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ORIGINAL RESEARCH PAPER

Heterologous expression of human interleukin-6 in *Streptomyces lividans* TK24 using novel secretory expression vectors

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Abstract *Streptomyces* is an attractive host for heterologous protein secretion. To further optimize its expression capacity, better expression vectors will be helpful. Here, based on pSGL1, a high copy number plasmid present in *Streptomyces globisporus* C-1027, we constructed a series of novel *E. coli*-*Streptomyces* shuttle expression vectors pIMB2–4. These vectors, which are compatible with pIJ-derived vectors, contain the strong promoter *ermE**p and signal sequence SP_{MelC1} of the first ORF of melanin operon in *S. antibiotics* (pIMB2), SP_{CagA} of C-1027 apoprotein in *S. globisporus* C-1027 (pIMB3 and pIMB4). Using these vectors, human interleukin-6 (IL-6) could successfully be expressed and secreted using *S. lividans* TK24 as host. Furthermore, replacement of a rare leucine codon TTA with CTG in SP_{CagA} enhanced IL-6 production.

Yuanjun Zhu and Lifei Wang equally contributed to this study.

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Introduction

Streptomyces are aerobic, filamentous Gram-positive soil bacteria, which produce more than half of the known antibiotics (Hopwood 1999). In addition, living in the soil, *Streptomyces* secrete many extracellular enzymes to decompose multiple substrates for facilitating their survival (Paradkar et al. 2003). The efficient protein secretion mechanism in *Streptomyces* has made them very attractive hosts for heterologous protein production (Vrancken and Anne 2009). During the last 20 years, there are many prokaryotic and eukaryotic proteins successfully expressed in *Streptomyces*, such as *Mycobacterium tuberculosis* alanine- and proline-rich antigenic protein (APA) (Vallin et al. 2006) and mouse TNF-α (Lammertyn et al. 1997). However, there are still a number of heterologous proteins especially eukaryotic proteins which are produced in *Streptomyces* only at very low levels, or not at all. For this reason, there is a need for further optimization of this expression system, such as new expression/secretion vectors and adaptation of codon usage of foreign genes.

The interest in the application of *Streptomyces* as host for the production of heterologous proteins has stimulated the development of an array of expression

vectors, which mainly are based on plasmids with a high copy number. Many *Streptomyces* vectors including pIJ702 are derived from pIJ101, a high copy number plasmid present in *S. lividans* ISP5434 (Kieser et al. 1982). Herai et al. (2004) reported a new pIJ101-derived P_{nitA}-NitR expression vector, in which expression of the gene of interest can be regulated by an inducer ε-caprolactam. In addition, Hatanaka et al. (2008) reported a pIJ101-derived *E. coli-Streptomyces* shuttle vector pTONA5, a hyperexpression vector in which a metalloendopeptidase (SSMP) promoter isolated from *S. cinnamoneus* TH-2 was used. Although some plasmids from other sources such as pFD666 have also been used for heterologous expression (Fink et al. 1991), new expression vectors with integrated features for secretory production of foreign proteins are still being pursued.

pSGL1 is a 7.4 kb plasmid isolated from *S. globisporus* C-1027 (Li and Li 1992), and its minimal replicon was identified to be within a 2 kb fragment (Hong and Li 1998). pSGL1 and its derived plasmids are compatible with pIJ101-derived plasmids and have a high copy number in *Streptomyces*, reaching 70–250 copies per genome (Hong and Li 1998). This vector was already successfully used for the efficient expression and secretion of soluble human interleukin-4 receptor in *S. lividans* TK24 (Zhang et al. 2004). This work suggested that pSGL1 can be a promising skeleton for the construction of new *Streptomyces* expression vectors.

In this report, we describe the construction of novel *E. coli–Streptomyces* shuttle vectors pIMB2–4, which can be conveniently used for secretory production of heterologous protein. These vectors employed the replicon of pSGL1, together with a strong promoter, different signal peptides and multiple cloning sites (MCS). These vectors were tested for the production of human interleukin-6 (IL-6), a pleiotropic cytokine. Post-translational modifications such as glycosylation have been shown to have little effect on the bioactivity of IL-6 (Tonouchi et al. 1988), and as a consequence a protein suitable to be expressed in a prokaryotic system. However, when expressed in *E. coli* IL-6 was produced as inclusion bodies (Tonouchi et al. 1988 and our unpublished data). Therefore, we attempted to express human IL-6 in *S. lividans* TK24 using novel vectors constructed in this work. Results obtained suggested that pIMB2–4 might be useful new vectors for the secretory production of heterologous proteins in *Streptomyces*.

Materials and methods

Bacterial strains and growth conditions

E. coli DH5α was used as host for cloning purposes. *E. coli* ET12567/pUZ8002 (Kieser et al. 2000) was used to transfer DNA into *S. lividans* by conjugation. Strains were grown either on solid or in liquid Luria–Bertani (LB) medium at 37°C. When applicable, antibiotics were used to select recombinant *E. coli* strains: 100 μg ampicillin (Ap) ml^{-1}, 50 μg kanamycin (Km) ml^{-1}, 25 μg chloramphenicol (Cm) ml^{-1} or 50 μg apramycin (Am) ml^{-1}. *S. lividans* TK24 was used as host strain for heterologous protein production. *S. lividans* TK24 and its derivatives were grown at 28°C on R2 agar (Kieser et al. 2000) for sporulation, mannitol soya flour (MS) agar (Kieser et al. 2000) for conjugation, in trypticase soy broth (TSB, BD) for isolation of genomic or plasmid DNA. When appropriate, apramycin was added at final concentrations of 50 μg ml^{-1} to solid medium, and at 10 μg ml^{-1} to liquid medium. For the production of heterologous protein, a preculture of *S. lividans* harboring expression plasmid was grown in 50 ml CM medium (Zhang et al. 2004; Qi et al. 2008) at 28°C, 220 rpm, for 48 h. About 30 glass beads ($D = 3$ mm) were added in the shake-flask to improve the condition of cell growth. 5 ml of this pre-culture was then inoculated into 50 ml of fresh CM medium, and the supernatant was harvest at the indicated time by centrifugation at 3,000 rpm for 20 min.

Construction of secretory expression vectors

The minimal replicon of pSGL1 (Li and Li 1992) was digested with *Sal*I and *Sac*I and subcloned into the *Sal*I–*Sac*I digested pUC19E (Supplementary Table 1). The resulted plasmid pUC19E-SG was digested with *Sac*I and *Pst*I to obtain the fragment containing the minimal replicon of pSGL1. pOJ446 (Kieser et al. 2000) was digested with *Eco*RI and then partially digested with *Pst*I to obtain the fragment containing *oriT* of RK2 for conjugation and *aac(3)IV* gene for apramycin resistance. pBluescript II KS(+)

(Stratagene) was digested with *Sac*I and *Eco*RI to obtain the fragment carrying *ori* (pUC) and ampicillin resistance gene *bla*. These three fragments were ligated together to generate pIMB1.

The *ermE** promoter (Bibb et al. 1994) fragment was amplified using primers 1 and 2 (Supplementary Table 2) from pL646 plasmid (Hong et al. 2007), and then ligated into the pGEM-T (Promega) resulting in pGEM-*ermE**p. The fragment containing the *melC1* (the first ORF in the melanin operon of *S. antibioticus*) gene (Bernan et al. 1985) including the promoter *melC1*p and signal sequence SP_{MelC1} was amplified with primers 3 and 4 (Supplementary Table 2) from pUC19-melC1 (Hong et al. 2003), and then ligated into pGEM-T resulting in pGEM-*melC1*p-SP_{MelC1}. *ermE**p fragment from pGEM-*ermE**p and *melC1*p-SP_{MelC1} fragment from pGEM-*melC1*p-SP_{MelC1} were ligated together into the *Hin*dIII–*Eco*RI gap of pIMB1 resulting in plasmid pIMB2. The multiple cloning sites (MCS) of pIMB2, including *Nde*I, *Sac*II, *Bgl*II, *Cla*I, *Bcl*I, *Eco*RV and *Eco*RI, was acquired through incorporation of primer 4 (Supplementary Table 2). The fragment containing *cagA* [an apoprotein gene in *S. globisporus* C-1027 (Sakata et al. 1992)] promoter *cagA*p and its wild type signal sequence $SP_{CagA(TTA)}$ was amplified from *S. globisporus* C-1027 total DNA with primer 5 and 6 (Supplementary Table 2), and ligated into pGEM-T resulting in pGEM-*cagA*p-$SP_{CagA(TTA)}$. The *cagA*p-$SP_{CagA(TTA)}$ fragment from pGEM-*cagA*p-$SP_{CagA(TTA)}$ and *ermE**p fragment were ligated together into the *Hin*dIII-*Eco*RI gap of pIMB1 as such obtaining plasmid pIMB3. The rare leucine codon TTA in $SP_{CagA(TTA)}$ was replaced by the preferred codon CTG through overlapping PCR using primer 7 and 8 (Supplementary Table 2). The fragment including *cagA*p and mutated signal sequence $SP_{CagA(CTG)}$ were ligated together into the *Hin*dIII-*Eco*RI gap of pIMB1, resulting in plasmid pIMB4 similarly as pIMB3. Through incorporation of primer 6 pIMB3 and pIMB4 also obtained a MCS consisting of *Nde*I, *Sac*II, *Eco*RV, *Bgl*II and *Eco*RI (Supplementary Table 2).

For cloning human IL-6 cDNA, total RNA of human peripheral blood mononuclear cells was extracted, and then was reversely transcribed into cDNA. Sequence for mature IL-6 coding region was amplified by PCR using primer 9 (Supplementary Table 2) containing the *Nde*I site and primer 10 (Supplementary Table 2) containing the *Bam*HI site downstream of the stop codon of IL-6 coding region. The resultant fragments were cloned into pGEM-T and confirmed by sequencing. The IL-6 cDNA fragments were then digested with *Nde*I and *Bam*HI, and then ligated into the *Nde*I-*Bgl*II (isocaudamer of *Bam*HI) gaps of pIMB2, pIMB3 and pIMB4 respectively, resulting in IL-6 expression plasmids pIMB2-IL6, pIMB3-IL6 and pIMB4-IL6.

SDS-PAGE and Western blot analysis for recombinant IL-6

Crude fermentation sample and purified recombinant protein were detected by SDS-PAGE and Western blot. The samples were loaded onto 12% SDS-PAGE gels and protein bands were visualized with Coomassie brilliant blue R-250 after electrophoresis. For Western blotting, proteins were separated by 12% SDS-PAGE and then blotted onto PVDF membrane (Millipore) using a semi-dry electroblotter (Bio-Rad). IL-6 was detected using mouse monoclonal anti-human IL-6 antibody (R&D Systems). Alkaline phosphate (AP) conjugate horse anti-mouse IgG (H + L) (Zhongshan Jinqiao) was used as secondary antibody. Immunoreactive bands were visualized using NBT/BCIP (Promega) substrate for AP.

ELISA of recombinant IL-6

The human IL-6 Quantikine immunoassay kit (R&D Systems), which employed the quantitative sandwich enzyme immunoassay technique, was used to detect IL-6 in the culture medium. A monoclonal antibody specific for IL-6 has been pre-coated onto a 96-well microplate. To each well, 100 μl of assay diluent which contains buffered protein, and 100 μl of IL-6 standard or sample were added. After incubation for 2 h at room temperature, the microplate was washed four times with 400 μl wash buffer. After adding 200 μl of IL-6 conjugate, which contained poly-antibody against IL-6 conjugated with horseradish peroxidase to each well, the microplate was incubated for 2 h at room temperature. The microplate was washed four times with 400 μl wash buffer, and then 200 μl of substrate solution was added to each well and kept at room temperature for 20 min. 50 μl of stop solution was added to each well, and the

absorbance at 450 nm was recorded as measurement of the reaction.

Purification of the recombinant IL-6 protein

The ÄKTA explorer system (GE Healthcare) was used for the purification of the recombinant IL-6. The lyophilized protein sample obtained through ammonium sulfate precipitation (90% saturation) was dissolved in buffer A (50 mM Tris–HCl, pH 8.5). After filtration with 0.4 μm membrane, the sample was loaded on a 1 ml cation exchange resin Q sepharose fast flow (GE Healthcare) column which was pre-equilibrated with at least 5 column volumes of buffer A. The column was washed with 5 column volumes buffer A and then eluted with about 100 column volumes buffer B (50 mM Tris–HCl, 1 M NaCl, pH 8.5) using a stepwise gradient (0–100%, the concentration of buffer B was increased by 2% when the absorbance at 280 nm returned to the baseline level) with a flow rate at 0.5 ml/min. The recombinant IL-6 was eluted from the cation exchange column at 8% buffer B fractions monitored at 280 nm. The purity of the recombinant protein was evaluated by 12% SDS-PAGE followed by Coomassie brilliant blue staining. The recombinant protein was dissolved in buffer C (50 mM Tris–HCl, 150 mM NaCl, pH 8.5) and then loaded on a superdex^{TM} 75 10/300 (GE Healthcare) gel filtration column which was pre-equilibrated with buffer C. The flow rate was maintained at 0.3 ml/min and the eluant was monitored at 280 nm. The purified product was lyophilized and kept at −20°C.

HPLC analysis of purified recombinant IL-6

Reversed-phase HPLC of recombinant IL-6 was performed with C-8 column (ZORBAX 300SB-C8, 4.6 × 50 mm, Agilent) using prominence LC-20A HPLC system (Shimadzu). A linear gradient of 20–80% (vol/vol) acetonitrile in water containing 0.1% (vol/vol) trifluoroacetic acid (TFA) for 30 min served as mobile phase at a flow rate of 1.0 ml/min. Chromatograms were recorded by UV detection at 280 nm.

N-terminal amino acid sequence analysis

The *N*-terminal amino acid residues of the purified recombinant IL-6 were identified by Edman degradation sequencing using Applied Biosystems Procise 491 (Applied Biosystems).

Biological activity assay

The purified recombinant IL-6 activity was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) proliferation assay using IL-6-dependent 7TD1 cells, which belong to murine B-cell hybridoma. 7TD1 cells (4×10^4 cells ml^{-1}) were seeded into a 96-well plate and cultured in the presence of four-fold serial dilutions of the IL-6 samples at 37°C, and recombinant human IL-6 (R&D product) was applied as standard control. After culturing for 72 h, 20 μl MTT was added to each well, and crystals of formazan were dissolved with 100 μl 10% SDS-10 mM HCl 4 h later. Cell growth was assessed by the absorbance at 570 nm. The results were processed with four parameters regression method, and the recombinant IL-6 bioactivity was calculated by comparing with the standard curve.

Results

Construction of novel secretory expression vectors pIMB2–4

Fragments containing the replicons for plasmid maintenance in *Streptomyces* and *E. coli*, promoter, signal peptides and selection markers were incorporated to construct a novel series of *E. coli–Streptomyces* shuttle vectors pIMB2–4 (Fig. 1). The minimum replicon of pSGL1 was employed as *Streptomyces* replication origin, and the *E. coli* replication origin was obtained from plasmid pBluescript II KS(+) (Stratagene) for convenient gene cloning in *E. coli*. pIMB2–4 also contain the RK2 *oriT* obtained from pOJ446 (Kieser et al. 2000) which allows conjugation between *E. coli* and *Streptomyces*. In these vectors, *ermE**p (Bibb et al. 1994) is used as a strong constitutive promoter for the expression of the target protein. The *melC1* promoter-signal sequence (Bernan et al. 1985) was located downstream of *ermE**p for protein secretion in pIMB2. As for pIMB3 and pIMB4, *cagA* promoter-signal sequence (Sakata et al. 1992) was employed in the same way as for pIMB2. The only difference between pIMB3 and 4 is that a rare codon TTA for leucine in

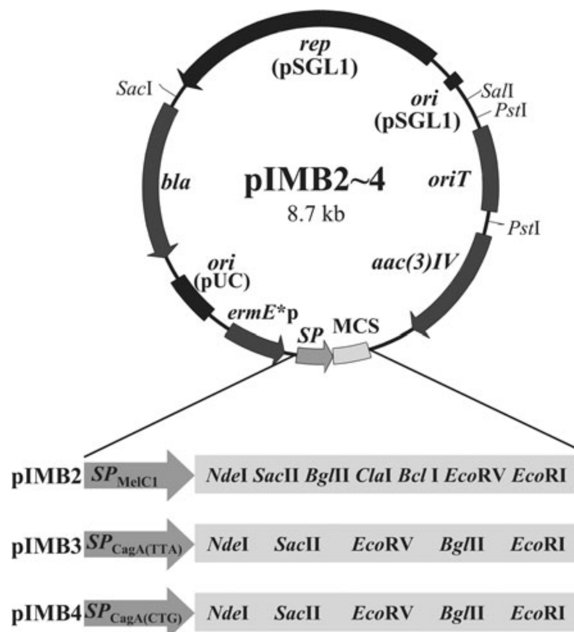

Fig. 1 The map of expression vectors pIMB2–4. *ori* (pSGL1), origin for *Streptomyces* plasmid pSGL1 replication; *rep* (pSGL1), replicase gene of pSGL1; *bla* ampicillin-resistance gene; *aac(3)IV* apramycin-resistance gene; *ori* (pUC), pUC origin of replication in *E. coli*; *oriT*, origin of DNA plasmid transfer during conjugation; *ermE**p *ermE* gene mutated promoter; SP_{MelC1} DNA sequence encoding the signal peptide of MelC1; $SP_{CagA(TTA)}$ wild type DNA sequence encoding the signal peptide of CagA, includes a rare leucine codon TTA; $SP_{CagA(CTG)}$ DNA sequence encoding the signal peptide of CagA, of which TTA was changed to CTG; *MCS* multiple cloning sites

the CagA signal peptide (SP_{CagA}) coding sequence was changed to CTG by site-directed mutagenesis. Gene cloning may be facilitated with the introduced multiple cloning sites (MCS), which is located just downstream the signal peptide cleavage site. The vectors pIMB2–4 contain the *aac(3)IV* gene originating from pOJ446, giving apramycin resistance, and *bla* corresponding to ampicillin resistance from pBluescript II KS(+) for selection in *Streptomyces* and *E. coli*, respectively. The complete nucleotide sequences of pIMB2–4 were confirmed by sequencing and that of pIMB4 has been submitted to the GenBank (accession number HM756283).

Expression of human interleukin-6 (IL-6) in *S. lividans* TK24

Mature human IL-6 coding sequence was amplified from cDNA of peripheral blood mononuclear cells, and then inserted into *Nde*I–*Bgl*II digested pIMB2–4 to generate pIMB2-IL6, pIMB3-IL6 and pIMB4-IL6 respectively. These recombinant IL-6 expression plasmids were conjugated from *E. coli* into *S. lividans* TK24, resulting in recombinant expression strains *S. lividans* [pIMB2-IL6], *S. lividans* [pIMB3-IL6] and *S. lividans* [pIMB4-IL6]. *S. lividans* [pIMB2] harboring the vector pIMB2 was used as a control. These *S. lividans* recombinant strains were cultured in CM medium at 28°C for 72 h. No apparent difference in the growth characteristics, such as dry weight of mycelia and pH of culture medium, was observed among these strains (data not shown). The secreted proteins in the culture medium after ammonium sulfate precipitation as well as the proteins in the cell lysates were determined by SDS-PAGE and Western blot analysis. A clear IL-6 specific band of about 20 kDa was detected in both *S. lividans* [pIMB3-IL6] and [pIMB4-IL6] culture medium samples (Fig. 2a, b lanes 3 and 4) but undetectable in their cell lysates (Fig. 2c, lanes 3 and 4), suggesting that the recombinant human IL-6 is expressed and secreted efficiently in both recombinant *S. lividans* strains. No IL-6 specific band was observed in *S. lividans* [pIMB2-IL6] culture medium (Fig. 2a, b lane 2), and Western blot detected an approximately 23 kDa IL-6 specific band intracellular (Fig. 2c, lane 2), which was consistent with the molecular weight of the IL-6 precursor. These results suggested that the MelC1 signal peptide (SP_{MelC1}) was inefficient for IL-6 secretion in *S. lividans* TK24.

To evaluate the secretion efficiency of recombinant IL-6, ELISA was used for quantitative measurement of IL-6 secreted by the *S. lividans* recombinant strains at different culture time. IL-6 reached a maximum expression level at 72 h (Fig. 3a, b), and by that time up to 0.50 and 0.61 mg/l IL-6 was detected in the culture media of *S. lividans* [pIMB3-IL6] and [pIMB4-IL6] respectively. However, no secreted IL-6 was detected in the culture media of *S. lividans* [pIMB2-IL6] (data not shown). These results demonstrated that replacing the rare codon TTA with CTG in SP_{CagA} coding sequence led to a relatively higher IL-6 protein production.

Purification of recombinant IL-6

The recombinant IL-6 protein expressed and secreted by *S. lividans* [pIMB4-IL6] was purified through a series of steps including ammonium sulfate

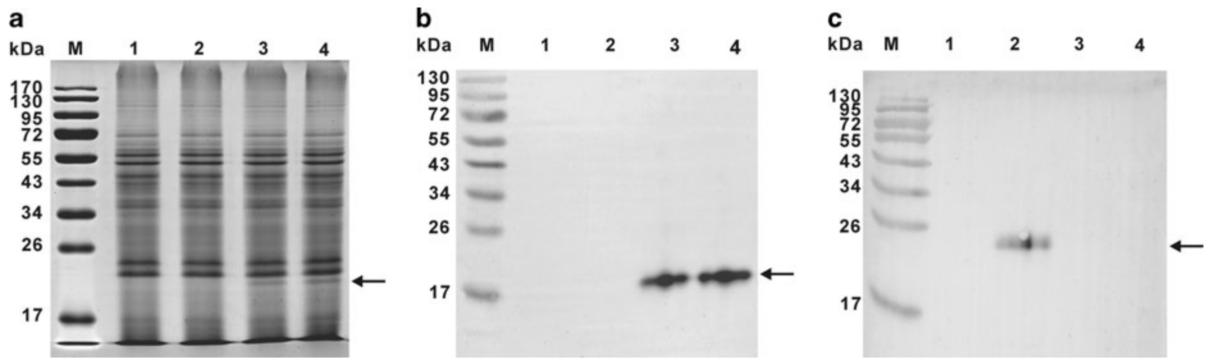

Fig. 2 Expression analysis of IL-6 in recombinant *S. lividans* strains. Proteins in the culture medium precipitated by ammonium sulfate, and the proteins in the cell lysates were detected by 12% SDS-PAGE and Western blot. Arrows indicate the band corresponding to the recombinant IL-6 protein. **a** SDS-PAGE analysis of recombinant proteins in the culture medium. **b** Western blot analysis of recombinant IL-6 in the culture medium. **c** Western blot analysis of IL-6 in the cell lysates. *Lane M* pre-stained protein marker; *lane 1 S. lividans* [pIMB2]; *lane 2 S. lividans* [pIMB2-IL6]; *lane 3 S. lividans* [pIMB3-IL6]; *lane 4 S. lividans* [pIMB4-IL6]

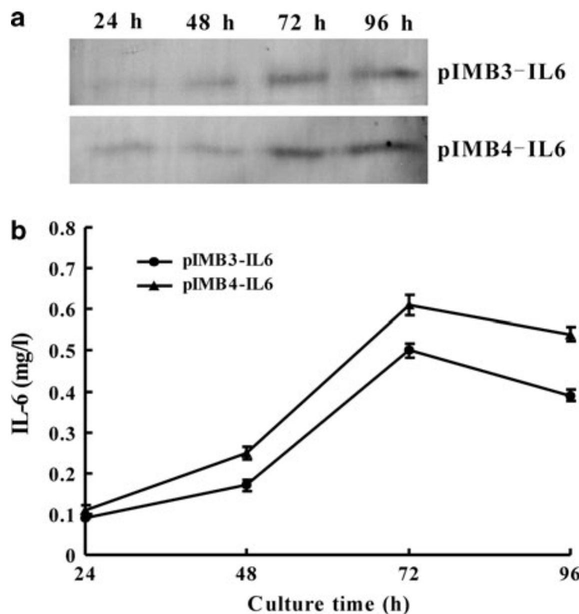

Fig. 3 Western blot and ELISA of recombinant IL-6 secreted by *S. lividans* [pIMB3-IL6] and [pIMB4-IL6] at different culture time. **a** Western blot analysis of recombinant IL-6 in the culture medium. A representative of three independent experiments was shown here. **b** ELISA analysis of IL-6. The human IL-6 Quantikine immunoassay kit was used to detect IL-6 in the culture medium. The values represent the means of three independent experiments (Mean ± standard error)

precipitation, cation exchange chromatography and gel filtration chromatography. The progress of purification was monitored by densitometric scanning of Coomassie brilliant blue stained SDS-PAGE gels (Fig. 4a) and Western blotting (Fig. 4b). The amount of recombinant IL-6 was about 0.5% of the total protein from ammonium sulfate precipitation fraction. After Q-sepharose cation exchange column, recombinant IL-6 reached about 24% of the total protein content in the pooled fractions. Further purification was carried out by superdex HR 75 column, and HPLC analysis showed that the purity of the recovered recombinant IL-6 was 90% (Fig. 4c). The profile of purification was summarized in Table 1. The biological activity of the purified recombinant IL-6 was determined by measuring the proliferation of IL-6-dependent 7TD1 cells by MTT assay, and its potency reached 7.7×10^6 U/mg.

The *N*-terminal amino acid residues of the purified recombinant IL-6 were determined as His-Met-Val-Pro-Pro-Gly-Glu-Asp-Ser by the Edman degradation method. Two additional residues (His-Met) at the *N*-terminal were identified when compared to the mature human IL-6, which was introduced by the *Nde* I site (CATATG). The result confirmed that no *N*-terminal degradation occurred in the process of protein expression and purification.

Discussion

Heterologous expression has been widely applied as an effective tool for the production of proteins of biopharmaceutical and therapeutic interest. *Streptomyces* have high secretion ability, and have been an attractive prokaryotic host for expression of

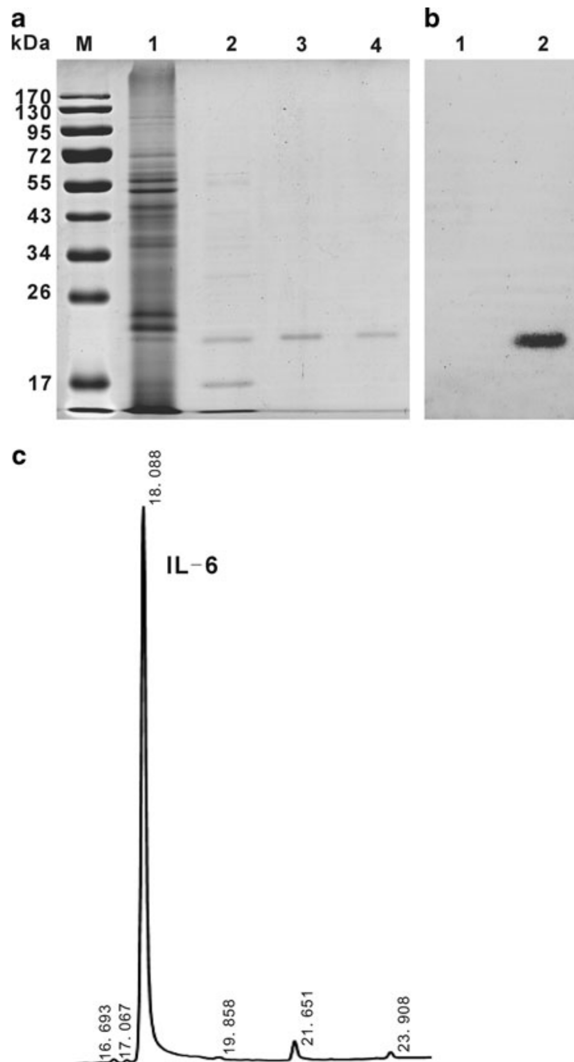

Fig. 4 Purification of recombinant IL-6 protein. **a** SDS-PAGE of proteins in different purification steps. The recombinant IL-6 protein secreted in *S. lividans* [pIMB4-IL6] at 72 h was purified through a series of steps. *Lane M* pre-stained protein marker; *lane 1* the crude sample precipitated by ammonium sulfate; *lane 2* sample purified after Q Sepharose FF chromatography; *lane 3* sample purified after Superdex^{TM} 75 10/300 chromatography; *lane 4* 0.5 μg IL-6 standard (R&D). **b** Western blot analysis of purified recombinant IL-6. *Lane 1* *S. lividans* [pIMB2]; *lane 2* purified recombinant IL-6. **c** HPLC analysis of purified recombinant IL-6

heterologous proteins. Although there are a few commonly used expression plasmids available, sufficient choice of convenient vectors still lacks in *Streptomyces* expression system. In this work, we constructed a series of novel *E. coli-Streptomyces* shuttle expression vectors pIMB2–4 derived of pSGL1, which has a high copy number and is compatible with pIJ101 (Hong and Li 1998). These vectors contain a strong constitutive promoter and efficient signal peptide sequence for directing secretory expression of foreign proteins. They also contain the *oriT* of RK2 for conjugative transferring plasmid from *E. coli* to *Streptomyces*, which may save time and effort compared to protoplast transformation.

For improving heterologous protein expression in *Streptomyces*, one of the effective ways is to increase the expression capacity of the host. Overexpression of some host proteins involved in expression or secretion, may lead to an increased production of foreign proteins. For example, Vrancken et al. (2007) overexpressed phage-shock protein A (PspA) in *S. lividans* TK24, and ultimately improved the secretory expression of several heterologous proteins in this way. Until now, most commonly used *Streptomyces* expression plasmids are derived from pIJ101 (Kieser et al. 1982), so it is necessary to develop vectors which can coexist with pIJ101 derived plasmids. Novel vectors pIMB2–4 derived from pSGL1 compatible with pIJ101 enlarge the number of existing vectors as such providing more options for the choice of expression plasmids. These vectors may also be useful in improving the production of the existing *Streptomyces* expression system which harbors pIJ101 derived plasmids.

In order to obtain high expression of foreign genes, an efficient promoter is needed. A strong constitutive promoter *ermE**p, which has been widely used to direct high level expression of foreign genes in *S. lividans* (Schmitt-John and Engels 1992), was introduced into the vectors pIMB2–4. For protein secretion two different signal peptides were used in pIMB2–4. The SP_{MelC1} has been used previously for the successful secretion of salmon calcitonin (Hong et al. 2003) and human glucagons (Qi et al. 2008) in *Streptomyces*. CagA is the apoprotein of antitumor antibiotic C-1027 in *S. globisporus* C-1027, and its promoter and signal peptide have been used to direct soluble human interleukin-4 receptor secretion in *S. lividans* TK24 (Zhang et al. 2004). In this work, recombinant human IL-6 was secreted into the culture medium guided by SP_{CagA}, while no recombinant IL-6 accumulated intracellularly, demonstrating that SP_{CagA} was efficient for recombinant IL-6 secretion. However, under the guidance of SP_{MelC1}, the secretion of IL-6 was unsuccessful, and IL-6 was detected as a precursor intracellularly. This result suggested that

Table 1 Purification of recombinant IL-6 secreted by *S. lividans* [pIMB4-IL6]

Purification steps	Total protein (mg/l)	Purity of IL-6 (%)	IL-6 (mg/l)	Yield (%)	Purification (fold)
Culture medium	ND	ND	0.61[a]	100	
Ammonium sulfate precipitation	100[b]	0.5[c]	0.50	82	1
Q-Sepharose FF	1.8[d]	24[c]	0.43	70	48
Superdex HR 75 10/300	0.3[d]	90[e]	0.27	45	180

ND Not determined

[a] Defined by ELISA

[b] Defined by BCA protein assay kit

[c] Defined by analysis of SDS-PAGE gel with quantity one software

[d] Defined by protein weight

[e] Defined by HPLC

different signal peptides vary greatly in secretion efficiency for a specific foreign protein, and the fusion of a signal sequence with proved high efficiency to secrete foreign protein will not certainly export another foreign protein effectively. Adopting different signal peptides in each vector respectively, pIMB2–4 may provide different options for heterologous protein secretion in *Streptomyces*.

In the process of protein export, the signal peptide of protein precursor is cleaved by the signal peptidase (SPase). The numbers and types of the amino acids between the signal peptide and mature foreign protein might have some influence on SPase processing. Lammertyn et al. (1997) kept two amino acids (Glu-Ala) of Vsi mature protein after the signal peptide cleavage site, which enhanced the accurate processing of the recombinant heterologous protein mTNF-α. In this work, for convenient construction of expression plasmids, multiple cloning sites were placed downstream of signal peptide and thus there were additionally two amino acids (His-Met) coded by *Nde* I between signal peptide and mature IL-6. Amino acid sequence analysis revealed that the SP_{CagA} was correctly recognized and cleaved off at the right site, suggesting that the insertion of His-Met did not affect the SPase cleaving efficiency.

Streptomycetes are characterized by a high GC-content genome (>70%) which results in a highly-biased codon-usage pattern. A rare leucine codon TTA is involved in regulating the expression of secondary metabolism related genes. In addition, the TTA codons within genes are tended to be close to the start of protein coding sequence, which might be more effective in affecting the translation of mRNAs (Leskiw et al. 1991). Ueda et al. (1993) have reported that, when preferred codons CTG and CTC for leucine in *Streptomyces* were changed to TTA, the expression of *ssl* gene was decreased. In this work, although the codons of IL-6 were not optimized, when a TTA at the start of SP_{CagA} coding sequence was replaced with CTG through site-directed mutagenesis, the result showed that this mutation led to a relatively higher level of IL-6 expression. These results suggested that codon optimization of recombinant genes could be an efficient approach to improve their expression in *Streptomyces*, and pIMB4 may therefore provide a better choice for other heterologous protein expression in *Streptomyces*.

Conclusion

Here we report the construction of a series of novel vectors pIMB2–4, which were shown to be convenient for protein secretory production in *Streptomyces*. Secretory production of active human IL-6 was obtained in *S. lividans* TK24 using pIMB3 and pIMB4 as vectors, in which enhanced IL-6 expression was achieved when the rare codon TTA of SP_{CagA} coding sequence was substituted by the preferred codon CTG. Since these vectors were compatible with pIJ101, they can be applied in combination with available pIJ101 derived vectors, which raises the prospect of a wider application.

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