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CLONING OF A TRANSGLUTAMINASE GENE FROM *Streptomyces thioluteus* TTA 02 SDS 14

Seprianto[1], Dewi Seswita Zilda[2*], Yusro Nuri Fawzya[2], Suharsono[1], Puspita Lisdiyanti[3] and Agustinus Robert Uria[2*]

[1]Departement of Biotechnology, Bogor Agricultural University, Indonesia,
Jalan Raya Dramaga Kampus IPB, Bogor 16680, Indonesia

[2]Research and Development Center for Marine and Fisheries Product Competitiveness and Biotechnology,
Jalan KS Tubun Petamburan VI, Central Jakarta 10260;

[3]The Research Center for Biotechnology, Indonesian Institute of Sciences

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Abstract

Microbial Transglutaminase (MTGase, EC 2.3.2.13) is an enzyme that catalyzes the transfer of acyl group. It has tremendous applications in binding meats together in texture improvement process. This research was aimed at cloning MTGase gene from *Streptomyces thioluteus* TTA 02 SDS 14. A genomic library with the average of 40 kb insert size was initially generated from the total genomic DNA of *S.thioluteus* TTA 02 SDS 14, and was subsequently screened using an MTGase-targeting primer pair. The genomic library construction was based on the fosmid pCC1FOS™ hosted in *E.coli* EPI300-T1^{R}. The isolated positive clone was verified by enzymatic digestion with *Not*I, *Eco*RV, and *Bam*HI, followed by PCR-amplification of MTGase fragment. The PCR product result of 360 bp was sequenced. The sequence analysis strongly suggests that the positive clone with 40-kb insert harbors an MTGase gene.

Keywords: MTGase *gene*, *S. thioluteus* TTA 02 SDS 14 , *genom library*

1. Introducton

Transglutaminase (TGase, E.C. 2.3.2.13) is an enzyme that catalyzes the transfer of acyl group. Indirectly, the a-carboxyamide groups of peptide-bond glutamine residues (Gln) act as the acyl donors, and the ε-amino groups of lysine residues (Lys) serve as acyl acceptors, intermolecular or intramolecular ε-(γ-glutamyl) lysine bonds that are formed, resulting in the polymerization of food proteins (Griffin et al., 2002). TGase has been used for texture improvement (e.g. gelling capacity, hardness and elasticity) of food protein-based products and fishery product processing, such as surimi or fish meat-based processed products. Indonesian product processing has grown rapidly in line with the increase of fish production and the development of fish products processing.

TGase is widely spread in nature, including in animals, plants, and microorganisms. In animals, it is involved in various biological functions, ranging from blood clotting to cell differentiation (Aeshclimann & Paulsson, 1994). This enzyme's limited sources and complicated separation and purification process make it very expensive (around US$ 80/unit) (Zhu et al.,1995). TGases of microbial origin (MTGases) have recently received increasing attention for industrial applications. The properties of MTGases offer more advantages compared to animal-derived transglutaminases, such as no requirement of cofactor Ca^{2+} for their activity, their stability across wide pH range, their ability to cross-link most food proteins, and lower production costs due to easy enzyme production and purification (Langston et al., 2006). Therefore, TGase production based on microorganisms is worthy to be pursued.

However, using wild-type microorganisms to product MTGase involves several drawbacks, such as slow cell growth, low yield, a large amount of non-target proteins interference to the target proteins, and the difficulty to purify native proteins that requires multiple purification steps (Soares et al., 2003). DNA recombinant technology is an emerging and well-established approach to provide bacterial enzymes in

*Corresponding author.
E-mail: seswitazilda@yahoo.com; agustinus.uria@gmail.com

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a sustainable way at industrial scale. This approach involves cloning and over expression of genes encoding bacterial proteins. Lin et al. (2004, 2006) reported the success of cloning and expression of MTGase-encoding genes derived from *S. platensis* and *S.ladakanum* in *S. lividans* host. The MTGase gene expression is regulated by a native promoter that controls MTGase gene from *S. lividans*. In this work, we reported the cloning of a 40-kb DNA fragment that contains gene coding for MTGase of an Indonesian *Streptomyce* strain. Obtaining this entire MTGase gene will then provide a basis for large-scale production through gene expression in easily culturable bacteria, such as *Escherichia coli*.

2. Material and Methods

2.1. Preparation of Genomic DNA

The Genomic DNA was prepared from *S. thioluteus* TTA 02 SDS 14 cells harvested from a 500-ml culture using slightly modified CTAB method (Piel et al., 2004). Briefly, the cell pellet was grounded in liquid nitrogen, followed by suspension in the buffer lysis (50 mM Tris-C pH 7.5, 50 mM EDTA, 700 mM NaCl, 2% SDS, 8 M EDTA), followed by a 15-min incubation at 60 °C. The cell suspension was extracted with an equal volume of phenol/chloroform/isoamylalcohol (in ratio 25:24:1) and centrifuged at 8.000 rpm for 10 min. The upper layer was transferred into an empty tube and the remaining phenol was removed by adding an equal volume of chloroform. The upper layer was taken and the DNA was recovered through ethanol precipitation.

2.2. Construction of a Plasmid-Based Genomic Library

High-molecular weight genomic DNA obtained from the isolate was fragmented by either ultrasonication or digestion with *Bgl*II (New England Biolabs). A total of 10 µl DNA digestion reaction was set up by mixing the following components: 30 µL of ddH2O, 5 µL of 10x CutSmart buffer, 10 µL of DNA sample, and 2 µL (1 unit) of *Bam*H1.The reaction mixture was incubated at 37 °C for 3 hours, followed by 10-minute incubation at 70 °C to terminate the reaction. The digestion product was electrophoretically run on 1% agarose gel in 1x TAE buffer. DNA fragments of 3-8 kb in length were gel-purified using Quick Gel Extraction Kit (Thermoscientific) and then repaired at both ends prior to DNA ligation using DNA end- itTM End-Repair Kit (Epicentre). The repaired DNA fragments were ligated with pJET1.2 blunt vector (Thermoscientific) using T4 DNA ligase at 4 °C for 12 hours. The ligation product was transformed into competent cells of *E. coli* TOP10 (Invitrogen). The transformation result was spread on Luria Bertani (LB) agar media that contains 100 µg/ml of ampicillin, followed with by an overnight incubation at 37 °C. The resulting colonies were individually transferred into 96-well microplates and screened by whole-cell PCR using specific primers to recognize the target transglutaminase gene.

2.3. Construction of a Fosmid-Based Genomic Library

The DNA fragments obtained were repaired at both ends to facilitate ligation with a dephosphorylated blunt-ended linear fosmid. Briefly, a total reaction volume of 60 µl was set up on ice, consisting of 6 µL of 10x end-repair buffer, 6 µL of 2.5mM dNTP, 6 µL of 10mM ATP, 39 µL DNA sample, and 3 µL of end-repair enzyme mix. After 45 minutes incubation at room temperature, the reaction was inactivated at 70 °C for 10 minutes and was electrophoretically separated on 1% *Low Melting Point* (LMP) gel. Then, the DNA fragments of ~40-kb in length were purified from the LMP gel using GELase (Epicentre).

DNA Fragments of ~40-kb were ligated into fosmid vector pCC1FOSTM (Epicentre) in the total volume of 10 µL that contains 1 µL of 10x Fast-Link Ligation Buffer, 1 µL of 10 mM ATP, 1 µL of pCC1FOS, 6 µL of DNA sample, and 1 µL Fast-Link DNA Ligase. Following a 4-hour incubation at room temperature, the ligation reaction was terminated at 70 °C for 10 minutes. The ligation mixture (2 µL) was added into 75 µl of competent *E. coli* EPI300-T1R, and mixed gently by three times vertical pipetting, was transferred to a 0.1-cm gapped electrophoretic cuvette, and was immediately subjected to electrophoretion at 1.8 kVolt on Micropulser (BioRad) (Sambrook & Russel, 2001). The transformed cell suspension was immediately mixed with 1 ml LB in an empty 1.5-ml microtube, followed by incubation at 37 °C in a shaking waterbath for 1 hour. Aliquote (200- 400 µL) of the transformed cell suspension was spread on an LB plate containing 12.5 ìg/mL chloramphenicol, 20 µg/ml X-gal, and 0.1 M IPTG, which then followed by overnight incubation at 37 oC for white-blue screening. Several white colonies were picked up and grown in 96-well microplates.

2.4. Library Screening for a 40-kb Fosmid Clone Harboring an Entire MTGase Gene

The genomic library grown in 96-well microplate format was screened through well pooling and whole-cell PCR detection. Briefly, 10 µl culture of individual wells in the same row was combined in a sterile eppendorf tube, designated as pools. The pools were individually detected by PCR-amplification, using primer set SDS-14F (5'-AGAAGTTCGA GCGGCT

GTCC-3') and SDS-14R (5'-TTACGGC TGCGTCG GTGT-3'). Subsequently, colonies or well-cultures corresponding with positive pool were individually screened by PCR using the same primer set above. The positive colonies were grown in 5 ml of liquid LB, supplemented with 12.5 µg/ml chloramphenicol and incubated at 37 °C for 16 hours (overnight) in shaking incubator. 0.1 % (v/v) of the 10-ml inoculum was added with 9 ml LB containing chloramphenicol and 20 µL of 1x induction solution (Epicentre) to induce the fosmid copy number. After 5-hour incubation at 37 °C, the culture was centrifuged at 8000 rpm for 10 minutes and fosmid was recovered using GeneJET Plasmid Miniprep Kit (Thermo Scientific). Insert presence was verified by cutting the fosmid with *Not*I, *BamH*I, and *EcoR*V. The digested fosmid was separated on 1% agarose gel, and fragments were individually extracted and PCR-verified for the presence of MTGase gene using PTGase4/5 and SDS 14F /14R primers set. The result is PCR products to be subcloned into pGEM-T Easy vector. Furthermore, sequencing is conducted on the inserts present in the vector.

3. Results and Discussion

The amplified MTGase gene was only in partial portion and not in full-length. To obtain information about the entire MTGase gene for its further heterologous expression, we constructed and screened genomic libraries. In our first attempt, a genomic library was generated using plasmid-based vector pJET1.2/Blunt that is capable of harboring inserts in 6-10 kb size range. This vector is equipped with a lethal gene that contains a multi cloning site (MCS). In the absence of foreign DNA fragment inserted into the MCS, the lethal gene would be expressed, causing *E. coli* colonies to be killed. The incorporation of a DNA fragment or an amplicon would disrupt the lethal gene, thereby making recombinant clones alive (Thermo Scientific). In our work, DNA fragments of 3-8 kb resulted from *Bgl*II digestion (Figure 1A) were initially extracted from 1% agarose gel and then repaired at both termini to facilitate direct ligation with pJET1.2/Blunt. After their transformation into *E. coli* TOP10, the resulting transformed clones account of only 58 colonies (Figure 1B), which are too few to represent the whole genome. All of the colonies were then transferred to a liquid medium in 96-well microplate format and to a new agar plate. PCR screening of the mixed cultured pooled clones in the same raw resulted in no positive colony, suggesting that the plasmid library size is too small, and therefore it is insufficient to find a single positive clone.

3.1. Construction of a Fosmid-Based Genomic Library

Previous report by Lin *et al*. (2006) showed that MTGase gene of 1254 bp in size was isolated from *S. plantesis* and heterologously expressed in *S. lividans*. This suggested that the construction and the screening of a genomic library with averagely 3 kb inserts could give a good chance of finding an entire MTGase gene from the producer. In this work, DNA fragments of approximately 40 kb were prepared from the isolate TTA 02 SDS 14 (Figure 2) and were subsequently used to construct a fosmid-based genomic library. Further screening of such library was expected to increase the chance of obtaining a full-length MTGase gene. As shown in Figure 2, the total DNA isolated in this work was mostly accumulated above 36-kb, suggesting that it can be used further to obtain DNA fragments of ~40-kb. The two ways gel-based extraction of ~40-kb DNA fragments employed in this

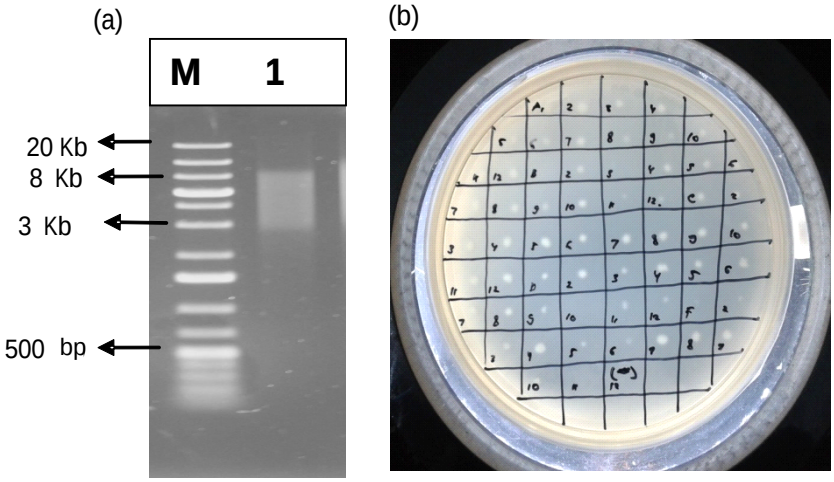

Figure 1. Plasmid –based library construction from Streptomyces genomic DNA**. (a)** DNA fragments of 3-8 kbin size derived from the Streptomyces genomic DNA. (**b)** *E. coli* transformants on the selection LB media containing ampicilin (100 µg/ml).

work were enzymatic treatment using GELaseTM (Epicentre) and column-based extraction using peqGOLD kit (peqLAB).For fosmid-based cloning, the quality of DNA fragments purified enzymatically looks better compared to that obtained using the column because almost no DNA smear was observed below 36 kb. Therefore, the DNA fragments obtained enzymatically (lane 1, Figure 2A) were chosen for ligation with fosmid pCC1FOSTM.The ligation product was subjected to packaging in bacteriophage particles, followed by its transfection into *E. coli* EPI300T1R. Unfortunately, no transfected *E. coli* colony was observed in the selection medium. It is most likely that the bacteriophage particles used in this work were not alive due to their inappropriate handling during shipping. To overcome this limitation, we decided to transform the ligation product into competent *E. coli* EPI300T1R directly, resulting in a fosmid library of approximately 700 members (Figure 2B). This library size is small, but it might be enough for the screening of a single positive clone harboring a full-length MTGase gene.

3.2. Screening of Genomic Library

For genomic library screening, the colonies grown on agar plates were individually transferred into 96-well microplates containing LB supplemented with chloramphenicol. Cultures in the same row were combined into a pool in a sterile eppendorf tube. Each pool was then screened by whole-cell PCR using the primer pair SDS-14F&SDS-14R that amplifies the partial MTGase gene. The PCR-based screening of all pools showed that two pools (F & H) are positively indicated with a target PCR product of ± 365 bp in the gel (Figure 3A). Further cultures' screening from the positive pools revealed some positive wells (Figure 3B).

3.3. Clone Analysis

Positive clone analysis was done to make sure that it carries a ~40-kb DNA fragment that contains a full-length MTGase gene. Prior to clone analysis, the number of the insert inside the clone was increased

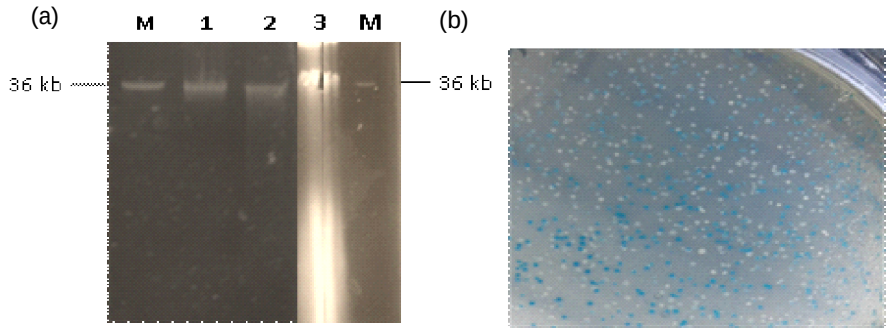

Figure 2. Gel-based selection and fosmid-based cloning of ~40-kb DNA fragments. **a**: Lane 1 indicated ~40 kb fragments purified enzymatically using GELase™(Epicentre). Lane 2 showed DNA fragments of ~40 kb with a bit smear prepared using peqGOLD Extraction Gel Kit (peqLab). Lane 3 is total HMW genomic DNA. M is a fosmid marker of 36 kb (Epicentre). **b**: *E. coli* transformants on the selection LB media supplemented with X-gal, IPTG, and chloramphenicol for blue-white screening.

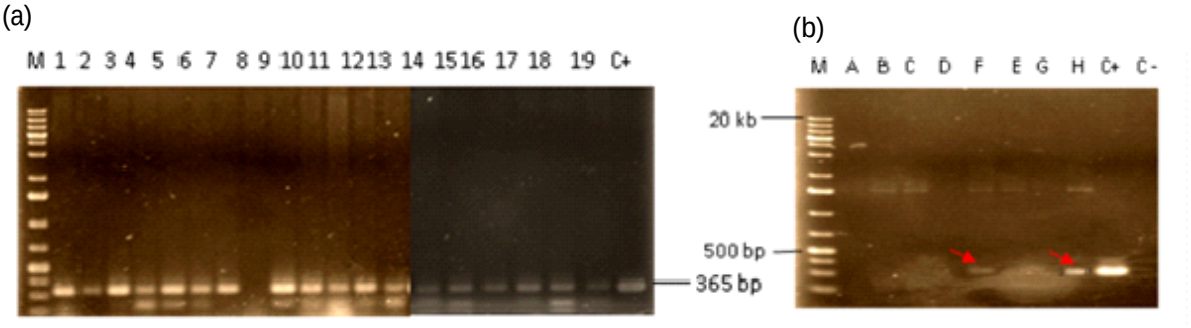

Figure 3. Screening of the genomic library in 96-well microplate format by clone pooling and whole-cell PCR. (**a**) PCR of individual pools derived from combining cultures in the same rows indicated two positive pools (marked with red arrows). (**b**) PCR of positive pools (F & H) indicated some positive wells with PCR product of 365 bp. Note: M, 1 kb plus marker; A-H, pools of cultures from the same rows; C+, positive control (genomic DNA SDS 14/LU); C-, negative control (*E. coli* colonies EPI300-T1^{R}).

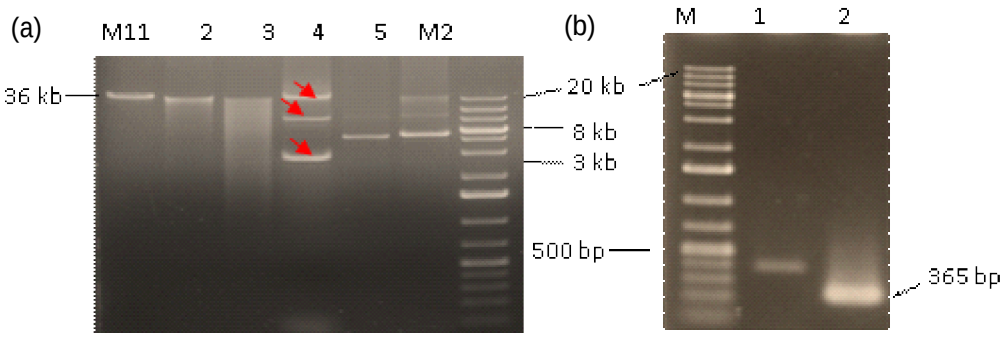

Figure 4. Analysis of a positive clone harboring a ~40-kb insert. (a) Enzymatic restriction with *Not*1 (lane 3), with *EcoR*V (lane 4), with *BamH*1 (lane 5). Note: lane M1, a 36-kb fosmid marker; lane M2, 1kb plus marker; lane 1, DNA fragments of~40kbprior to cloning; and lane 2, DNA fragments of~40kb digested with *Not*1. (b) PCR-detection for the presence of MTGase gene using the primers PTGase4/5 (lane 1) and the primers SDS 14F/R (lane 2).

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        T7_promoter                                              NcoI
  1  TAATACGACTCACTATAGGGGGCCAATCCATGCTCCGGCCGCCATGGCGGCCGCGG
       EcoRI
 59  GAATTCGATTTACGGCTGCGTCGGTGTCACCTGGGTCAACTCGGGTCCCTACCCGAC
  1            L  R  L  R  R  C  H  L  G  Q  L  G  S  L  P  D

117  GAACAAACTGGCGTTCGCGTTCTTCGACGAGAACAGATACAAGAACGACCTCAAGAA
 20  E  Q  T  G  V  R  V  L  R  R  E  Q  I  Q  E  R  P  Q  E

174  CACCAGCCCCCGCCCCGACGAAACGCGGGCGGAGTTCGAGGGTCGCATCGCCAAGGA
 40  H  Q  P  P  P  R  R  N  A  G  G  V  R  G  S  H  R  Q  G

235  CAGTTTCGACGAGGGGAAGGGGTTCAAGCGGGCGCGCGATGTGGCGTCCCTCCTCAA
 59  Q  F  R  R  G  E  G  V  Q  A  G  A  R  C  G  V  P  P  Q

293  CAAGGCCCTGGAAGGTGCCCACGACGAGGGGACGTACATCGACAACCTCAAGACGGA
 78  Q  G  P  G  R  C  P  R  R  G  D  V  H  R  Q  P  Q  D  G

351  GCTCACGAACAGCAATGACGCTCTGCTCCTCGGGGACAGCCGCTCGAACTTCTACTC
 97  A  H  E  Q  Q  -  R  S  A  P  R  G  Q  P  L  E  L  L  L

408  GGCGCTGAGGAACACACCGTCCTTCAAGGAAAGAGACGGAGGCAATCACGACCGTCA
116  G  A  E  E  H  T  V  L  Q  G  K  R  R  R  Q  S  R  P
                      NotI
467  CAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAAC

524  GCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATC

581  ATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCAC
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Figure 5. Sequences analysis of partial MTGase gene on the vector pGEMT -MTG1 (V): insert area, (italics): stop codon, (yellow block): sequence of partial MTGase gene, (not block): most of the sequences of pGEMT-Easy vector.

up to 200 copies by adding induction solution (L-arabinose) into the growth medium. The induced construct was then recovered and digested separately with *Not*I, *EcoR*V, and *BamH*I. *Not*I is a rare cutting enzyme, in which its restriction sites flank the insert in the recombinant pCC1FOS. As shown in Figure 4A (lane 3), *Not*I-digestion of the clone gave three bands. One band (around 8 kb) was the pCC1FOS backbone. The other two bands (~37-kb and ~3-kb) were parts of a ~40-kb insert restricted by *Not*I. *Not*1 restriction enzyme digestion of the fosmid DNA released and produced inserts that are more than 40 kb in size (Choi et al., 2012). The ~3-kb band was then extracted from the gel. The PCR-amplification of the extracted ~3-kb fragment using MTGase-targeting primer set showed positive result (Figure 4B), suggesting that the isolated fragment may harbor a full-length MTGase gene. The results of PCR products are to be subcloned

into pGEM-T Easy vector. Furthermore, the insert present in the recombinant vector is sequenced.

3.4. Sequence Analysis

The recombinant vector of pGEMT-MTG1 is validated by DNA sequencing to determine the insert fragment. The results of DNA sequencing pGEMT-MTG1 vector showed that fragment in the size of ~ 400 bp was successfully inserted into nucleotide 69, which ends at nucleotide 460 of pGEMT-*Easy* vector (Figure 5). Nucleotide sequence of 69 is the end of T-overhang on the vector, so it requires A-overhang on the DNA inserts to allow litigation. A-overhang on the DNA inserts is obtained by the A-tailing or by adding the inserts of adenine in DNA using PCR method (Brown 2006). Sequence analysis of PCR product showed 92% similarity to MTGase genes in *Steptomyces cinnamoneus* deposited in GenBank with the accession number of HQ853022.1 (Duran et al.,1998). Obtaining an entire MTGase gene from this *Streptomyces* would provide a basis for its heterologous expression in an appropriate host and would allow a large-scale production of MTGase.

4. Conclusion

Streptomyces thioleteus TTA 02 SDS 14 obtains a full-length MTGase gene for its further heterologous expression. A plasmid-based library with the insert size of 3-8 kb was constructed and screened. However, due to the very small size of the library, no positive clone was observed. To increase the chance of obtaining a DNA fragment harboring an entire MTGase gene, a fosmid-based genomic library that consists of ~700 clones was subsequently generated. Further screening of such fosmid library led to the isolation of positive clone. Subsequent analysis of such clone confirmed that it harbors ~40-kb insert with an entire MTGase gene present in it. Sequence analysis of PCR product showed 92% similarity to MTGase genes in *Steptomyces cinnamoneus* (HQ853022.1).

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