

Improvement in Hyperexpression with a Combination of Truncated Scmp Promoter and *Streptomyces Lividans*

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Research Article

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Abstract

We have previously reported a powerful promoter from the *Streptomyces cinnamoneus* TH-2 strain named 'scmp' and created an expression vector of pTONA5a based on pIJ702 for expression using *S. lividans* (Hatanaka et al. (2008) Protein Expr Purif 62: 244-248). The full-length scmp promoter sequence consists of 424 bp upstream of a metalloendoprotease gene in the *S. cinnamoneus* TH-2 genome. The promoter works in the presence of inorganic phosphate and glucose. In this study, we present the essential region of the scmp promoter (promoter C), which lacks 358 bp of the 5' region of the full-length promoter. Promoter C was very short and contained only 63 bp. Moreover, there was no restriction on the medium component by combining the promoter and the *S. lividans* 1326 strain. Using promoter C and *S. lividans*, we succeeded in the extracellular production of the *Streptomyces* enzymes leucine aminopeptidase, ferulic acid esterase, and transglutaminase, which possessed signal peptides for secretion via the second pathway, at high levels.

Introduction

Actinomycetes are gram-positive bacteria that are useful tools for the biological production of several secondary metabolites, including antibiotics. Actinomycetes are also known to produce various enzymes, such as proteases (Moriyama et al. 1969; Hatanaka et al. 2005; Uesugi et al. 2011), cellulase, and hemicellulase (Kumar et al. 2020). In recent years, genome analysis technology has been developed, and there are many opportunities to select and express these useful proteins from genomic information. However, the production of recombinant proteins from actinomycetes using *Escherichia coli* as a host sometimes results in the failure to form inclusion bodies. One of the reasons for this is GC-rich (ca. 70%) in their genomes. Actinomycetes have a good ability to secrete proteins into the culture medium because they do not have an outer membrane. Thus, Actinomycetes are a suitable host for the production of secreted proteins from actinomycetes (Anné et al. 1993; Anné et al. 2014). We have previously reported a metalloendoprotease (SCMP) from *Streptomyces cinnamoneus* (previously named *S. septatus*) TH-2 strain (2). SCMP was highly expressed and secreted using a medium that was rich in glucose and inorganic phosphate, and the yield was 0.3 g /L (2). To utilize this hyperproduction, we cloned the upstream region of the scmp gene as a promoter and created an expression vector of pTONA5a, which was based on pIJ702, for expression in *S. lividans*. The vector harbors ori and rep for streptomycetes and thiostrepton-resistant genes (Hatanaka et al. 2008). By combining this vector with the *S. lividans* 1326 strain, we succeeded in the extracellular production of several *Streptomyces* enzymes (Hatanaka et al. 2008; Uraji et al. 2018; Wan et al. 2019). In this study, we attempted to identify an essential region of the scmp promoter to enhance its expression. We confirmed that the truncated promoter showed higher protein expression than the full-length promoter. Furthermore, we succeeded in protein expression by using the truncated promoter in medium that was not rich in glucose and phosphate and in commercial tryptic soy broth (TSB).

Materials And Methods

Culture conditions and transformation of *S. lividans*

Escherichia coli JM109 was used as the host cell for vector construction. *S. cinnamoneus* TH-2 was used as the source of the promoter. We used a secreted leucine aminopeptidase (LAP) from *S. griseus* as a reporter gene and the *S. lividans* 1326 strain as an expression host. The PG medium contained 2.0% glucose, 0.8% K₂HPO₄, 0.05% MgSO₄·7H₂O, 0.5% polypeptone, and 0.5% yeast extract. In the 0.1% Pglu medium, the K₂HPO₄ concentration was changed to 0.1% in PG medium, and glucose was replaced with glycerol in PG medium in the Pgly medium. Tryptic soy broth was purchased from Japan Becton, Dickinson, and Company (Tokyo, Japan). Protoplast preparation and transformation were performed according to the method described by Hopwood et al. (Hopwood et al. 1985). The constructed plasmids were transformed into protoplasts of the *S. lividans* 1326 strain, and transformants were selected by thiostrepton resistance. The obtained transformants were inoculated in LB medium containing 1.0% trypton, 0.5% yeast extract, and 1.0% NaCl and cultured until the stationary phase. The cells were then collected and washed with 0.9% NaCl, and the cells were inoculated into the medium.

Construction of expression plasmids containing the truncated scmp promoters

The pTONA5a plasmid with the leucine aminopeptidase gene (pTONA5a-*lap*) was constructed as described previously (Hatanaka et al. 2008). PCR was performed to obtain genes with truncated promoters. To amplify the fragments of A, C, and D, the forward primers with *Asel* site (ATTAAT) were constructed at the -277 bp, -66 bp, and -46 bp sites of scmp, and the reverse primers were constructed at the end of the inserted genes with *HindIII*. The fragment of A was amplified with the following primers: 5'-ATTAATCGGCAACCAGCCGCGGACGG-3' (the underlying indicates an *Asel* site) and 5'-AAGCTTAGGTGGGCGGTTGCGCCGGTG-3' (the underling indicates a *HindIII* site). The fragment of C was amplified with the following primers: 5'-ATTAATCACCACACCTGCACCACAGG-3' (the underling indicates an *Asel* site) and 5'-AAGCTTAGGTGGGCGGTTGCGCCGGTG-3' (the underling indicates a *HindIII* site). The fragment of D was amplified with the following primers: 5'-ATTAATGCGGTCGTACAACCGGCTGC-3' (the underlying indicates an *Asel* site) and 5'-AAGCTTAGGTGGGCGGTTGCGCCGGTG-3' (the underlying indicates a *HindIII* site). The fragment of B was amplified with the following primers: 5'-ATTAATCGGCAACCAGCCGCGGACGG-3' (the underlying indicates an *Asel* site) and 5'-CATATGTGTCTCCTATGAGGGGGTTGG-3' (the underlying indicates an *NdeI* site). The pTONA5a-*lap* plasmid was used as the template. The amplified fragments were cloned in pCR-Blunt II-TOPO (Thermo Fisher Scientific, Tokyo, Japan), and the inserted sequences were confirmed. Clones A, C, and D were digested with *Asel* and *HindIII*, and the fragments were ligated into the *Asel-HindIII* gaps of pTONA5. Clone B was digested with *Asel* and *NdeI*, and the fragment was ligated into the *Asel-NdeI* gap of pTONA5.

Construction of expression plasmids for ferulic acid esterase (FAE) and transglutaminase (TG)

The FAE gene from *S. cinnamoneus* NBRC12852 was cloned and amplified with the following primers: 5'-CATATGATCATGATCAGCTCGATGAGGAAGACATC-3' (the underling indicates an *NdeI* site) and 5'-

AAGCTTCACTTCATGAAGGTGACCTCGGGGT-3' (the underlying indicates a *Hind*III site). The PCR product was cloned into pCR-Blunt II-TOPO, and cloning was confirmed by sequencing. The resulting FAE gene was cloned into the gap of *Nde*I-*Hind*III of pTONA containing promoter C. TG construction from *S. mobaraensis* NBRC13864 has already been reported (Tokai et al. 2020).

Assay of LAP activity

LAP activity was determined using Leu-*p*NA (Sigma-Aldrich Japan, Tokyo, Japan) as a substrate. The sample (0.1 mL) and 0.1 mL of the substrate (32 mM) were added to 0.8 mL of a 100 mM Tris-HCl (pH 8.0) and 1 mM CaCl₂ solution. The amount of *p*-nitroaniline released was determined by measuring the absorbance at 405 nm using a spectrophotometer (UV2800, Hitachi Ltd.). The initial velocity was determined from the linear region of the optical density profile ($\epsilon_{405\text{nm}} = 10,600 \text{ M}^{-1} \text{ cm}^{-1}$) (Arima et al. 2006).

DNA accession numbers.

The accession numbers assigned to the sequences in the DNA Data Bank of Japan (DDBJ) database are as follows: SCMP, AB189036; LAP, AB125217; FAE, AB921569; and TG, AY615816.

Results

LAP activity and expression by full-length *scmp* promoter in PG medium

We have reported that SCMP, which is a metalloendoprotease from *S. cinnamomeus* TH-2 strain controlled under the *scmp* promoter, required 2% glucose and 0.8% phosphate to be secreted into the culture as an active form (Hatanaka et al. 2005). Based on our findings, we examined four media for secreted LAP expression using the transformant harboring the pTONA5a-*lap*. After 4 days of culture at 30°C, the LAP activity of the supernatant was analyzed, and protein expression was detected by SDS-PAGE. The supernatant of the culture in PG medium showed LAP activity and protein expression (Fig. 1). In contrast, low phosphate medium (0.1% K₂HPO₄ in PG), Pgly medium (2% glycerol instead of glucose in PG), and TSB showed lower LAP activity and expression than PG medium (Fig. 1).

LAP activity and expression by truncated *scmp* promoters

To identify the essential region of the *scmp* promoter, we constructed expression vectors combined with truncated promoters and the *lap* gene (Fig. 2). pTONA5a-*lap*, which included the full-length *scmp* promoter (FL), and expression vectors with four types of truncated *scmp* promoters (A, B, C, and D) were transformed into the *S. lividans* 1326 strain, and the obtained transformants were cultivated in PG medium. As shown in Fig. 3A, the A promoter, which lacks 148 bp of the 5' region of the *scmp* promoter, showed three-fold higher LAP activity than FL. In addition, promoter C, which lacked 358 bp of the 5' region of the *scmp* promoter, showed four-fold higher activity than that of FL. Promoter D, which harbors 46 bp of the 3' region in the *scmp* promoter, also showed two-fold higher LAP activity than FL. Promoter B

lacked 206 bp of the 3' region of the *scmp* promoter. The secreted LAP proteins were detected except for promoter B, and the content of LAP proteins increased in order from A and C to D compared with FL (Fig. 3B).

Truncated promoter C required no glucose and phosphate for *lap* expression

From the results of the truncated promoter in PG medium (Fig. 3), we expected that promoter C would require no glucose and phosphate and would be easy to use. Thus, we compared the secreted LAP activities using FL and truncated promoters A and C under the conditions of low phosphate concentration (0.1% phosphate), the exchanged carbon source of glucose with glycerol (Pgly), and TSB. The LAP activity using the FL promoter was the highest in the PG medium compared to the other three cultivation conditions (Fig. 4A). The LAP activity of promoter A was remarkably increased in the low-phosphate medium. This value was three times higher than that of FL. Furthermore, the LAP activity of promoter C was higher than that of FL and A under all conditions (Fig. 4A). Moreover, the protein expression levels under the four conditions by the C promoter were also increased compared with those under the FL promoter under PG (Fig. 4B). In particular, the LAP protein was strongly secreted by the combination of the C promoter and Pgly medium (Fig. 4B). In addition, the levels of other secreted proteins were reduced by using promoters C and TSB, although the content of secreted LAP protein was slightly lower than that in PG or Pgly (Fig. 4B).

Expression of other actinomycetes proteins by promoter C

We attempted to produce other proteins with promoter C. FAE is an enzyme that releases ferulic acid from plant biomass (Uraji et al. 2013). When the *Streptomyces* FAE gene (Uraji et al. 2018) was cloned downstream of promoter C and expressed in PG and TSB media, enzyme production was confirmed in both (Fig. 5). To utilize the full-length *scmp* promoter, it was necessary to use PG medium; however, when cultured in PG medium, the expression of proteins other than the target protein was induced, and multiple proteins were often expressed simultaneously. It was necessary to purify the culture supernatant and extract only the target protein. In the case of FAE expressed by promoter C, the expression of contaminating proteins was suppressed in both PG and TSB medium, and the expression of other proteins was particularly reduced in TSB medium (Fig. 5). TG mainly connects proteins (glutamine side chain) to proteins (lysine side chains) by covalent bonds; by utilizing this property, it can be used in the processing of marine products, modification of meat, and processing of dairy products (Tokai et al. 2020). Similar to the FAE, TG was expressed by promoter C in PG or other media. The enzyme is released into the culture's supernatant due to its secretory signal; there is a prosequence downstream of the secretory signal, which is cleaved by another enzyme (peptidase) secreted after TG. By removing the prosequence, the enzyme is converted into an active mature form of TG. When TG was expressed by promoter C in PG medium, a mature protein mixed with the prosequence form was expressed (Fig. 5). On the other hand, when TG was expressed in Pgly medium, conversion to the mature form of TG was completed. These results indicate that using promoter C facilitates the secretion of several types of proteins because the promoter does not restrict the carbon source.

Discussion

In this study, we identified the essential region (63 bp, promoter C) of the *scmp* promoter. By using pTONA containing promoter C and *S. lividans*, we succeeded in the extracellular expression of three *Streptomyces* enzymes, LAP, FAE, and TG, which possessed signal peptides for secretion via the second pathway.

The full-length *scmp* promoter consists of 424 bp upstream sequences of the SCMP gene in the *S. cinamomeus* TH-2 genome. To date, we have not determined the sequence similarity to the genome information of other organisms or actinomycetes. Because the promoter is powerful, it is used to commercially produce two *Streptomyces* enzymes. However, there is a restriction on the medium component of enzyme expression using the promoter. This study aimed to remove the restriction on the use of the *scmp* promoter by identifying the essential region. We presented the essential region of the *scmp* promoter (promoter C), which lacks 361 bp of the 5' region of the full-length promoter. Promoter C was very short and consisted only of 63 bp. Moreover, there was no restriction of the medium component by the combination of the promoter and the *S. livians* 1326 strain. This system will be useful for producing and characterizing streptomycete proteins.

In *Streptomyces*, protein expression in secondary metabolism and morphological changes are controlled by several transcriptional factors (Romero-Rodriguez et al. 2015). The *scmp* gene is homologous to *sgmA* genes that encode a metalloendoprotease (SGMP) involved in morphological changes in *Streptomyces griseus*, whose expression is controlled by AdpA (Ohnishi et al. 2005). AdpA is an important transcriptional regulator that binds to DNA, including the motif -TGGCSNGWWY- (Ohnishi et al. 2005). Kato *et al.* reported the loss of AdpA binding ability in the *sgmA* promoter region led to the loss of SGMP protein expression (Kato et al. 2002). First, we estimated that the *scmp* promoter also controlled the AdpA protein or a similar regulation system of AdpA. We found four AdpA-binding motif-like sequences, -CTGCCCGAAC-, -AGGTGCGTTT-, -ATGCCCGAAC-, and -CGTCCGGTAT-, in approximately 250 bp of the *scmp* promoter. However, the truncated *scmp* promoters C and D, which lacked more than 300 bp in the 5' region of the full-length *scmp* promoter, were active (Fig. 3). These results suggest that the working mechanism of the *scmp* promoter is different to that of SGMP by AdpA.

The feature of the *scmp* promoter is that a large amount of protein is secreted extracellularly. By using PG medium, the content of secreted proteins reached the maximum at least 4 days of culture, this cultivation time resulting in the secretion of many proteins, which might be produced by secondary metabolism (Fig. 3B). Using promoters C and TSB, the content of secreted proteins was more intense in the target protein of LAP than in PG medium (Fig. 4B). It was indicated that promoter C might be regulated differently to be compared with the full-length promoter. Further research is needed to reveal how the promoter functions in streptomycetes.

Because the medium component was not restricted, extracellular production of recombinant proteins using the truncated promoter C was more convenient than the full-length promoter. In Fig. 5, we represented FAE and TG expression using promoter C in PG and other media. By using promoter C and the PG medium, this conversion was insufficient, and zymogen and active TG coexisted in the culture

(Fig. 5(B)). The conversion was complete using promoter C and Pgly media. From these results, it can be inferred that the expression of the host peptidase was active in the Pgly medium, and conversion to the mature form was performed more rapidly than that in the TSB and PG media. This result suggests that it was difficult to select various media with the conventional full-length scmp promoter, but by using the 63-base promoter, the medium's components were no longer restricted and the enzyme to be expressed was expanded.

Promoter C is very short and convenient, exerting no restriction on the medium component. A similar short promoter of *kasOp** (97 bp) from *S. coelicolor* has been reported. The promoter was engineered by random mutagenesis between sites -10 and -35, and screened using the Lux reporter. The *kasOp** exhibited showed higher activity at the transcription and protein levels than those of known strong promoters of *ermEp** and SF14p (Wang et al. 2013). Further work is in progress to compare the activities of promoter C and the *kasOp** promoter in *Streptomyces* protein expression.

Conclusions

In this study, we present the essential region of the scmp promoter (promoter C), which lacks 361 bp of the 5' region of the full-length promoter. Promoter C was very short and only consisted of 63 bp. Moreover, there was no restriction on the medium component by the combination of the promoter and *the S. livians* 1326 strain. This system will be a useful tool for producing and characterizing streptomycetes proteins.

Abbreviations

SCMP

metalloendoprotease from *Streptomyces cinnamoneus*

TSB

tryptic soy broth

LAP

leucine aminopeptidase

FAE

ferulic acid esterase

TG

transglutaminase.

Declarations

Acknowledgement

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Authors' contributions

MU and TH constructed the plasmids. MU and LY conducted expression experiments. MU and TH wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

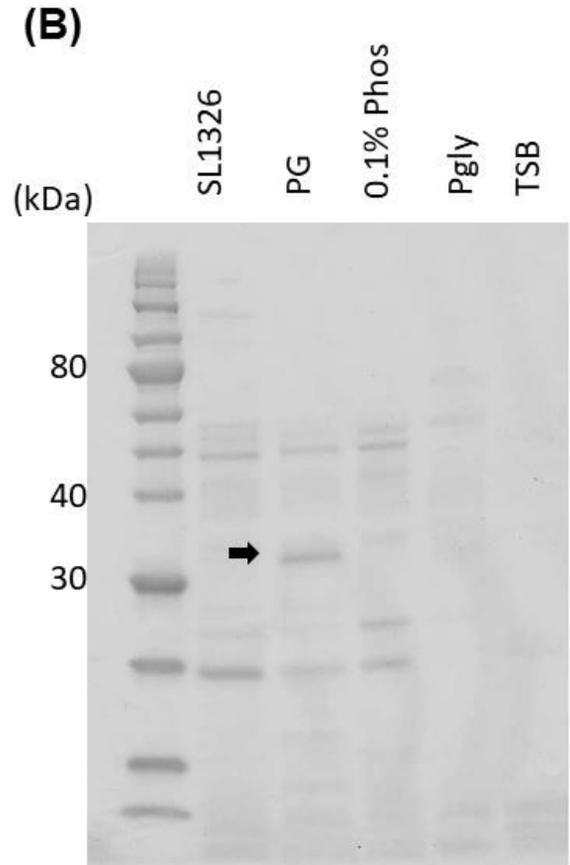
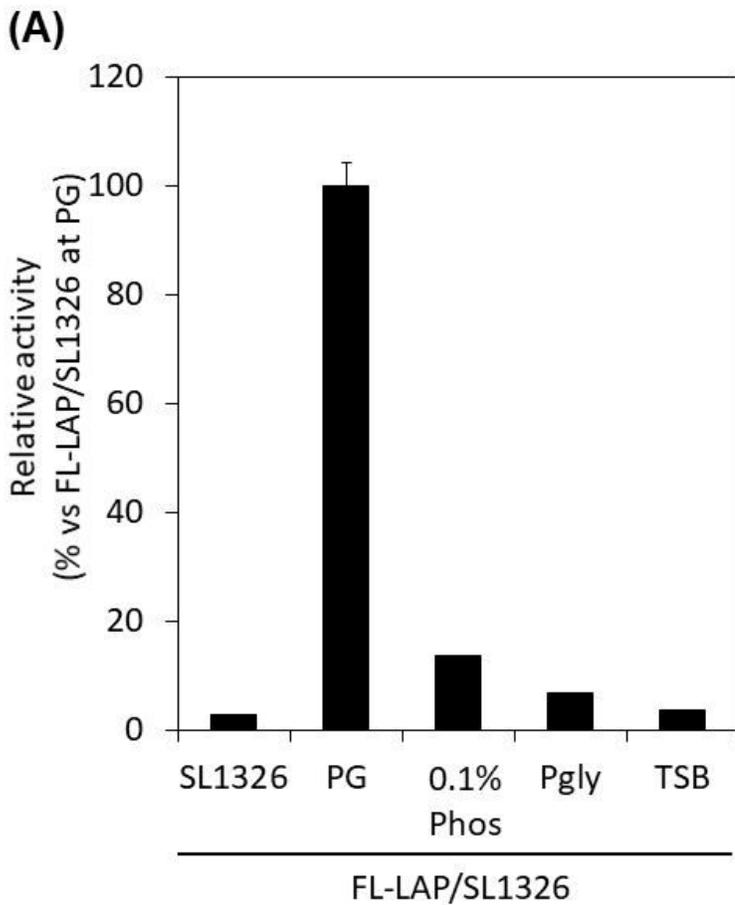


Fig. 1

Figure 1

LAP activities and expression by the full-length scmp promoter.

(A) LAP activities by the promoter cultured in different media. (B) SDS-PAGE analysis of LAP expression by the promoter cultured in different media. The gel used was 10% polyacrylamide and was stained with Coomassie Brilliant Blue. All lanes were loaded with 10 μ L of the culture supernatant. Arrows indicate the locations of the LAP proteins.

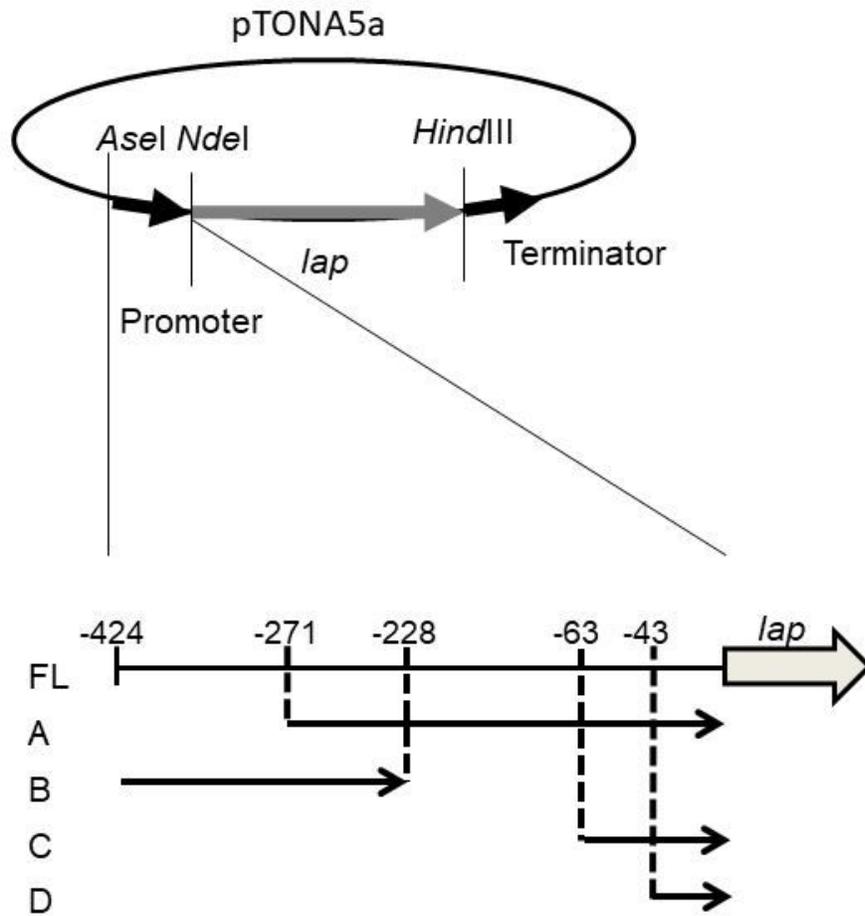


Fig. 2

Figure 2

Schematic representation of pTONA5a plasmid construction, including truncated SCMP promoters and the *lap* gene; promoter; scmp promoter, terminator; kibilysin terminator, *lap*; leucin aminopeptidase, FL; full length scmp promoter.

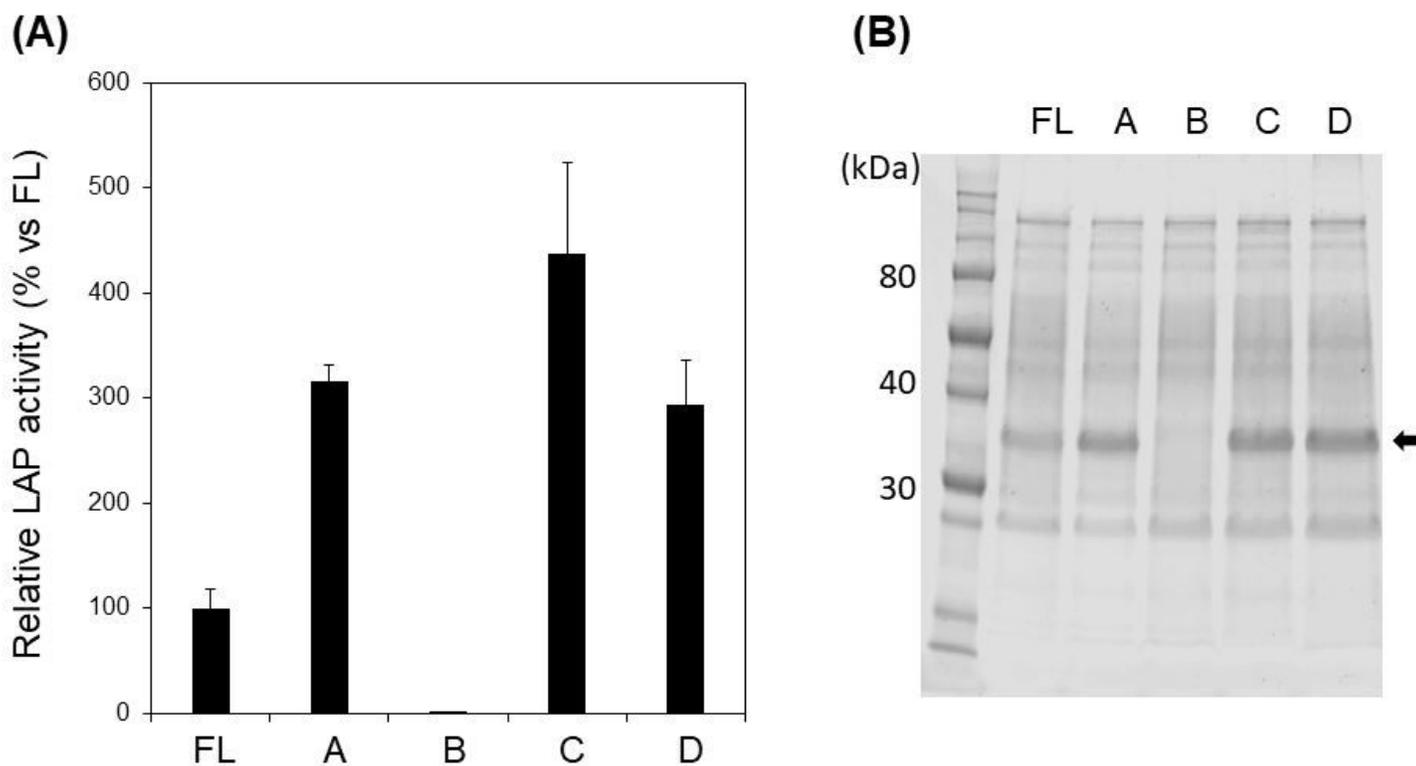


Fig. 3

Figure 3

LAP activities and expression using SCMP promoters and PG medium.

(A) Comparison of LAP activities in culture supernatants of the full length (FL) and truncated (A-D) scmp promoters. The activity was normalized to the FL values. The average value from three independent experiments is shown. Error bars represent the SE. (B) SDS- PAGE analysis of culture supernatants by FL and A-D truncated scmp promoters. The gel used was 10% polyacrylamide and was stained by Coomassie Brilliant Blue. All lanes were loaded with 10 μ L of culture supernatant. Arrows indicate the locations of the LAP proteins.

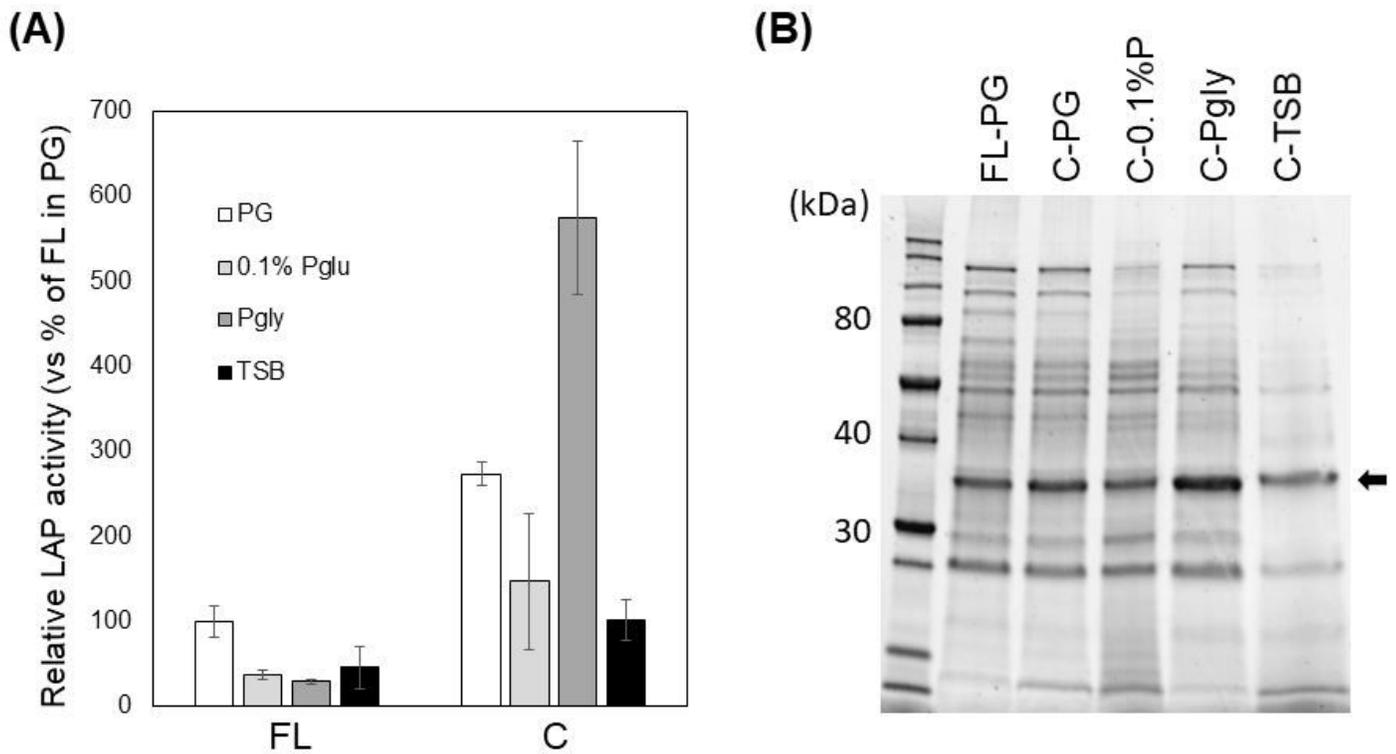


Fig. 4

Figure 4

LAP activities and expression of SCMP promoters in different media.

(A) Comparison of LAP activities in culture supernatants of full length (FL) and truncated promoter C in different media. PG; PG medium (0.8% KH_2PO_4 and 2% glucose), 0.1% Pglu; 0.1% KH_2PO_4 and 2% glucose, Pgly; 0.8% KH_2PO_4 , and 2% glycerol. The activity was normalized to that of FL in PG medium values. The average value from three independent experiments is shown. Error bars represent the SE. (B) SDS-PAGE analysis of culture supernatants with FL in PG medium and C truncated scmp promoters in different media. The gel used was 10% polyacrylamide and was stained by Coomassie Brilliant Blue. All lanes were loaded with 10 μL of culture supernatant.

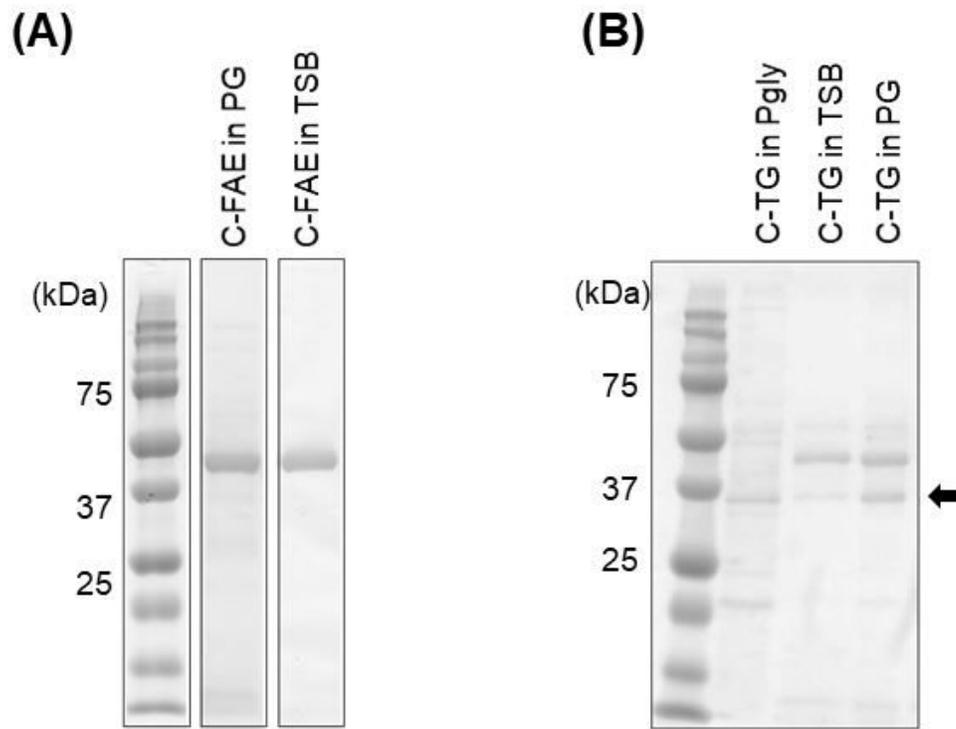


Fig. 5

Figure 5

FAE and TG expression by promoter C in different media

(A) FAE expression by promoter C in PG and TSB media. (B) TG expression by promoter C in PG, TSB, and Pgly media. The gel used was 10% polyacrylamide and was stained by Coomassie Brilliant Blue. All lanes were loaded with 10 μ L of culture supernatant. An arrow indicates the mature form of TG protein.

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