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Plasmid pRARE as a Vector for Cloning to Construct a Superproducer of the Site-Specific Nickase N.*Bsp*D6I

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Abstract—The gene of methylase M.*Scc*L1I that protects DNA against hydrolysis with the nickase N.*Bsp*D6I was inserted into plasmid pRARE carrying genes of tRNA, which are rare in *E. coli*. The insertion of the gene *sscML1I* into pRARE was reasoned by incompatibility of pRARE and the plasmid carrying the gene *sscML1I*, because both plasmids contained the same *ori*-site. Upon transformation of *E. coli* TOP10F′ cells with both the recombinant plasmid pRARE/MSsc and the expression vector pET28b containing the nickase gene *bspD6IN* under the phage T7 promoter, a strain of *E. coli* was obtained which produced $7 \cdot 10^5$ units of the nickase N.*Bsp*D6I per 1 g wet biomass, and this yield was two orders of magnitude higher than the yield of the enzyme from the strain free of pRARE/MSsc.

Key words: site-specific nickases, restriction–modification systems, superproduction, rare codons, pRARE

Site-specific nickases, or nicking endonucleases, are a new class of enzymes and a new component of restriction–modification systems. Similarly to type II restriction endonucleases, nickases recognize a short specific sequence on double-stranded DNA and cleave DNA at a fixed distance from it, but, unlike restriction endonucleases, nickases produce a nick only in one, definite, chain of DNA. At present, four nickases isolated from bacterial strains are known [1-4]. Although these nickases are isolated from different sources, they are isoschizomers, i.e., they recognize the same site (5′-GAGTC-3′/5′-GACTC-3′) and cleave DNA in the same fashion with respect to the site: directed to the 3′-end, they cleave only the chain containing the sequence 5′-GAGTC-3′ at the distance of four nucleotides from the site. Similarly to restriction endonucleases, nickases are sensitive to methylation of the recognizable site [5], and the gene of DNA methylase is located near the nickase gene [6]. Although nickases provide a break in only one chain, they can, similarly to restriction endonucleases, cleave DNA with production of homogenous fragments. This occurs when two sites with opposite orientation are located closely to one another. In this case, different chains are cleaved, and if the length of protruding ends is insufficient to provide their hybridization, a double-stranded break is detected. Nickases were revealed just due to this feature. And because of this feature, all known nickases are isoschizomers: DNA of phage T7 which is traditionally used as a substrate in searches for restriction endonucleases contain four pairs of closely located inverted sites.

In the natural strain *Bacillus* sp. D6 we found earlier the nickase N.*Bsp*D6I, and its gene was cloned into the expression vector pET28b with which *E. coli* TOP10F′ cells were transformed. Upon induction, a specific nicking activity was recorded in the lysates, but by electrophoresis of the lysate proteins, no intensive inducible protein band was found in the region of 70 kD [7]. The low yield of the nickase we explained by difference in the frequency of codons used in *E. coli* and *Bacillus* cells.

This article describes our approach for construction of a superproducer of the nickase N.*Bsp*D6I using the plasmid pRARE carrying genes of transfer RNAs rare for *Escherichia coli*.

MATERIALS AND METHODS

In this work we used plasmids pET28b, pRARE (Novagen, USA), p15MSsc [8] and *E. coli* strain TOP10F′: *F′* [*lacI*^{q}, *Tn10(Tet*^{R}*) mcrA Δ(mrr-hsdRMS-*

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mcrBC) φ80 lacZ ΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7679 galU galK rpsL(Str^R) endA1 nupG]. Enzymes, such as DNA ligase of phage T4, the Klenow fragment of *E. coli* DNA polymerase I, and *Taq* polymerase, were prepared by us. Restriction endonucleases *Bam*HI and *Eco*RI were from Fermentas (Lithuania), and *Ssc*L1I and *Bsp*LU4I (the isoschizomer of *Ava*I) were prepared by us earlier [9, 10].

Reactions of restriction, ligation, and "building" of protruding ends with the Klenow fragment and isolation of plasmid DNA were performed using standard techniques [11]. Polymerase chain reaction from the colonies was performed as described earlier [12].

Electrocompetent cells for transformation were prepared as proposed in [13], with some modifications. *E. coli* cells were grown in 50 ml of medium 2YT (1.6% bactotrypton, 1% yeast extract, 0.5% NaCl, pH 7.0) to the middle of the logarithmic growth phase (A_{600} = 0.6 unit), cooled on ice, centrifuged at 4000*g* for 10 min, and resuspended in 50 ml of 1 mM HEPES (pH 7.0). The suspension was centrifuged for 10 min at 4000*g*, the precipitate was suspended once more in 50 ml of the same buffer, and centrifuged under the same conditions, then resuspended in 50 ml of 10% glycerol in water, centrifuged, and the biomass was resuspended in 3 ml of 10% glycerol, divided into 80-μl aliquots, and stored at −70°C.

The competent cells were transformed with ligase mixture using an *E. coli* Pulser electroporator (BioRad, USA) at the field strength of 15 kV/cm and pulse duration of 1 msec, then incubated for 1 h in 1 ml of medium SOC (1% bactotrypton (Difco, USA), 0.5% yeast extract (Difco), 1% NaCl, pH of the final medium 7.0) supplemented with 20 mM glucose. Then the cells were planted onto dishes with an appropriate antibiotic. The grown-up colonies were replanted, and these cells were used later for mini-preparative isolation of plasmids. Clones containing the insertion-bearing plasmid were selected by PCR and used in further work.

To express the nickase gene cloned into the plasmid pET28b under the control of the phage T7 promoter, *E. coli* TOP10F′ cells were used which contained pRARE/MSsc as the second plasmid. The expression was induced by infecting the cells with phage λCE6, which carried the gene of phage T7 RNA polymerase, under the control of the phage λ promoter P_L. The nickase producer cells were grown during 5 h in 5 ml of medium LB (1% bactotrypton, 0.5% yeast extract, 1% NaCl, pH of the final medium 7.3) in the presence of kanamycin (35 μg/ml) and chloramphenicol (10 μg/ml). Then this culture was planted into 7 liters of medium LB containing the same antibiotics and grown under intensive aeration at 37°C until optical density of 0.6 unit at wavelength of 590 nm. The nickase expression was induced by infection with phage λCE6 at the multiplicity of 10. After incubation for 3 h at 37°C, the cells were cooled and precipitated by centrifugation (10 min, 6000*g*, 4°C). The precipitate (37 g) was resuspended in a lysis-providing buffer (20 mM potassium phosphate, pH 7.5, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride). The resulting suspension was ultrasonicated on ice using a UZDN-A disintegrator (thrice, 45-sec pulse, with intervals of 1 min, at 0.7 of full power). The lysate was cleared by centrifugation at 25,000*g* for 30 min. The supernatant fluid was examined for the presence of the nickase by the enzymatic activity and used to isolate the nickase by column chromatography.

All stages of the chromatographic purification were performed at 4°C. Columns of 100 ml in volume with phosphocellulose P11 (Whatman, England) and 20-ml columns with ceramic hydroxyapatite (BioRad) were used. The phosphocellulose-containing column was preequilibrated with buffer A (20 mM potassium phosphate, pH 7.5, 50 mM KCl, 1 mM DTT) supplemented with 1 mM EDTA. The hydroxyapatite-containing column was equilibrated with buffer B (20 mM potassium phosphate, pH 7.5, 150 mM KCl, 1 mM DTT). The application and elution on both columns were performed at the same rate of 30 ml/h. Proteins from the phosphocellulose-containing column were eluted with a linear gradient of KCl in buffer A. From hydroxyapatite, proteins were eluted with a linear gradient of potassium phosphate.

The presence of nickase in the cell lysates and the column fractions was determined by cleavage of phage T7 DNA with aliquots of the lysates or the fractions which resulted in a specific set of fragments. The reaction products were analyzed by electrophoresis in 0.7% agarose gel. The column fractions were also analyzed by electrophoresis in 0.1% SDS/8% polyacrylamide gel by the Laemmli technique [14].

The nickase quantity in 40 μl of buffer for nickase (10 mM Tris-HCl, pH 7.5, 150 mM KCl, 10 mM $MgCl_2$), which provided the cleavage of 1 μg phage T7 DNA during 1 h at 55°C, was taken as the unit of the enzyme activity.

The optical density of the preparations was determined with a Hitachi 220A spectrophotometer at the wavelength of 280 nm. The absorption of 0.1% nickase N.*Bsp*D6I corresponded to 1.036 optical units (this value was taken from the program ExPASy-ProtParam Tool).

The presence of the active M.*SSc*L1I methylase in TOP10F′ cells with pRARE/MSsc was tested by protection against the cleavage with the restriction endonuclease *Ssc*L1I of the plasmid pRARE/MSsc isolated from these cells.

RESULTS AND DISCUSSION

Creation of a genetic construction, which concurrently carried genes of rare transfer RNAs and DNA-methylase *Ssc*L1I. The genetic code is degenerate, each amino acid is encoded by, on average, three codons, and various

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1    makkvnwyvs cspRspekiq pelkvLanfe Gsywkgvkgy kaqeafakel
51   aalpqflgtt ykkeaafstr dRvapmktyg fvfvdeegyl rIteagkmla
101  nnrRPkdvfl kqlvkwqyps fqhkgkeyPe eewsInplvf vlslLkkvgg
151  lskldiamfc ltatnnnqvd eiaeeImqfr nerekIkGqn kkleftenyf
201  fkRfekiyGn vgkiregksd sshkskietk mRnardvada ttRyfrytgL
250  fvaRgnqlvl npeksdlide iissskvvkn ytRveefhey yGnpslpqfs
301  fetkeqlldL ahRIrdentR Laeqlvehfp nvkveIqvle diynslnkkv
351  dvetlkdviy hakelqLelk kkklqadfnd prqleevidl levyhekknv
401  ieekikarfi ankntvfewl twngfiilGn aleyknnfvi deelqpvtha
451  agnqpdmeiI yedfivlgev ttskGatqfk mesepvtRhy lnkkkelekq
501  GvekeLyclf iapeinkntf eefmkynivq ntRiiplslk qfnmlLmvqk
551  kliekGRRls sydiknlmvs lyrttIeceR kytqikagle etlnnwvvdk
601  evRf
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Fig. 1. Amino acid sequence of the site-specific nickase N.*Bsp*D6I (No. AJ534336 in the DDBJ/EMBL/GenBank databank). The amino acid residues corresponding to the rare codons are shown with bold capital letters.

organisms preferentially use different codons. Therefore, codons, which are standard for *Bacillus*, e.g., AGA, are rare for *E. coli*, and *vice versa*. When constructing producers of various proteins, researchers often find the problem of a low yield of the protein because its gene contains many rare codons. We have also found this problem when creating the overproducer of the nickase N.*Bsp*D6I.

The amino acid sequence of the nickase N.*Bsp*D6I contains 42 residues corresponding to codons rare for *E. coli*, and 13 of them are AGA (arginine) (Fig. 1). Amino acid residues corresponding to the rare codons are shown with bold capital letters.

The codon adaptation index (CAI) for the nickase N.*Bsp*D6I in *E. coli* is 0.226, which correlates to proteins with low expression. The codon adequacy index was determined according to a program written by Kirienko [15]. Moreover, two rare codons are located in tandem in the N-end (amino acid residues (aa) 104 and 105) and three rare codons are located successively in the C-terminal region (aa 556, 557, and 558). Such location of the rare codons is especially unfavorable for effective expression. There are two solutions for the problem of rare codons: either to construct a producer based on *Bacillus*, or to additionally insert genes of rare tRNAs into cells of the producer (*E. coli*). It was decided to insert into *E. coli* cells the plasmid pRARE, which carries genes of tRNAs rare in *E. coli*.

As noted above, nickases can fragment DNA. We have shown that adenine methylase M.*Ssc*L1I, which recognizes the site GANTC, protects DNA against cleavage with nickase. The gene of this methylase was earlier cloned by us into the plasmid p15SK. This vector was constructed based on the *ori*-site of the plasmid p15a and carried the gene of resistance to chloramphenicol as a genetic marker. In the plasmid pRARE the site *ori* p15a and the gene of resistance to chloramphenicol are also used. Because plasmids with the same sites *ori* cannot coexist in the same cell, we decided to combine the genes of rare tRNAs and methylase in the same plasmid, i.e., to subclone the methylase gene into pRARE.

The plasmid pRARE constructed by Novagen (Fig. 2) [16] contains the genes, which encode tRNA for the rare in *E. coli* codons AGA, AGG (Arg), GGA (Gly),

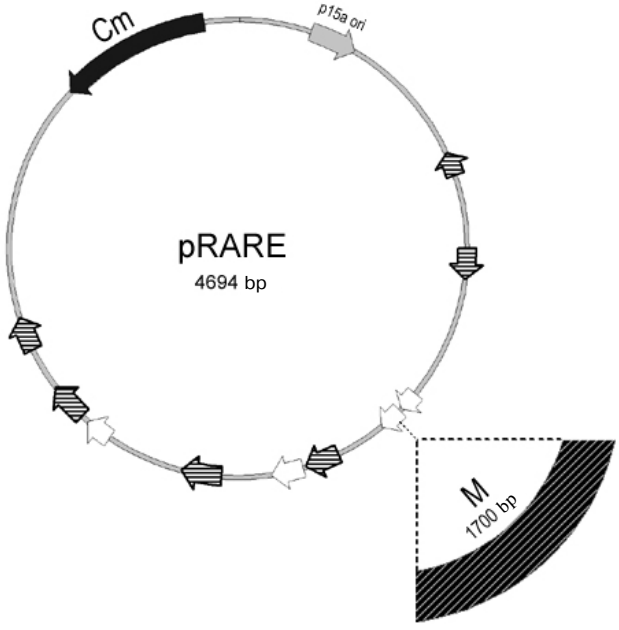

Fig. 2. Scheme of the plasmid pRARE/MSsc. Hatched arrows present the genes, which encode tRNAs rare in *E. coli*. Unhatched arrows present the genes encoding frequent tRNAs. M, the gene of methylase M.*Ssc*L1I. No scale is followed.

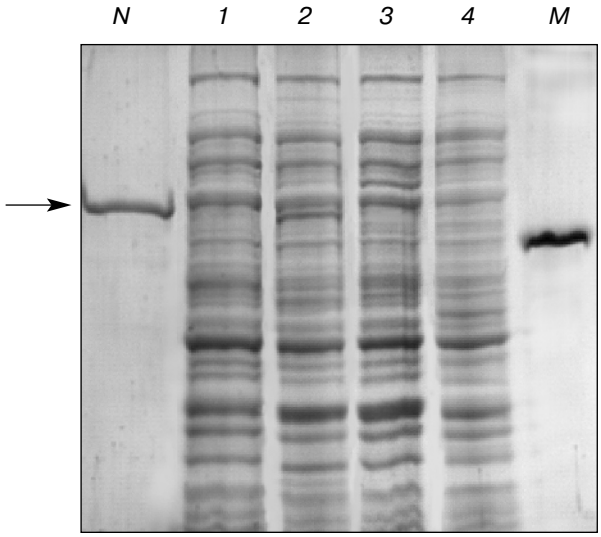

Fig. 3. Effect of the introduction of RARE/MSsc into cells on induction of the nickase. Lanes: *1*, *2*) the lysate of the cells containing pRARE/MSsc; *3*, *4*) the lysate free of pRARE/MSsc; *1*, *3*) uninduced cells; *2*, *4*) cells after induction. *N*, nickase (1.5 μg); *M*, BSA.

AUA (Ile), CUA (Leu), CCC (Pro). But this plasmid is not designed to be used as a vector, in particular, it has no polylinker, and we failed in obtaining information about its nucleotide sequence. The nucleotide sequence at the replication start and those of the genes encoding tRNAs and resistance to the antibiotic were analyzed, and they all were found to contain sites of widely used restriction endonucleases. Only the gene encoding the frequent in *E. coli* tyrosine tRNA (Fig. 2) was found to have two sites *Ava*I at the distance of 100 bp between them. Sites *Ava*I are absent in the sequence of the site *ori* and in the genes of rare tRNAs and resistance to the antibiotic. The cleavage of pRARE with the restriction endonuclease *Ava*I produces only one hydrolysis product in the electrophoregram, and the size of this product is similar to that of the linear form of the plasmid. Such a cleavage pattern suggests the absence of *Ava*I sites also in the intergenic sequences of the plasmid.

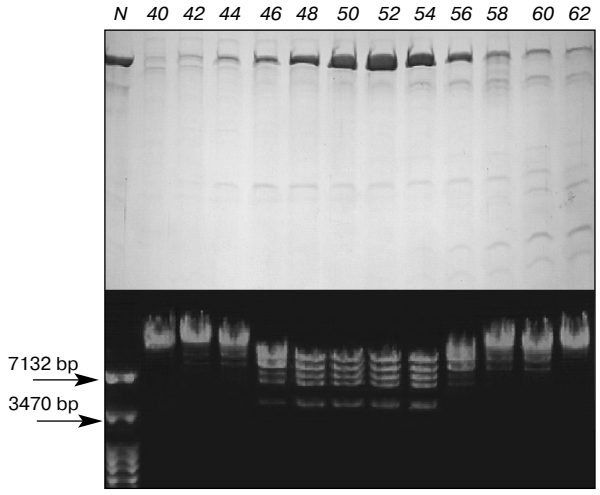

Fig. 4. Analysis of fractions eluted from phosphocellulose. At the top electrophoresis in denaturing polyacrylamide gel is shown; at the bottom the cleavage is presented of phage T7 DNA with 100-fold diluted aliquots of the fractions resulting by electrophoresis in agarose gel. The figures show numbers of the fractions. *N*, nickase.

Thus, the plasmid pRARE was linearized using *Ava*I, and p15MSsc was treated with *Bam*HI and *Eco*RI to cut out the gene of DNA methylase together with the promoter region. The vector and insertion ends were built to blunt Klenow fragments and ligated. The presence of the insertion was determined by resistance of the recombinant plasmid to hydrolysis with the restriction endonuclease *Ssc*L1I. The selected clones were transformed with the expression vector pET28b, which carried the nickase gene under the control of the phage T7 promoter. The resulting clones were examined for synthesis of the nickase. As a result, several clones, which produced the nickase, were selected and one of them was used for preparative isolation of the nickase (Fig. 3).

Isolation of the nickase N.*Bsp*D6I. The clarified cell lysate was applied onto a column with phosphocellulose equilibrated with buffer A. Phosphocellulose P11 does not bind nucleic acids; therefore, the lysate required no special treatment to remove nucleic acids. Then the column was washed with two volumes of buffer A and eluted with a linear gradient of KCl (50 mM-1 M). The fractions were studied for presence of the nickase activity and by electrophoresis in polyacrylamide gel. By electrophoresis in polyacrylamide gel, the most intense bands with molecular weight of about 70 kD and the nickase activity were recorded in the fractions 40-62, which corresponded to KCl concentration of 450-500 mM (Fig. 4).

Upon the purification on phosphocellulose, the nickase preparation was in a rather concentrated solution of KCl; under these conditions, only a few carriers, in particular hydroxyapatite, can bind proteins; therefore, it was chosen as the second carrier. The fractions 46-56 were combined, and although hydroxyapatite can bind proteins under high ionic strength, we decreased the concentration of the salt to 150 mM diluting the combined fractions in buffer (20 mM potassium phosphate, pH 7.5, 1 mM DTT). Then the preparation was applied onto a column with hydroxyapatite. Elution was performed with a linear gradient of potassium phosphate (0.02-0.5 M). The nicking activity was found in fractions 19-30 with the maximum in fractions 22-25, which corresponded to the potassium phosphate concentration of 120-150 mM. By electrophoresis, only a protein with the molecular weight of about 70 kD was detected in the fractions, and the maximal quantity of the protein was found in the most active fractions (Fig. 5).

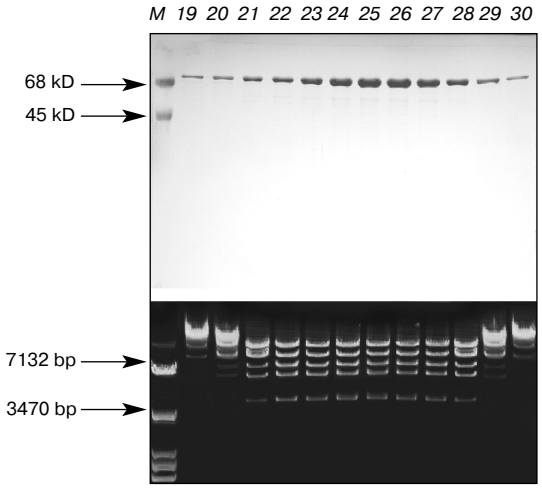

Fig. 5. Analysis of the fractions eluted from hydroxyapatite. Designations as in Fig. 4.

Thus, the electrophoretically pure nickase was prepared. The yield was $7 \cdot 10^5$ units per 1 g of wet biomass, which is two orders of magnitude higher than the yield of the enzyme from the strain free of pRARE/MSsc. Such activity shows that the introduction of the genes of rare tRNAs allowed us to construct a superproducer of the nickase N.*Bsp*D6I. We have also shown that the plasmid pRARE can be cloned into it by the site *Ava*I.

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