Research Article

Expression by *Streptomyces lividans* of the Rat α Integrin CD11b A-Domain as a Secreted and Soluble Recombinant Protein

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We already reported the use of a long synthetic signal peptide (LSSP) to secrete the *Streptomyces* sp. TO1 amylase by *Streptomyces lividans* strain. We herein report the expression and secretion of the rat CD11b A-domain using the same LSSP and *S. lividans* as host strain. We have used the *Escherichia coli/Streptomyces* shuttle vector pIJ699 for the cloning of the A-domain DNA sequence downstream of LSSP and under the control of the constitutive *erm*E-*up* promoter of *Streptomyces erythraeus*. Using this construct and *S. lividans* as a host strain, we achieved the expression of 8 mg/L of soluble secreted recombinant form of the A-domain of the rat leukocyte β2 integrin CD11/CD18 alpha M subunit (CD11b). This secreted recombinant CD11b A-domain reacted with a function blocking antibody showing that this protein is properly folded and probably functional. These data support the capability of *Streptomyces* to produce heterologous recombinant proteins as soluble secreted form using the "LSSP" synthetic signal peptide.

1. INTRODUCTION

Streptomyces species are saprophytic gram-positive soil bacteria that posses a large range of extracellular hydrolytic enzymes such as α-amylases, agarases, cellulases, xylanases, nucleases, and lipases [1]. The potential of *S. lividans*, as a host for the expression of heterologous recombinant bacterial and eukaryotic proteins, has extensively been investigated [2]. Indeed, this microorganism has several features that make it a suitable host for efficient recombinant protein expression among which are the well-established genetic manipulation procedures [3] and the absence of extensive restriction modification systems that are generally present in other *Streptomyces* species. Furthermore, *S. lividans* has very low endogenous extracellular proteolytic activity when compared to other *Streptomyces* species [4] and does not elicit the formation of inclusion body of recombinant protein in the cytoplasm, a problem encountered in the majority of the expression systems used for eukaryotic and prokaryotic proteins in *E. coli*. Indeed, overproduction of some proteins in *E. coli* led to their incorporation into insoluble inclusions

bodies. While it is possible to solubilize the inclusions bodies, there is no guarantee that attempted renaturation will lead to significant level of active protein. The high level of solubility is a general characteristic of proteins made in *Streptomyces* [3]. For these diverse reasons, *S. lividans* is the host of choice for the secretory production of heterologous proteins and a very attractive biotechnology platform.

Over the past two decades, a growing number of studies have demonstrated that *S. lividans* is a model host for the production of secreted heterologous recombinant proteins [5–12]. In most cases, genes of interest were fused to well-characterized signal peptide sequences of naturally highly secreted *Streptomyces* proteins [11, 13, 14]. Alternatively, synthetic signal peptides were used such as the 35 amino acids long synthetic signal peptide (LSSP) that we have previously used successfully for the secretion of the *Streptomyces* sp. TO1 amylase [10] which is a naturally secreted protein. This signal peptide was deduced by alignment of several *Streptomyces* signal peptides and carried an additional ribosome binding site as well as a second initiation codon. The addition of a second ribosome binding site and a second initiation codon

increased the yield of secreted protein according to Pagé et al. [15].

Several *Streptomyces* secretion systems have successfully been developed for the production of eukaryotic proteins especially those of medical interest [9]. For examples, we can mention the efficient production in *S. lividans* of the murine tumor necrosis factor (TNF) alpha fused to the secretory signal peptide of the subtilisin inhibitor protein from *S venezuelae* [8, 16]. Expression of biologically active human interferon alpha 2 was also achieved in *S. lividans* [17].

The $\beta 2$ integrins (CD11/CD18) are surface heterodimeric glycoproteins and represent one of the most important families of the leucocytes adhesion molecules. This family is composed of 4 glycoprotein complexes each one consists in an α/β heterodimer containing two distinct chains: the alpha (CD11a, CD11b, CD11c, and CD11d) and the beta (CD18) subunits. They have a widespread vital function in development, embryogenesis, tissue organization, wound healing and immune response [18]. The $\beta 2$ integrins recognize multiple ligands and mediate several important cell-substrate and cell-cell adhesive interactions. Binding of integrins to their physiological ligands is a dynamic, divalent cation-dependent, and tightly regulated process. The α subunits display an A-domain in their amine termini that mediates proinflammatory functions [19]. These are mainly the adhesive interaction with vascular endothelial cells that lead to leukocyte transmigration and diapedesis [20]. The A-domain of the αM subunit (CD11b) of the rat leukocyte $\beta 2$ integrin CD11/CD18, which was used for this work, was already cloned and expressed in *E. coli* in the intracellular compartment as a soluble recombinant fusion protein with GST. Recombinant CD11b A-domain was released from the fusion protein by thrombin cut. This recombinant domain retained its adhesive function as shown by its binding in vitro with members of the immunoglobulin superfamily [20]. Furthermore it was used to demonstrate that it is capable to protect the skeletal muscle from inflammatory injury in vivo using a rat model of crush syndrome [21]. In this paper, we report for the first time the heterologous expression and efficient secretion, by *S. lividans*, of the soluble recombinant rat CD11b A-domain; using the constitutive *erm*E-*up* promoter (P*erm*E) from *Streptomyces erythraeus* [22] and the long synthetic signal peptide (LSSP) [10].

2. MATERIAL AND METHODS

2.1. Bacterial strains and media

S. lividans 1326 was used as a host cell for transformation. Protoplasts preparation and transformation procedures were performed as described by Hopwood et al. [23]. The R2YE medium was used for protoplasts regeneration and thiostrepton (the antibiotic selection marker carried by the *E. coli*/*Streptomyces* shuttle vector pIJ699 [24]) was added at 50 μg/mL for selection of transformed *S. lividans* cells. For growth of *S. lividans* harbouring pMS115, thiostrepton was added at a concentration of 15 μg/mL in liquid medium or at 20 μg/mL in solid medium and cultures were grown on TSB or NMMP media [23] at 30°C for 48 h with shaking.

E. coli strains used were TOP10 for cloning of PCR products into pCR-Blunt vector (Invitrogen) and DH5α as host strain for plasmid propagation. Cultures were grown at 37°C in Luria-Bertani medium in the presence of ampicillin (50 μg/mL) when required.

2.2. Plasmids and cloning procedures

The 276 bp *Sac*I-*Bam*HI fragment from pIJ4070 harbouring the constitutive *erm*E-up promoter [22] was subcloned in the pMS39 plasmid [10] carrying the long synthetic signal peptide (LSSP) giving the pMS41 plasmid. Then the 410 bp *Kpn*I-*Hin*cII fragment (P*erm*E-up-LSSP) from pMS41 was cloned in the *Kpn*I-*Hin*cII sites of pIJ4070 yielding the pMS51 plasmid (P*erm*E-up-LSSP).

The sequence coding for the A-domain of the rat leukocyte $\beta 2$ integrin CR3 alphaM subunit (CD11b) was amplified by PCR using the following two primers: S43 5′-CTC**GATATC**TGTCCTCAGCAGGAGAGCAAC-3′ harbouring an *Eco*RV site and S44 5′-CT**CTGCAG**CGTCAGT-CAGTCACGAT-3′ harbouring a *Pst*I site and as template the pGEX-2T plasmid containing the CD11b A-domain fused to the glutathione S-transferase (GST) [20]. (PCR conditions were 5 min at 94°C followed by 35 cycles of 30 s at 94°C, 45 s at 68°C, and 35 s at 72°C. Amplification was carried out using the Pfu DNA polymerase from Stratagene.) The 622 bp PCR fragment corresponding to the A-domain coding sequence was cloned into the pCR-Blunt vector to produce the pMS52 plasmid. Then the 610 bp *Eco*RV-*Pst*I fragment from pMS52 was cloned in the *Eco*RV-*Pst*I sites of pMS51 giving the pMS62 plasmid. Finally, the 1030 bp *Bgl*II fragment from pMS62 carrying the cassette "P*erm*E-up-LSSP-A-domain" was ligated to the pIJ699 vector cut by *Bgl*II [24]. After transformation of the *S. lividans* 1326 protoplasts, the recombinant strain carrying the resulting pMS115 plasmid was called *S. lividans* 1326/pMS115.

2.3. DNA isolation and manipulation

Plasmid preparation and DNA manipulation from the *S. lividans* 1326/pMS115 strain were carried out according to Hopwood et al. [23] and from *E. coli* as described by Sambrook et al. [25].

2.4. RNA isolation and RT-PCR

The *S. lividans* 1326/pMS115 strain was precultured at 30°C on TSB medium [23] then inoculated at 1/10 in the same conditions. Cultures were stopped at the end of the exponential phase (36 h), early stationary phase (50 h), and late stationary phase (60 h). Then the cells were harvested by centrifugation (7500 xg, for 15 min at 4°C). RNAs were prepared from these cultures as described by Hopwood et al. [23] except that a DNase I (Promega) treatment was used in addition to salt precipitation. This RNA preparation was used in RT-PCR experiments. cDNA corresponding to the A-domain

was synthesized from the isolated RNAs (treated with DNase) using the AMV reverse transcriptase (AMV-RT) from Amersham as follows: 2 μL of RNA (2 μg), 2.5 μL of primer S259 at 10 μM, and 9 μL H$_2$O RNase-free. Reactions were incubated in a thermocycler at 65°C for 5 min then placed on ice and the following components were added in each sample: 4 μL of AMV-RT 5x buffer, 1 μL of AMV-RT (1 U/μL), 1 μL of RNasin (Amersham), and 0.5 μL of dNTP 10 mM each. Reactions were incubated at 42°C for 90 min and then at 70°C for 15 min.

10% of the synthesized cDNA (2 μL) was amplified by PCR using the internal primers S258-forward, 5′-CAGGA-GAGCAACATTGCCTTC-3′, and S259-reverse, 5′-GTGAT-CACCAGCTGGCTTAGA-3′. As control, we have performed a PCR reaction using as matrix 1 μg of RNA treated with DNase. The cycling parameters were 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 52°C for 45 s, and 72°C for 30 s. In all cases, 10 μL from 50 μL of the PCR reactions were analyzed on a 1.2% agarose gel.

2.5. Western blot analysis

S. lividans 1326/pMS115 strain was precultured on TSB medium then inoculated at 1/10 in NMMP medium [23] with of 1% (W/V) of glucose and grown for 48 h at 30°C. After culture centrifugation (7500 xg, for 15 min at 4°C), supernatant was lyophilized, resuspended in a minimum amount of phosphate buffered saline (PBS) at pH 7.4, and then dialyzed for 24 h at 4°C against the same buffer.

Protein content was determined using Bradford's method with serum albumin as the standard.

15 μg of secreted proteins were loaded and resolved on a 15% SDS denaturing polyacrylamide gel run in a Tris glycine electrophoresis buffer as described by Laemmli [26].

The GST/CD11b A-domain fusion protein and the A-domain released from the fusion protein by thrombin cut were used as controls [20]. The gel was transferred to an immobilon-P membrane. Unspecific binding of the antibodies was prevented by soaking the membrane in PBS buffer-tween 20 containing 5% of dried skimmed milk for one hour at 37°C. The membrane was then incubated overnight at 4°C with the function blocking monoclonal antibody anti-rat CD11b (clone OX42) purchased from Pharmingen and diluted to 1/1000 in TBS containing 1% dried skimmed milk. Immunoreactive bands were revealed using peroxidase conjugated anti-rat IgG antibodies.

3. RESULTS AND DISCUSSION

3.1. The ermE-up promoter drives an efficient expression of the rat α integrin CD11b A-domain in S. lividans

To investigate the potential of *S. lividans* 1326 to express α integrin A-domain as a secreted recombinant protein, the A-domain of the rat leukocyte β2 integrin αM subunit (CD11b), coding sequence was amplified by PCR and fused to the long synthetic signal peptide (LSSP) [10] of the pMS51 plasmid harbouring the *erm*E-*up* promoter as
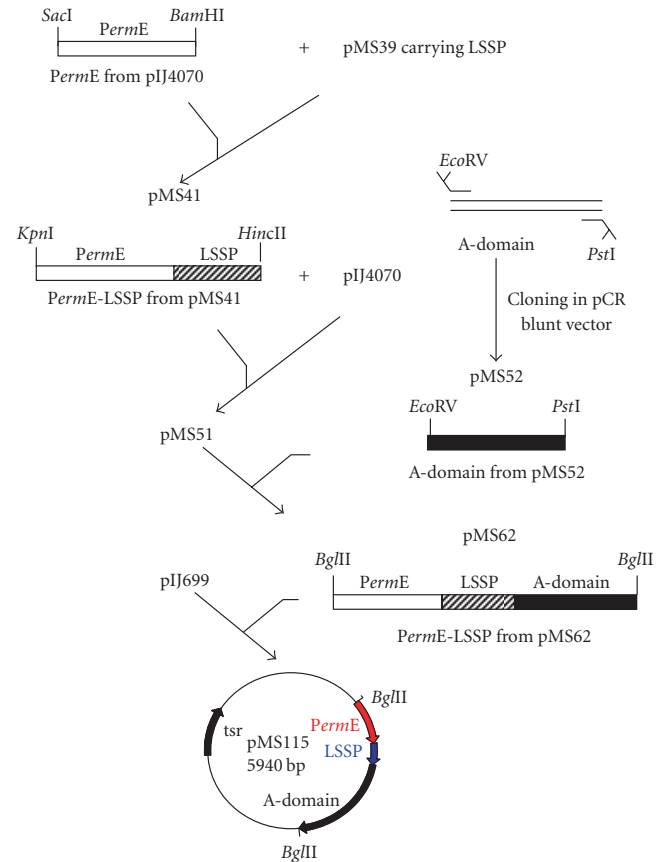

Figure 1: Construction of plasmid pMS115. The constitutive *erm*E-up promoter from pIJ4070 was subcloned as a *Sac*I-*Bam*HI fragment in the pMS39 plasmid carrying the long synthetic signal peptide (LSSP) giving the pMS41 plasmid. Then the *Kpn*I-*Hin*cII fragment carrying P*erm*E-up-LSSP from pMS41 was cloned in pIJ4070 yielding the pMS51 plasmid. PCR product of the rat CD11b A-domain was cloned into the pCR-Blunt vector to produce the pMS52 plasmid. The fragment E*co*RV-*Pst*I corresponding to the A-domain from pMS52 was cloned in pMS51 giving the pMS62 plasmid. Finally, the *Bgl*II fragment from pMS62 carrying the cassette "P*erm*E-up-LSSP-A-domain" was cloned in the pIJ699 vector yielding the pMS115 plasmid.

described in "Materials and Methods" (Figure 1). Finally the cassette "P*erm*E-up-LSSP-A-domain" was introduced in *S. lividans* 1326 using the *E. coli/S. lividans* shuttle vector pIJ699 [24] giving the pMS115 plasmid (Figure 1).

To demonstrate the successful expression of the A-domain in *S. lividans* 1326/pMS115, RT-PCR was carried out with RNA prepared from this strain at different growth phases (late exponential phase (36 h), early stationary phase (50 h), and late stationary phase (60 h)) and treated with DNase. As it is shown in Figure 2, an expected 495 bp fragment internal to the A-domain was effectively amplified and presented throughout all the growth stage tested with the same intensity. This observation confirms the constitutivity of the *erm*-up promoter that governs the expression of the A-domain. This amplification results from the cDNA
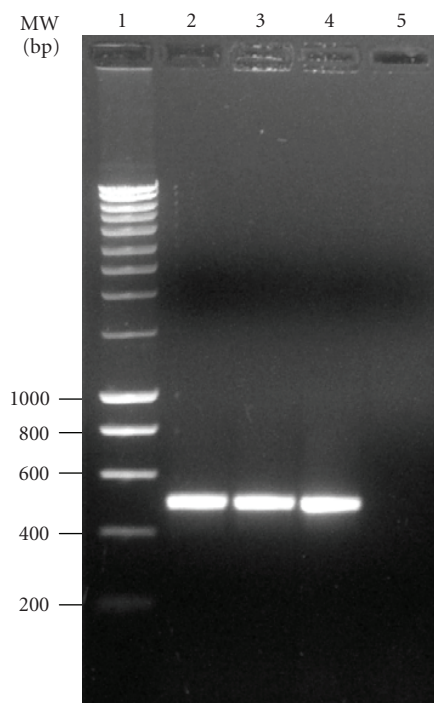
FIGURE 2: RT-PCR analysis of the A-domain expression in *S. lividans* 1326/pMS115. Lane 1: smart ladder molecular weight (MW) marker (Eurogentec). Lanes 2, 3, and 4: RT-PCR performed with cDNA synthesised from 2 μg of RNA treated with DNase and prepared from the *S. lividans* 1326/pMS115 strain grown on TSB medium for 36 h, 50 h, and 60 h, respectively. Lane 5: PCR performed directly with 2 μg of RNA treated with DNase (control).

obtained from A-domain messengers since no amplification was observed when PCR reaction was performed directly with DNase-treated RNA. This result confirms the expression in *S. lividans* 1326/pMS115 of the rat αM subunit (CD11b) A-domain sequence under the control of the constitutive *erm*E-*up* promoter from *Streptomyces erythraeus* [22]. Indeed, this strong and constitutive promoter was widely used for gene expression in various *Streptomyces* species [10, 27–31].

3.2. The LSSP signal peptide permits the production of the CD11 A-domain as an extra cellular protein in S. lividans

The *S. lividans* 1326/pMS115 was grown for 48 hours on NMMP medium with glucose as the carbon source. Then, the supernatant culture was lyophilized, dialyzed against PBS buffer and electrophorized on an SDS polyacrylamide gel. As shown in Figure 3, the size of the recombinant A-domain (21 kDa) expressed by *S. lividans* was the same of its homologous expressed in *E. coli* as a GST/A-domain fusion protein (48.5 kDa) and released by thrombin cut [20]. Since it is known that *E. coli* is unable to glycosylate the polypeptide, and given the fact that the size of the recombinant protein
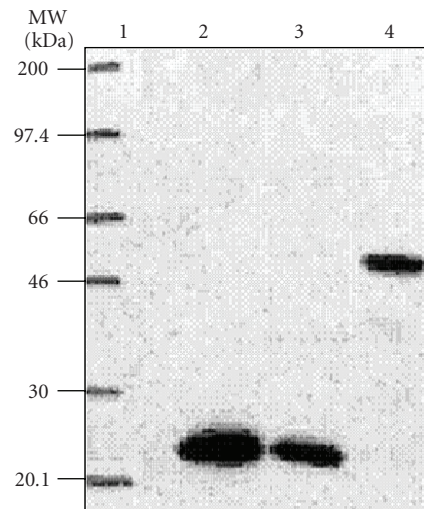

FIGURE 3: Western blot analysis of the A-domain secretion in *S. lividans* 1326/pMS115. Supernatant of the *S. lividans* 1326/pMS115 strain grown on NMMP medium was concentrated 50x, lyophilized, resuspended in PBS, then dialyzed as described in "Materials and Methods." Proteins were run on a 15% SDS polyacrylamide gel. Lane 1: molecular weight standards (MW) from Amersham; lane 2: 15 μg of supernatant proteins from *S. lividans* 1326/pMS115; lane 3: 2 μg of the recombinant A-domain (21 kDa) released from the GST/CD11b A-domain fusion protein (48.5 kDa) by thrombin cut; lane 4: 2 μg of the GST/CD11b A-domain fusion protein (48.5 kDa).

expressed in each system is similar, we can conclude that *S. lividans* did not glysosylate the A-domain, even though the sequence of the rat CD11b displays a putative N glycosylation site.

Furthermore, the yield of the recombinant rat CD11b A-domain secreted by *S. lividans* was estimated at 8 mg per litre. This yield is based on the comparison of band intensity as shown in Figure 3 and takes into consideration the 50x concentration of the supernatant prior to loading on to electrophoresis gel. The estimated A-domain represents 8% of the total extracellular proteins (100 mg/L). This relatively low extracellular protein level could be explained by the use of a minimal medium (NMMP) useful for the further analysis. Western blot analysis of the supernatant of the *S. lividans* culture showed (Figure 3) a protein band with a molecular mass of about 21 kDa corresponding to the secreted rat CD11b A-domain expressed in *S. lividans*. Binding of the function blocking antibody implied that this secreted recombinant form is properly folded and suggests strongly that the protein would be functional as previously shown with its counterpart produced in *E. coli*.

As described by Mhiri et al. [10], the long synthetic signal peptide (LSSP) contains two positive charges on the N-terminal region and carries two ribosome binding sites (RBS) as well as two translational initiation codons which are known to contribute to increasing the yield of the secreted protein [15, 32]. This signal peptide was successfully used for the secretion of the *Streptomyces* sp. TO1 amylase

[10]. The efficient secretion in the *S. lividans* 1326/pMS115 culture supernatant of the A-domain of the rat leukocyte αM subunit (CD11b) with the LSSP signal peptide argue in favour of expanding the use of this signal peptide for secretion of prokaryotic and eukaryotic proteins of interest in *S. lividans*. The ability to produce the CD11 A-domain as an extra cellular protein is of great interest for performing further structure-function studies as well as for applications of this recombinant domain as potential anti-inflammatory agent.

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REFERENCES

[1] S. T. Williams, M. Goodfellow, G. Alderson, E. M. Wellington, P. H. Sneath, and M. J. Sackin, "Numerical classification of *Streptomyces* and related genera," *Journal of General Microbiology*, vol. 129, no. 6, pp. 1743–1813, 1983.

[2] N. Nakashima, Y. Mitani, and T. Tamura, "Actinomycetes as host cells for production of recombinant proteins," *Microbial Cell Factories*, vol. 4, p. 7, 2005.

[3] T. Kieser, M. J. Bibb, M. J. Buttner, K. F. Chater, and D. A. Hopwood, *Practical Streptomyces Genetics*, John Innes Foundation, Norwich, UK, 2000.

[4] C. Binnie, J. D. Cossar, and D. I. H. Stewart, "Heterologous biopharmaceutical protein expression in *Streptomyces*," *Trends in Biotechnology*, vol. 15, no. 8, pp. 315–320, 1997.

[5] B. Hong, Y. Li, G. Van Mellaert, and J. Anné, "Expression of biologically active human tumor necrosis factor β by *Streptomyces lividans*," *Yi Chuan Xue Bao*, vol. 30, no. 3, pp. 209–214, 2003.

[6] M. Lara, L. Servín-González, M. Singh, et al., "Expression, secretion, and glycosylation of the 45- and 47-kDa glycoprotein of *Mycobacterium tuberculosis* in *Streptomyces lividans*," *Applied and Environmental Microbiology*, vol. 70, no. 2, pp. 679–685, 2004.

[7] C. Ogino, M. Kanemasu, Y. Hayashi, et al., "Over-expression system for secretory phospholipase D by *Streptomyces lividans*," *Applied Microbiology and Biotechnology*, vol. 64, no. 6, pp. 823–828, 2004.

[8] C. Pozidis, E. Lammertyn, A. S. Politou, et al., "Protein secretion biotechnology using *Streptomyces lividans*: large-scale production of functional trimeric tumor necrosis factor α," *Biotechnology and Bioengineering*, vol. 72, no. 6, pp. 611–619, 2001.

[9] L. Van Mellaert, C. Dillen, P. Proost, et al., "Efficient secretion of biologically active mouse tumor necrosis factor α by *Streptomyces lividans*," *Gene*, vol. 150, no. 1, pp. 153–158, 1994.

[10] S. Mhiri, M. Mezghani, L. Mellouli, M. Ben Ali, K. Belguith, and S. Bejar, "Efficient synthetic signal peptides for *Streptomyces*," *Biotechnology Letters*, vol. 22, no. 16, pp. 1305–1310, 2000.

[11] G. Sianidis, C. Pozidis, F. Becker, et al., "Functional large-scale production of a novel *Jonesia* sp. xyloglucanase by heterologous secretion from *Streptomyces lividans*," *Journal of Biotechnology*, vol. 121, no. 4, pp. 498–507, 2006.

[12] R. Morosoli and C. Dupont, "Secretion of xylanase A2 in *Streptomyces lividans*: dependence on signal peptides length, number and composition," *FEMS Microbiology Letters*, vol. 179, no. 2, pp. 437–445, 1999.

[13] E. Lammertyn and J. Anné, "Modifications of *Streptomyces* signal peptides and their effects on protein production and secretion," *FEMS Microbiology Letters*, vol. 160, no. 1, pp. 1–10, 1998.

[14] E. Lammertyn, S. Desmyter, S. Schacht, L. Van Mellaert, and J. Anné, "Influence of charge variation in the *Streptomyces venezuelaeα*-amylase signal peptide on heterologous protein production by *Streptomyces lividans*," *Applied Microbiology and Biotechnology*, vol. 49, no. 4, pp. 424–430, 1998.

[15] N. Pagé, D. Kluepfel, F. Shareck, and R. Morosoli, "Increased xylanase yield in *Streptomyces lividans*: dependence on number of ribosome-binding sites," *Nature Biotechnology*, vol. 14, no. 6, pp. 756–759, 1996.

[16] E. Lammertyn, L. Van Mellaert, S. Schacht, et al., "Evaluation of a novel subtilisin inhibitor gene and mutant derivatives for the expression and secretion of mouse tumor necrosis factor alpha by *Streptomyces lividans*," *Applied and Environmental Microbiology*, vol. 63, no. 5, pp. 1808–1813, 1997.

[17] D. Pulido, J. A. Vara, and A. Jimenez, "Cloning and expression in biologically active form of the gene for human interferon α2 in *Streptomyces lividans*," *Gene*, vol. 45, no. 2, pp. 167–174, 1986.

[18] D. Sheppard, "In vivo functions of integrins: lessons from null mutations in mice," *Matrix Biology*, vol. 19, no. 3, pp. 203–209, 2000.

[19] M. Michishita, V. Videm, and M. A. Arnaout, "A novel divalent cation-binding site in the A domain of the β2 integrin CR3 (CD11b/CD18) is essential for ligand binding," *Cell*, vol. 72, no. 6, pp. 857–867, 1993.

[20] K. Zerria, N. Bebbiche, M. R. Barbouche, K. Dellagi, and D. M. Fathallah, "Cloning of the rat CR3 αM (CD11b) subunit, expression and binding assay of recombinant isolated CD11b VA (A-domain) and ICAM-1 Ig modules," *Archives de l'Institut Pasteur de Tunis*, vol. 79, no. 1–4, pp. 11–17, 2002.

[21] K. Zerria, E. Jerbi, S. Hammami, et al., "Recombinant CR3 alpha A-domain Blocks PMN recruitment and protects against skeletal muscle inflammatory injury in rat," *Immunology*. In press.

[22] M. J. Bibb, G. R. Janssen, and J. M. Ward, "Cloning and analysis of the promoter region of the erythromycin resistance gene (*ermE*) of *Streptomyces erythraeus*," *Gene*, vol. 38, no. 1–3, pp. 215–226, 1985.

[23] D. A. Hopwood, M. J. Bibb, K. F. Chater, et al., *Genetic Manipulation of Streptomyces, A Laboratory Manual*, John Innes Foundation, Norwich, UK, 1985.

[24] T. Kieser and R. E. Melton, "Plasmid pIJ699, a multi-copy positive-selection vector for *Streptomyces*," *Gene*, vol. 65, no. 1, pp. 83–91, 1988.

[25] J. Sambrook, E. F. Fritsch, and T. Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, 2nd edition, 1989.

[26] U. K. Laemmli, "Cleavage of structural proteins during the assembly of the head of bacteriophage T4," *Nature*, vol. 227, no. 259, pp. 680–685, 1970.

[27] N. Parajuli, H. T. Viet, K. Ishida, et al., "Identification and characterization of the afsR homologue regulatory gene from *Streptomyces peucetius* ATCC 27952," *Research in Microbiology*, vol. 156, no. 5-6, pp. 707–712, 2005.

[28] R. Kawachi, Y. Koike, Y. Watanabe, et al., "Development of a genetic system in chitinase-producing *Streptomyces* and the application of an allosamidin-insensitive chitinase gene to homologous overexpression," *Molecular Biotechnology*, vol. 26, no. 3, pp. 179–186, 2004.

[29] B. A. Traag, G. H. Kelemen, and G. P. Van Wezel, "Transcription of the sporulation gene ssgA is activated by the IclR-type regulator SsgR in a whi-independent manner in *Streptomyces coelicolor* A3(2)," *Molecular Microbiology*, vol. 53, no. 3, pp. 985–1000, 2004.

[30] C. J. Wilkinson, Z. A. Hughes-Thomas, C. J. Martin, et al., "Increasing the efficiency of heterologous promoters in actinomycetes," *Journal of Molecular Microbiology and Biotechnology*, vol. 4, no. 4, pp. 417–426, 2002.

[31] L. Xiang and B. S. Moore, "Inactivation, complementation, and heterologous expression of encP, a novel bacterial phenylalanine ammonia-lyase gene," *Journal of Biological Chemistry*, vol. 277, no. 36, pp. 32505–32509, 2002.

[32] S. H. Faß and J. W. Engels, "Influence of specific signal peptide mutations on the expression and secretion of the α-amylase inhibitor tendamistat in *Streptomyces lividans*," *Journal of Biological Chemistry*, vol. 271, no. 25, pp. 15244–15252, 1996.