Heterologous Expression of SARS-CoV ORF10 and X5 Genes in *E. coli* and *Streptomyces lividans* TK24

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In previous studies a variety of novel accessory genes has been identified that were interspersed among the structural genes of the SARS-CoV (severe acute respiratory syndrome coronavirus) genome. The predicted unknown proteins (PUPs) encoded by the accessory genes, which are considered to be unique to the SARS-CoV genome, might play important roles in the SARS-CoV infection. Two of these genes, called ORF10 and X5, were synthesized and introduced into *E. coli* and *Streptomyces lividans* TK24, respectively. SDS-PAGE and Western blot revealed that the ORF10 and X5 genes have been expressed in the two hosts. This is the first report of heterologous expression of ORF10 and X5 genes in *E. coli* and *S. lividans* TK24. This work makes it possible to study the structure and potential functions of proteins encoding by these two genes.

Key words: SARS-CoV, ORF10 and X5, Heterologous Expression

Introduction

The severe acute respiratory syndrome (SARS) is an epidemic caused by a novel coronavirus designated as SARS-CoV (Marra *et al*., 2003). SARS-CoV is a positive-strand RNA virus. The SARS-CoV genome is about 30 kb in length and encodes 3 groups of proteins (Marra *et al*., 2003; Rota *et al*., 2003; Zeng *et al*., 2003). Group 1 proteins mainly refer to replicase polyproteins expressed by two open reading frames (ORF1a and ORF1b) that are linked together by a ribosomal frameshift and undergo cotranslational proteolytic processing (Marra *et al*., 2003; Rota *et al*., 2003). Group 2 proteins include four structural proteins: the spike protein (S), envelope protein (E), membrane glycoproteins (M), and the nucleocapsid protein (N). These structural proteins are common to all known coronaviruses (Marra *et al*., 2003; Rota *et al*., 2003). In addition to these two groups of proteins, there are nonstructural proteins that encode by novel accessory genes. These proteins are varying in length from 39 to 274 amino acids (aa) and are with no significant sequence homology to proteins in other coronaviruses. These group 3 proteins, named predicted unknown proteins (PUPs), are unique to SARS-CoV (Marra *et al*., 2003; Rota *et al*., 2003).

The number of the PUPs is not consistently reported by different studies. Marra *et al*. (2003) identified nine, while Rota *et al*. (2003) only identified five PUPs that are longer than 50 aa. These proteins are nonconserved and may have novel functions in virus replication and pathogenesis or modulate immune responses to infection. As reported recently, X4 protein, also known as U122 or 7a (Tan *et al*., 2004a) encoded by a gene named ORF8 (Marra *et al*., 2003) or ORF7a (Thiel *et al*., 2003), was showed to inhibit the growth of Balb/c3T3 cells and to interact with other SARS proteins including ORF3, M, N and S proteins. It was also showed that these five proteins could form a large molecule during virus infection (Tan *et al*., 2004a). In addition, the X4 protein could induce apoptosis via the caspase pathway in various cell types (Tan *et al*., 2004a).

The ORF3 protein, also known as U274 (Tan *et al*., 2004b), X1 (Rota *et al*., 2003) or ORF3a protein (Snijder *et al*., 2003; Thiel *et al*., 2003), could interact with S proteins functionally (Zeng *et al*., 2004). Moreover, the ORF3 protein can interact with M and N proteins and induce apoptosis in Vero E6 cells (Law *et al*., 2005) as well. Recently, some results indicated that the X1 protein might serve as an ion channel for virus release (Lu *et al*., 2006).

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Previous studies suggested that these PUPs might play important roles in SARS-CoV infection. Therefore, identification and characterization of new functional proteins from these PUPs will be helpful in understanding the pathogenesis of SARS-CoV and identifying potential targets for drug and vaccine designs against SARS-CoV. The successful heterologous expression of PUPs would make one step further towards this purpose.

ORF10 gene is 120 bp long and encodes a predicted 39aa protein. X5 gene, also known as ORF11 (Marra *et al*., 2003), encodes a predicted protein of 84 aa. The functions of ORF10 and X5 proteins are unclear (Rota *et al*., 2003). In the present study, we heterologously expressed the ORF10 and X5 genes in *Escherichia coli* and *Streptomyces lividans* TK24. To our knowledge, this is the first study reporting the heterologous expression of ORF10 and X5 genes, especially in *S. lividans* TK24. The successful heterologous expression of virus genes in *S. lividans* TK24 proves that *Streptomyces* is a possible host for different genes from various organisms including animals, plants, bacteria and viruses. Moreover, the successful heterologous expression of the two nonstructural ORFs will help characterizing of their functions.

Results

Expression of recombinant ORF10 and X5 proteins in E. coli

ORF10 and X5 genes were expressed in *E. coli*. As showed by SDS-PAGE, the expressed *E. coli* [pET32a-ORF10] and *E. coli* [pET32a-X5] corresponded to the protein bands 29.3 kDa (His-X5 fusion protein) and 23.9 kDa (His-ORF10 fusion protein), respectively (Fig. 1). The Western blotting showed that the 29.3 kDa and 23.9 kDa bands reacted positively to the His-Tag monoclonal antibody. No band, however, was observed in the negative control plasmid (Fig. 2). It was demonstrated that there were indeed ORF10 and X5 proteins expressed in the *E. coli* culture.

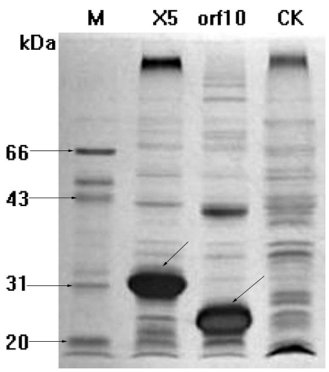

Fig. 1. SDS-PAGE analysis of *E. coli* [pET32a-ORF10] and *E. coli* [pET32a-X5]. M, protein molecular marker; X5, *E. coli* [pET32a-X5]; orf10, *E. coli* [pET32a-ORF10]; CK, *E. coli* [pET32a]. The arrows show the expressed recombinant ORF10 and X5 proteins, respectively.

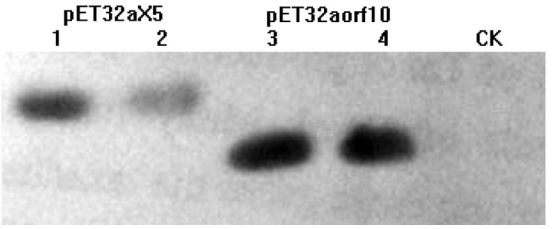

Fig. 2. Western blot analysis of ORF10 and X5 proteins. 1, 2, Western blot results of *E. coli* [pET32a-X5]; 3, 4, Western blot results of *E. coli* [pET32a-ORF10]; CK, Western blot result of *E. coli* [pET32a].

The expression conditions were optimized. The best expression for *E. coli* [pET32a-X5] could be obtained when cells were grown at 37 °C until OD600 reached 0. 8. IPTG (isopropyl-β-D-thiogalactopyranoside) was then added with the final concentration of 0.4 mM to induce the expression of SARS genes. The best expression was achieved after 2 h induction at 37 °C for *E. coli* [pET32a-X5]. For *E.coli* [pET32a-ORF10], optimal expression conditions were similar to *E. coli* [pET32a-X5] except that the induction temperature was 30 °C and the induction time was 4 h. The expression products were applied to SDS-PAGE analysis. The SDS-PAGE scanning showed that ORF10 and X5 proteins accounted for 68.5% and 20% of the total proteins, respectively.

Purification of recombinant ORF10 and X5 proteins

The ORF10 and X5 genes were expressed in *E. coli* under optimal conditions. A total of 50 ml expression cultures of *E. coli* [pET32a-ORF10] and *E. coli* [pET32a-X5] were harvested, respectively, by centrifugation at 12000 × *g* for 2 min. All purification procedures were carried out at 0–4 °C. Every 2 g of cell pellet were resuspended in 5 ml PBS buffer (20 mM sodium phosphate, 50 mM NaCl, pH 7.8) and sonicated in an ice bath for 10 min. The lysed cells were centrifuged at 12000 × *g* for 30 min

and the pellets were discarded. The residual supernatant was filterted though a 0.45 μm filter membrane and then loaded onto a ProBond resin column (Invitrogen, CA). Fractions were collected after being eluted from a His-Bond column with a linear gradient of imidazole from 0 to 500 mM in 8 ml of elution buffer (20 mM sodium phosphate, 500 mM NaCl, pH 6.0) and applied to SDS-PAGE analysis. SDS-PAGE revealed that two recombinant proteins had been purified which could be studied further.

The purified fractions, corresponding to the desired recombinant proteins, were collected. The concentrations of the purified proteins were determined as 7 mg/ml for the ORF10 protein and 3 mg/ml for the X5 protein based on the standard concentration curve of bovine serum albumin (BSA) [$Y = 0.05119 + 0.00864\ X$, where Y refers to OD595 and X means the concentration of proteins (μg/ml)].

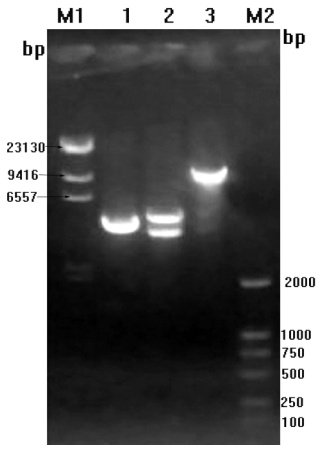

Fig. 4. Restriction site analysis of pSGLgppM. M1, λDNA/*Hin*dIII marker; 1–3, *Msc*I, *Sac*II, and *Swa*I analysis of pSGLgppM; M2, DL2000 marker.

Restrictional analysis of Streptomyces vector pSGLgppM

pSGLgpp is one of the *Streptomyces* vectors carrying thiostrepton and neomycin resistance genes (Zhang and Li, 2000). The vector has been used to heterologously express many animal genes (Zhang *et al*., 2004; Jiang and Li, 2002). The vector, however, was not sequenced completely and only with very few restriction enzyme sites for cloning of heterologous DNA. For heterologous gene subcloning, the vector has been modified in this experiment. After inserting a fragment with 44 enzymatic sites into the pSGLgpp, the vector pSGLgppM with more cloning sites was obtained. For clarification of the enzyme sites, different restriction enzymes were selected to digest the pSGLgppM. The results showed that *Spe*I, *Nhe*I, *Bgl*II, *Eco*RV, *Swa*I, *Eco*RI and *Hin*dIII are one-site enzymes in the vector that could be used to insert heterologous genes (Figs. 3 and 4). Therefore, *Swa*I and *Spe*I were chosen as the cloning sites for the insertion of ORF10 and X5 genes into pSGLgppM in order to generate the SARS expression vectors pSGLgppMORF10 and pSGLgppMX5.

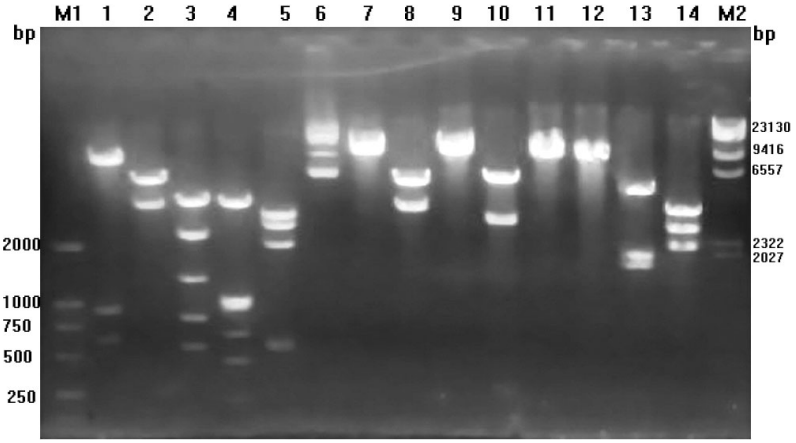

Fig. 3. Restriction site analysis of pSGLgppM. M1, λDNA/*Hin*dIII marker; 1–14, *Age*I, *Bln*I, *Nae*I, *Apa*I, *Nco*I, *Cla*I, *Spe*I, *Stu*I, *Nhe*I, *Sal*I, *Bgl*II, *Eco*RV, *Not*I, and *Sph*I analysis of pSGLgppM; M2, DL2000 marker.

Expression of ORF10 and X5 genes in S. lividans TK24

ORF10 and X5 genes were expressed in *S. lividans* TK24. The expressed products from *S. lividans* [pSGLgppMORF10] and *S. lividans* [pSGLgppMX5] were subjected to 16% tricine-SDS-PAGE (Schägger and Jagow, 1987), yielding protein bands corresponding to 10.2 kDa (His-ORF10 fusion protein) and 14.6 kDa (His-X5 fusion protein) (Figs. 5 and 6). Western blot analysis showed that the 10.2 kDa and 14.6 kDa bands reacted positively to the His-Tag monoclonal antibodies. No band was observed in the negative control plasmid. It was demonstrated that there were indeed ORF10 and X5 proteins expressed in the *S. lividans* TK24 culture.

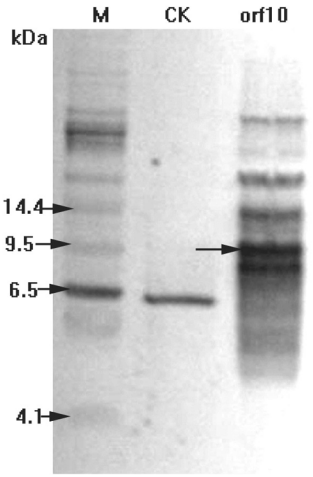

Fig. 5. Tricine-SDS-PAGE analysis of *S. lividans* [pSGLgppMORF10]. M, protein marker; CK, tricine-SDS-PAGE result of *S. lividans* [pSGLgppM]; orf10, tricine-SDS-PAGE result of *S. lividans* [pSGLgppMORF10]. The arrows show the recombinant ORF10 protein.

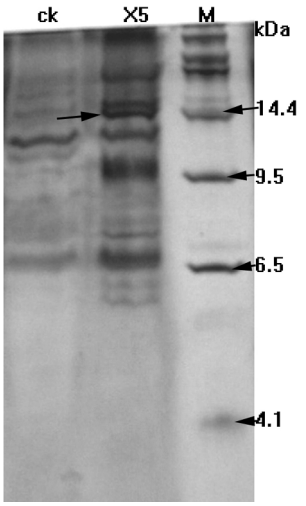

Fig. 6. Tricine-SDS-PAGE analysis of *S. lividans* [pSGLgppMX5]. CK, tricine-SDS-PAGE result of *S. lividans* [pSGLgppM]; X5, tricine-SDS-PAGE result of *S. lividans* [pSGLgppMX5]; M, protein marker. The arrows show the recombinant X5 protein.

Discussion

In our preliminary experiments (data not shown), the recombinant SARS-CoV ORF10 and X5 proteins were not detectable in *E. coli* with IPTG induction. It could be explained by either unsuitable conditions for SARS-CoV genes expression or the toxicity of the expressed proteins. To solve this problem, modifications of the induction procedure were tested. The first change was to replace ampicillin by carbenicillin. Since β-lactamase secreted into the medium by *E.coli* cells will destroy all of the ampicillin; use of ampicillin as a selective antibiotic needs special care. In addition, ampicillin is susceptible to the lower pH value that is caused by cell metabolism. This means that plasmid-lacking cells will proliferate as ampicillin hydrolyzes during the bacteria cell growth. Use of carbenicillin, which is stable at lower pH values, instead of ampicillin helps to prevent an overgrowth of plasmid-free cells.

Second, the content ORF10 and X5 expression products was increased and reached the maximum during a 4-hour induction. The contents of recombinant proteins, however, decreased rapidly with the further lapse of culture time. After 13 h of incubation, the expression proteins were no more detectable (data not shown). Therefore, a short induction time, such as 2 or 4 h, was critical in solving this problem.

Finally, the effect of acidifying the medium by cell metabolism should be concerned. The metabolism of cells will make the average pH value of cell cultures to be decreased and the antibiotic to be unstable. Therefore, the cell pellets were washed twice with double distilled H_2O and resuspended in fresh medium before induction.

S. lividans is a suitable host for human genes (Binnie *et al.*, 1997). To our knowledge, neither expression of SARS-CoV PUPs encoding genes nor expression of other virus genes has been reported in *Streptomyces* so far. One of the reasons

might be the toxicity of virus proteins to the host. To alleviate the toxicity, a *Streptomyces* secretion vector, pSGLgpp, which is widely used to express human or animal genes, has been chosen and modified to perform the experiments (Zhang and Li, 2000; Zhang *et al*., 2004). Our experiment indicated that the vector could be used to express virus genes as well.

In summary, in the present study, two PUPs genes, ORF10 and X5, were introduced into *E. coli* and *Streptomyces* cells and induced to express recombinant SARS proteins. The results suggested that *S. lividans* could be a host strain not only for ORF10 and X5, but also potentially for other SARS genes. More studies on the heterologous expression of SARS genes will provide opportunities to study the biochemistry and structure of SARS-CoV proteins and to develop anti-SARS drugs.

Materials and Methods

Strains and plasmids

Escherichia coli TG1 was used as a host strain for subcloning. The expression vector pET32a and the bacterial strains BL21 trxB(DE3) were from Novagen (Madison, USA). *Streptomyces lividans* TK24 was a generous gift from Prof. Y. G. Wang (Institute of Medicinal Biotechnology, Peking Union Medical College and Chinese Academy of Medical Sciences). *Streptomyces* expression vector pSGLgpp was kindly provided by Prof. Y. Li (Institute of Medicinal Biotechnology, Peking Union Medical College and Chinese Academy of Medical Sciences). Cloning vector pMECA was kindly supplied by Dr. Parrott (University of Georgia, Athens, USA).

Enzymes and chemicals

DNA polymerase, DNA ligase, and restriction enzymes were purchased from Takara Shuzo Co. Ltd. (Kyoto, Japan). All other fine chemicals were of a analytical grade and commercially available.

Microorganisms and culture conditions

The transformants of *E. coli* strains were cultured in Luria Bertani (LB) medium containing 500 μg/ml carbenicillin at 37 °C. The transformants of *S. lividans* were selected on R2YE agar plates (Thompson *et al*., 1980) and induced to express on CM medium (Zhang *et al*., 2004) supplemented with thiostrepton (50 μg/ml) and neomycin (50 μg/ml) at 28 °C.

Construction of E. coli expression vector for expression of ORF10 and X5

ORF10 and X5 genes with restriction sites at the 5′-end and 3′-end (*Eco*RI and *Xho*I for ORF10 gene; *Bam*HI and *Xho*I for X5 gene) were chemically synthesized according to the genome sequence of Tor2 isolate (GenBank accession number AY274119.3). After sequence confirmation, the genes were subcloned into the vector pET32a, which was double digested either by *Eco*RI/*Xho*I or *Bam*HI/*Xho*I. The resulting plasmids, pET32a-ORF10 and pET32a-X5, were used to transform *E. coli* BL21 trxB(DE3) cells to express the ORF10 and X5 genes in the form of a His_6-Tag fusion protein.

Construction of Streptomyces expression vector for expression of ORF10 and X5

The cloning vector pMECA was digested by *Eco*RI and *Hin*dIII. The resulting fragment containing 44 sites was subcloned into the pUC18, which was also digested by *Eco*RI and *Hin*dIII, and formed the recombinant vector pUCM. *Hpa*I and *Hin*dIII were used to double digest pUCM, and the excised fragment was inserted into the *Eco*RI and *Hin*dIII sites of pSGLgpp, in which the *Eco*RI site was blunted by T4 DNA polymerase. The constructed expression vector pSGLgppM was digested by several restrictional enzymes to clarify the restriction enzyme cleavage sites. pET32a-ORF10 and pET32a-X5 were digested by *Sph*I and *Xho*I, respectively, and then inserted into the *Sph*I and *Xho*I double digested pMECA vector to generate pMECA-ORF10 and pMECA-X5. The *Msc*I and *Spe*I fragments cleaved from pMECA-ORF10 and pMECA-X5 were reintroduced into the region between *Swa*I and *Spe*I of pSGLgppM, respectively, to yield the expression vectors pSGLgppMORF10 and pSGLgppMX5. The vectors pSGLgppMORF10 and pSGLgppMX5 were transformed into *S. lividans* TK24 by protoplast transformation to express secreted ORF10 and X5 recombinant proteins with His_6-Tag in their *N*-terminus.

Expression and purification of recombinant ORF10 and X5 from E. coli

The transformed cells were grown at 37 °C in 2 ml of Luria-Bertani (LB) medium plus carbenicillin (500 μg/ml) for 5–7 h. 100 μl of cells were transferred into 8 ml fresh LB medium containing carbenicillin (500 μg/ml); they grew until OD600

reached 0.2–0.6. The 8 ml cultures of *E. coli* [pET32a-ORF10] and *E. coli* [pET32a-X5] were collected by centrifugation at 12000 × *g* for 2 min and the supernatants were discarded. The cells were washed with double distilled H_2O twice and centrifuged at 12000 × *g* for 2 min. The cell pellets were reinoculated into 50 ml LB medium plus 500 μg/ml carbenicillin and grew until OD600 reached 0. 8. Then the 50 ml cultures of *E. coli* [pET32a-ORF10] and *E. coli* [pET32a-X5] were collected by centrifugation, washed with double distilled H_2O and reinoculated into fresh 50 ml LB medium supplemented with 500 μg/ml carbenicillin successively. Induction of ORF10 and X5 protein expression was carried out immediately by addition of IPTG into the fresh 50 ml medium with a final concentration of 0.4 mM. The culture of *E. coli* [pET32a-ORF10] was induced for another 4 h at 30 °C, while the culture of *E. coli* [pET32a-X5] was induced for 2 h at 37 °C. The cells were harvested from the culture by centrifugation at 12000 × *g* for 2 min and then cell pellets were resuspended in 5 ml of 20 mM PBS buffer (20 mM sodium phosphate, 50 mM NaCl, pH 7.8). The cell suspensions were sonicated and then centrifuged again at 12000 × *g* for 30 min to remove the residue. After being filtered through 0.45 μm membranes, supernatants were applied to a 2 ml ProBond Resin (Invitrogen) column that had been pre-equilibrated by binding buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.8). The ProBond columns were washed with 8 ml of binding buffer containing additional 10 mM imidazol and then 8 ml wash buffer (20 mM sodium phosphate, 500 mM NaCl, pH 6.0). The expressed ORF10 and X5 proteins were then eluted with consecutively 8 ml of elution buffer (20 mM sodium phosphate, 500 mM NaCl, pH 6.0) in increasing imidazole concentration of 10–500 mM. The fractions containing recombinant protein were combined and examined by SDS-PAGE with silver staining. The concentrations of purified ORF10 and X5 proteins were determined using the Bradford method with BSA as standard.

Expression of pSGLgppMORF10 and pSGLgppMX5 in S. lividans TK24

The transformed cells were cultivated in CM liquid medium [3% of glucose, 1% of casein enzymatic hydrolysate, 1% of bacto yeast extract, 1% of trace element solution (v/v)] supplemented with 50 μg/ml neomycin at 28 °C for 2–3 d. The culture was inoculated again in fresh CM liquid medium plus neomycin (50 μg/ml) in the ratio 1:50, and cultured for another 2–3 d. The culture was centrifuged at 12000 × *g* for 10 min. The supernatant was harvested and subjected to ammonium sulfate fractionation for 24 h. At 80% saturation, all of desired proteins were precipitated. The ammonium sulfate precipitation was centrifuged at 12000 × *g* for 10 min. The resulting protein precipitate was dissolved in water and then dialyzed against 20 mM PBS buffer for 24 h. After these treatments, the resulting dialysate was lyophilized to powder. The powder was dissolved in a small volume of water, subjected to tricine (*N*-[tris(hydroxymethyl)methyl]glycine)-SDS-PAGE (Schägger and Jagow, 1987) and visualized by silver staining.

Immunoblot analysis of expressed ORF10 and X5 recombinant proteins from E. coli and S. lividans TK24

The expressed and concentrated samples from *E. coli* were subjected to 12% SDS-PAGE and then electroblotted onto a polyvinyliden difluoride (PVDF) microporous membrane (Millipore, USA) at 42 V (150 mA) for 2.5 h. Membranes were then blocked with 30 ml of TBS (10 mM Tris-HCl, 150 mM NaCl, pH 7.6) buffer, containing 1–5% nonfat milk powder for 3 h at 4 °C. After washing with TBS buffer for 3 times, 5 min each, the membrane was incubated with the monoclonal 6×His antibody (Merck; 1:1000 dilution with TBS) overnight at 4 °C. The membrane was washed with TTBS [10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20 (v/v), pH 7.6] buffer for 3 times, 10 min each. The membrane was then hybridized with secondary antibodies (peroxidase conjugated goat anti-rabbit IgG, diluted to 1:5000; Rockland Immunochemicals, Gilbertsville, USA) for 2 h at 4 °C. The membrane was finally washed with TTBS for 5 times, 10 min each, before being developed by ECL (enhanced chemiluminescence) detection reagents (Amersham, UK). The immunoblot analysis of *S. lividans* [pSGLgppMORF10] and *S. lividans* [pSGLgppMX5] was carried out according to the protocol described above except for 16% tricine-SDS-PAGE separation.

Acknowledgements

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