(^{-}\) (to generate the \( \Lambda 20 \) clones). The PCR products were digested with NdeI and XhoI and inserted into pETDuet-1 or pRSFDuet-1 (Novagen, Germany) digested with NdeI and XhoI.

The \( hsdR \) genes in all clones were fully sequenced; The \( hsdS \) gene was amplified from pVMC3 by PCR using oligonucleotides 5\(^{-}\) GCGTAAGTCATATGAGTTCCCAATTG -3\(^{-}\) and 5\(^{-}\)-GCGTAAGTCATCGAGTCAGGATTTTACGTGAGGC -5\(^{-}\). The PCR product was digested with NdeI and XhoI and inserted into pETDuet-1 digested with NdeI and XhoI. The \( hsdM \) gene was amplified from pVMC3 by PCR using oligonucleotides 5\(^{-}\)-GCGTAAGTCATATGATGAATAAATCCAATTT TGAAT-3\(^{-}\) and 3\(^{-}\)-GCGTAAGTCATATGAAATATTTGGACAGGC -5\(^{-}\). The PCR product was digested with NdeI and XhoI and inserted into pETDuet-1 digested with NdeI and XhoI. The \( hsdM \) gene was amplified from pVMC3 by PCR using oligonucleotides 5\(^{-}\)-GCGTAAGTCATATGAGTTCCCAATTG -3\(^{-}\) and 5\(^{-}\)-GCGTAAGTCATATGATGAATAAATCCAATTT TGAAT-3\(^{-}\) and 3\(^{-}\)-GCGTAAGTCATATGAAATATTTGGACAGGC -5\(^{-}\). The PCR product was digested with NdeI and XhoI and inserted into pETDuet-1-HsdS clone from the step above, digested with NcoI and EcoRI and inserted into the pETDuet-1-HsdS clone from the step above, digested with NcoI and EcoRI. QuikChange mutagenesis (Stratagene, USA) with oligonucleotides 5\(^{-}\)-CTTTAAAGAGATATACCAG AACAATAACGATCTG -3\(^{-}\) and 5\(^{-}\)-CAGATCGTTATTGATCATGTTATATCCTCCTATTAA
AG-3’ was used to mutate the NcoI site to revert the hsdM gene to the correct sequence (2). The complete sequences of the hsdM and hsdS genes were confirmed.

**Protein Expression and Purification**

Expression trials for EcoKI HsdR were performed using the following *E. coli* strains (all from Novagen): BL21(DE3), ArcticExpress(DE3) RIL, B834(DE3), BL21(DE3) pLysS, BL21(DE3) Star, NovaBlue(DE3), Rosetta 2(DE3) and Tuner(DE3). Cells were transformed with the pRSF-based expression clones and then grown in 30 mL LB containing 30 μg/mL kanamycin. For ‘standard’ expression, cultures were grown at either 27 ºC, 30 ºC or 37 ºC to an OD of 0.5 and then protein expression induced with 0.5 mM IPTG and growth continued at 27 ºC overnight. For Tuner(DE3) cells, IPTG was added between 25-500 mM. Alternatively 30 mL LB was supplemented with Overnight Express Auto Induction System 1 (Novagen) components and cultures were grown overnight at 30 ºC.

EcoKI HsdRΔ20 was purified from *E. coli* BL21(DE3) cells transformed with the appropriate HsdR recombinant clone. Cultures were grown in 0.5 L of LB medium containing 30 μg/mL kanamycin supplemented with Overnight Express Auto Induction System 1 components at 30 ºC for ~18 hours. Cells were harvested and resuspended in 50 mL Buffer A [50 mM Tris, pH 8.0, 10 mM MgCl₂, 0.1 mM EDTA, 10 % (v/v) glycerol, 7 mM 2-mercaptoethanol, 1 mM benzamidine, 0.1 mM PMSF] supplemented with EDTA free protease inhibitor cocktail (Roche). The cells were lysed by sonication and the clarified by centrifugation at 7,358g for 10 minutes followed by 106,255g for 1 hour 50 minutes. The supernatant was filtered at 0.45 μm and loaded onto a 60 mL DEAE Sepharose column (GE Healthcare UK Ltd) equilibrated with Buffer B (50 mM Tris, pH 8.0, 10 mM MgCl₂, 0.1 mM EDTA, 7 mM 2-mercaptoethanol). Bound protein was eluted with a 500 mL NaCl gradient (0-500 mM) in Buffer B. Fractions containing HsdR were pooled and concentrated using Amicon Ultra-15 centrifugal filter devices (10,000 MWCO) (Millipore), and then applied to a High Load 16/60 Superdex 200 column (GE Healthcare UK Ltd) equilibrated with Buffer C (50 mM Tris, pH 8.0, 10 mM MgCl₂, 0.1 mM EDTA, 7 mM 2-mercaptoethanol, 200 mM NaCl). Fractions containing HsdR were pooled and concentrated as before. HsdR was stored in 50% (v/v) glycerol at −20 ºC. Protein concentration was determined by absorbance at 280 nm, using ε = 114,233 M⁻¹ cm⁻¹ (3).
EcoKI MTase was purified from *E. coli* BL21(DE3) cells (Novagen) transformed with the HsdM-HsdS recombinant clone. Cultures were grown in 1 L of LB medium containing 50 μg/mL ampicillin at 37 °C to an OD of 0.4 – 0.5 at 600 nm. Protein expression was then induced with 0.5 mM IPTG and the cells grown at 27 °C for 20 hours. Cells were harvested and resuspended in 50 mL Buffer A and lysed by sonication. The cell extract was clarified by centrifugation at 7,358g for 10 minutes followed by 106,255g for 1 hour 50 minutes. The supernatant was filtered at 0.45 μm and loaded onto a 60 mL DEAE Sepharose column (GE Healthcare UK Ltd) equilibrated with Buffer B. Bound protein was then eluted with a 500 mL NaCl gradient (0 – 500 mM) in Buffer B. Fractions containing MTase were pooled and dialysed against Buffer B for >2 hours, and then applied to a HiPrpep 16/10 Heparin FF column (GE Healthcare Uk Ltd), equilibrated with Buffer B. Bound proteins were eluted with a 200 mL NaCl gradient (0 – 500 mM) in Buffer B. The fractions containing MTase were pooled and dialysed as before, then applied to a MonoQ 5/50 GL column (GE Healthcare UK Ltd), equilibrated with Buffer B and eluted with a 20 mL NaCl gradient (0 – 500 mM) in Buffer B. Two separate peaks were produced that contained MTase; one was identified as the active M<sub>2</sub>S<sub>1</sub> species and the other as an inactive M<sub>1</sub>S<sub>1</sub> species. The M<sub>2</sub>S<sub>1</sub> species was applied to a High Load 16/60 Superdex 200 column (GE Healthcare UK Ltd) equilibrated with Buffer C. The fractions containing the MTase were pooled and concentrated using Amicon Ultra-15 centrifugal filter devices (10,000 MWCO). EcoKI MTase was stored in 50% (v/v) glycerol at – 20 °C. Protein concentration was determined by absorbance at 280 nm, using ε = 143,140 M<sup>-1</sup> cm<sup>-1</sup> (3).

**Subunit analysis**

To quantify the subunit components, SDS-PAGE gels stained with colloid-blue stain (BioRad BioSafe) were quantified using Lab Works Image Acquisition and Analysis Software (UVP, CA, USA) to calculate the intensity of each band using a linear scale. Intensity values were corrected on the basis of the molecular weight of each of the protein subunits relative to HsdM and assuming a linear relationship between dye binding and molecular weight.
SUPPLEMENTARY RESULTS

Cloning and purification of EcoKI as separate HsdR and MTase pools
To assess the subunit dynamics of EcoKI using our previous approaches (4), we needed to have separate preparations of the MTase and HsdR so that the two could be varied independently, rather than holoenzyme preparations where the MTase and HsdR are co-expressed and the \( R_2M_2S_1 \) complex purified directly. Previous work on the isolated EcoKI HsdR subunit used protein expressed and purified from cells transformed with a multicopy plasmid containing \( hsdR \) and its natural promoter, \( \text{P}_{\text{res}} \) (3). The reported yields using this approach were \(~1 \text{ mg of } 95\% \text{ homogenous protein from } ~15 \text{ g of cell paste. To try to improve the yield, the EcoKI HsdR gene was cloned into the Novagen DUET series of plasmids, under control of IPTG-inducible } \text{P}_{\text{T7lac}} \). (A number of clones were generated with different truncations and N-terminal extensions – see below). Previous attempts to over-express HsdR from an inducible promoter have failed (3,5). Using pET-DUET which encodes an ampicillin resistance marker, we were also unable to detect any measurable levels of protein using SDS-gels and colloidal Coomassie staining (data not shown). However, by using pRSF-DUET which encodes a kanamycin resistance marker and which has a moderately higher copy number than pET-based vectors, measurable levels of over-expression were obtained. It is possible that the poor expression using ampicillin resistant plasmids is because of plasmid instability as a result of HsdR toxicity; since \( \beta \)-lactamase is excreted into the media, the ampicillin concentration would be reduced allowing cells which have lost the plasmid to out-compete the more slowly growing cells carrying the plasmid. This would not occur for the kanamycin resistance cassette.

Despite the increased levels of protein observed, expression trials using \( \text{E. coli} \) BL21 (DE3) and LB broth resulted in relatively poor cell growth and thus low final cell mass. To increase cell mass and to also attempt to further increase expression, alternative competent cell strains and growth conditions were examined (Supplementary Materials and Methods). Overall, the conditions that improved HsdR expression the most were using BL21(DE3) with Novagen Overnight Express autoinduction system 1 at 30 °C (Supplementary Figure 1A).

Initial expression and purification attempts were conducted using a clone of \( hsdR \) as described by Loenen et al. (2), which we will term “full length”. However, Dryden and co-workers showed that EcoKI HsdR expressed from \( \text{P}_{\text{res}} \) is present as a mix of two truncated
proteins that are 19 and 20 amino acids shorter at the N terminus (3), which we will term ΔN19 and ΔN20, respectively. In our initial HsdR preparations based on the full length gene, N-terminal sequencing (Will Mawby, Proteomics Facility, University of Bristol) indicated that there was a mixture of full length and truncated versions of HsdR in agreement with the previous findings (3). In cloning the HsdR gene in the DUET series, the ATG start codon of the full length gene was placed at an ideal distance from a consensus Shine Delgarno (SD) sequence. It may be that the truncated proteins result from either initiation at an alternative SD within the full length sequence or that the full length protein is processed. Since the truncated HsdRs are the proteins most likely present in the cell (3), additional clones were generated in which ATG start codons for ΔN20 or ΔN19 were cloned at the idealised pRSF-DUET translation start site. The truncated HsdRs were also expressed and purified in the same way as the full length protein (data not shown). Whilst N-terminal sequencing of the ΔN20 protein indicated the expected N-terminus, the ΔN19 clone gave a mixture of the ΔN19 and ΔN20 proteins (Will Mawby, Proteomics Facility). As the ΔN20 version represents a single enzyme species following over-expression, this was used for all experiments described in the main text.

A strategy for purification of EcoKI HsdR has been previously described (3). However, this was found to give very low yields from our over-expression system and a modified protocol was adopted (Supplementary Materials and Methods). The final ΔN20 preparations of HsdR were ~75% pure, as judged by SDS-PAGE densitometry (Supplementary Figure 1A). Therefore, despite the increased over-expression (~ 1 mg of HsdR from 4 g of cells), there has been a loss in purity compared to the previously described method. Nonetheless, the preparation was found to be free of contaminating activities that interfered with our assays and was used without further purification. The concentration of HsdR was calculated to include a correction for the degree of purity. The validity of this was checked by comparing to an EcoKI endonuclease preparation that was independently-purified and supplied by David Dryden and co-workers (University of Edinburgh) – a good correspondence between the concentrations of the HsdR subunits was noted (Supplementary Figure 1B – see below).

To over-express the EcoKI MTase, the HsdS and HsdM proteins must be co-expressed as otherwise the HsdS protein is insoluble. The individual hsdS and hsdM genes were cloned at the separate T7 promoters of pET-DUET (Materials and Methods). We developed a modified purification strategy compared to that published (Materials and Methods). The expression of
EcoKI MTase from BL21(DE3) transformed with the pET-DUET clone using standard LB media resulted in a large over-expression of HsdM but a very weak expression of HsdS (data not shown), most likely reflecting the instability of the HsdS subunit. Therefore care was taken to purify a complex with the correct M$_2$S$_1$ stoichiometry. The relative subunit composition was judged by densitometry of SDS-PAGE gels, making the assumption that staining is proportional to protein molecular weight (Figure 1B). This gave a ratio between the two subunits of 2.00 M: 1.27 S (normalised to the M subunit, Table 1). The final yield of the MTase was ~1 mg from ~4 g of cells, with contaminants comprising less than 5% of the total.

A direct comparison of the relative subunit concentrations was made between the MTase preparation, a reconstituted endonuclease where the MTase was mixed with a two-fold molar excess of HsdR without further purification, and the holoenzyme preparation of EcoKI supplied by David Dryden (Supplementary Figure 1B, Supplementary Table 1). We estimated the relative concentration of each subunit by densitometry. Because of co-migration of contaminants from the HsdR preparation at the same apparent molecular weight as the HsdS subunit (Figure 1A), we normalised the data relative to the HsdM subunit which by comparison is less contaminated by co-migrating proteins. This gave similar R:M:S ratios for the holoenzyme and for the reconstituted enzyme (Supplementary Table 1). Within the error of these estimates (~ ±0.2), this indicates that both enzyme preparations have a similar composition and that our estimates of the HsdR concentration are sufficiently accurate.

SUPPLEMENTARY REFERENCES

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Supplementary Table 1. Relative intensities of HsdS, HsdM and HsdR bands calculated from colloidal-blue stained SDS gels (Supplementary Materials and Methods, Supplementary Figure 1B). Standard errors in determining the band intensities are in the range ±0.20. *Values are calculated by correcting the intensities for relative differences in MW assuming a linear relationship between mass and staining intensity and the HsdM value was nominally set to a value of 2.

|                     | HsdR | HsdM* | HsdS |
|---------------------|------|-------|------|
| Holoenzyme (Edinburgh) | 2.41 | 2     | 0.97 |
| Reconstituted (Bristol) | 2.28 | 2     | 1.12 |
| MTase (Bristol)       | ---  | 2     | 1.27 |
**Supplementary Figure 1.** Expression and purification of EcoKI. (A) SDS-PAGE gels showing expression of EcoKI HsdR under ‘standard’ conditions compared to expression using media supplemented with Overnight Express Media, “OE”. Samples taken at different stages of the purification are also shown (U: before induction with IPTG, I: after induction with IPTG). See Supplementary Materials and Methods, and Supplementary Data text for further details. (B) Colloid-blue stained SDS-PAGE gels comparing different preparations of EcoKI: an R<sub>2</sub>M<sub>2</sub>S<sub>1</sub> complex purified as a complete endonuclease, “holoenzyme”, courtesy of David Dryden (University of Edinburgh); an R<sub>2</sub>M<sub>2</sub>S<sub>1</sub> complex reconstituted from separately prepared HsdR and MTase, “Reconstituted”; and the MTase preparation, “MTase”. The results of quantification are presented in Supplementary Table 1.