CLONING AND EXPRESSION OF FUSION PROTEIN GLUTATHION S TRANSFERASE-STREPTAVIDIN ON *Bacillus subtilis* SPORES AND EVALUATION OF ITS SPECIFIC BINDING WITH BIOTINYLATED ANTIBODY CETUXIMAB

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ABSTRACT

We cloned fusion gene (*cotB-gst-streptavidin*), encoding three sequential proteins including membrane spore coat protein CotB, Glutathion S Transferase (GST), and streptavidin, into the chromosome of *Bacillus subtilis* PY79 to create recombinant strain named SA2. Expression of streptavidin on the outer coat of SA2 spores were confirmed by immunofluoresence using specific anti-streptavidin rabbit IgG and anti-rabbit IgG labeled Alexa546. The specific bindings of biotinylated cetuximab on spores of *Bacillus subtilis* SA1 (expressing CotB-streptavidin) and on *Bacillus subtilis* SA2 were detected by Western Immunoblotting. The data indicates that binding of biotinylated cetuximab on SA2 spores was slightly 1.4 fold higher than that on SA1 spores, suggesting limited role of GST in enhancing the exposure of streptavidin for interaction with biotinylated cetuximab.

Keywords: Bacillus subtilis spore; Glutathion S Transferase (GST), streptavidin, biotinylated cetuximab.

1. INTRODUCTION

The *Bacillus subtilis* spores have been used as models for expression of target proteins fused with membrane protein, such as CotB, CotC, CotE on the spore surface [1 - 3]. Due to the unique, distinguishing heat-stable feature of spores, a spore-based display system provides advantages relative to those based on the use of bacterial cells. There has been number of antigens selected as model proteins and successfully displayed on the spore surface by fusion to *cotB*: i) the non-toxic 459 amino acid C-terminal fragment of the tetanus toxin (TTFC), encoded by the *tetC* gene of *Clostridium tetani* [4]; ii) the 103 amino acid B subunit of the heat-labile

toxin of enterotoxigenic strains of *Escherichia coli* (LTB), encoded by the *eltB* gene [4]; iii) toxin A peptide of *Clostridium difficile* [5].

Recently, we have been successful in cloning and expression of a gene encoding a fusion protein of CotB and streptavidin on the surface of *B. subtilis* SA1 spores [6, 7]. The SA1 spores is bound specifically with biotinylated cetuximab, a chimeric IgG1 monoclonal antibody that targets the extracellular domain of Epidermal Growth Factor Receptor (EGFR) over expressed on the surface of 30 – 85 % colon cancer cell types, including cetuximab-sensitive HT29 cell lines [4, 8 - 10]. The binding constant of SA1 with cetuximab biotinylated is determined to be about 10^7 M^{-1}, indicating the specific binding of streptavidin with biotinylated cetuximab [7].

In this work, we further create another recombinant *B. subtilis* SA2 strain expressing fusion proteins of CotB, Glutathion S Transferase, and streptavidin (CotB-GST-streptavidin). GST has been known as famous tag protein which can increase solubility of its fused proteins. Here, GST is designed in connection between CotB and streptavidin, with the aim to enhance exposure of streptavidin on spore surface, toward the external environment for interaction with biotin or biotinylated molecules. The specific binding of biotinylated cetuximab on SA1 and SA2 is compared to prove actual role of GST in enhancing streptavidin-biotin interaction.

2. MATERIALS AND METHODS

2.1. Materials

B. subtilis wild type strain PY79 (*spo*$^+$) was used. All recombinant strains described here are isogenic derivatives of PY79. Plasmid amplification for nucleotide sequencing, sub-cloning experiments and $CaCl_2$-mediated transformation of *E. coli* competent cells were performed in the *E. coli* strain DH5α as described in Sambrook et al., 1989 [11]. Methods for *Bacillus* including the two-step transformation of *B. subtilis* were those outlined in Cutting *et al*., 1990 [12].

2..2 Methods

2.2.1. Construction of gene fusions

Using laboratory *B. subtilis* strain containing *cotB-gst* (kindly provided by Prof. Simon Cutting) and *Streptomyces avidinii* strain 11996 (NCIMB Ltd., Aberdeen, UK) as a chromosomal template, the complete ORF of *cotB-gst,* and ORF *of streptavidin* were amplified using respective primers (cotB forward: 5'-cgcggatccACGGATTAGGCCGTTTGTCCT-3' having a restriction site for *Bam*HI, GST reverse: 5'-cccaagcttATCCGATTTTGGAGGATGGTTC-3' having a restriction site for *Hin*dIII, Strep forward, 5'-aaaaagcttGACCCCTCCAA GGACTCGAAG-3' having a restriction site for *Hin*dIII, and Strep reverse 5'-aaagaattc CTACTGCTGAACGGCGTCGAG-3' having a restriction site *Eco*RI). Purified PCR-amplified *cotB-gst* having the expected size of 1.7 kb was cleaved with *Bam*HI and *Hin*dIII and cloned into pDG364. Then, purified PCR-amplified *streptavidin* DNA having the expected size of 500 bp was cleaved with *Hin*dIII and *Eco*RI and cloned into pDG364-cotB-gst. This created an in-frame fusion of *cotB-gst* with *streptavidin* at the *Hin*dIII site. The clone was verified using DNA sequencing across the fusion site and the plasmid was linearized by digestion with *Pst*I.

Linearised DNA was then used to transform competent cells of *B. subtilis* strain PY79 with selection of chloramphenicol-resistant colonies. Transformants carried a stable, double crossover, insertion of the *cotB-streptavidin* chimera at the amylase gene (*amyE*) and the resulting clone named SA2 (Figure 1).

2.2.2. Preparation of spores

Sporulation of *B. subtilis* PY79 (*spo*$^+$), the *B. subtilis* strains SA1 harbouring *cotB-streptavidin* [6, 7], and SA2 harbouring *cotB-gst-streptavidin (cotB-gst-SA)* were made in DSM (Difco sporulation media) at 37 ^oC using the exhaustion method. Sporulating cultures were harvested 60h after the initiation of sporulation and suspensions of spores purified using lysozyme treatment to break any residual sporangial cells followed by washing in 1 M NaCl, 1M KCl and water [13]. The number of spores was calculated by serial dilution and plate counting. Spores were killed by autoclaving (120 ^oC, 15 psi, 20 mins). Water lost by evaporation was calculated and sterile water added to restore to the original volume before autoclaving using microscopic counting of spore particles using a hemocytometer to determine spore counts. 100 % spore killing was validated by serial dilution and plate counting.

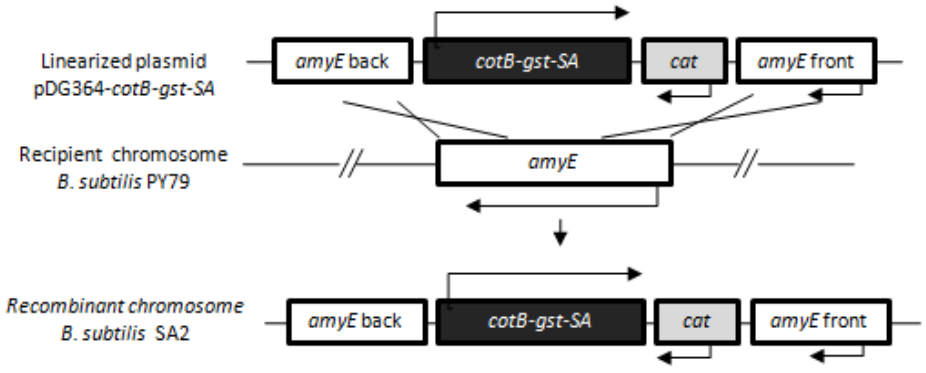

Figure 1. Strategy for the chromosomal integration of the *cotB-gst-streptavidin (cotB-gst-SA)* gene. Arrows indicates direction of transcription.

2.2.3. Immunofluorescent staining and imaging

1 × 10^9 SA1 spores, 1 × 10^9 SA2 spores, and 10^9 PY79 spores were fixed with ice-cold paraformaldehyde 1 % for 15 min, then washed three-times with PBS (145 mM, pH 7.4). Next, the spores were incubated with polyclonal streptavidin-specific antibody at 10 mM for 45 min at RT, followed by anti-rabbit IgG conjugated Alexa 546 at 10 mM for 45 min at RT. Incubations with antibodies were performed in the presence of 2 % BSA and three washes with PBS (pH 7.4; NaCl 137 mM) plus 0.5 % BSA after the first antibody. The spores were imaged under an excitation of 525 nm (green laser) using a confocal fluorescence microscope Carl Zeiss LSM510.

2.2.4. Western analysis of cetuximab binding

Biotinylated cetuximab were prepared in similar way to what described previously by Nguyen et al in 2012 [7]. Different amounts of biotinylated cetuximab which were prepared as previously described ranging from 0.25 µg, 1 µg, 4 µg to 8 µg were bound either on 1×10^9 SA1 and 1×10^9 SA2 spores in PBS (pH7.4; NaCl, 137 mM). Incubations were made in 300 µl buffers for less than 1 hour and the unbound biotinylated cetuximab was removed using three washes with PBS (0.01 M, pH 7.4) before SDS-PAGE analysis. Biotinylated cetuximab bound with 2×10^8 spores (one fifth of the initial amount of spores incubated) was checked for the presence of a 60 kDa species indicating that DTT-reduced IgG by Western blotting using anti-human IgG-conjugated alkaline phosphatase (Promega), and the purple color reaction determined using the NBT/BCIP substrate. 100 ng of biotinylated cetuximab was used as a positive control. The intensity of 60 kDa bands was analyzed using Scion Image® software.

3. RESULTS AND DISCUSSION

3.1. Cloning *cotB-gst-streptavidin* fusion gene into *B. subtilis* PY79 genome

We started with amplification of the *cotB-gst* gene from the developed recombinant *B. subtilis* strain *cotB-gst* as mentioned in the Materials and Methods. The amplified band which was about 1.7 kb (data not shown) after being digested with *Bam*HI and *Hin*dIII was then cloned into the vector pDG364 having the same protruding ends *Bam*HI and *Hin*dIII.

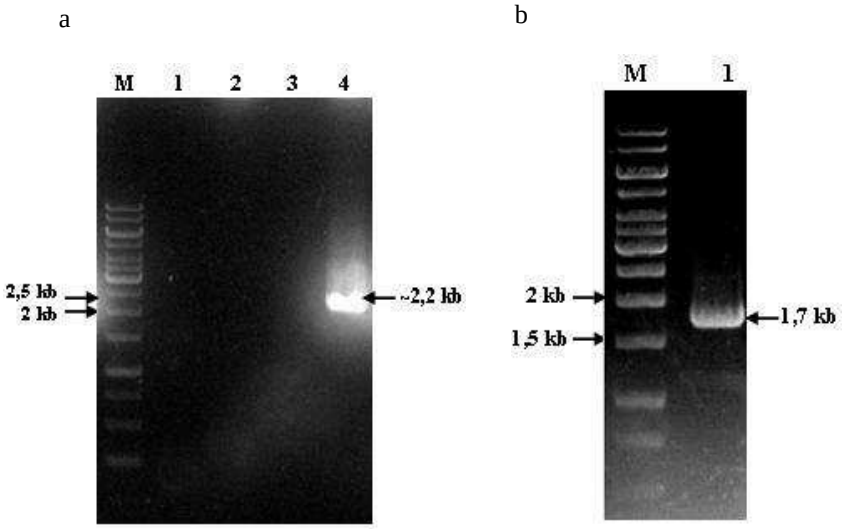

Figure 2. Screening a positive clone carrying pDG364-cotB-GST plasmid. (a) DNA electrophoresis on 1 % agarose gel of directly PCR product from four colonies using universial primers for plasmid pDG364 (364 Fw and 364 Rv). Lane M: 1 kb ladder, Lane 1 - 3: negative colonies, Lane 4: positive colonies. (b) DNA electrophoresis on 1 % agarose gel of a directly 1.7 kb PCR product from colony 4 using primers specific for cotB forward primer (cotB Fw) and specific for GST reverse primer (GST Rv). Lane M: 1 kb ladder, Lane 1: colony 4.

We screened the positive recombinant pDG364-cotB-gst using direct PCR method of colonies growth on LB plate containing ampicillin. As shown in the Figure 2a of screening four colonies by PCR using universial primers of pDG364 (364 Fw and 364 Rv), we could obtain a

clear band of about 2.2 kb in the case for colony 4. To confirm the presence of *cotB* and *gst* genes in the colony 4, we used cotB Fw and GST Rv primers to amplify specific band for *cotB-gst* and obtained a PCR product of about 1.7 kb (Fig. 2b, lane 1) corresponding to the total size 1.1 kb of *cotB* and 0.6 kb of *gst*. Thus, we successfully amplified and cloned *cotB-GST* gene into pDG364 vector to form a recombinant pDG364 cotB-gst.

In similar way, we sequentially cloned streptavidin gene into plasmid pDG364-cotB-gst via restriction sites *Hin*dIII and *Eco*RI. We amplified streptavidin gene of about 550 bp (Fig 3a) and cloned the enzyme digested PCR product into the pDG364-cotB-gst having the same restriction sites *Hin*dIII and *Eco*RI. We screened a positive colony using sets of primers specifically to *cotB-gst*, pDG364 plasmid, and *streptavidin*. As expected, we can amplified the band of 1.7 kb, 2.2 kb, and 0.55 kb, respectively, confirming the presence of the fusion gene *cotB-gst-streptavidin* in the plasmid pDG364 (Figure 3b). The exact sequence of *cotB-gst-streptavidin* was also confirmed by DNA sequencing (data not shown).

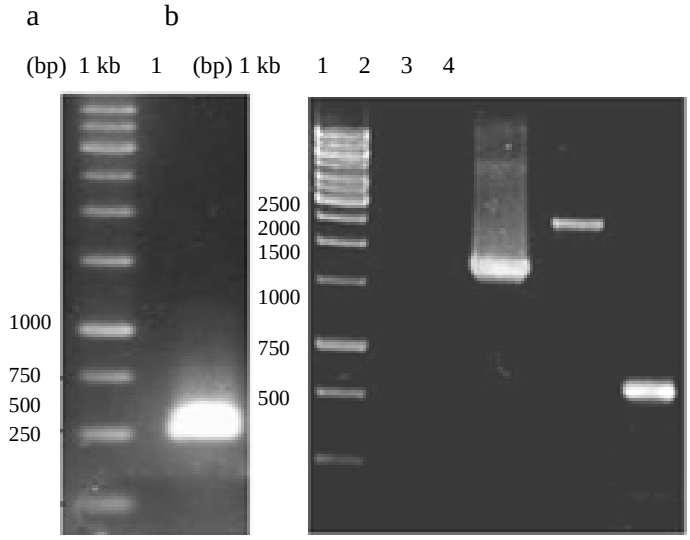

Figure 3. Cloning of *streptavidin* into pDG364-cotB-GST plasmid. (a) Lane M: 1 kb ladder, Lane 1: Electrophoresis on 1 % agarose gel of amplified *streptavidin* gene from genome of *Streptomyces avidini*. (b) Electrophoresis on 1 % agarose gel of PCR product amplified directly from colonies. Lane M: 1kb ladder, Lane 1: PCR product from colony without pDG364 cotB-gst-streptavidin plasmid using 364 Fw and 364 Rv, Lane 2-4: PCR product from a colony harbouring pDG364-CotB-gst-streptavidin using cotB Fw and GST Rv, 364 Fw and 364 Rv, and primers specific for *streptavidin*, respectively.

Based on the capability to synthesize amylase of the *B. subtilis* wild strain and the inability of the recombinant *B. subtilis* because of *amyE* gene in *B. subtilis* chromosome was interrupted by the insertion of recombinant *CotB-gst-streptavidin* gene we examined this colony feature on LB 1 % starch plates and tested with Lugol solution. As a result, we observed the amylolytic ring of the wild strain PY79 whereas we did not observe any ring of the colonies of *B. subtilis* SA2 (Fig. 4). The data confirm that *cotB-gst-streptavidin* gene was successfully integrated into the position of *amyE* sequence in chromosome of *B. subtilis* SA2.

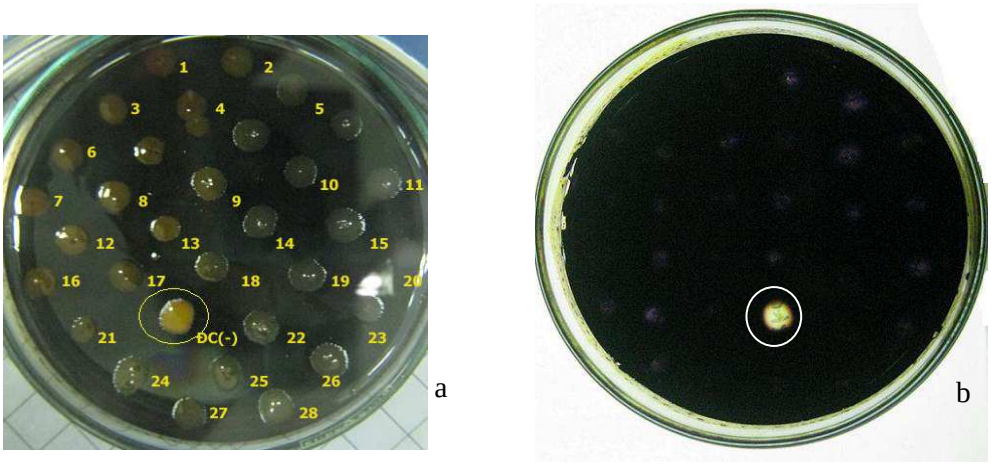

Figure 4. Twenty eight colonies of *B. subtilis* SA2 and one colony of *B. subtilis* PY79 grown on LB agar containing 1 % starch overnight, then stained with Lugol solution (b), and finally being removed off the plate to observe amylotic ring (b). The circle in (a) indicates the position of the PY79 strain colony, which is corresponding to the position of clear white zone of amylotic ring in (b).

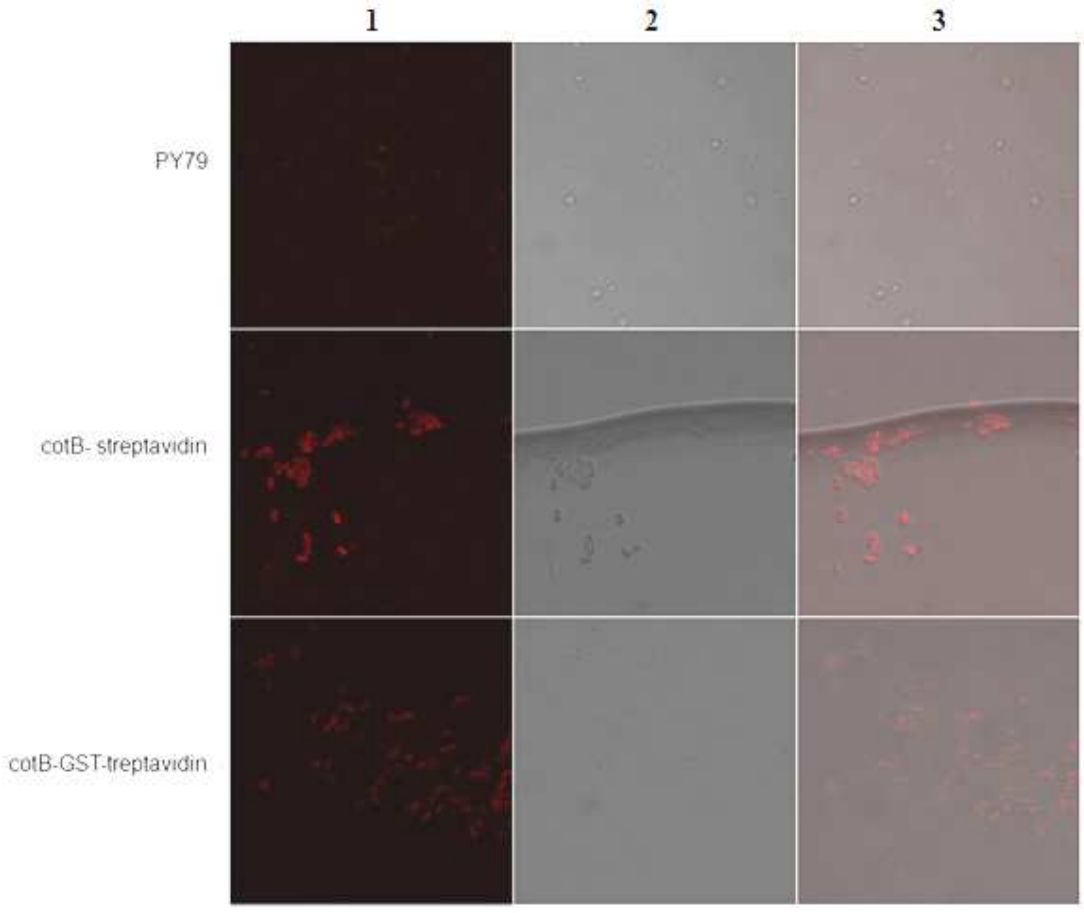

Figure 5. Immunofluorescence staining of spores (with or without) expressing streptavidin using primary anti-streptavidin rabbit IgG and secondary anti-rabbit IgG Alexa 546, and observation of fluorescent signals using confocal microscopy. (1) Images of emissive signals of Alexa 546 nm under green laser excitation, (2) Images of signals viewed under the white light, (3) Images of merged signals from (1) and (2).

3.2. Expression of streptavidin on the surface of recombinant *B. subtilis* SA2 spores

Immunofluorescent analysis of the expression of streptavidin on the spore surface of PY79 wild type strain, recombinant strains SA1 (CotB-streptavidin) and SA2 (CotB-GST-streptavidin) was performed using primary anti-streptavidin polyclonal antibody, and secondary anti-rabbit IgG labeled Alexa 546 (excitation: 546 nm, emission: 570 nm). We observed clear signals as presented by red color of Alexa 546 fluorescent dye on the spores SA1 and SA2 under green laser excitation. Simultaneously, these signals were merged in the position of spores observed under white light. By contrast, we did not observe any signal on the PY79 spores. The acquired results allowed us to surely affirm that the presence of streptavidin on the spore outer surface of both strains SA1 and SA2 with similar expression level

3.3. Binding of biotinylated cetuximab on SA2 spores in comparison to on SA1 spores

SA1 and SA2 spores (1×10^9) were firstly killed by autoclaving, and then tested for binding with cetuximab, an inhibitor of EGFR. Biotinylated anti-EGFR monoclonal IgG (cetuximab) at 250 ng, 1 µg, 4 µg, 8 µg were bound to spores. The unbound cetuximab was washed off and then 2×10^8 cetuximab bound SA1 spores were examined by SDS-PAGE fractionation and Western blotting (Figure 6). The DTT in the SDS-PAGE loading buffer would reduce the disulfide bridge of IgG, resulting in two fragments corresponding to the light and heavy chains.

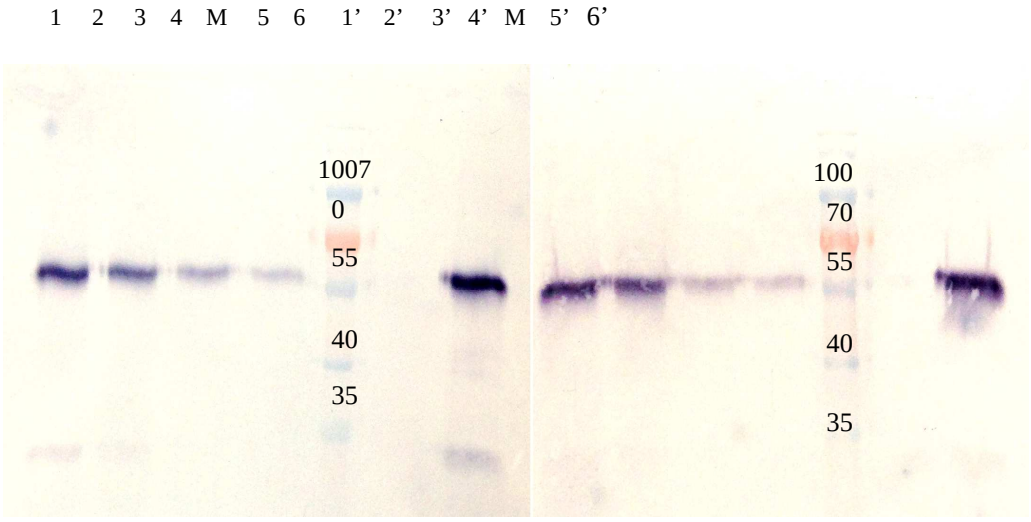

Figure 6. Western analysis of binding of biotinylated cetuximab to spores expressing streptavidin. Biotinylated mouse-human chimeric monoclonal IgG cetuximab at different amounts 8 µg (lane 1 and 1’), 4 µg (lane 2 and 2’), 1 µg (lane 3 and 3’), 250 ng (lane 4 and 4’) were bound to 1×10^9 killed spores SA1 (lane 1 - 4), and killed spores SA2 (lane 1’-4’) and total extracted proteins of 2×10^8 spores were applied to each well, and then were fractionated on 12 % SDS-PAGE gels and transferred to membranes and probed with anti-human IgG conjugated with Alkaline Phosphatase (Promega). A positive control was 100 ng biotinylated cetuximab applied directly to the well and run in parallel (lane 3). A negative control was PY79 spores bound with 1 µg biotinylated cetuximab performed at parallel experiment. Molecular weight markers (kDa) are indicated.

As shown in the Figure 6, we could clearly observe a band of about 60 kDa indicating the size of cetuximab which was bound to the spores SA1 (Figure 6, lane 1-4) and SA2 (Figure 6, lane 1'-4'). The intensity of bands was in correspondence with the initial amount of biotinylated cetuximab incubated with the spores. The intensity of the band in Figure 6, lane 1 at 8 μg biotinylated cetuximab incubated with SA1 spores was equal to 50 % intensity of the control 100 ng biotinylated cetuximab (Figure 6, lane 1), indicating that the amount of cetuximab bound on 2×10^8 SA1 spores was about 50 ng, which implied that only about 250 ng from the 8μg of incubated cetuximab had bound to 1×10^9 SA1 spores. The intensity of the band in Figure 6, lane 1' at 8 μg biotinylated cetuximab incubated with SA2 spores was equal to 70 % intensity of the control 100 ng biotinylated cetuximab (Figure 6, lane 1), indicating that the amount of cetuximab bound on 2×10^8 SA2 spores was about 70 ng, which implied that about 350 ng from the 8μg of incubated cetuximab had bound to 1×10^9 SA2 spores. Thus, the binding on SA2 was improved about only 1.4 fold compared to SA1. The data indicates that the binding of biotinylated cetuximab on SA2 spores was slightly improved compared to that on SA1 spores. On the other hand, PY79 did not show any observable band (Figure 6, lane 5 and 5') demonstrating specific binding of biotinylated cetuximab to the streptavidin expressed as fusion proteins with CotB and CotB-GST on the spores SA1 and SA2, respectively.

4. CONCLUSION

In sum, we successfully amplified the DNA fragments of genes coding *cotB-gst* and *streptavidin*-encoding gene using PCR, sequentially cloned the amplified *cotB-gst* and *streptavidin* gene into the pDG364 *cotB* plasmid and integrated the *cotB-gst-streptavidin* construct into *B. subtilis* genome. The expression of the streptavidin protein on the surface of *B. subtilis* SA2 spores was confirmed by Western blotting. The binding of biotinylated cetuximab with SA2 (cotB-GST-streptavidin) was just slightly 1.4 fold higher than that on SA1 (cotB-GST-streptavidin) spores, indicating limited role of GST in enhancing exposure of streptavidin for interacting with biotinylated cetuximab.

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TÓM TẮT

NHÂN DÒNG VÀ BIỂU HIỆN PROTEIN DUNG HỢP GLUTATHION S TRANSFERASE-STREPTAVIDIN TRÊN BÀO TỬ *Bacillus subtilis* VÀ ĐÁNH GIÁ KHẢ NĂNG GẮN ĐẶC HIỆU VỚI KHÁNG THỂ CETUXIMAB BIOTINYL HÓA

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Chúng tôi đã nhân dòng gen dung hợp (*cotB-gst-streptavidin*) mã hóa cho ba protein nối tiếp nhau bao gồm protein lớp vỏ áo của bào tử CotB, Glutathion S Transferase (GST), và streptavidin vào nhiễm sắc thể của *Bacillus subtilis* PY79 để tạo nên chủng tái tổ hợp SA2. Biểu

hiện của streptavidin trên lớp vỏ của bào tử SA2 spores được kiểm chứng bởi phương pháp miễn dịch huỳnh quang, sử dụng kháng thể sơ cấp IgG thỏ kháng streptavidin và kháng thể thứ cấp kháng thỏ gắn chất huỳnh quang Alexa546. Gắn bám đặc hiệu của biotinylated cetuximab lên bào tử của *Bacillus subtilis* SA1 (biểu hiện cotB-streptavidin) và lên *Bacillus subtilis* SA2 được phát hiện bởi phương pháp thẩm tách miễn dịch. Kết quả chỉ ra khả năng gắn của biotinylated cetuximab lên bào tử SA2 tăng lên không nhiều, khoảng 1,4 lần so với gắn lên bào tử SA1, gợi ý vai trò khiêm tốn của GST trong việc tăng cường sự bộc lộ của streptavidin trên bề mặt bào tử để tương tác với biotinylated cetuximab.

Từ khóa: bào tử *Bacillus subtilis*, Glutathion S Transferase (GST), streptavidin, biotinylated cetuximab.