Evaluation of a Novel Subtilisin Inhibitor Gene and Mutant Derivatives for the Expression and Secretion of Mouse Tumor Necrosis Factor Alpha by *Streptomyces lividans*

E. Lammertyn, L. Van Mellaert, S. Schacht, C. Dillen, E. Sablon, A. Van Broekhoven, and J. Anne

Laboratories of Bacteriology and Immunobiology, Rega Institute, Katholieke Universiteit Leuven, B-3000 Leuven, and Innogenetics NV, B-9052 Ghent, Belgium

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In order to evaluate the expression and secretion signals of the highly secreted subtilisin inhibitor of *Streptomyces venezuelae* CBS762.70 (VSI) for the production of heterologous proteins by *Streptomyces lividans*, mouse tumor necrosis factor alpha (mTNF) was chosen as a model protein. The mTNF cDNA was fused to the vsi signal sequence. The analysis of secretion by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and biological activity measurements revealed an efficient translocation of mTNF. Up to 300 mg of secreted biologically active mTNF per liter could be obtained in shaker-flask cultures. By analyzing the effects of mutations in the N region of the VSI signal peptide on secretion, we found that decreasing the +3 charge of the wild-type protein to +2 resulted in a 3- to 10-fold increase in secretion.

Although *Streptomyces* is principally known for the extracellular production of many valuable compounds, such as antibiotics and other pharmacologically useful molecules, herbicides, industrial enzymes, etc., this organism is increasingly gaining interest of many research groups due to its ability to secrete eukaryotic proteins in a biologically active form (8). High-level production of heterologous proteins is readily obtained with the expression and secretion signals of abundantly secreted homologous proteins. In this respect, the signals of several subtilisin inhibitors have proven to be very effective. The subtilisin inhibitors of *Streptomyces albostricoides* and *Streptomyces longiporius* have already been efficiently used for the extracellular production of several heterologous proteins (6, 19-22). Following a recent screening of several *Streptomyces* strains for highly secreted proteins, a novel subtilisin inhibitor (VSI) was isolated from *Streptomyces venezuelae* CBS762.70 (23a). VSI contains a typical streptomycete signal peptide of 28 amino acids carrying three positively charged residues (two arginines and one lysine) within its amino-terminal region. In addition, the vsi promoter has been shown to be very effective in initiating transcription.

The primary objective of the present study was to establish the secretory production of mouse tumor necrosis factor alpha (mTNF) in *Streptomyces lividans* with the expression and secretion signals of this novel subtilisin inhibitor. The second objective was to investigate the importance of positive charges in the N region of the signal peptide. Several roles have been proposed for these positively charged residues in the N regions of all prokaryotic signal peptides (14). These roles include electrostatic interaction with the negatively charged membrane and recognition of SecA (1) or another component of the secretory pathway. Mutation analysis of the N terminus from signal peptides in various organisms shows that, in general, basic amino acids are not absolutely essential to obtain secretion, although they seem to enhance the efficiency of the process. In this study, the number of positive charges in the VSI signal peptide was modified from 0 to 5 by in vitro mutagenesis and the effects on protein synthesis and secretion were analyzed.

**MATERIALS AND METHODS**

Bacterial strains and growth conditions. *S. lividans* was grown at 27°C with continuous shaking at 300 rpm in nutrient broth 2 (Lab M, Bury, United Kingdom) buffered at pH 7 with 0.05 M MOPS (3-[N-morpholino]propanesulfonic acid). When necessary, thiolestrept (10 μg/ml) was added. Prototrophy and subsequent transformation of *S. lividans* were carried out as described by Hopwood et al. (12). *Escherichia coli* strains were grown at 37°C (300 rpm) in Luria broth in the presence of ampicillin (50 μg/ml) or tetracycline (15 μg/ml). For solid media, 15 g of agar was added per liter.

**Vector constructions.** All DNA manipulations used in this work were performed by standard techniques. Restriction endonucleases and other DNA-modifying enzymes were purchased from Boehringer (Mannheim, Germany), Eurogentec (Seraing, Belgium), and Life Technologies (Gaithersburg, Md.). Oligonucleotides were obtained from Eurogentec.

For initial construction of pCBSmTNFec and pCBS2mTNFec, the vector pBSDK, a derivative of pBluescriptII KS which lacks the unique HI restriction site, was used as a basic vector. A 0.6-kb Smal fragment of the *S. venezuelae* CBS762.70 chromosome containing the vsi promoter, the ribosome binding site, the signal sequence, and a part of the vsi signal region was cloned in pBluescriptII KS and subsequent transformation of *S. lividans* was used to obtain the recombinant plasmid. For in vitro transfection of the *S. lividans* chromosome containing the vsi promoter, the ribosome binding site, the signal sequence, and a part of the vsi signal region was cloned in pBluescriptII KS and subsequent transformation of *S. lividans* was used to obtain the recombinant plasmid. For the construction of pCBSmTNFec, pBS-CBSS was digested with HI/BamHI and the ends were blunted with Klenow fragment polymerase. Again, the Smb/EcoRI fragment of the vsi signal sequence, the mTNF expression cassette was transferred to the streptomycete multicopy plasmid pJ486 (24) as a BamHI/EcoRI fragment, resulting in pCBSmTNFec. For the construction of pCBS2mTNFec, pCBS-CBSS was digested with Drai and the ends were blunted with Klenow fragment polymerase. Again, the Smb/EcoRI mTNF fragment was ligated to this vector and pCBS2mTNFec was obtained in a manner similar to that described for pCBSmTNFec. For the construction of pCBS10SmTNFec, the pACYC184 derivative containing the complete vsi gene on an NcoI restriction fragment. After 105 codons of the usielonin inhibitor gene, an AvrII/EcoRI linker was introduced for in-frame fusion. This linker encoded a FactorXa recognition sequence and also contained a NheI restriction site. This unique NheI site was then used for ligation of the mTNF cDNA, and the complete expression cassette was obtained in a manner similar to that described for pCBSmTNFec. In this study, the number of positive charges in the VSI signal peptide was modified from 0 to 5 by in vitro mutagenesis and the effects on protein synthesis and secretion were analyzed.

**ACKNOWLEDGMENTS**

This work was supported by grants from the National Research Foundation for the Promotion of the Scientific Research and of the University Research (Belgium). We thank Eric Lamotte and Marc Vandekerckhove for helpful discussion.

**REFERENCES**


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were constructed as described in Materials and Methods and designed with the respective conservation of 0, 2, and 105 Streptomyces plasmids.

SECRETOY PRODUCTION OF mTNF BY S. LIVIDANS

RESULTS

Secretion of biologically active mTNF. To establish the secretory expression of mTNF under the control of the newly characterized promoter, ribosome binding site, and signal sequence of the vsl gene, three vsl-mTNF gene fusions were designed with the respective conservation of 0, 2, and 105 amino acids of VSI after the signal peptidase cleavage site. The last two constructs were made with the intention of conserving the signal peptidase cleavage site, which is supposed to enhance accuracy processing (2, 23). The corresponding vectors were constructed as described in Materials and Methods and were designated pCBSmTNF, pCBS2mTNF (Fig. 1), and pCBS105mTNF, respectively. For each construct, several S. lividans TK24 transformants were analyzed for biologically active mTNF, and the following yields were obtained: 1×10^10 to 2.5×10^10 U/liter for S. lividans(pCBSmTNF), 2.5×10^10 to 10^10 U/liter for S. lividans(pCBS2mTNF), and 0.25×10^10 to 1.25×10^10 U/liter for S. lividans(pCBS105mTNF). For cultures containing pCBSmTNF and pCBS2mTNF, these values mean an increase in the yield of secreted mTNF by a factor of 10 to 100, compared to the yield of mTNF secreted under the control of the α-amylase regulatory sequences and signal sequence (23). With a specific activity of about 2.5×10^8 U/mg of mTNF protein, as estimated by immunoblot analysis, for S. lividans(pCBS2mTNF) cultures, levels of extracellular mTNF reached 300 mg/liter.

Protein analysis and N-terminal amino acid sequencing. Immunoblot analysis of culture fluids of S. lividans (pCBSmTNF) and (pCBS2mTNF) with anti-mTNF antisera showed two immunoreactive bands of about 17 kDa corresponding to the calculated molecular mass for mTNF. For S. lividans (pCBS105mTNF), a fusion protein consisting of almost the entire VSI gene with conservation of 105 codons of the mature vsl gene on an Nco I restriction fragment. This work

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<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source or reference</th>
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<tr>
<td>pIG2mTNF</td>
<td>Te+, mTNF cDNA</td>
<td>Innogenetics</td>
</tr>
<tr>
<td>pIG2mTNF560B1</td>
<td>pIG2mTNF derivative with 560B1 site at the first codon of mTNF cDNA</td>
<td>This work</td>
</tr>
<tr>
<td>pBSDK</td>
<td>pBlueScriptIKS derivative lacking the unique DraII site</td>
<td>This work</td>
</tr>
<tr>
<td>pBS-CBSS</td>
<td>pBSDK derivative containing the vsl promoter, the vsl ribosome binding site, the vsl signal sequence, and a part of the mature vsl gene</td>
<td>This work</td>
</tr>
<tr>
<td>pCBSmTNFe</td>
<td>pBS-CBSS derivative with mTNF cDNA directly fused to the signal sequence of the vsl gene</td>
<td>This work</td>
</tr>
<tr>
<td>pCBS2mTNFe</td>
<td>pBS-CBSS derivative with mTNF cDNA fused to the vsl gene with conservation of two codons of the mature vsl gene</td>
<td>This work</td>
</tr>
<tr>
<td>pCBS105mTNFe</td>
<td>pBS-CBSS derivative with mTNF cDNA fused to the vsl gene with conservation of 105 codons of the mature vsl gene</td>
<td>This work</td>
</tr>
<tr>
<td>pACYC184</td>
<td>pACYC184 derivative containing the complete vsl gene on an NcoI restriction fragment</td>
<td>This work</td>
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**Streptomyces plasmids**

<table>
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<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source or reference</th>
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<tr>
<td>pIJ486</td>
<td>Multiple cloning site, Tsr</td>
<td>24</td>
</tr>
<tr>
<td>pCBSmTNF</td>
<td>pIJ486 derivative containing the mTNF expression cassette of pCBSmTNFe</td>
<td>This work</td>
</tr>
<tr>
<td>pCBS2mTNF</td>
<td>pIJ486 derivative containing the mTNF expression cassette of pCBS2mTNFe</td>
<td>This work</td>
</tr>
<tr>
<td>pCBS105mTNF</td>
<td>pIJ486 derivative containing the mTNF expression cassette of pCBS105mTNFe</td>
<td>This work</td>
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# Ap*, ampicillin resistant; Te*, tetracycline resistant; Cm*, chloramphenicol resistant; Ap+, ampicillin sensitive; Tsr*, thioestrepton resistant.

Sites in vitro mutagenesis kit from Promega as described by the manufacturer. The following oligonucleotides were used: 5’-GGCCTCGAGCTGCATTGGAATTACGTCGACGCATGGTGAAC-3’ for the mutant that yielded no charge (mut0), 5’-GGCTCCACGGCCTGCAGGTCGACGCATGGTGAAC-3’ for mut1, 5’-GGCTCCACGGCCTGCAGGTCGACGCATGGTGAAC-3’ for mut2. 5’-CCAC CGCTTTGAGAGCTGAGCAGCATGGTGAAC-3’ for mut3 and 5’-CCACCG GCCCTGGAGAGCTGAGCAGCATGGTGAAC-3’ for mut5 (Fig. 1).

After subcloning of the corresponding fragments in pUC19, the in-frame fusions and inserted mutations were confirmed by DNA sequence analysis according to the dideoxy chain termination method.

**RNA analysis.** Streptomyces cells were grown for 24 or 36 h. After the cells were harvested by centrifugation (6,000 × g, 10 min), total RNA was extracted by the method of Hopwood et al. (12). After isolation, control experiments to ensure the quality of the RNA samples were carried out by gel analysis and ethidium bromide staining. For dot blotting, the RNAs were applied to a Hybond N membrane (Amersham, Buckinghamshire, United Kingdom) in several dilutions. The RNA samples were hybridized according to the method described by Engler-Blum et al. (4) with either the digoxigenin-labeled mTNF cDNA available on plasmid pIG2mTNF (Innogenetics NV, Ghent, Belgium) or a digoxigenin-labeled PCR fragment of the chromosomal stl gene. Detection of the hybridizing signals was done with 0.25 mM CDP-Star (Tropix Inc., Bedford, Mass.) (11). The final X-ray films were scanned with a Desktop Plus densitometer (PDI).

Detection and quantification of mTNF. After being sampled, culture supernatants of S. lividans (pCBS2mTNF) were immediately stored at −80°C. Amounts of biologically active mTNF were determined using an Immobilon P membrane, N-terminal sequences were analyzed by gel analysis and ethidium bromide staining. For dot blotting, the RNA samples were applied to a Hybond N membrane (Amersham, Buckinghamshire, United Kingdom) in several dilutions. The RNA samples were hybridized according to the method described by Hopwood et al. (12). After isolation, control experiments to ensure the quality of the RNA samples were carried out by gel analysis and ethidium bromide staining. For dot blotting, the RNAs were applied to a Hybond N membrane (Amersham, Buckinghamshire, United Kingdom) in several dilutions. The RNA samples were hybridized according to the method described by Hopwood et al. (12). After isolation, control experiments to ensure the quality of the RNA samples were carried out by gel analysis and ethidium bromide staining.
determined. The N terminus of the upper protein (EAVRSS SQNS) corresponded to the N terminus of the mature fusion protein, which proves that the fusion protein is correctly cleaved by the signal peptidase of *S. lividans*. The second band contained different forms of the mTNF protein; i.e., they lacked 2, 3, or 4 amino acids. This observation was also made previously (23), and the different forms of the protein were found to be the consequence of aminopeptidase activity. Finally, the 13-kDa band appeared to be the homologous *S. lividans* trypsin inhibitor STI-1 (whose N terminus is SLYAPSAL), which also inhibits subtilisin (18).

**Influence of positive charges in the signal peptide on protein secretion.** To investigate the importance of the three positive charges in the N-terminal region of the signal peptide for effecting secretion and to analyze whether the level of secreted mTNF could still be improved, several mutants of pCBS2mTNF were made by in vitro mutagenesis as indicated in Fig. 1. As arginine residues are more commonly used than lysines in streptomycete signal peptides, extra positive charges were added by introducing CGT or CGC. The three positive charges of the wild-type signal peptide were gradually replaced by glutamine residues. In this way, we obtained signal peptide mutants with positive charges ranging from 0 to 5. After transformation of *S. lividans* with the different constructs, mTNF was measured both by a biological activity test and by Western blotting. For each of the mutants, eight different transformants were sampled for the presence of intracellular and extracellular mTNF after 30 and 48 h of growth. The average results after 48 h of growth are shown in Fig. 3A. It is clear that charge variation has an effect on the export of mature biologically active mTNF, since the differences in amounts of secreted mTNF are significant. Taking the amount of mTNF secreted under the influence of positive charges in the signal peptide on protein secretion.
ence of the wild-type signal peptide as a reference (100%), we found changes in the amounts of secreted mTNF to the following values for mutants with the indicated charges: 16% for 0, 80% for +1, 320% for +2, 80% for +4, and 50% for +5. This variation was also visualized by SDS-PAGE followed by Coomassie staining (Fig. 3B).

Immunoblot analysis of the recombinant mTNF produced by S. lividans transformants, each carrying a different mutant VSI signal peptide, showed the mature form and a few degradation products in the culture fluid. Cell lysates contained both the processed and the unprocessed form of mTNF together with a protein of intermediate size, which may be a degradation product of the precursor protein (Fig. 4). Whether the mature mTNF present in the cell extracts originates from the

![Bar graph showing mTNF production](image)

**FIG. 3.** (A) Intracellular, extracellular, and total amounts of mTNF produced by S. lividans under the influence of the different signal peptide mutants as obtained by biological activity measurements. (B) Coomassie staining of secreted proteins of S. lividans cultures harboring the different signal peptide mutants after trichloroacetic acid precipitation and SDS-PAGE. Equivalents of 50 μl of culture fluid were applied to the gel. Molecular mass markers are noted at the right. WT, wild type. (C) Graphical representation of efficiencies of secretion calculated as secreted mTNF/(secreted mTNF + intracellular mTNF) × 100.

![Immunoblot image](image)

**FIG. 4.** Immunoblot of secreted (lanes a) and intracellular (lanes b) mTNF. For each mutant (whose charges are noted above the lanes), an equivalent of 20 μl of culture fluid and a corresponding amount of cell lysate were used for SDS-PAGE and subsequent blotting. —, S. lividans(pIJ486).
cytoplasm or is attached to the cell wall or cell membrane was not further investigated.

Calculating the real efficiencies of secretion (Fig. 3C) reveals that although the wild type is most efficient in directing export of mTNF, the +2 mutant is able to secrete more. We found that 76% and 60% of the synthesized mTNF is secreted by the wild type and the +2 mutant, respectively.

Positive secretion effect correlates with increased mRNA level. To analyze a possible effect of the introduced mutations at the transcription level, total RNA was prepared from S. lividans cultures harboring the different mutant constructs, including the wild type. Results obtained after slot blotting and hybridization with an mTNF cDNA-specific probe together with an internal control to ensure that the cultures were located at approximately the same position on the growth curve are shown in Fig. 5. Therefore, a chromosomal stil-1 probe was used. As can be concluded from this figure in combination with Fig. 3A, the level of mTNF mRNA is different for each mutant and seems to be closely correlated with the total amount (intracellular and extracellular) of synthesized mTNF.

DISCUSSION

In this study, the promoter, the ribosome binding site, and the signal sequence of a recently isolated novel subtilisin inhibitor were used for the efficient production of extracellular mTNF. Up to 300 mg of biologically active mTNF per liter could be obtained. These yields equal the highest levels thus far described for the extracellular production of eukaryotic proteins by S. lividans (8). It should be noted that these values were obtained from shaken-flask cultures and can undoubtedly be increased in a more reproducible way by growing the strains under fermentation conditions. In the second part of our work, the number of positive charges in the VSI signal peptide were altered by in vitro mutagenesis. Subsequently, the effects of these mutations on transcription and secretion of mTNF were analyzed. Although none of the mutations resulted in a total obstruction of secretion, a clear variation in yield could be observed. The +2 mutant was able to secrete 3- to 10-fold more mTNF than the wild-type signal peptide (+3). Nevertheless, the wild-type signal peptide was most effective in secreting the produced mTNF. We can therefore conclude that the presence of positive charges in the N region is not absolutely required for protein export. This conclusion accords with those of previous reports for other organisms, such as E. coli (reviewed in reference 7) and Bacillus subtilis (3), and with that of a recent report on secretion of tendamistat by S. lividans (5).

Results of an analogous study for the production of another cytokine, which is secreted by the same system but in lesser amounts, also revealed a higher production of extracellular protein with two positive charges in the signal peptide (unpublished results). The variation in levels of mTNF when secretion was under the control of the different signal peptide mutants could be observed at the level of transcription as well. Our observations do not support the recent results of Fass and Engels (5), who observed an analogous increase in secretion with a +2 mutant of the tendamistat signal peptide, although they did not detect a difference in the amount of mRNA. They attributed the observed variation in secretion of tendamistat to a modulation at the level of precursor translation. From their results, i.e., the absence of any effect of the introduced mutations at the transcriptional level and the absence of precursor proteins, they suggested that tendamistat, being a small molecule of 74 amino acids, can be secreted by a prokaryotic equivalent of the eukaryotic signal recognition particle-dependent pathway. A role for the hydrophilic part of the signal peptide in this coupling mechanism between secretion and protein synthesis was suggested. This mechanism could be uncoupled by some mutations introduced in the hydrophilic region, which resulted in the accumulation of the precursor in the cells. However, in our work with mTNF, a polypeptide of 156 amino acids, the precursor could be detected in the cell lysates for each of the mutants. Contradictory results concerning a possible role of the signal peptide N region in a coupling between translation and translocation can be found in the literature. This hypothesis is supported by several studies of E. coli (9, 13, 16). On the other hand, Puziss et al. (17) and Chen and Nagarajan (3), working, respectively, on the export of maltose binding protein in E. coli and the secretion of levanucrase in B. subtilis, recently postulated that an effect of introduced mutations on translation, and subsequent export, is more likely to be a consequence of, for example, subtle alterations in the structure of mRNA which, in turn, may influence the efficiency of translational initiation or of some other, as yet undescribed, mechanism. Puziss et al. (17) suggested several lines of evidence for some kind of coupling mechanism between protein export and translation, but in a manner that does not involve the positively charged N region. As we are dealing with a eukaryotic protein, it is of interest to determine whether the same phenomenon is observed when a combination of the mutant VSI signal peptides is used together with the subtilisin inhibitor itself.

It is difficult to draw conclusions concerning the mechanisms responsible for effecting mTNF synthesis from our results. As we did not find significant changes in the secondary structure or stability of mRNA by analyzing the sequences using the FOLD algorithm, further experiments are needed to investigate the reasons for the variation in mRNA. From an industrial point of view, the results described above for the VSI secretory system are promising. Furthermore, modulating the signal peptide by changing the present charges may be a valuable tool to increase production of heterologous proteins in Streptomyces or other host organisms.
REFERENCES


