An alternative approach for screening active Bam HI variants: Overexpression in T-7 RNA polymerase based system

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The type II restriction endonuclease, BamHI, has been overexpressed in E. coli by cloning the BamHI gene in frame with an E. coli Ribosome Binding Site (RBS) under the T7 promoter of an E. coli expression vector pRSET A. The expression level of BamHI endonuclease using this construct was found to be higher than that reported of the overexpressing clone pAEK14. Our overexpressing clone, pAABRw in BL21 cells in presence of BamHI methylase in pMAP6 following induction with IPTG yields about 9.2x10^6 units per gram wet cell paste. In vivo activity of the recombinant endonuclease could be confirmed by the SOS induction assay in JH139 cells even in the absence of T7 polymerase and cognate BamHI methylase because of leaky expression in E.coli. This provides an alternate way to screen the active endonuclease and its variants.

Overexpression of recombinant proteins encounters various problems which is further compounded if the protein is toxic to the cell. In this paper, we describe a strategy which can be generally used for overexpression of endonucleases. Recombinant endonucleases cannot be expressed unless cognate methylase gene is also present and special genetic screens are used to detect the clones expressing viable proteins. To screen binding proficient but catalytically deficient variants of EcoRI, mutants were isolated that damage DNA despite methylation of EcoRI site; these mutants display reduced sequence specificity. This method was extended to BamHI endonuclease, where the three temperature sensitive mutants with reduced phage restriction were identified. We reported an alternative strategy for screening and overexpression, where the endonuclease gene was cloned with its own RBS under the T7 promoter (pPMBR.0) and was observed to express in E. coli even in the absence of T7 polymerase and the cognate methylase. A low level of leaky expression by the host polymerase gave rise to SOS response which assisted in screening catalytically active mutants. BamHI was finally expressed in a complete system which included constitutive expression of BamHI methylase and heat inducible T7 polymerase. The expression level was, however, very low even though the gene was under the control of a strong promoter like T7. This was probably because the native Bacillus RBS of BamHI gene, GGGGG, was unusual and inefficient in the E. coli host. So, it was decided to clone the gene at NdeI - EcoRI site of the expression vector pRSET A which bears the same T7 promoter, so that the endonuclease gene replaces the gene 10 of phage T7 present in frame with the E. coli RBS. Here, we present the results of this overexpression strategy.

One specific advantage in our system is that the recombinant protein under the control of T7 promoter can be exclusively labelled with 35S-methionine by inducing the T7 RNA polymerase in cells growing in minimal medium under conditions when the host E. coli RNA polymerase is inhibited with rifampicin. Secondly, T7 promoter is possibly recognized weakly by E. coli polymerase leading to low level transcription, which is inefficiently translated in E. coli when the RBS is of Bacillus origin. This leaky low level expression gives us a handle to screen for catalytic variants through in vivo SOS based assay because DNA damage is not severe but enough to elicit SOS response.

Materials and Methods

Bacterial strains, plasmid and media

The following bacterial strains and plasmids were used: DH10B (mcr-) harbouring the plasmid pMAP6, a derivative of pACYC184 (tet) containing the BamHI methylase gene cloned earlier in our laboratory under it’s own promoter at the EcoRI site. BL21 (DE3) was used for the expression of BamHI R gene under the T7 promoter. JH139 which carry...
dinD1:: lacZ fusion was obtained from Dr. P Model and used to detect in vivo SOS induction. pRSET A (Invitrogen) was used for subcloning the BamHI R gene from the recombinant clone pMPBPamR.0 for overexpression. Luria Bertani (LB) medium and Luria agar were supplemented with ampicillin (Amp) at 100 μg/ml, tetracycline (Tet) at 10 μg/ml or kanamycin (Kan) at 50 μg/ml as and when required. 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Sigma) was used at a concentration of 40 μg/ml.

**Expression and purification of recombinant BamHI protein**

For expression of the recombinant BamHI endonuclease, the recombinant plasmid pAABRw was co-transformed along with pMAP6 into BL21(DE3) cells. The transformant (single colony) was inoculated into 3 ml LB containing antibiotics ampicillin (100 μg/ml) and tetracycline (10 μg/ml) and grown overnight at 37°C in the shaker. The overnight grown culture was used to inoculate 10 x100 ml media containing appropriate amounts of the two antibiotics. The transformants were grown till OD600 reached 0.6 and induced with IPTG to a final concentration of 0.4 mM. The cells were allowed to grow for 3 hr and then harvested at 4°C and sonicated in lysis buffer (10 mM potassium phosphate (pH 7.0), 1 mM DTT, 1 mM EDTA, 200 mM NaCl, 1 mM PMSF and 100 μg/ml lysozyme) using Ultrasonics (model no. W385) sonicator. The sonicated cell suspension was centrifuged to pellet down the cell debris and the supernatant was loaded onto the phosphocellulose column, already activated and equilibrated with the equilibration buffer (10 mM potassium phosphate (pH 7.0), 200 mM NaCl, 1 mM EDTA, 1 mM DTT and 10% glycerol). The bound proteins were eluted with a 200 ml linear gradient of NaCl (0.2-1 M) in equilibration buffer. The eluate was collected in 2 ml fractions, 1 μl of each fraction was checked for BamHI activity using plasmid DNA, pRSET A. The fractions showing BamHI endonuclease activity were pooled and aliquots of this were used to measure the protein concentration using the Bradford dye binding micro assay with BSA as standard to find the specific activity after phosphocellulose column purification. The rest of the pooled fraction was loaded onto hydroxyapatite column equilibrated with the equilibration buffer. The column was then washed and eluted with a 100 ml gradient of potassium phosphate (pH 7.0) from 0.01-0.5 M. Peak endonuclease activity got eluted between 0.15 to 0.25 M potassium phosphate. The fractions showing activity were pooled and again the protein content was quantified using the Bradford’s dye binding micro assay. Subsequently the BamHI endonuclease assay was performed to calculate the specific activity. The pooled fraction after hydroxyapatite step was concentrated and the buffer content changed to storage buffer (300 mM KCl, 10 mM Tris-HCl (pH 8.0), 1 mM DTT, 1 mM EDTA and 10% glycerol) using centricon YM-10 assembly.
(Millipore) (10 kDa). The purified enzyme was stored at 4°C. Enzyme samples collected after each step were analysed on 12% SDS-PAGE.

**BamHI endonuclease assay**

For this, 1 μl of the eluted fraction was added to 10 μl digestion mix containing 100 ng pRSET A which has a single site for BamHI, in BamHI buffer [10 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 100 mM NaCl and 1 mM DTT] and incubated at 37°C for 1 hr.

**Determination of BamHI enzyme units**

Lambda (λ) DNA (Promega) was used as substrate for determining units of BamHI endonuclease activity present in the pooled fraction as well as the specificity of mutant endonucleases. One μg of λ DNA was digested with decreasing amounts of pooled fraction in 20 μl reaction mix containing BamHI buffer at 37°C for 1 hr. The minimum amount of solution capable of complete digestion of 1 μg λ DNA in 1 hr was defined as one enzyme unit.

**Results and Discussion**

**Construction and characterisation of the recombinant BamHI clone**

The BamHI R gene was cloned in frame under the T7 promoter of the E. coli expression vector pRSET A as described in Methods. The construct was designated as pAABRw. The schematic diagram of the construction of pAABRw is shown in Fig. 1.

The recombinant plasmid, pAABRw was characterized through restriction analysis. The BamHI clone was further verified by sequencing.

**In vivo SOS induction assay**

In the absence of the cognate methylase, BamHI endonuclease would cleave at the BamHI sites on the host DNA. Such an action in E. coli induces SOS response when a set of genes is triggered on to repair the damage. SOS response is monitored using strains which carry the lactose operon fused to the damage inducible (din) promoter. The original din::lacZ fusion strains are Amp. Since our plasmid pAABRw is also Amp, we used a derivative of the original strain, JH139, developed by Heitman and Model which is Kan' instead of Amp'. Even in the absence of T7 polymerase, there is some leaky expression of BamHI endonuclease from T7 promoter which produce DNA breaks in vivo, in the absence of BamHI methylase, inducing the SOS DNA repair response. The construct, pAABRw when transformed into JH139 cells, did induce SOS response. In fact, the extent of DNA damage was so much that the cells did not survive. No colonies could be seen, rather a blue patch of lysed cells was seen on the plate. Cells transformed with only the vector pRSET A gave white colonies which turned faint blue on keeping the plate at 4°C for 2-3 days. Early lysis of the transformed cells was also evident in the growth curve of transformed cells. We believe this is due to higher level of expression in pRSET A vector since the original BamHI clone containing the native RBS in pT7-6, pPMBamR.0 could grow stably and did form blue colonies on X-gal plates.

Even in the presence of cognate methylase expressing plasmid (pMAP6), the DH10B cells containing the recombinant pRSET A plasmids which expressed active BamHI endonuclease, were unstable and lost the recombinant plasmids on storage, whereas the original pT7-6 clone containing the native RBS was stable and did not lose the recombinant plasmids on storage. This observation suggests that even in the presence of the cognate methylase, the level of BamHI endonuclease produced due to leaky expression of the T7 promoter in E. coli is proving toxic to the cells leading to plasmid loss. The few transcripts that are made from pRSET A are very efficiently translated to raise the level of toxic protein which kills the cells.

**Expression and purification of the recombinant BamHI endonuclease**

The E. coli host strain DH10B in which the two plasmids, pMAP6 and pAABRw, have been transformed lacks T7 polymerase. The BamHI gene which is under the T7 promoter will not normally express in this host strain. For overexpression, therefore, both the plasmids were co-transformed into BL21(DE3) competent cells which contain an inducible T7 RNA polymerase gene. Fig. 2 shows the improved level of expression of BamHI endonuclease in pAABRw as compared to the level of expression in pPMBamR.0.

It was observed, however, that the BL21 transformants, on storage in the form of glycerol stock, showed lower expression of the recombinant BamHI endonucleases. In fact, cells stored for 2-3 months completely lost the ability to express the BamHI endonuclease on induction with IPTG. It is also observed that the level of expression decreases as the culture volume increased, the amount of BamHI induced per unit volume is lower in 200 ml culture as compared to that in 10 ml culture and in 1 litre culture, it is still lower (all of them were induced at
the same OD). We, therefore, grew the cells in ten flasks of 100 ml LB medium each. It is known that BL21(DE3) cells have a problem of plasmid instability when the expressed gene product is toxic to the host cell. So each time we wanted to overexpress BamHI protein, it was essential to freshly transform the BL21(DE3) cells with the appropriate clone.

The growth curves of the BL21 (DE3) transformants of the wild type clones, pAABRw and pPMBamR.0 in the presence of pMAP6 were checked. We found that the BL21 cells transformed with pPMBamR.0 grew normally, reaching the stationary phase at about 1.25 OD_{600}, whereas the cells transformed with pAABRw reached the stationary phase quite early, the maximum OD_{600} recorded was 0.61 (Fig. 3). This indicates that even in the presence of BamHI methylase, the basal level of BamHI endonuclease produced (before IPTG induction) was toxic to the cells. To check if we could improve the stability of the expressing clones, pAABRw was transformed into BL21(DE3)pLysS cells. This strain contains a plasmid, pLysS, which

![Diagram of plasmid construction](image)

**Fig. 1 — Construction of the overexpressing wild type BamHI clone (pAABRw).**
carries the gene encoding T7 lysozyme, a natural inhibitor of T7 RNA polymerase which is supposed to inhibit the basal level expression of the target genes. The growth of the transformants, BL21(DE3) and BL21(DE3)pLysS were monitored. Not much difference was observed, the cells containing pLysS grew to a maximum OD$_{600}$ of 0.72 and the cells which did not contain pLysS grew to a maximum OD$_{600}$ of 0.62 (Fig. 3 inset).

BamHI endonuclease was purified by chromatography as described in Methods. Fig. 4 shows the protein content after each purification step.

The specific activity of the purified endonuclease after each purification step was checked using λ DNA. Table 1 shows the total protein content, number of units and specific activity obtained after each purification step. Due to interference by other nucleases, we could not estimate correctly the specific activity in the crude extract. The expression level of the wild type BamHI clone, pAABRw, was found to be greater than that of the overexpressing clone pAEK14 reported by Jack et al. The overexpressing clone pAEK14 in ADK21 cells following induction with IPTG has been reported to yield about $5 \times 10^6$ units per gram wet cell paste whereas the clone pAABRw in BL21(DE3) cells in presence of pMAP6 following induction with IPTG yields about $9.2 \times 10^6$ units per gram wet cell paste.

Table 1 — Purification status of wild type BamHI endonuclease from 2 g of wet cell paste of overexpressing clone, pAABRw, in BL21(DE3) cells

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Sp. Activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>112</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Phosphocellulose</td>
<td>13.6</td>
<td>$18.4 \times 10^6$</td>
<td>$1.35 \times 10^6$</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>6.9</td>
<td>$12.7 \times 10^6$</td>
<td>$1.84 \times 10^6$</td>
</tr>
</tbody>
</table>
Fig. 3 — Growth curves of BL21(DE3) cells containing pPMBR.0 and pMAP6 (●) and pAABRw and pMAP6 (▲). [The inset shows the growth curves of BL21(DE3) cells (o) and BL21(DE3)pLysS cells (△) transformed with pAABRw and pMAP6.]

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References