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Heterologous Expression of Bovine Lactoferricin in *Pichia methanolica*

HaiKuan Wang[1,2]*, XinHuai Zhao[1], and FuPing Lu[2]

[1]*Key Lab of Diary Science, Ministry of Education, Harbin, PO 150030, P. R. China; fax: (864515) 519-0340; E-mail: hkwang@tust.edu.cn*
[2]*Tianjin Key Laboratory of Industrial Microbiology, College of Biotechnology, Tianjin University of Science and Technology, Tianjin, PO 300457, P. R. China; fax: (86226) 060-2298; E-mail: haikuanwangcn@yahoo.com.cn*

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Abstract—According to the bias of codon utilization of *Pichia methanolica*, a fragment encoding bovine lactoferricin has been cloned and expressed in the *P. methanolica* under the control of the alcohol oxidase promoter, which was followed by the *Saccharomyces cerevisiae* α-factor signal peptide. The α-factor signal peptide efficiently directed the secretion of bovine lactoferricin from the recombinant yeast cell. The recombinant bovine lactoferricin appears to be successfully expressed, as it displays antibacterial activity (antibacterial assay). Moreover, the identity of the recombinant product was estimated by Tricine-SDS-PAGE.

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Bovine lactoferricin (LfcinB) is a 25-residue peptide that is excised through pepsin cleavage in the stomach from the intact 80-kD bovine milk protein lactoferrin. Bellamy et al. [1] reported that a 25-residue peptide derived from a highly cationic region near the N-terminus of bovine lactoferrin had a markedly increased antimicrobial potency compared to the parent protein. This basic peptide contains a single disulfide cross-link and is considerably more active as an antimicrobial peptide than the intact protein. LfcinB was active against both Gram-positive (*Bacillus*, *Listeria*, and *Streptococcus*) and Gram-negative (*Escherichia coli*, *Klebsiella*, *Salmonella*, *Proteus*, and *Pseudomonas*) microorganisms *in vitro*. A pathogenic intestinal bacterium, *E. coli* 0111, was additionally found to be susceptible [2-4]. In addition, the peptide has shown antiviral [5, 6] and antitumor [7, 8] activities. Moreover, the peptide is capable of stimulating the adaptive immune response and has antiinflammatory properties [9, 10]. One of the major impediments to the progress of research and application of LfcinB is the cost of production from either bovine lactoferrin digestion or chemical synthesis.

Xingjun Feng et al. [11] reported that a synthetic fragment encoding LfcinB and have successfully expressed LfcinB in *E. coli*, with the antibacterial activity of recombinant LfcinB proved in this study, but it was in *E. coli* as a fusion protein and the purification process was complicated and inefficient. There is no report on the expression of LfcinB in the *Pichia methanolica* as we know so far. *Pichia methanolica* is a relatively new system and a homothallic organism that offers many of the advantages of eukaryotic expression systems such as protein processing and protein folding, while being as easy to manipulate as *E. coli* or *Saccharomyces cerevisiae*. It is faster, easier, and less expensive to use than other eukaryotic expression system such as baculovirus or mammalian tissue culture and generally gives higher expression levels. As a yeast, it shares the advantages of molecular and genetic manipulations with *Saccharomyces*, and it has the added advantage of 10- to 100-fold higher heterologous protein expression levels. These features make *P. methanolica* very useful as a protein expression system.

The present study addresses the production of a recombinant LfcinB in *P. methanolica*.

Abbreviations: AUG1) alcohol oxidase; LfcinB) bovine lactoferricin.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Strains, media, and culture condition. *Escherichia coli* DH5α was used in all DNA manipulations and grown in Luria–Bertani medium (tryptone, 10 g/liter; NaCl, 10 g/liter; yeast extract, 5 g/liter; agar, 16 g/liter).

The host strain used for heterologous expression was *P. methanolica* pMAD16. *Pichia methanolica* was grown in YPAD (yeast extract, 10 g/liter; peptone, 20 g/liter; dextrose, 20 g/liter; adenine, 0.1 g/liter; agar, 20 g/liter).

The vector pMETα A (Invitrogen, USA) was used for expression in *P. methanolica*. Expression of the insert in the vector is controlled by the methanol-inducible AUG1 (alcohol oxidase) promoter. The pMETα A possesses the α-secretion factor from *S. cerevisiae*.

Gene cloning and construction of vectors. The lactoferricin gene was obtained by a recursive PCR (rPCR) strategy using two lapping oligonucleotides that represented the partial sequence of the sense and antisense strands of the DNA sequence according to the bias for preferred codons of *P. methanolica*. Briefly, both of the oligonucleotides had 53 nucleotides, with 13 bp overlapping at the 40 end of each. The PCR was performed using the primers LfcinB-F (5′-CCGGAATTCTTCAAGTGTAGAAGATGGCAATGGAGAATGAAGAAGTTGGGTGC-3′) and LfcinB-R (5′-CCGGGATCCGAAAGCTCTTCTAACACAACTAATAGATGGAGCACCCAACTTCT-3′). Both the forward and reverse primers contained *Eco*RI and *Bam*HI restriction sites. PCR conditions employed: 94°C for 1 min (once), 94°C for 30 sec, 51°C for 30 sec, 72°C for 2 min (30 cycles), 72°C for 5 min (once). The purified PCR products were cloned into the pUC19 vector, subsequently excised using *Eco*RI and *Bam*HI, followed by purification from an agarose gel and insertion into pMETα A, resulting in the vector pMETα A–LfcinB. After transformation into *E. coli* and isolation of plasmid DNA, the presence of the inserts was determined both by PCR and by restriction enzyme digestion followed by agarose-gel electrophoresis. Sequence analysis was performed on pMETα A–LfcinB to ensure the integrity of the constructs.

Yeast transformation and identification. Yeast transformation was performed as described by Choi et al. [12]. pMETα A–LfcinB plasmid DNA was digested with *Asc*I prior to transformation into electrocompetent cells of *P. methanolica*. The vector pMETα A without inserts was simultaneously transformed into *P. methanolica* and used as controls. Transformant colonies were picked from minimal glucose (MD) plates and transferred to plates with either MD or minimal methanol (MM) as the carbon source, for examination of methanol utilization. Some integrants were checked for the presence of the expression cassette in their genome by PCR using primer specific for the lactoferricin gene.

Induction and expression of the foreign gene. *Pichia methanolica* Mut^+ and Mut^s strains were cultured on 25 or 100 ml of buffered dextrose-complex medium (yeast extract, 10 g/liter; peptone, 20 g/liter; 100 mM potassium phosphate buffer, pH 6.0; YNB, 13.4 g/liter; biotin, 400 μg/liter; dextrose, 20 g/liter) in 250-ml or 1-liter flasks for approximately 24 h. For putative Mut^s strains, 50 ml of culture was placed in 100 ml centrifuge tubes, centrifuged at 3000*g* for 5 min, and the cells were resuspended in 15 ml of buffered methanol-complex medium (yeast extract, 10 g/liter; peptone, 20 g/liter; 100 mM potassium phosphate buffer, pH 6.0; YNB, 13.4 g/liter; biotin, 400 μg/liter; methanol, 5 ml/liter), and transferred to 250-ml flasks. For putative Mut^+ strains, 3.75 ml was centrifuged and resuspended in 15 ml of the same medium. Mut^+ and Mut^s strains carrying only the integrated pMETα A vector with no insert were used as controls. Flasks were incubated at 30°C, and 0.5% (v/v) methanol was added daily. Every 24 h, samples were withdrawn to carry out an activity assay. Several parameters that affected the level of protein expression were assayed. Antibacterial activity was assayed by measuring zones of growth inhibition in thin agar plates with *E. coli* K12D31 as described by Hultmark et al. [13]. One hundred microliters of each condensed expression supernatant was applied in the wells. The liquid culture was centrifuged at 10,000*g* for 10 min, and 100 ml of the cell-free supernatant from cultures was freeze-dried and dissolved in 5 ml sterile water. 16.5% Tricine-SDS-PAGE was performed [14], followed by staining with Coomassie blue. Protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad, Sweden), based on the Bradford dye-binding procedure, with bovine serum albumin (BSA) as a standard.

Purification of recombinant LfcinB. The rapid procedure described by manufacturer's instructions (Qiagen, USA) for the purification of LfcinB from the cell-free supernatant was essentially employed in this study. One milliliter of the 50% Ni-NTA slurry was added to 4 ml of 20× concentrated supernatant from cultures and the mixture was gently shaken for 15-60 min at room temperature. The supernatant–resin mixture was carefully loaded into an empty column with the bottom cap still attached. The column was washed twice with 4 ml wash buffer and centrifuged for 10 sec at 1000*g* between washes, and then the supernatant was carefully removed. The protein was eluted 3 times with 0.5 ml elution buffer and centrifuged for 10 sec at 1000*g* between each elution step. The flow-through was collected for antibacterial activity and Tricine-SDS-PAGE analysis.

RESULTS AND DISCUSSION

Construction of vectors for the expression of lactoferricin in *P. methanolica*. The schematic of construction of the recombinant pMETα A–LfcinB plasmid is shown in Fig. 1. Plasmid pMETα A–LfcinB is composed of the

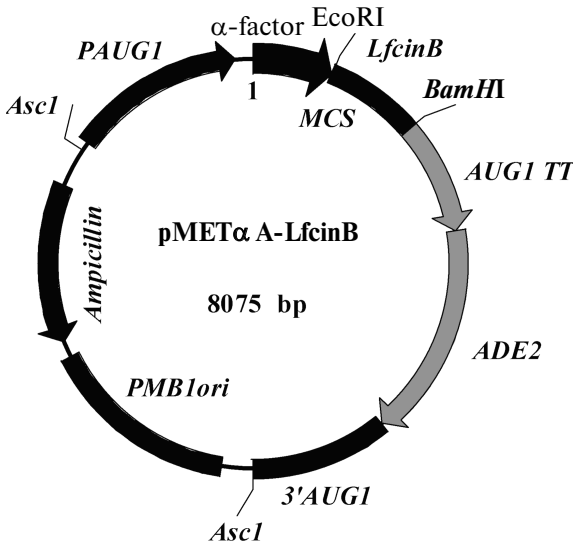

Fig. 1. Plasmid constructed for the expression of LfcinB in *P. methanolica*. The LfcinB gene was cloned into pMETα A downstream of the native *S. cerevisiae* α-factor secretion signal. *PAUG1* and *AUG1 TT*, promoter and transcription termination region of the alcohol oxidase gene from *P. methanolica*, respectively; *ADE2*, *S. cerevisiae* wild-type gene coding for phosphoribosyl-aminoimidazole carboxylase and used to complement *P. methanolica ade*2-11 strains; *PMB1ori*, *E. coli* replication origin.

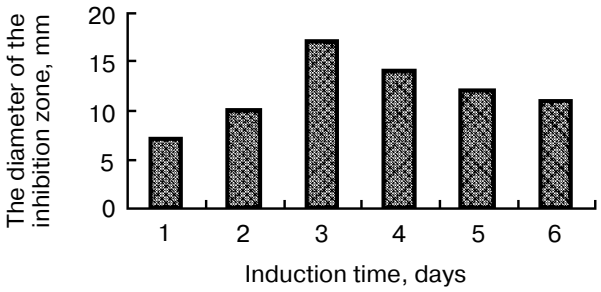

Fig. 2. Time course for the expression of LfcinB in *P. methanolica*. Induction conditions: 24°C, pH 6.0, 0.5% methanol induction for 1-6 days.

inducible promoter AUG1, *S. cerevisiae* α-factor secretion signal, and a termination transcription signal. The LfcinB gene was achieved through a recursive PCR (rPCR) strategy. The PCR product of the LfcinB gene was 75 bp. After transformation, the transformants were screened through the restriction enzymes, *Eco*RI and *Bam*HI, and were sequenced. The results of restriction analysis and DNA sequencing showed that the LfcinB gene was inserted correctly into the expression vector. Transformation with an *Asc*I-linearized version of pMETα A–LfcinB favored its insertion into the yeast genome by homologous recombination. From 50 to 100 transformants were obtained after selection of recombinants on an MD plate. The use of genomic PCR analysis ensured the isolation of pure clones of transformants bearing the genomically integrated copies of pMETα A–LfcinB plasmids. PCR amplification results showed that the transformants produced a 75-bp product.

Expression of lactoferricin in *P. methanolica*. Sixty clones were used for expression in a shake flask. Secretion of the recombinant LfcinB into the supernatant was monitored by an antibacterial activity assay. The clone with the best activity was chosen for upscaled protein production. This work on LfcinB expression in *P. methanolica* has identified temperature, pH, and oxygen as factors that affect lactoferricin production. The production of LfcinB was favored by lower cultivation temperature. Cultures were first grown in buffered dextrose-complex medium at 30°C and then transferred to buffered methanol-complex medium at 24°C with increasing the diameter of the inhibition zone by 150%. The pH of unbuffered growth medium decreased from 3- to approximately 2-fold activity. The use of buffered methanol-complex medium maintained the pH 6.0 for expression of LfcinB and has been shown to be beneficial for LfcinB activity. In addition, all cultures were grown in baffled flasks with shaking to ensure an adequate supply of oxygen, which is necessary for the oxidation of methanol to formaldehyde by alcohol oxidase. The relationship between induction time and the diameter of the inhibition zone is indicated in Fig. 2.

Under the best expression conditions, approximately 120 mg LfcinB was secreted into 1 liter of medium. Recombinant LfcinB was purified by means of affinity chromatography. The yield of purified recombinant LfcinB was about 90 mg/liter. The purified peptide pro-

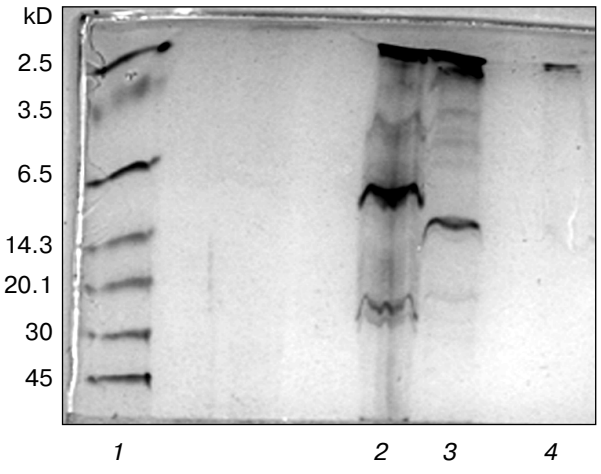

Fig. 3. Tricine-SDS-PAGE analysis of proteins secreted by *P. methanolica*. Lanes: *1*) low molecular weight markers; *2*, *3*) 20× concentrated supernatant from cultures; *4*) purified LfcinB expressed in *P. methanolica*. Conditions of 16.5% Tricine-SDS-PAGE electrophoresis: 10 mA constant current per 0.75 mm gel was applied until the dye front reached the top of the separating gel. Then current was increased to 20 mA per gel and maintained at this level until the bottom of the gel was reached.

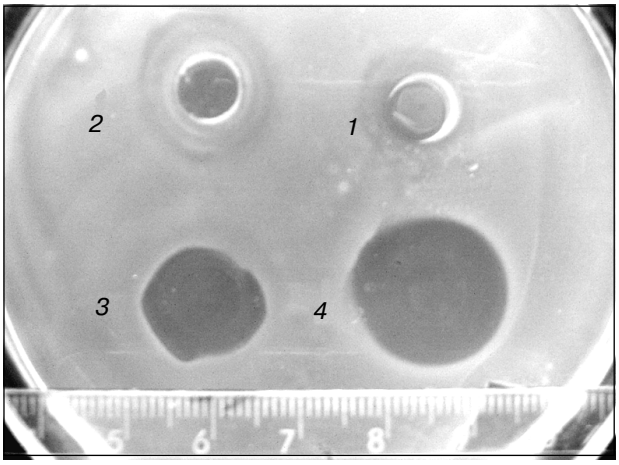

Fig. 4. Antibacterial assay of purified LfcinB: *1*) control with no LfcinB; *2-4*) purified LfcinB.

duced a single homogeneous band from Coomassie blue stained Tricine-SDS-PAGE (Fig. 3). As shown in Fig. 4, the recombinant lactoferricin exhibited antibacterial activity.

The *P. methanolica* system employing the AUG1 methanol promoter is capable of producing yield of 2500 mg secreted protein per liter. Although the lactoferricin expression level is 120 mg lactoferricin per liter with the pMETα A expression vectors used, lactoferricin expression could be improved further by using the pMAD16 strain in a fermenter and increasing in the copy number of the gene. In recent years, more attention is focused on the isolation from natural sources of LfcinB and the study of its structure–function relationship. It is anticipated that LfcinB or its derivatives will find more diverse applications in the future. Successful expression of LfcinB in *P. methanolica* may provide a valuable method for producing large quantities of active antimicrobial peptide. At the same time, the successful expression of functionally active LfcinB provides us at least with a biochemical tool to get various LfcinB-derived peptides that will certainly facilitate the study of its structure–function relationship.

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REFERENCES

1. Bellamy, W., Takase, M., Wakabayashi, H., Kawase, K., and Tomita, M. (1992) *J. Appl. Bacteriol.*, **73**, 472-479.
2. Yamauchi, K., Tomita, M., Giehl, T. J., and Ellison, R. T. (1993) *Infect. Immun.*, **61**, 719-728.
3. Shin, K., Yamauchi, K., Teraguchi, S., Hayasawa, H., Tomita, M., and Otsuka, Y. (1998) *Lett. Appl. Microbiol.*, **26**, 407-411.
4. Ulvatne, H., Samuelsen, O., Haukland, H. H., Kramer, M., and Vorland, L. H. (2004) *FEMS Microbiol. Lett.*, **237**, 337-384.
5. Andersen, J. H., Osbakk, S. A., Vorland, L. H., Traavik, T., and Gutteberg, T. J. (2001) *Antiviral Res.*, **51**, 141-149.
6. Berkhout, B., Wamel, J. L. B., Beljaars, L., Meijer, D. K. F., Visser, S., and Floris, R. (2002) *Antiviral Res.*, **55**, 341-355.
7. Yoo, Y. C., Watanabe, S., Watanabe, R., Hata, K., Shimazaki, K., and Azuma, I. (1998) *Adv. Exp. Med. Biol.*, **443**, 285-291.
8. Eliassen, L. T., Berge, G., Sveinbjornsson, B., Svendsen, J. S., Vorland, L. H., and Rekdal, O. (2002) *Anticancer Res.*, **22**, 2703-2710.
9. Kreig, A. M., Yi, A. K., Matson, S., Waldschmidt, T. J., Bishop, G. A., and Teasdale, R. (1995) *Nature*, **374**, 546-549.
10. Britigan, B. E., Lewis, T. S., Waldshcmidt, M., McCormick, M. L., and Krieg, A. M. (2001) *J. Immunol.*, **167**, 2921-2928.
11. Xing-jun Feng, Jian-hua Wang, An-shan Shan, Da Teng, Ya-lin Yang, Yi Yao, Guan-pin Yang, Yan-chun Shao, Shuo Liu, and Fan Zhang (2006) *Protein Express. Purif.*, **47**, 110-117.
12. Choi, J., and Kim, E. E. (2002) *Antiviral. Res.*, **55**, 279-290.
13. Hultmark, D., Engstrom, A., and Bennich, H. (1982) *Eur. J. Biochem.*, **127**, 207-217.
14. Schagger, H., and von Jagow, G. (1987) *Analyt. Biochem.*, **166**, 368-379.