

Protein Secretion Biotechnology Using *Streptomyces lividans*: Large-Scale Production of Functional Trimeric Tumor Necrosis Factor α

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Abstract: We evaluated the feasibility of large-scale production of biopharmaceuticals expressed as heterologous polypeptides from the Gram-positive bacterium *Streptomyces lividans*. As a model protein we used murine tumor necrosis factor alpha (mTNF α). mTNF α fused C-terminally to the secretory signal peptide of the subtilisin-inhibitor protein from *Streptomyces venezuelae*. Under appropriate fermentation conditions, significant amounts of mature mTNF α (80–120 mg/L) can be recovered from spent growth media. Efficient downstream processing allowing rapid purification of mTNF α from culture supernatants was developed. Importantly, the protein is recovered from the spent growth medium in its native trimeric state as judged by biophysical analysis. Further, mTNF α secreted by *S. lividans* is significantly more active in an in vitro apoptosis tissue culture assay than a corresponding polypeptide produced in *Escherichia coli*. This pilot study provides the first validation of *S. lividans* protein secretion as an alternative bioprocess for large-scale production of oligomeric proteins of potential therapeutic value. © 2001 John Wiley & Sons, Inc. *Biotechnol Bioeng* 72: 611–619, 2001.

Keywords: tumor necrosis factor alpha; protein translocase; signal peptide; secretion; *Streptomyces*

INTRODUCTION

Streptomyces lividans has attracted significant interest over the past years as a potential host for heterologous polypep-

ptide secretion (Anné and Van Mellaert, 1993; Binnie et al., 1997a; Gilbert et al., 1995; Morosoli et al., 1997). Secretion by-passes the problem of inclusion body formation in the cytoplasm, very common in the better characterized *E. coli* system. Additional advantages of *S. lividans* include very efficient secretion directly into the growth medium, the absence of lipopolysaccharides and simple genetic manipulation (Hopwood et al., 1985), and low-protease activity. *Streptomyces lividans* has been used for the heterologous secretion of several polypeptides of bacterial and eukaryotic origin. In most cases, heterologous genes are fused to signal sequences from highly expressed/secreted endogenous *Streptomyces* proteins. Signal peptides act as address tags allowing secretory proteins to specifically recognize the Sec translocase on the target membrane (Economou, 1998; 2000; Izard and Kendall, 1994; Lammertyn and Anné 1998). Systematic mutational analysis of signal peptides has revealed them to be crucial determinants that can modulate the efficiency of secretion of heterologous proteins in *Streptomyces* (Lammertyn and Anné, 1998). Additional information from the well-understood molecular mechanism of protein secretion through the *E. coli* Sec pathway (Economou, 1998; 2000) is hoped to allow the optimization of many components of the *Streptomyces* secretion pathway.

Previously, we evaluated murine tumor necrosis factor alpha (mTNF α) as a heterologous secretion substrate and have demonstrated secretion of active mTNF α into the growth medium (Lammertyn et al., 1998; van Mellaert et al., 1994). In the course of this work we used the signal peptide and transcription elements of the *S. venezuelae* CBS762.70 subtilisin-inhibitor gene (*vs*). A mutation in the signal peptide that reduced the overall positively charged

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amino acid residues from +3 to +2 gave optimal mTNF α secretion (Lammertyn et al., 1998). The availability of *S. lividans* strains which can secrete high levels of mTNF α protein in analytical-scale experiments has allowed us to explore scaled-up growth of *S. lividans* carrying the mTNF α gene and to initiate the development of purification schemes from spent media. Most importantly, it allowed us to examine whether the mTNF α produced by *S. lividans* was biochemically and biologically active. This question was particularly important because mTNF α is trimeric (Smith and Baglioni, 1987; Wingfield et al., 1987) and it was not known how efficiently such a complex protein may assemble into its active form in *Streptomyces* spent media. Biological activity is not in itself a sufficient marker because even monomeric forms of mTNF α can be partially active (Smith and Baglioni, 1987).

We now present data for the development of large-scale secretion and downstream processing of mTNF α from *Streptomyces* cells and demonstrate that the heterologous protein thus produced is in a fully native state. These data are discussed in the context of a wider use of *Streptomyces lividans* as an alternative host for production of biopharmaceuticals.

RESULTS

Testing of Different Media for Optimal mTNF α Secretion in *Streptomyces lividans*

To determine the feasibility of large-scale mTNF α production optimal fermentation conditions were sought (Fig. 1). For rapid quantitation of mTNF α during growth and purification steps, a highly specific polyclonal anti-mTNF α antiserum was prepared (lane 7; see Materials and Methods). *Streptomyces lividans* cells were grown in five rich-growth media using shake-flask cultures: Luria-Bertani broth (LB; lane 2), Phage medium (lane 3), NM (Van Mellaert et al., 1994; lane 4), a modified Trypticase Soy broth medium (ST; lane 4; Binnie et al., 1997b) with (lane 5) or without (lane 6) supplementary dextrose (2.5 g/L). Supernatants were harvested after 4 d and the final yield in secreted mTNF α was determined. Cells grown in phage medium (Korn et al., 1978) showed no secretion of mTNF α either in Coomassie blue-stained gels (lane 3) or after immunostaining (data not shown). All other media tested gave rise to significant levels of a secreted protein migrating with an apparent molecular weight of 17,000 Da (lanes 2 and 4–6) and representing 40–80% of extracellular protein. This protein was shown by immunodetection using the anti-mTNF α antiserum and aminoterminal sequencing to correspond to murine TNF α (data not shown). When secreted protein yields are expressed as a percentage of cell mass produced, the ST medium can be shown to lead to optimal final yields of mTNF α (up to 150 mg/L: secreted proteins as % of cell mass). Secreted protein yields did not coincide with growth efficiency: LB medium allows the highest biomass yield (7.9 g/L) but displays the lowest levels of secreted material.

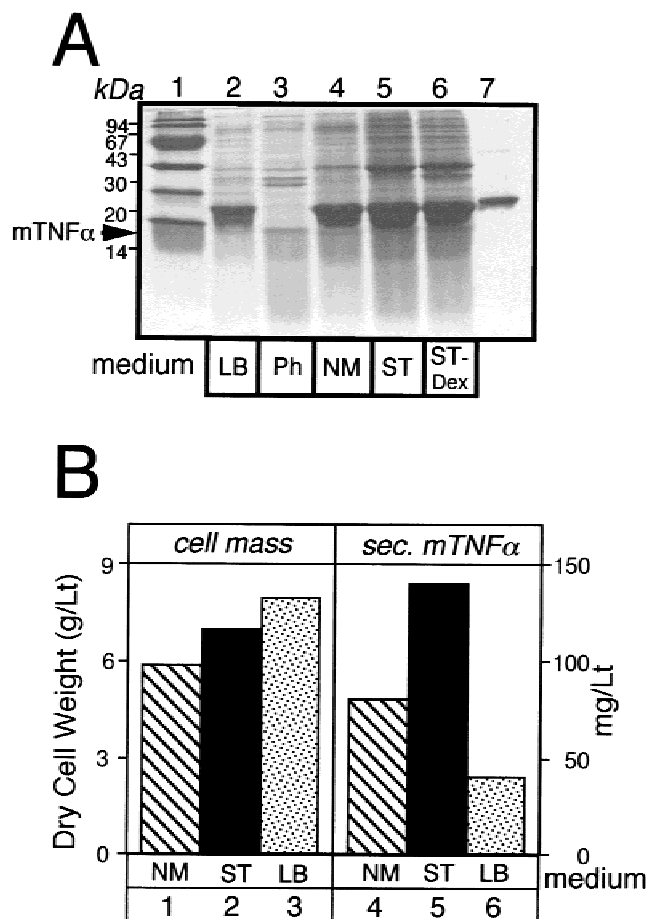


Figure 1. Evaluation of rich media for mTNF α secretion from *S. lividans*. Polypeptides (20 μ g/lane) from culture supernatants from cells grown for 4 days were harvested by precipitation with TCA (15%), analyzed by SDS-PAGE, and stained with Coomassie blue. (A) Lane 1, molecular weight markers: Phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), α -lactalbumin (14.4 kDa). Secreted mTNF α is indicated. Lane 7, His mTNF α . (B) Quantitation of secreted mTNF α in three of the previously tested media.

Growth Time-Course Studies and the Effect of Dextrose

Growth and secretion time-course experiments were used to determine optimal culture times. We define optimal time as that required for the highest possible mTNF α yields with the least possible contamination from other secreted proteins (or those derived from cell lysis) and with the lowest possible proteolytic degradation from extracellular proteases and aminopeptidases. In simple ST medium the optimal culture time was found to be between 4 and 6 d (Fig. 2). In this period of time the pH of the culture was found to increase steadily from an initial value of 7.0 to a final value of 9.5 after 9 d of growth. Final yield of secreted mTNF α in this period of time can be significantly increased by addition of exogenous carbon source known to affect production of other heterologous proteins (Kim et al., 1998; Parro and Mellado, 1994). Addition of dextrose at different concen-

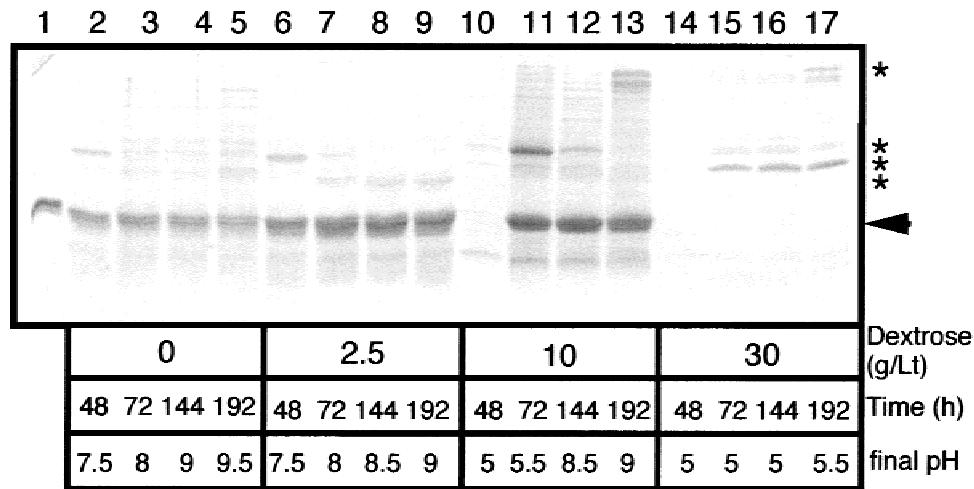


Figure 2. Time course of mTNF α secretion and the effect of additional carbon source. Polypeptides from culture supernatants from cells grown for 4 days were analyzed by SDS-PAGE and stained with Coomassie blue. (A) Lane 1, MW markers. Secreted mTNF α (filled arrow) and polypeptides expressed only under certain growth conditions (asterisks) are indicated. (B) Comparison of pH changes in the various media tested in A.

trations of up to 10 g/L led to a significant increase in the amount of secreted mTNF α (Fig. 3A, lanes 2–13). ST media containing 10 g/L of dextrose gave the highest observed yield of secreted mTNF α (200 to 300 mg/L in different experiments) and significantly shortened the optimal fermentation time to less than 72 h. In this medium we observed an initial lag time after inoculation that was very prominent (lane 10). This lag correlates well with the low pH measured in the medium under the same conditions. Strikingly, cells grown in the presence of 30 g/L dextrose had an even more pronounced secretion defect (lanes 14–17). Only a few secreted proteins could be detected (lane 17; indicated by asterisks) and these did not include the mTNF α polypeptide. Reduced secretion coincided with an acidic culture pH that was maintained throughout the monitored growth period. Because cell growth is slower but not arrested under these conditions, excess carbon source and/or low pH in the medium differentially affect the biosynthesis/export of secretory proteins. In contrast to previous observations (Kim et al., 1998), fed-batch introduction of the dextrose did not alleviate suppressed growth (data not shown).

Purification of mTNF α Secreted from *Streptomyces lividans*

Having defined optimal growth conditions for high-yield secretion of recombinant mTNF α , it was possible to proceed in establishing a purification scheme for secreted mTNF α (Fig. 3 and Table I). The first crucial step in isolating mTNF α from spent *S. lividans* culture medium was achieved by means of ultrafiltration. This step allowed a routine concentration of 10 L spent supernatants down to 0.2 L within a period of 4 h with a loss of TNF of less than 5% (lane 4). Moreover, we chose to use an ultrafiltration membrane with a 30 kDa exclusion limit and therefore this

step led to a modest (1.1-fold) purification by removing several low-molecular-weight contaminants, such as the prominent subtilisin-inhibitor protein (lane 3). This was possible because the TNF proteins are trimers of a 17 kDa subunit (Smith and Baglioni, 1987; Wingfield et al., 1987; and see below). An additional advantage of ultrafiltration was the removal of low-molecular-weight secondary metabolites and pigments produced during culture as well as media components that interfere with chromatography (data not shown). Ultrafiltration retentates containing mTNF α were further concentrated and purified 2.2-fold by ammonium sulfate precipitation (lane 5). More than 90% of TNF was isolated in the 60% ammonium sulfate saturation fraction and several proteinaceous contaminants thus removed. Salt removal and additional purification were achieved by gel filtration (lane 6). Ion-exchange chromatography allowed the isolation of mTNF α of more than 98% purity as judged by Coomassie-stained SDS-PAGE gels (lane 7). The purified protein was shown by aminoterminal sequencing (data not shown) and by anti-mTNF α staining

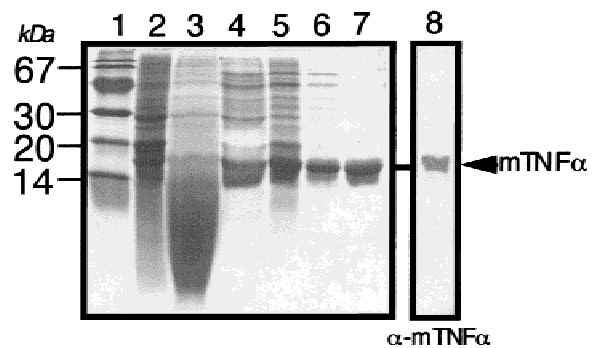


Figure 3. Large-scale purification of mTNF α from *S. lividans* culture supernatants. Protein samples (20 μ g/lane) from the various purification steps described in Table I were analyzed as in A and were stained with Coomassie blue. Lane 8, anti-mTNF α staining on western blots.

Table I. Purification of mTNF α secreted from *Streptomyces lividans*.

Purification step	Total protein (mg)	Purification factor
Culture supernatant	2,500	1.0
Ultrafiltration retentate	2,270	1.1
(NH ₄) ₂ SO ₄ precipitation	1,140	2.2
Sephacryl S-200	950	2.6
Mono Q HR 10/10	800	3.2

of Western blots (lane 8) to be correctly processed murine TNF α .

Biophysical and Biochemical Characterization of Secreted mTNF α

To evaluate the quality of mTNF α produced as a *S. lividans*-secreted protein it was important to determine whether the purified material is structurally and functionally intact. Size-exclusion chromatography revealed that the mTNF α protein has a native molecular weight of ~52 kDa (Fig. 4, panel A) that is in good approximation with the expected molecular weight of the native trimeric molecule (Smith and Baglioni, 1987; Wingfield et al., 1987). In contrast, the *E. coli*-produced His mTNF α protein has a calculated apparent molecular weight of ~34 kDa as determined using the same conditions of size-exclusion chromatography and is therefore dimeric. Far-UV circular dichroism analysis (panel B) of the two mTNF α proteins in the range of 200–250 nm reveals a curve that is of relatively low intensity with a trough centered at 220 nm, an overall shape typical of an all β -sheet protein and identical to that reported for native and recombinant murine and human TNF α (Hlodan and Pain, 1994; Narhi et al., 1996; Wingfield et al., 1987). Thermal denaturation curves are in good agreement with previous observations and reveal that the intensity of the negative ellipticity increases dramatically with increasing temperature. Both mTNF α proteins unfold cooperatively, but not reversibly with an apparent transition midpoint in the range of 67–68°C (Fig. 4C). Such a melting point is consistent with values reported in the past for murine TNF α (66°C at pH 7; Narhi et al., 1996).

We conclude that mTNF α secreted from *Streptomyces lividans* acquires the secondary, quaternary and, presumably, the tertiary organization of the native molecule.

Assay of mTNF α Biological Function

Purified mTNF α was tested for its ability to induce apoptosis in mouse fibrosoma cell lines as described previously (Heremans et al., 1990; Lattime and Stutman, 1992). *Streptomyces lividans*-produced mTNF α was found to be very active, because it inhibited cell growth and caused extensive cell death at concentrations as low as 1 ng/mL (Fig. 5A). The *S. lividans* recombinant mTNF α is approximately one order of magnitude more efficient than commercially avail-

able mTNF α produced in *E. coli* (Fig. 5B, compare lane 2 with lane 3) or the His mTNF α we synthesized (lane 1). We conclude that mTNF α produced as a secreted polypeptide from *S. lividans* cells is biologically fully functional.

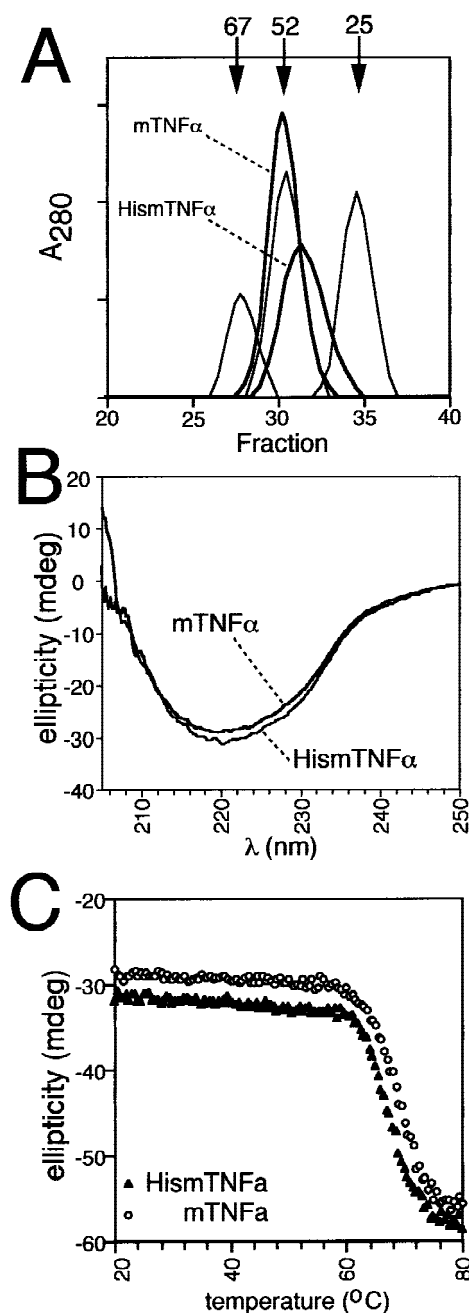


Figure 4. Structural characterization of *S. lividans*-secreted mTNF α . (A) Secreted mTNF α is dimeric. Purified mTNF α (30 μ g) and His mTNF α (35 μ g) were chromatographed through a gel-filtration column and their native molecular weights determined by calculating their relative migration compared to that of proteins of known molecular weight (arrows indicate the positions of three of the marker proteins used: bovine serum albumin [67 kDa]; ovalbumin [43 kDa]; chymotrypsin [25 kDa]). (B) Far-UV-CD spectra of mTNF α proteins. Experiments were carried out at ambient temperature. (C) Thermal denaturation curves. Purified mTNF α (100 μ g) and His mTNF α (100 μ g) were exposed to gradual temperature rise and spectra determined at 222 nm as described in Materials and Methods.

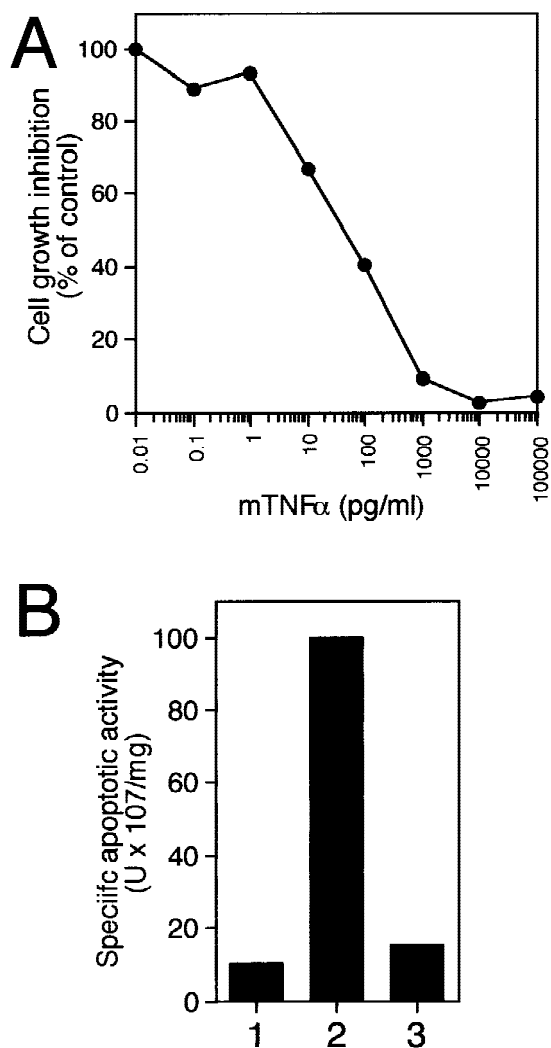


Figure 5. Biological activity of *S. lividans*-secreted mTNF α . (A) Assessment of dose-response biological effect of recombinant mTNF α on WEHI-164-13 mouse fibrosoma cells (Lattime and Stutman, 1992). Exponentially growing mouse fibrosoma WEHI-164-13 cells were plated in 96-well flat-bottom plates and incubated without and with varying concentrations of recombinant mTNF α produced by *S. lividans*. At the end of this period cell cultures were assessed for cell growth using the MTT assay that determines the proportion of dead cells as described in the Material and Methods section. (B) The apoptotic potential of mTNF α produced *S. lividans* (lane 2), HismTNF α produced in *E. coli* (lane 1) on WEHI-164-13 cells was compared to that of mTNF α produced in *E. coli* (without a Hexahistidine tag) and supplied commercially (lane 3).

DISCUSSION

It has been argued that secretion in the growth medium by *S. lividans* cells can be a viable alternative to more traditional methods for heterologous production of high-added-value polypeptides. We tested this possibility and present the first pilot scheme for large-scale production of functional murine TNF α protein expressed heterologously by *S. lividans*. Several heterologous proteins of bacterial (Anné and Van Mellaert, 1993; Gilbert et al., 1995; Morosoli et al., 1997) and a few of mammalian origin (Bender et al., 1990; Binnie et al., 1997a, 1997b; Fornwald et al., 1993; Noack et

al., 1988; Taguchi et al., 1995; Ueda et al., 1993) can be secreted from *Streptomyces* cells usually in small-scale experiments. We find that the mTNF α protein can be produced at high levels under various fermentation conditions. In certain cases the secreted form represents more than 60% of the total protein present in the spent growth media (Figs. 1, 2). Accumulation of the protein was observed under prolonged incubations (up to 190 h) suggesting that, unlike other heterologous polypeptides (DelaCruz et al., 1992), secreted mTNF α is very stable. Nevertheless, extended incubation may also lead to accumulation of contaminating secretory proteins, proteolytic degradation or products of cell lysis (Fig. 2). This may result in an increase in purification efforts and time for biopharmaceuticals that require a high degree of purity. Addition of optimal carbohydrate amounts can lead to maximal product yields in less than 72 h (Fig. 2). Our data indicate that further reduction of this time period can be achieved by fine-tuning the fermentation protocol. It is obvious that the ability to reduce incubation times can prove valuable in the production of inherently unstable enzymes and reduce processing costs. The remarkable enrichment of the recombinant protein in the culture supernatant allows mTNF α purification to near-homogeneity using a simple and rapid chromatographic scheme with final yields exceeding 80%. The ST medium used offers a significantly better yield than the previously tested ones (Lammertyn et al., 1997) and, moreover, it drastically reduces aminoterminal cleavage of the secreted mTNF α (data not shown).

To develop *Streptomyces*-secretion biotechnology it is important to understand how different physiological conditions affect protein secretion. During development of fermentation protocols we identified growth media that block secretion of some polypeptides, including recombinant mTNF α , but not of others (Figs. 1, 2). This was particularly prominent in the presence of excess carbon source known to cause medium acidification (DelaCruz et al., 1992; Kim et al., 1998; Madden et al., 1996). Using the anti-mTNF α antibody we did observe accumulation of mTNF α intracellularly in acidified medium (data not shown). This would suggest that acidification may, at least in part, influence the protein-secretion pathway. Excess glucose or batch addition of large amounts of nutrients inhibits secretion but not biosynthesis of bacterial and fungal enzymes from *Streptomyces* (DelaCruz et al., 1992; Kim et al., 1998; Parro and Mellado, 1994). Secretion may be affected by the level (Oh and Liao, 2000) or activity of SecA, the only translocase subunit whose biosynthesis is regulated (Economou, 1998) and by the activities of the four signal peptidases (Lammertyn and Anné, 1998; Parro et al., 1999). Genome-wide approaches such as DNA arrays (Oh and Liao, 2000) are expected to contribute to the molecular understanding of the interplay between the *Streptomyces* metabolic processes and protein secretion. The wide variation in the profile of endogenous *Streptomyces*-secreted proteins under different growth conditions, makes the establishment of strict culture

conditions very important for reproducibility of subsequent purification schemes for heterologous proteins.

Bioprocess development for secreted proteins from *S. lividans* shows some challenging differences compared to the more common intracellular production of proteins in other unicellular hosts. Fermentation times are longer and the mycelial nature of the cells poses distinct problems such as clotting of filters and inefficient cell removal by centrifugation. Despite the wealth of knowledge concerning *Streptomyces* fermentation targeted an antibiotic production, additional efforts will be required to optimize heterologous protein secretion in this host. Secreted proteins from *Streptomyces* can undergo hydrolytic attack from proteases and/or aminopeptidases (Butler et al., 1995; 1996; Lammertyn et al., 1997) and it is not clear how efficiently heterologous proteins can be folded upon secretion. To address this we examined the quality and biochemical state of the secreted polypeptide. Recombinant mTNF α secreted by *S. lividans* in large-scale preparations is properly folded, has acquired a correct quarternary organization and is highly active in biological assays. Such extensive characterization is particularly important in view of the fact that even monomeric (Smith and Baglioni, 1987) or dimeric (Fig. 4A) mTNF α retain partial activities (Fig. 5B) of the fully assembled trimeric molecule. These results suggest that it may be possible to produce other complex polypeptides from *S. lividans* cells in large-scale processes in an active form.

In conclusion, the secretion-based approach developed here for mTNF α offers significant bioprocessing advantages. Procedures established for intracellular inclusion-body isolation from *E. coli* expression are frequently complex, multistage, require polypeptide denaturation with a chaotrope and refolding. We propose that the alternative process developed here could be applicable to other polypeptides of biopharmaceutical importance at an industrial scale.

MATERIALS AND METHODS

Bacterial Strains and Recombinant DNA Experiments

Escherichia coli strains DH5a, JM109, BL21 (DE3) and BL21.19 (Mitchel and Oliver, 1993) carrying various plasmids were used. Growth and manipulations were as described (Karamanou et al., 1999). DNA manipulations for *E. coli* were as described by Ausubel et al., (1994) and those for *Streptomyces* as described by Hopwood et al., (1985). The identity of all constructs was confirmed by DNA sequencing and/or restriction analysis. *Streptomyces lividans* strain TK24 carrying plasmid pCBS2mTNF α +2 that expresses mTNF α (Lammertyn et al., 1997) was used throughout this study. In this construct the *N*-terminus of mTNF α is fused to a mutant derivative of the signal peptide subtilisin inhibitor of *Streptomyces venezuelae* CBS762.70.

Construction of HismTNF α

The coding sequence of mTNF α was amplified with primers MamTNF (5'-tatcaacatgaccatcaccaccatcacgtaagatcaagtagtcaaaattcg-3' (left) and mTNFSTOP (5'-gactccaaagtagacctgcc-3') (right) using PCR with pSelm TNF α as template. pSelmTNF α is a pAlter-1 (Promega) derivative containing the mTNF α cDNA as a *Bam*HI-*Eco*RI restriction fragment. The left primer introduces an *Nde*I site and a sequence encoding the 6His-tag. The generated product was digested with *Nde*I and *Eco*RI and the isolated fragment was ligated into vector pET3a (Novagen) digested with *Nde*I and *Eco*RI yielding pETmTNF α .

Bacterial Growth and Fermentation

All *Streptomyces* growth was in the presence of thiostrepton (5 μ g/mL) to select for plasmid maintenance. Media used in this study were: phage medium (Korn et al., 1978) (per liter: 10 g glucose, 5 g tryptone, 5 g yeast extract, 5 g LabM, 0.74 g CaCl₂ · 2H₂O, 0.5 g MgCO₄ · 7H₂O, pH: 7.2), NM medium (Van Mellaert et al., 1994) (per liter: 25 g LabM, 0.05M MOPS pH 7.2), LB and TS, a modification of the trypticase soy broth (Binnie et al., 1997b) (per liter: 17 g tryptone, 3 g bacto soytone, 5 g NaCl, 2.5 g K₂HPO₄, pH: 7.2–7.5). Shake-flask studies were conducted in 2-L Erlenmeyer flasks containing 1-L liquid medium. Fermentor growth was carried out in bioengineering 50-L (30-L working capacity) or 20-L (15-L working capacity) fermentors. All cultures were grown at 28°C (for shake-flask cultures a refrigerated shaking incubator was used; New Brunswick). Spore suspensions of *S. lividans* TK24 cells were stored at –80°C in 20 v/v glycerol. Inocula were prepared by transferring spore suspensions (0.3–1% v/v depending on the density of the spore suspension) in appropriate culture media. Dry cell weight determination was carried out by spotting culture aliquots on preweighted Whatman filter paper followed by drying at 100°C.

Chemicals and Biochemicals

Dithiothreitol (DTT), sodium dodecyl sulfate (SDS), lysozyme, nucleases, Coomassie brilliant blue, thiostrepton (minimum 90%, HPLC-grade), detergents, and imidazole were from Sigma (St. Louis, MO). Bacto soytone from Difco Laboratories (Detroit, MI), LabM Nutrient Broth No2 from B.P. (England). Restriction enzymes, T4 ligase, calf alkaline phosphatase were from New England Biolabs (Beverly, MA) or MINOTECHbiotechnology; oligonucleotides from Amersham Pharmacia Biotech. dNTPs were from Promega (Madison, WI). Sequenase and ³⁵S-labeled dNTPs were from Amersham Pharmacia Biotech. Commercially available mTNF α was supplied by Innogenetics.

Protein Purification and Chromatography

All chromatography resins and molecular weight markers were from Amersham Pharmacia Biotech unless otherwise stated. Purified proteins were stored at –20°C.

Synthesis of HismTNF α in *Escherichia coli*

HismTNF α (see above) cloned in *E. coli* was overexpressed (Karamanou et al., 1999). A protein with the anticipated molecular weight of 17,000 Da corresponding to HismTNF α is produced in high amounts as seen in Coomassie blue-stained SDS-polyacrylamide gels (>20% total protein). More than 80% of the protein is in a soluble form and was purified to homogeneity using a single-step metal-chelate chromatography on nickel-nitrilotriacetic acid agarose (QIAGEN) according to manufacturer's instructions. This protein was shown by immunostaining using an anti-hexahistidine-specific antibody (Clonotech) to carry the N-terminal hexahistidine tag.

mTNF α Purification from *Streptomyces* Culture Supernatants

The protocol that follows has been optimized for 800–1000 mg purified mTNF α protein derived from a 10 L culture of *S. lividans*. On rare occasions, protein yield was 3–4-fold reduced due to genetic instability. Cells were grown for 4 d at 28°C. Supernatants were collected after harvesting and discarding the cells.

Step 1—Ultrafiltration The 10 L supernatant was concentrated at 4°C down to 0.1 L with the Minitan II Ultrafiltration System (MILLIPORE; mounted with PTTK Filter Plates, 30,000 NMWL, polysulfone). The Minitan apparatus was rinsed with 100 mL of 20 mM Tris-HCl pH: 7.4 to recover all proteins retained.

Step 2—Ammonium Sulphate Fractionation Secreted polypeptides from concentrated spent growth media were precipitated on ice by slow addition of finely ground (NH₄)₂SO₄ under constant stirring and collected by centrifugation (Sorval, JA20 rotor, 4°C, 20 min, 10,000 rpm). Pellets were resuspended in 5 mL of buffer A (20 mM Tris-HCl pH: 8.0, 0.3M NaCl).

Step 3—Size-Exclusion Chromatography (SEC) Resuspended samples were chromatographed on a Sephacryl S-200HR 26/100 column equilibrated with 2 column volumes of buffer A and fractions were analyzed by SDS-PAGE.

Step 4—Ion Exchange Chromatography SEC Fractions containing mTNF α were pooled and diluted with 20 mM Tris-HCl pH: 8.0 to bring the final concentration of NaCl down to 0.08M and were loaded on a Mono Q HR10/10 (equilibrated with buffer B: 20 mM Tris-HCl pH: 8.0, 0.08M NaCl). The column was washed with 10 column volumes of buffer B and then proteins were eluted with a 900 mL 0.1–0.6M NaCl linear gradient.

Molecular Weight Determination Using SEC A pre-packed Superdex 200HR 10/30 column (10–600 kDa separation range) mounted on a FPLC system at 4°C was used (flow rate 0.4 mL/min; back-pressure ~1 Mpa). The column was equilibrated with 50 mM Tris-HCl pH: 7.2, 300 mM NaCl, 5 mM β -mercaptoethanol. Samples were injected via a V-7 valve, using a 200- μ L loop. MW determination of

mTNF α using the migration distances of naive marker proteins (LMW kit) and of Blue Dextran 2000 (void volume determination) was as recommended (Amersham Pharmacia Biotech).

Circular Dichroism-Thermal Stability

Circular dichroism (CD) spectra in the far ultraviolet range were recorded on a Jasco J-715 spectropolarimeter supplied with a Peltier element as described (Karamanou et al., 1999). Spectra were recorded at 20°C, pH: 7.6 and represent the average of six scans. Thermal denaturation curves were obtained by monitoring the ellipticity at 222 nm upon heating of the protein samples at 60°C/h. This wavelength was chosen because it is very close to the minimum ellipticity for the native protein and, more importantly, lies in a region of the spectrum where noise does not increase appreciably with temperature.

mTNF α Functional Assay

The amount of biologically active mTNF- α in the supernatants of *S. lividans* cultures or in the purified samples was determined as described (Heremans et al., 1990) and by using recombinant mTNF- α produced in *E. coli* (Innogenetics N.V., Ghent, Belgium) as a standard. Briefly, mouse Fibrosoma WEHI-164-clone 13 cells (Lattime and Stutman, 1992; purchased from ATCC, USA) were plated in 96-well flat-bottom plates and incubated in a humidified atmosphere at 37°C with 5% CO₂. Cytotoxicity of mTNF α towards exponentially growing WEHI-164-cells (3×10^4) was spectrophotometrically evaluated in triplicate series of experiments via the in situ reduction of 500 ng/mL MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Thiazolyl blue]. Reduced MTT was measured spectrophotometrically with an automated plate reader (Stat Fax-2100, Awareness Technology Inc., USA) at 570 nm after lysis of cells with 200 μ L DMSO. Control wells containing medium plus cells (total absorbance) or medium alone (background absorbance) served as internal controls. Cell growth was calculated as a percentage of MTT reduction as follows: %growth = (mean experimental absorbance/mean control absorbance) \times 100.

Miscellaneous Techniques

Protein concentration was determined either by using the Bradford reagent (Bio-Rad) with BSA as a standard, by UV absorbance, or by amino acid analysis. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (17.5% gels) was performed as described by Karamanou et al. (1999). Anti-mTNF α antisera were prepared as described (Hadzidakis et al., 1993). Quantitative immunoblotting was as described (Manting et al., 2000) using HismTNF α as control.

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References

- Anné J, Van Mellaert L. 1993. *Streptomyces lividans* as host for heterologous protein production. *FEMS Microbiol Lett* 114:121–128.
- Ausubel FM, Brent R, Kingston RE, Moore DD, Smith JA, Seidman JG, Struhl K. 1994. *Current protocols in molecular biology*. New York: John Wiley & Sons.
- Bender E, Koller KP, Engels JW. 1990. Secretory synthesis of human interleukin-2 by *Streptomyces lividans*. *Gene* 86:227–232.
- Binnie C, Cossar JD, Stewart DI. 1997a. Heterologous biopharmaceutical protein expression in *Streptomyces*. *Trends Biotechnol* 15:315–320.
- Binnie C, Jenish D, Cossar D, Szabo A, Trudeau D, Krygsman P, Malek LT, Stewart DI. 1997b. Expression and characterization of soluble human erythropoietin receptor made in *Streptomyces lividans* 66. *Protein Expr Purif* 11:271–278.
- Butler MJ, Binnie C, DiZonno MA, Krygsman P, Soltes GA, Soostmeyer G, Walczyk E, Malek LT. 1995. Cloning and characterization of a gene encoding a secreted tripeptidyl aminopeptidase from *Streptomyces lividans* 66. *Appl Environ Microbiol* 61:3145–3150.
- Butler MJ, Aphale JS, Binnie C, DiZonno MA, Krygsman P, Soltes G, Walczyk E, Malek LT. 1996. Cloning and analysis of a gene from *Streptomyces lividans* 66 encoding a novel secreted protease exhibiting homology to subtilisin BPN'. *Appl Microbiol Biotechnol* 45:141–147.
- DelaCruz N, Payne GF, Smith JM, Coppella SJ. 1992. Bioprocess development to improve foreign protein production from recombinant *Streptomyces*. *Biotechnol Prog* 8:307–315.
- Economou A. 1998. Bacterial preprotein translocase: mechanism and conformational dynamics of a processive enzyme. *Mol Microbiol MS* 27:511–518.
- Economou A. 2000. Bacterial protein translocase: A unique molecular machine with an army of substrates. *FEBS Lett* 476:18–21.
- Fornwald JA, Donovan MJ, Gerber R, Keller J, Taylor DP, Arcuri EJ, Brawner ME. 1993. Soluble forms of the human T cell receptor CD4 are efficiently expressed by *Streptomyces lividans*. *Biotechnology (NY)* 11:1031–1036.
- Gilbert M, Morosoli R, Shareck F, Kluepfel D. 1995. Production and secretion of proteins by streptomycetes. *Crit Rev Biotechnol* 15: 13–39.
- Hatzidakis G, Katrakili K, Krambovitis E. 1993. Development of a direct and specific enzyme immunoassay for the measurement of oestrone sulfate in bovine milk. *J Reprod Fertil* 98:235–240.
- Heremans H, Van Damme J, Dillen C, Dijkmans R, Billiau A. 1990. Interferon- γ , a mediator of lethal lipopolysaccharide-induced Schwartzman-like shock reactions in mice. *J Exp Med* 171: 1853–1869.
- Hlodan R, Pain RH. 1994. Tumour necrosis factor is in equilibrium with a trimeric molten globule at low pH. *FEBS Lett* 343:256–260.
- Hopwood DA, Bibb MJ, Chater KF, Kieser T, Bruton CJ, Kieser HM, Lydiate DJ, Smith CP, Ward JM, Schrepf H. 1985. *Genetic manipulation of Streptomyces: A laboratory manual*. Norwich, CT: John Innes Foundation.
- Izard JW, Kendall DA. 1994. Signal peptides: Exquisitely designed transport promoters. *Mol Microbiol* 13:765–773.
- Karamanou S, Vrontou E, Sianidis G, Baud C, Roos T, Kuhn A, Politou A, Economou A. 1999. A molecular switch in SecA protein couples ATP hydrolysis to protein translocation. *Mol Microbiol* 34: 1133–1145.
- Kim E, Shin DH, Irwin DC, Wilson DB. 1998. Fed-batch production of *Thermomonospora fusca* endoglucanase by recombinant *Streptomyces lividans*. *Biotechnol Bioeng* 60:70–76.
- Korn F, Weingärtner B, Kutzner HJ. 1978. A study of twenty actinophages: Morphology, serological relationship and host range. In: Freerksen E, Tarnak I, Thumin JH, editors. *Genetics of actinomycetales*. Stuttgart & New York: Fisher G. p 251–270.
- Lammertyn E, Van Mellaert L, Schacht S, Dillen C, Sablon E, Van Broekhoven A, Anné J. 1997. Evaluation of a novel subtilisin inhibitor gene and mutant derivatives for the expression and secretion of mouse tumor necrosis factor alpha by *Streptomyces lividans*. *Appl Environ Microbiol* 63:1808–1813.
- Lammertyn E, Anné J. 1998. Modifications of *Streptomyces* signal peptides and their effects on protein production and secretion. *FEMS Microbiol Lett* 160:1–10.
- Lammertyn E, Desmyter S, Schacht S, Van Mellaert L, Anné J. 1998. Influence of charge variation in the *Streptomyces venezuelae* alpha-amylase signal peptide on heterologous protein production by *Streptomyces lividans*. *Appl Microbiol Biotechnol* 49:424–430.
- Lattime EC, Stutman A. 1992. WEHI-164 clone 2F: In vitro antitumor effects of tumor necrosis factor and gamma-interferon. *Nat Immun* 11:34–45.
- Madden T, Ward JM, Ison AP. 1996. Organic acid excretion by *S. lividans* TK24 during growth on defined carbon and nitrogen sources. *Microbiology* 142:3181–3185.
- Manting EH, van Der Does C, Remigy H, Engel A, Driessen AJ. 2000. SecYEG assembles into a tetramer to form the active protein translocation channel. *EMBO J* 19:852–861.
- Mitchell C, Oliver D. 1993. Two distinct ATP-binding domains are needed to promote protein export by *Escherichia coli* SecA ATPase. *Mol Microbiol* 10:483–497.
- Morosoli R, Shareck F, Kluepfel D. 1997. Protein secretion in streptomycetes. *FEMS Microbiol Lett* 146:167–174.
- Narhi LO, Philo JS, Li T, Zhang M, Samal B, Arakawa T. 1996. Induction of alpha-helix in the beta-sheet protein tumor necrosis factor-alpha: thermal- and trifluoroethanol-induced denaturation at neutral pH. *Biochemistry* 35:11447–11453.
- Noack D, Geuther R, Tonew M, Breitling R, Behnke D. 1988. Expression and secretion of interferon-alpha 1 by *Streptomyces lividans*: Use of staphylokinase signals and amplification of a neo gene. *Gene* 68: 53–62.
- Oh MK, Liao JC. 2000. Gene expression profiling by DNA microarrays and metabolic fluxes in *Escherichia coli*. *Biotechnol Prog* 16: 278–286.
- Parro V, Mellado RP. 1994. Effect of glucose on agarase overproduction by *Streptomyces*. *Gene* 145:49–55.
- Parro V, Schacht S, Anné J, Mellado RP. 1999. Four genes encoding different type I signal peptidases are organized in a cluster in *Streptomyces lividans* TK21. *Microbiology* 145:2255–2263.
- Pöhling S, Piepersberg W, Wehmeier UF. 1997. Protein secretion in *Strep-*

- Streptomyces griseus* N2-3-11: Characterization of the *secA* gene and its growth phase-dependent expression. FEMS Microbiol Lett 156:21–29.
- Smith RA, Baglioni C. 1987. The active form of tumor necrosis factor is a trimer. J Biol Chem 262:6951–6954.
- Taguchi S, Misawa S, Yoshida Y, Momose H. 1995. Microbial secretion of biologically active human transforming growth factor alpha fused to the *Streptomyces* protease inhibitor. Gene 159:239–243.
- Ueda Y, Tsumoto K, Watanabe K, Kumagai I. 1993. Synthesis and expression of a DNA encoding the Fv domain of an anti-lysozyme monoclonal antibody, HyHEL10, in *Streptomyces lividans*. Gene 129:129–134.
- Van Mellaert L, Dillen C, Proost P, Sablon E, DeLeys R, Van Broekhoven A, Heremans H, Van Damme J, Eyssen H, Anné J. 1994. Efficient secretion of biologically active mouse tumor necrosis factor alpha by *Streptomyces lividans*. Gene 150:153–158.
- Wingfield P, Pain RH, Craig S. 1987. Tumour necrosis factor is a compact trimer. FEBS Lett 211:179–184.