Periplasmic expression of a restriction endonuclease in *Escherichia coli* and its effect on the antiviral activity of the host

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One possible mechanism preventing phage infection of the bacterial cells is the presence of an effective restriction-modification system (R-M) which allows restriction of the invading DNA. However, there are some limitations to the absolute restriction of foreign DNA. Since there is a serious conflict between increase in the restriction-modification genes expression level and cell viability, we examined the antiviral effect of EcoRI restriction endonuclease after its translocation to the periplasmic space of the cell. We assumed that such reconstructed R-M system could be able to degrade foreign DNA at the stage of its passage through the cell envelope of Gram-negative bacteria, before its penetration into the bacterial cytoplasm. The Tat secretion pathway of *Escherichia coli* was used to export R.EcoRI fused to the TorA leader peptide across the cytoplasmic membrane. However, although we observed a huge accumulation of the TorAss-R.EcoRI pre-protein in the cytoplasm the Tat system did not provide an efficient transport across the cytoplasmic membrane. Moreover, our data strongly suggest that endonuclease cannot function under the conditions prevailing in periplasmic space, therefore, the transported endonuclease could not contribute to an increase in restriction properties of the host.

Key words: Tat secretion, EcoRI endonuclease, TorA, DsbA, periplasmic expression, DNA restriction

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INTRODUCTION

Many strains of *Escherichia coli* (*E. coli*) and other Gram-negative bacteria are used for the production of heterologous proteins of vital practical importance and measurable commercial market. In any biotech venture, to make it reproducible and cost-effective, in addition to the optimization of culture growth conditions and to control of target gene expression it is necessary to create a proper protection that prevents cell lysis. Virulent bacteriophages are one of the major risks when carrying bacterial cultivation in the laboratory and in industrial processes. Bacteriophages are able to induce cell lysis even in large scale cultures, Restriction-modification systems (R-M) protect bacterial cells against the invasion of viral DNA (Makarova *et al.*, 2013). They exist in almost all bacterial strains and consist of two types of enzymes: a restriction endonuclease and a methyltransferase. The endonuclease specifically recognizes target nucleotide sequence and cuts it while akin DNA methyltransferase protects the sequence from being cut through methylation of specific bases. This allows the bacteria to survive in the hostile phage-abundant environment. Bacteriophages have several mechanisms to counteract the restriction of their own DNA in the bacterial cell at the early stages of infection (Toek and Dryden, 2005; Labrie *et al.*, 2010; Samson *et al.*, 2013). In the natural environment, restriction of foreign DNA reduces the possibility of viral infections by 2–3 orders of magnitude but does not eliminate them completely.

The aim of this project was to evaluate whether the transfer of the process of foreign DNA restriction into the bacterial periplasm could be effective in degradation of the invading DNA. A model EcoRI restriction endonuclease was translocated to the periplasmic space by the Tat (twin-arginine translocation) secretion system (Bogsch *et al.*, 1998; Santini *et al.*, 1998; Sargent *et al.*, 1998) utilizing native and cleavable *E. coli* signal sequences - from pre-trimethylamine N-oxide (TMAO) reductase TorA (Mejean *et al.*, 1994; Santini *et al.*, 1998). Cells expressing the hybrid proteins were challenged by λ virus bacteriophage infection.

MATERIALS AND METHODS

**Bacterial strains and plasmids.** *E. coli* K-12 strains used in this work included DH15a (F– λ– endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR mcrABK224Δ (lacIQZ∆M15 D(lacZYA+argF)U169, hsdR17(tK–M–)*) (New England Biolabs) for the recovery and propagation of the recombinant plasmids, and as a source of torA (TMAO reductase) signal sequence; ER1992 (F–Δ(argF-lac)U169 supE44 cI4Δ dinD1 Mu d1734 KanR, LacZY) rpsD1 tnrD1 endA1 speI132 thi-1 Δ(mcrC-mrr)14 ΔS510) (Fomenkov *et al.*, 1994) (New England Biolabs) and DH10B (F–Δ torA Δ(mcrA-badRMS-mcrBC) Q80lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara leu) 7697 galU galK rpsL mcrG λ–) (Grant *et al.*, 1990) both used in phage restriction assays. Moreover, Tat- strains: DADE (as MC4100 Δtat:ABCD ΔattE, (Wexler *et al.*, 2000) and B1LK0 (ΔtatC, (Sargent *et al.*, 1998) which were gifts from prof. T. Palmer (University of Dundee) and the periplasmic protease deficient strain HMI140 (F– ΔlacX74 galE galK thi rpsL, ΔphoA degP, ppr ompT cda tsp rpoN11 Str R Km R Tet R Cm R, (Meerman and Georgiou, 1994) were used as the controls of Tat-dependent fusion gene expression. *E. coli* SOS-reporter strains MP060 (MG1655 ΔattHK022::lacIqP) and MP064 (MP060 ΔrecA) (Pieška *et al.*, 2016) were used for self-restriction evaluation. *E. coli* cells were grown in LB me-
dia when necessary supplemented with antibiotics at the following concentrations: ampicillin (Ap) at 50 μg/ml, chloramphenicol (Cc) at 12.5 μg/ml, kanamycin (Km) at 15 μg/ml and tetracycline (Tc) at 15 μg/ml. L-arabinose (Sigma) and isopropyl β-D-thiogalactopyranoside (IPTG, Sigma) were used as indicated in the experiments. Plasmids (Supplementary Table 1 at https://ojs.ptbioch.edu.pl/index.php/abp) were introduced to the cells by a standard chemical procedure (Sambrook, 1989).

**ecoRIR fusion construction.** Construction of *ecoRIR* endonuclease gene in fusion with trimethylamine N-oxide reductase TorA signal peptide (44 aa N-terminal, with -AQAAR remaining after the cleavage at the fusion site (Mejean et al., 1994; Thomas et al., 2001) allowed secretion of the resulting product to the periplasmic space. In order to test the functionality of the secretion and/or posttranslational modification of such a hybrid first, we constructed the *torA*-*ecoRIR*-gfp fusion gene under control of an arabinose-inducible *P*~arab~ promoter of pBAD24 plasmid vector (Guzman et al., 1995). Using specific primers for the leader sequence of the *torA* gene, *torA*for and *torA*rev (Supplementary Table 1 at https://ojs.ptbioch.edu.pl/index.php/abp), the PCR products (145 bp) with NcoI-SalI restriction sites on their ends were obtained and then cloned into the corresponding sites of the pBAD24, resulting in pBadT. Next, proximal fragment of the *ecoRIR* gene was PCR-amplified using R EcoRIBgl2 and P1457 primers and P1M-RRM plasmid as a template (Mrusk et al., 2011), than cut with BglII and HindIII (204 bp), and cloned into the BglII and HindIII sites of the pBadT. Thus, obtained vector was named pBadTR and contained a fragment spanning 2-70 aa of the R.EcoRI protein N terminus. Then, PCR was used to generate a *Aegyptus victoria* gfp F64l/S65T double mutant gene using Gfpup and Gfpdown primers and pGreenTIR vector as a template (Miller and Lindow, 1997). The resulting gfp gene was flanked by HindIII restriction sites with a synonymous serine codon mutation at the 2nd position, and then was cloned into the HindIII site of pBadTR. The final construct was named pBadTRG (Fig. 2). Plasmid pBadTR carrying the complete sequence of the *ecoRIR* gene in fusion with *torA*-ls was obtained by cloning a PCR-generated DNA product (by using RecoBgl2 and RecoHind primers cut by HindIII (641 bp) and inserted downstream of a proximal part of *ecoRIR* gene in pBadTR to reinsert the whole gene. Taq DNA polymerase (Fermentas) was used in all PCR reactions.

Analogously, *dsbA*-*ecoRIRM* under control of arabinose promoter was constructed in three steps. First, two partially overlapping 58 nt oligos coding *dsbA* leader sequence (22 codons) were annealed and filled in by polyclarase DNA I Klenow fragment. Resulted 86-bp ds DNA fragment was restricted with EcoRI and SalI and ligated to the similar sites of pBAD24. Then, BglII-HindIII fragment of the *ecoRIR* gene from pBadTR was subcloned. Finally, HindIII-HindIII fragment with *ecoRIR*-ecoRIRM from pBadTRM was joined, resulting in plasmid pBADdsbR (Supplementary Table 1 at https://ojs.ptbioch.edu.pl/index.php/abp).

The pSALectEcoRI vector carrying the *ecoRIR* translational fusion gene between N-terminal *torA* leader and C-terminal β-lactamase fusion reporter (*bla* gene) for periplasmic transport via the Tat secretion system was constructed by cutting the pSALectDmdNK+1 (Lutz et al., 2002) with NdeI/SpeI and insertion of a PCR-created *ecoRIR* endonuclease gene. The expression of fusion gene was induced with 1 mM IPTG at 37°C for 1 h. All fusions were verified by DNA sequencing.

**Preparation of subcellular fractions.** Overnight culture of DH5α carrying an appropriate plasmid (pBadTRG or others) was diluted with fresh LB medium supplemented with ampicillin. Cell pellet obtained from 1.5 ml of culture was collected and suspended in 400 μl of a sucrose buffer to induce osmotic shock (20% sucrose, 200 mM Tris-HCl (pH 7.6), 1 mM EDTA). Fractionation was done according to the method described by (Harrison et al., 1997). The spheroplast pellet (cytoplasmic fraction) was suspended in 120 μl of ice water and frozen. Only 20 μl of the total volume was applied to each well of the gel for analysis. The supernatant (periplasmic fraction) was concentrated 2-fold by evaporation under mild conditions, before being subjected to electrophoresis. For endonuclease activity determination 0.4 μg of λ DNA was digested for 1.5 hour with 3 μl of each cell extract.

**Ion-exchange protein fractionation.** The R.EcoRI and ssTorA-R.EcoRI protein lysates obtained from DH5α strains carrying pBadTR, were separated by ion exchange chromatography. Bacterial pellet obtained from 100 ml culture after 2 h of 0.03% L-arabinose induction was suspended in 6 ml of buffer S (10 mM KPO4, pH 7.4, 20 mM KCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, 5% glycerol, containing 0.1 mM phenyl-methyl-sulfonyl-fluoride (PMSF) as a protease inhibitor) and disrupted by sonication at 4°C in 60–10-s bursts. The sonicated cells were centrifuged at 14,000 rpm for 30 min. The resulting supernatant was applied to a phosphocelulose P11 column (Whatman (2.5×2.5 cm) equilibrated with buffer S without PMSF. The column was washed with 200 ml of buffer S and the enzymes were eluted with a 100 ml linear gradient ranging from 20 mM to 1000 mM KCl in the same buffer. 1.75 ml fractions were collected and assayed for restriction activity and assessed to determine protein concentration.

**SDS-PAGE and western blot analysis.** Proteins were analyzed after 10% SDS-PAG electrophoresis by Coomassie Blue staining or were transferred onto a nitrocellulose membrane. The lysates were diluted in electrophoresis sample buffer and heated at 95°C for 5 min. Electrottransfer was conducted at room temperature overnight at 20 V in Tris-glycine buffer (25 mM Tris/192 mM glycine, pH 8.3). The membrane was blocked with TBS buffer (25 mM Tris-HCl pH 7.8, 0.5 M NaCl) with 3% skimmed milk, and then incubated with rabbit polyclonal anti-R.EcoRI and anti-M.EcoRI antibodies (kindly provided by I. Kobayashi, University of Tokyo). Anti-R. EcoRI and anti-M.EcoRI sera were diluted 1:4000 before use. Then, the nitrocellulose membrane was washed with TBS with 3% milk and incubated with anti-rabbit IgG antibody conjugated to alkaline phosphatase (diluted 1:20000, Sigma) or anti-rabbit goat IgG (diluted 1:80000, Sigma). To detect specific targets the substrates for alkaline phosphatase (only in experiment illustrated in Supplementary Fig. 2 at https://ojs.ptbioch.edu.pl/index.php/abp) nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) were used (Fermentas). To detect the peroxidase activity, a Pierce ECL Plus Substrate (Thermo Scientific) was used. Nitrocellulose membrane was exposed to X-ray film to capture chemiluminescent signal.

For detection of the GFP protein, a mouse anti-GFP (Santa Cruz Biotechnology, dilution 1:1000) primary antibody was used, followed by incubation with an anti-
mouse antibody conjugated to horseradish peroxidase (Sigma, 1:150000 dilution of 1mg/ml stock solution).

**Phage restriction assays.** The restriction activity of E. coli cells carrying the *ecoRI* genes was measured by determination of the plating efficiency of the λb2a phage (Kellenberger et al., 1960). All tests were performed with freshly transformed cells. Serial dilutions of λb2a phage in TM buffer (10 mM MgSO₄, 100 mM NaCl) were prepared ranging from 1 to 10⁸. Qualitative assay: 4 ml of top agar preheated to 45°C with 300 μl bacterial culture was poured on LA plates and allowed to set for 15 min. Then, 10-μl spots of the phage dilutions were applied with following overnight incubation at 37°C. Quantitative assay: mixtures of appropriate phage dilutions (100 μl) and host bacteria (300 μl) were incubated for 15 min at room temperature and then 4 ml of top agar was added and the whole mixture was poured onto the LA plate and incubated overnight at 37°C. The efficiency of plaque formation was calculated as a quotient of plaque-forming units obtained with cells carrying the tested plasmid and plaque-forming units obtained with a restriction-negative strain.

### RESULTS AND DISCUSSION

The rationale of the project

The key factor ensuring effectiveness of the invading DNA restriction is the shift of the balance between DNA methyltransferase and its cognate endonuclease towards the restriction activity. As it was reported previously, using an immunocytochemical microscopy technique EcoRI endonuclease molecules were localized predominantly within the envelope part of E. coli cells, including the periplasmic space (Kohring & Mayer, 1987). Similarly, HsdR subunit of the heteromultimeric EcoKI type I R-M enzyme was found to be associated with the cytoplasmic membrane, having access to the periplasmic space (Holubova et al., 2000). Thus, it could be assumed that periplasmic localization is well-adapted for the restriction of foreign DNA. Apart from this, there is still an open question whether the restriction endonuclease could be active in the periplasm.

We first attempted to construct an E. coli strain with higher resistance to viral infection using an arabinose-inducible EcoRI R-M system on pBAD24 plasmid (Guzman et al, 1995) (Fig. 1, Supplementary Table 1 at https://ojs.ptbioch.edu.pl/index.php/abp). However, overexpression of the R.EcoRI led to exposure of the chromosome to restriction cleavage, as shown by using E. coli P₆₅₅₃₅₇₅₃ SOS-reporter strains (Pleška et al., 2016) (Supplementary Fig. 1 at https://ojs.ptbioch.edu.pl/index.php/abp). We continued to use a convenient tool of expression control of the EcoRI R-M genes by P₆₅₅₅₇₅₇₅₃ promoter/araC regulatory unit of pBAD24 in order to test a possibility to export a fraction of the endonuclease enzyme (and, in fact, to relocate the whole process of DNA restriction) into the bacterial periplasm, expecting its higher effectiveness in milieu free of DNA methylation. To make the optimal choice of an appropriate combination of promoter and secretion pathways we decided to use the Tat-dependent secretion system which provides an interesting alternative among others because in this system only properly folded proteins are exported, which assures their activity also in the cytoplasm (DeLisa et al., 2002; Bruser, 2007). We used E. coli torA leader sequence which has a prominent potential to export many useful proteins

![Figure 1. The scheme of the plasmid constructs key features.](image-url)

Set of EcoRI R-M expression vectors for the export of hybrid proteins to the periplasmic space via Tat secretion system: pBadTEG for TorAss-R.EcoRI, arabinose-inducible GFP (pre-protein/mature – 39.1/34.5 kDa), pBadTRM for TorAss-R.EcoRI (35.7/31.2 kDa), and pSAlectEcoRI for TorAss-R.EcoRIβ-lactamase (64.5/60.3 kDa), or SRP secretion system: pBADdsbRM for DsbAss-R.EcoRI (32.7/31.7 kDa). In addition, amino-acid and nucleotide sequence of fusion junctions with Spase I cleavage sites (arrow) and BglII and SpeI restriction sites (underlined) are shown.

(Mergulhao et al., 2005; Matos et al., 2012). Since we used the arabinose-inducible P₆₅₅₅₇₅₇₅₃ promoter, all the fusion constructs containing the ecoRI gene with appropriate signal leader sequence together with ecoRIM gene were made as pBAD24 derivatives (Fig. 1, Supplementary Table 1 at https://ojs.ptbioch.edu.pl/index.php/abp). As the result the expression level of such modified endonuclease and its restriction effectiveness could be tested by measuring the efficiency of infection (EOP) by the virulent lambda phage.

**Tat-dependent export of R.EcoRI endonuclease/GFP reporter hybrid**

Next, we chose the twin-arginine translocation (Tat) system as the most useful and advantageous secretion pathway for correctly folded heterogenous proteins (DeLisa et al., 2002; Kudva et al., 2013). Transport across the cytoplasmic membrane to the periplasmic space requires an N-terminal signal peptide which is recognized by membrane transport system consisting of TatA/E, TatB and TatC proteins (DeLisa et al., 2002). A wide variety of recombinant proteins were successfully targeted to periplasm using the TorA signal sequence (Bruser, 2007; Matos et al., 2012). So far, only one attempt of secretion of endonuclease to the periplasmic space and extracellular medium was reported (Toksoy et al., 1999; Toksoy et al., 2001). However, the efficient transport of the hybrid protein by the Sec general secretory pathway was detected after only 12–18 h of induction.

To examine the relative efficiency of the secretion and post translational periplasmic processing of TorAss-R.EcoRI hybrid, we initially constructed the *torA*–*ecoRI* fusion gene under arabinose-inducible P₆₅₅₅₇₅₇₅₃ promoter in the pBAD24 vector (Guzman et al., 1995) (Fig. 1). The GFP reporter protein is convenient to test the Tat system functionality and monitor the fate of the hybrid protein
Figure 2. Tat translocation of TorAss-R.EcoRIΔ71-277-GFP (TR’G) hybrid protein.
(A) Expression of the torAss-ecoRIR'-gfp fusion gene in E. coli DH5α carrying the pBadTRG plasmid analyzed by fluorescence microscopy. A representative experiment is shown (i) pBadTRG non-induced (exposed for 1.5 ms), (ii) pBadTRG induced for 2 h with 0.03% L-arabinose (exposed for 0.48 ms), (iii) cells carrying pGreenTIR induced for 2 h with 0.1mM IPTG (exposed for 0.48 ms). Arrowheads (panel ii) indicate pole-located protein aggregates. Scale bar: 5 mm. (B) Subcellular distribution of TR’G protein analyzed by immunodetection with an anti-GFP serum, after cell fractionation. Top panel: visualization of the non-induced (lanes 2–4) or L-arabinose-induced (0.03% for 2 h) (lanes 5 and 6) cell lysates after resolving on 10% SDS-PAGE and staining with Coomassie Brilliant-Blue. Lane 1: size markers; lane 2: whole lysate from non-fractionated cells; lanes 3 and 5: cytoplasmic (C) fractions; lanes 4 and 6: periplasmic (P) fractions (six times more vol/vol when compared to fraction C). Bottom panel: western blot analysis using mouse anti-GFP antibody. White arrows indicate the position of mature R.EcoRIΔ71-277-GFP (34.5 kDa), black arrows – the position of the TR’G pre-protein hybrid (39.1 kDa).

Figure 3. Export of the EcoRI endonuclease fused to the TorAss signal sequence.
(A) The dynamics of the expression of the wild-type R.EcoRIR (pBAD-RM) and hybrid TorAssR.EcoRIR (pBadTRM) proteins in DH5α cells after 1, 2, 3, and 4-hour induction with 0.04% L-arabinose. The same induction conditions were applied for all experiments described below. Western blot analysis performed using anti-M. E. coli and anti-R.EcoRI antibodies. (B) Detection of the mature form of TorAssR.EcoRIR by comparison of the lysates from the Tat transport defective cells (DADE and B1LK0) after 4-hour expression. White arrow – position of the mature R.EcoRIR. (C) Detection of the mature form of TorAssR.EcoRIR by comparison of lysates from cells producing EcoRIR methylase alone. Lane 1: lysate of cells producing wild-type EcoRI R-M (pBAD-RM), five times diluted; lanes 2 and 3: lysates of the DH5α cells expressing the TorAssR. EcoRI hybrid. The same induction conditions were applied for all experiments described below. Western blot analysis performed using anti-M. E. coli and anti-R.EcoRI antibodies. (D) Restriction activity present in the spheroplast (S) and periplasmic (P) fraction of the cells, before (−) and after (+) L-arabinose induction of the cells carrying pBadTRM plasmid. 0.4 μg of λ DNA was digested for 1.5 hours with 3 μl of cell extracts after fractionation. (E) Western blot analysis of fractionated extracts from cells expressing TorAss-R.EcoRIR-β-lactamase (pSAlectEcoRIR). *IPTG – indicates following fractions: spheroplast (S) and periplasmic (P) after 1-hour induction with 1 mM IPTG. Arrows show the pre-protein (p) and mature (m) form of the hybrid. DNAa, DnAK, alkaline phosphatase (PhoA*) and β subunit of the E. coli RNA polymerase (β-RNAP) are shown as spheroplast/ periplasmic markers. (F) Right panel: BglII restriction analysis of pSAlectEcoRIR recombinant DNA on 0.8% agarose gel. Positive recombinants (#2–5 in bold) were linearized (4.03 kb). Inverted colors of the gel are shown. Left panel: The ampicillin resistant phenotype of the positive recombinants (#2–5) bearing the pSAlectEcoRIR plasmid on Ap− selective medium after 48-h growth at 37°C. Negative recombinant is indicated as #1.

brane, it was a promising indication for further experiments with the TorAss-R.EcoRI full-length hybrid.

The effectiveness of phage DNA restriction by the endonuclease translocated into the periplasmic space

The periplasmic localization of the EcoRIΔ71-277-GFP hybrid demonstrated by a fractionation assay and subsequent immunodetection with an anti-GFP serum
Periplasmic expression of EcoRI restriction endonuclease

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To get a clear answer about the Tat-dependent R.EcoRI endonuclease export across the membrane, we used a pSALect vector-based system employing an N-terminal TorAss leader which directs the post-translation export of the fusion protein with a C-terminally fused β-lactamase (Bla) reporter, to the periplasm (Lutz et al., 2002). The Bla protein is only functional when it is translated in-frame with the target protein and only after its translocation across the inner membrane to the periplasm where disulfide bond formation takes place. Thus, the pSALectEcoRI plasmid was constructed, which encoded the TorAss-R.EcoRI-Bla hybrid. Such construct can only be maintained in cells with the presence of the pLM271c plasmid serving as the source of M.EcoRI methyltransferase, whose gene is constitutively expressed from its own promoter (Mruk et al., 2011). It was shown that only positive recombinants with the endonuclease/β-lactamase hybrid transported across the membrane were able to grow on LB-agar plates containing 50 μg/ml ampicillin even without IPTG induction of the gene expression (Fig. 3F). β-lactam resistance phenotype testified conclusively to the fact that the transport of the R.EcoRI-β-lactamase hybrid into the periplasm occurred, but western blot analysis of the IPTG-induced cell lysates revealed its very low efficiency. The presence of the immature hybrid was observed mainly in the cytoplasmic space. We did not observe an increase of export in the HM140 strain deficient in known cell envelope proteases (Supplementary Fig. 3) at https://ojs.ptbioch.edu.pl/index.php/abp (Gentz et al., 1988; Meerman & Georgiou, 1994).

Tat-dependent translocation of the hybrid R.EcoRI with pSALect vector

(3F). As described earlier, usually, the translocation of the recombinant Tat substrates is not complete, therefore the precursor as well as the mature-sized proteins may accumulate in the cytoplasm (Blaudeck et al., 2001; Sturm et al., 2006; Richter et al., 2007). The k_w phase DNA restriction assay demonstrated that cells exhibited a restriction activity level 10 times higher than in case of the restriction-negative control strain. We found that the transportation outcome was not limited by the degradation in the periplasmic space. We did not observe an increased export in the HM140 strain deficient in known cell envelope proteases (Supplementary Fig. 3) at https://ojs.ptbioch.edu.pl/index.php/abp (Gentz et al., 1988; Meerman & Georgiou, 1994). However, as already shown, the rate of phage DNA transport across bacterial membranes varied was rather high and reached a value several-fold higher when compared to conjugation or transformation processes (Letellier et al., 1999). Moreover, the transferred DNA crosses the bacterial envelope linearly through the protein/lipid channel, therefore, it is protected from the periplasmic nucleases (Letellier et al., 2003) (Roessler and Ihler, 1986). Different forms of DNA channels created with the participation of phage proteins concern both T-odd (T3, T5, T7) and T-even (T4) phages (Letellier et al., 1999). Probably for this reason there is no noticeable effect of periplasmic DNase endonuclease I presence on the effectiveness of phage lambda infection (Dürwald & Hoffmann-Berling, 1968). However, early experiments on T-phage series showed that DNA degradation appeared to be mediated by endonuclease I only during superinfection in conjunction with temporal exclusion phenomenon, the phage–induced inhibition of DNA penetrating the cell envelope (Fielding & Lunt, 1970; Anderson & Egner, 1971). Another aspect is that most of phages choose the cellular poles of Gram-negative bacteria as the preferred place for DNA injection, which can possibly limit its contact with periplasmic nucleases (Edgar et al., 2008).

Since the Tat components are present at relatively low levels in E. coli cells, we tried to co-express the tat/abc genes (pEXT-TatABCs plasmid) to reinforce the flux by this secretion pathway (Barrett et al., 2003) As shown in Supplementary Fig. 3 (at https://ojs.ptbioch.edu.pl/index.php/abp) this did not increase the export capacity under the applied conditions in comparison to the wild-type Tat levels (4 h and overnight, lanes 3 and 4 vs. lanes 5 and 6, respectively). There are two difficulties in transporting this cytoplasmic endonuclease across the membrane. Firstly, the likely saturation of the Tat pathway by increasing production of TorAss-R.EcoRI; secondly, the protein instability. We observed a high level of stable pre-protein in the cytoplasm and only a few percent of mature protein after the transport. Our hybrid proteins exhibited most, if not all, of their restriction activity in the cytoplasm. We found that the transportation outcome was not limited by the degradation process in the periplasmic space. We did not observe an increase of export in the HM140 strain deficient in known cell envelope proteases (Supplementary Fig. 3) at https://ojs.ptbioch.edu.pl/index.php/abp (Gentz et al., 1988; Meerman & Georgiou, 1994).

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tion factors DnaA and DnaK (a significant fraction of this chaperone can be released outside the cell by osmotic shock, (el Yaagoubi et al., 1994), a transcriptional machinery protein – the β-subunit of the RNA polymerase and a periplasmic protein – alkaline phosphatase, PhoA (Fig. 3E).

Signal recognition particle (SRP) co-translational export of DsbAssR:EcoRI hybrid

In order to direct R.EcoRI to the SRP pathway we fused it to the DsbA protein signal sequence of E. coli (periplasmic disulfide bond oxidoreductase) (Schierle et al., 2003). This co-translational translocation would prevent the formation of a secondary structure and therefore precluded enzyme’s activity in the cytoplasm. E. coli ER1992 cells carrying pBADdsbRM construct (Fig. 1) after L-arabinose induction accumulated high amount of pre-DsbAssR.EcoRI hybrid protein but the efficiency of the export to the periplasm was very low (Supplementary Fig. 4A at https://ojs.ptbioch.edu.pl/index.php/abp). Although EcoRI restriction activity could be easily detected in cell extracts (Supplementary Fig. 4B at https://ojs.ptbioch.edu.pl/index.php/abp), the ability of those cells to restrict lambda phage DNA was almost none (Supplementary Fig. 4C). Using phosphocellulose chromatography fractionation we showed that pre-DsbAssR.EcoRI protein had EcoRI-specific restriction activity (Supplementary Fig. 4D at https://ojs.ptbioch.edu.pl/index.php/abp) suggesting it underwent of proper folding in the cytoplasm. However, the overproduction of the hybrid precursor did not induce SOS response (Supplementary Fig. 4E, F at https://ojs.ptbioch.edu.pl/index.php/abp).

Analyzing expression of the ecoRIR and ecoRIM from the pBADTRM plasmid which depends on the arabinose promoter, we observed an equal and high level of endonuclease and methyltransferase production, which did not allow the efficient restriction of the phage DNA. Thus, we tried to separate the R-M gene expression control by employing a two-plasmid system, maintaining a constitutive expression of the EcoRI methyltransferase gene from its natural P<sub>r</sub> promoter (pACYC<sub>A+</sub> plasmid), and having an arabinose-inducible <i>tor</i><sub>A+</sub>/endonuclease fusion gene (pBADTR). By induction with 0.03% L-arabinose we were able to increase the DNA restriction to the same level as in the case of the pBAD-RM plasmid, but not higher (Supplementary Table 2 at https://ojs.ptbioch.edu.pl/index.php/abp). This was most probably caused due to the excess of cellular restriction over methylation which caused a cell survival problem (Naito et al., 1995) manifested by growth inhibition and loss of the pBADTR plasmid (data not shown).

CONCLUSIONS

Collectively, our results suggest that restriction endonuclease may not be a suitable tool for restriction of foreign DNA in the periplasmic space. The key problem in this case is jamming of the Tat translocation machinery and also lack of optimal conditions for DNA restriction in the periplasm. Low endonuclease concentration in the periplasm caused omission the DNA target (number of infected phages per cell was limited up to several per cell under the applied conditions), or lack of access to the target DNA (phage DNA sheltered by tail and/or phage proteins creating the channel).

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

All Supplemental materials are at https://ojs.ptbioch.edu.pl/index.php/abp

REFERENCES


https://ojs.ptbioch.edu.pl/index.php/abp
Periplasmic expression of EcoRI restriction endonuclease


