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Promoter constructions for efficient secretion expression in *Streptomyces lividans*

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Summary. Promoters from different *Streptomyces* genes were cloned in front of the Tendamistat gene from *S. tendae*, in order to study secretion-expression in *S. lividans* using a pIJ702 plasmid vector system. Besides the promoters we cloned a transcriptional terminator downstream of the Tendamistat gene to improve transcription efficiency. The promoters we selected were: (1) a synthetic *Escherichia coli*-like consensus promoter; (2) the *aph*I promoter of the neomycin resistance gene from *S. fradiae*; (3) an *erm*E-up promoter mutant from *Saccharopolyspora erythraea*; (4) the *mel*C promoter of the tyrosinase operon from *Streptomyces antibioticus*. In addition, we tested the thiostrepton-inducible *tip*A promoter from *S. lividans* in our Tendamistat secretion system. The promoters were cloned upstream of the Tendamistat ribosome binding site in order to conserve the original translation initiation. The Tendamistat secretion mediated by the different promoter constructions above varied dramatically in up to 10 mg/l in the case of the synthetic promoter and the *aph* promoter, and up to 500 mg/l mediated by the *erm*E-up promoter. The *mel*C promoter allowed about 200 mg/l Tendamistat secretion and the *tip*A promoter proved to be inducible from less than 0.5 mg/l up to 40 mg/l of Tendamistat secretion. Based on the amount of secreted Tendamistat and on the analysis of mRNA levels, we conclude that transcriptional activity regulates the efficiency of our secretion-expression system.

Introduction

Tendamistat is a small, stable exoprotein from *S. tendae* that specifically inhibits α-amylases. It consists of 74 amino acids with two disulphide bridges. The inhibitor gene was isolated from *S. tendae*, cloned and sequenced by Koller et al. (1984, 1989). The gene encodes a signal sequence of 30 amino acids preceded by a typical ribosome binding site. The availability of its three-dimensional structure determined in parallel by X-ray cristallography by Pflugrath et al. (1986) and by nuclear magnetic resonance (NMR) techniques, ^{1}H-NMR by Kline et al. (1986) and ^{13}C-NMR by Kessler et al. (1990), make it an ideal model for protein design studies. The regulatory sequences of the Tendamistat gene were used to construct a secretion-expression system in *S. lividans* for the production of heterologous genes such as human interleukin-2 (Bender et al. 1990a), hirudin (Bender et al. 1990b) and human proinsulin (Koller et al. 1989). The heterologous genes were fused directly with the Tendamistat signal sequence, or with the complete inhibitor sequence in the case of proinsulin.

The gene products were secreted by transformed *S. lividans* cells, but in very low amounts and only part of the protein was found to be correctly processed and to be biologically active. Other examples for secretion-expression of heterologous genes by *Streptomyces* have been described for tumour necrosis factor (Chang and Chang 1988), interferon α-1 (Noack et al. 1988) and interleukin-1β (Lichtenstein et al. 1988). In order to evaluate our secretion system, we decided to keep the Tendamistat ribosome binding site and the leader sequence constant and to vary the promoter region. The results of Koller et al. (1989) had already shown that Tendamistat secretion is possible in high amounts, and thus we argue that translation and secretion are not limiting factors. Furthermore, the Tendamistat gene is an ideal secretable detector gene for the analysis of promoter activity, because it is easy to detect and to quantify by activity test, HPLC analysis or immunoblotting.

A synthetic transcriptional terminator was fused downstream of the inhibitor gene, to define the 3′-end of the transcripts (Pulido and Jimenez 1987). The following promoters were chosen:

1. A synthetic promoter based on a consensus sequence for *Streptomyces-E. coli*-like promoters (Hopwood et al. 1986) (Fig. 1).
2. The promoters P1 and P2 of the neomycin resistance gene (*aph*I) from *S. fradiae* (Thompson and Gray 1983; Janssen et al. 1989) (Fig. 1).

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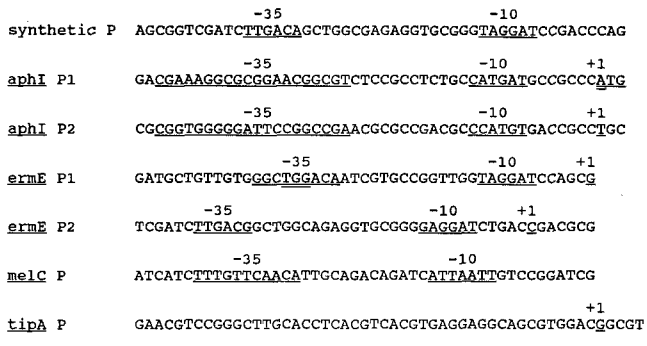

Fig. 1. Promotor sequences. The sequences of the different promoters are shown: the synthetic promoter, *aph*I (Thompson and Gray 1983), *erm*E (Bibb et al. 1985b), *mel*C (Bernan et al. 1985) and *tip*A promoter (Murakami et al. 1989). Transcriptional startpoints, −10 and −35 region are *underlined*. The 3-bp deletion (TGG) of the *erm*E P1 promoter is *double underlined*

3. The P1 and P2 promoters of the erythromycin resistance gene (*erm*E) from *Saccharopolyspora erythraea* (Bibb et al. 1985b), where the *erm*E P1 promoter carries a 3-bp deletion in the −35 region (Bibb and Janssen 1986) causing a promoter-up mutation (Fig. 1) and
4. The *mel*C promoter of the tyrosinase gene from *Streptomyces antibioticus* (Katz et al. 1983; Bernan et al. 1985) (Fig. 1).
5. The thiostrepton-inducible *tip*A promoter from *S. lividans* (Murakami et al. 1989) (Fig. 1), obtained from C. J. Thompson was tested.

Materials and methods

Bacterial strains, transformation and culture methods. Standard media and methods of culture and transformation of *Streptomyces* are described in Hopwood et al. (1985). *S. lividans* TK 24 (Hopwood et al. 1983) was used as host strain for all expression vectors that were derivatives of the *Streptomyces* high copy number plasmid pIJ702 (Katz et al. 1983). *S. lividans* was transformed by protoplast transformation (Chater et al. 1982) and transformants were selected by thiostrepton. The medium used for transformation was R2YE as described by Hopwood et al. (1985). The thiostrepton-resistant *S. lividans* clones were plated on R2YE containing 50 µg/ml of thiostrepton. Tendamistat expression was measured in 50 ml liquid culture containing 5% glucose, 2% casamino acids, 0.2% ammonium sulphate, 15 mM phosphate buffer, pH 6.9, 3 mM magnesium sulphate, trace elements and thiostrepton (5 µg/ml). The dry weight of *Streptomyces* cells was determined by collecting the cells from 1 ml culture in weighed Eppendorf caps, washing and drying at 80° C. *E. coli* K12 strain BMH 7902 was grown in 2YT medium according to Maniatis et al. (1982). Cloning and DNA sequencing was done in pUC18 plasmids described by Yanisch-Perron et al. (1985).

E. coli was transformed by previously described methods (Maniatis et al. 1982) and the transformants were selected on 2YT agar containing ampicillin. To detect β-galactosidase activity, agar plates were flooded with 40 µl DMF containing X-gal and IPTG. For in-vitro mutagenesis, M13 mp18 phage was used as vector (Messing and Vieira 1982; Kramer et al. 1984) and *E. coli* K12 strain BMH 71-18 was used as the host.

DNA isolation, manipulation and characterization. Large-scale preparation of *Streptomyces* plasmids are described in Hunter (1985); small-scale preparations were performed according to MacNeil (1986). *E. coli* plasmids and double-stranded M13 phage DNA were isolated as described in Maniatis et al. (1982). Oligodeoxynucleotides were synthesized on an Applied Biosystems (Foster City, Calif., USA) DNA synthesizer 380A using the cyanoethoxyphosphoramidite method (Sinha et al. 1983). Restriction endonucleases, T4 DNA ligase, Klenow enzyme, Taq DNA polymerase and T4 polynucleotide kinase were purchased from New England Biolabs (Beverly, MA, USA) and T7 DNA polymerase from Pharmacia (Uppsala, Sweden). All enzymes were used according to the manufacturer's recommendations. Sequencing of plasmid DNA or M13 phage DNA was performed according to Sanger et al. (1977), using [^{35}S]-α-dATP (1000 µCi/mmol, Amersham, Braunschweig, FRG) and T7 DNA polymerase or Taq DNA polymerase. All described constructions were sequenced on M13 phage-, pUC18- or on pTT1 level and were controlled by restriction length analysis.

Protein assays, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. Tendamistat expression was measured in the supernatants of liquid cultures using the modified dinitrosalicylate (DNSS) method according to Bergmeyer (1970). Culture supernatants (10 µl) were analysed by SDS-PAGE according to Schägger and von Jagow (1987), followed by Coomassie-blue staining or transfer to Immobilon membranes (Millipore, Freehold, NJ, USA) according to Plough et al. (1989).

Immunodetection was carried out using a Tendamistat-specific polyclonal rabbit antibody. A goat antirabbit immunoglobulin C conjugated with alkaline phosphatase (Sigma, München, FRG) was used as second antibody. Enzyme activity was detected with 5-bromo-4-chloro-3-indolyl phosphate-toluidine salt (Biorad, Richmond, Virgin., USA) and *p*-nitro blue tetrazolium chloride (Biorad) according to the manufacturer's recommendations.

RNA isolation and Northern blots. Cells from 1 ml samples of *S. lividans* liquid cultures were collected, washed with 1 × phosphatebuffered saline (PBS) (150 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.9 mM KH_2PO_4) and resuspended in 200 µl buffer I (160 mM TRIS, pH 7.6, 20 mM $MgCl_2$, 20 mM β-mercaptoethanol). Then 200 µl proteinase K solution (2 mg/ml in 2 × SEPH-buffer (0.5% SDS, 10 mM EDTA, 2 mM 1,10-phenanthroline, 0.2 mg/ml of heparin, pH 6.0)) was added and the solution was incubated at 37° C for 20 min. After 10 min, the solution was sheared with a hypodermic needle. After incubation the solution was extracted twice with phenol/chloroform. The nucleic acid was precipitated from the aqueous phase with 2.5 vol ethanol and 0.1 vol 3 M NaOAc, pH 5.3, at −20° C. The precipitate was collected, dried and resuspended in 50 µl DNase mix: 50 mM NaAc, 25 mM $MgCl_2$, 4 mM $CaCl_2$, 20 mM Ribonucleoside-Vanadyl-Complex (New England Biolabs), 15 units RNase-free DNaseI (Boehringer, Mannheim, FRG) and incubated for 20 min at 37° C. The quality of the resulting RNA preparation was assessed by agarose gel electrophoresis, quantified spectrophotometrically and stored at −20° C for several months.

The RNA isolated was subjected to agarose gel electrophoresis and transferred to a Hybond N-Nylon membrane (Amersham) according to the method of Southern (1975). The RNA was fixed on dried filters by UV radiation (5 min, wavelength 302 nm). At this stage the 23S and 16S rRNA and the RNA molecular mass markers (Boehringer) could be detected. The membrane was transferred to the prehybridisation solution and incubated for 5 min at 65° C (Church and Gilbert 1984). The hybridisation probe (a 5′ ^{32}P-labelled 52-bp oligonucleotide) was added to the solution. After 4 h hybridisation at 65° C, the membrane was washed five times with 0.1 × SSC, 1% SDS at 60° C, dried at room temperature and subjected to autoradiography (about 4 days at −80° C in a Kodak X-OMATIC-cassette, Kodak AR film).

Construction of Tendamistat expression vectors. The construction of the different promoter-Tendamistat fusions and the insertion of a synthetic transcriptional terminator is described in Fig. 2.

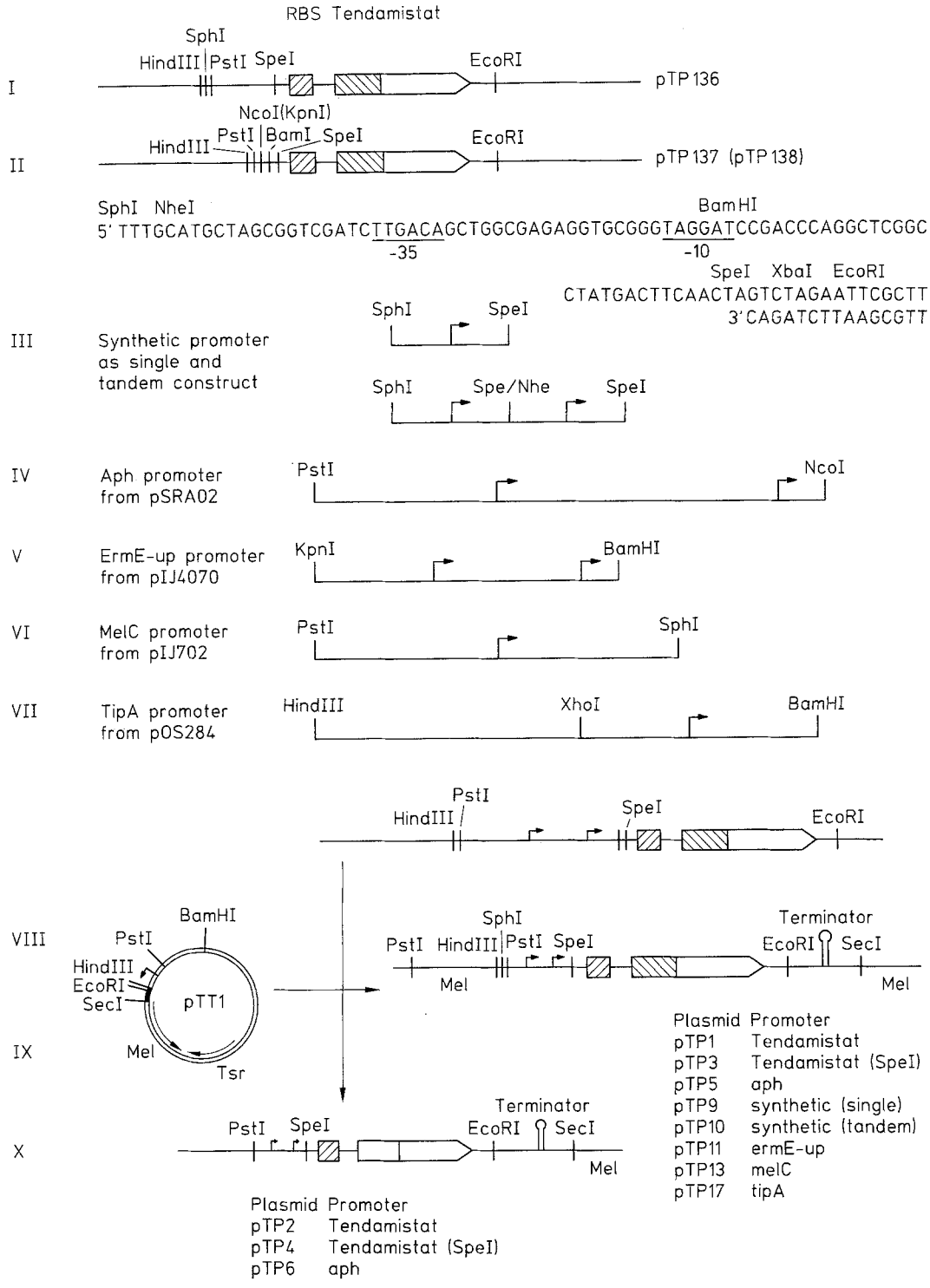

Fig. 2. Construction of the different promoter Tendamistat fusions. The 670-bp *Hin*dIII-*Eco*RI Tendamistat-fragment (Koller et al. 1989) was inserted into the phage-vector M13mp18: *I*, a single *Spe*I site was generated 15 bp upstream of the Tendamistat ribosome binding site by oligonucleotide-directed M13 mutagenesis and the gene was transferred into pUC18 (pTP136); *II*, the Tendamistat promoter region was exchanged by two different synthetic linkers (a *Pst*I-*Nco*I-*Bam*HI-*Spe*I-linker in pTP137 and a *Pst*I-*Kpn*I-*Bam*HI-*Spe*I-linker in pTP138); *III*, the synthetic consensus promoter was synthesized as a single 112-bp oligonucleotide and was made double-stranded, by using the "self priming method" (Uhlmann 1988). The double strand was cut by *Sph*I and *Spe*I and was inserted as single and tandem construct upstream of the Tendamistat ribosome-binding site into pTP136; *IV*, the *aph*I promoter region was cloned as the 644-bp *Pst*I-*Nco*I fragment in front of the Tendamistat gene (pTP137); *V*, the *erm*E-up promoter was cloned as the 270-bp *Kpn*I-*Bam*HI fragment into pTP138; *VI*, the *mel*C promoter region is already present in pTT1 (see VIII). So the promoterless Tendamistat gene derived from pTP137 was directly cloned downstream of the *mel*C promoter into pTT1. The resulting pTP13-plasmid (see IX) carries only the *mel*C promoter upstream of the Tendamistat ribosome-binding site; *VII*, the *tip*A promoter was cloned as a 600-bp fragment including the upstream arranged *fd* terminator in front of the Tendamistat gene; *VIII*, into pIJ702 a synthetic transcriptional terminator was introduced. The resulting *Streptomyces* vector pTT1 carries three single cloning sites marked by *arrows* (*Pst*I, *Hin*dIII and *Eco*RI); *IX*, the different promoter Tendamistat fusions were isolated from the pUC18 derivatives (II–VII) and cloned into pTT1 by *Hin*dIII and *Eco*RI (the resulting plasmids and promoters are *listed* below the diagram). In these cases the additional *mel*C-promoter is restored upstream of the insert; *X*, the different constructions were cloned by *Pst*I and *Eco*RI into pTT1 under loss of the additional *mel*C-promoter region (plasmids and promoters are *listed* below the diagram)

Cultivation of Streptomyces clones. Transformants of *S. lividans* TK 24 secretion-expression vectors were selected first by thiostrepton resistance and then by using a Tendamistat plate test. Strain were grown in liquid culture for 10 days, taking daily aliquots. The collected cells were subjected to dry weight determination. The culture supernatants were applied to the Tendamistat activity test (DNSS-test; Bergmeyer 1970) for quantification of the secreted inhibitor and to SDS-PAGE. Additional 1 ml samples were taken on the 3rd or 4th day of culture for RNA preparation. A *S. lividans* clone carrying the pTT1 plasmid was cultivated as a negative control.

Results and discussion

Constructions containing the original Tendamistat gene (pTP1 and pTP2) and the gene with the novel *Spe*I site (pTP3 and pTP4) secreted about 15–40 mg/l of α-amylase inhibitor. On SDS-PAGE only a poor band is visible (Fig. 3) and on Northern blot a single signal was detected (Fig. 4). Without the synthetic transcriptional terminator inserted downstream of the Tendamistat gene no distinct transcription band was observable. Contrary to the results reported by Pulido and Jimenez (1987) for the hIFN α-2 gene, the terminator insertion did not increase significantly the Tendamistat expression.

The synthetic *E. coli*-like consensus promoter (pTP9 and pTP10) mediated less than 10 mg/l of α-amylase inhibitor production (Table 1). This low secretion could only be detected on Coomassie-stained SDS-gels if fivefold concentrated culture supernatant was used. On Northern blot a very poor signal was observed (Fig. 4). Experiments by Takahashi et al. (1988) and Buttner et al. (1990) lead to the conclusion that more than one sigma-factor is recognizing *E. coli*-like promoters in *Streptomyces*. This leads to the assumption that more than

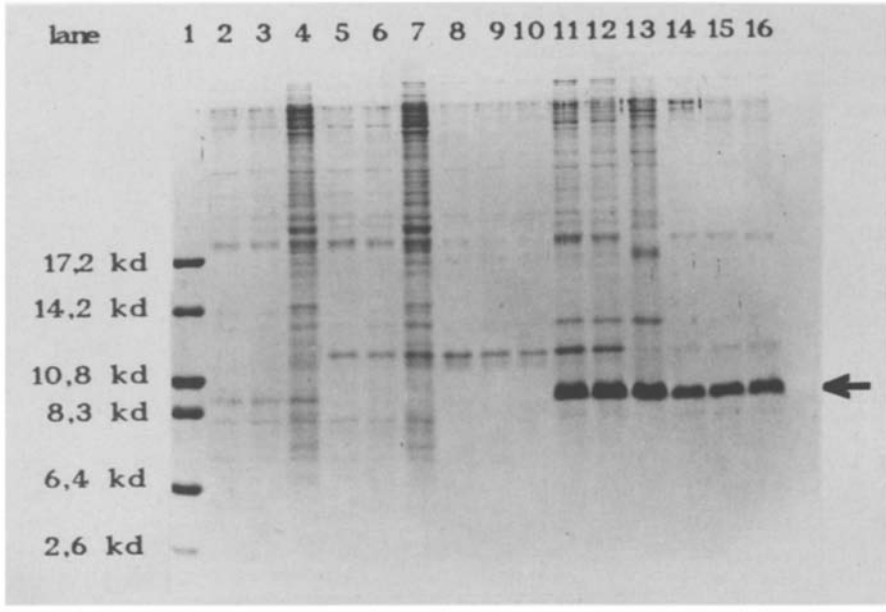

Fig. 3. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with culture supernatants of Tendamistat-producing *Streptomyces lividans* clones. To this SDS-PAGE 10 µl culture supernatant of different clones of the 5th, 6th and 7th culture days were applied: *lane 1*, the molecular mass markers (in kilodaltons); *lanes 2–4*, wild-type Tendamistat construction pTP1; *lanes 5–7*, the *aph*I promoter construction pTP5; *lanes 8–10*, the synthetic promoter; *lanes 11–13*, the *erm*E-up promoter construct pTP11; *lanes 14–16*, the *mel*C promoter plasmid pTP13. The Tendamistat band is marked by *arrows*

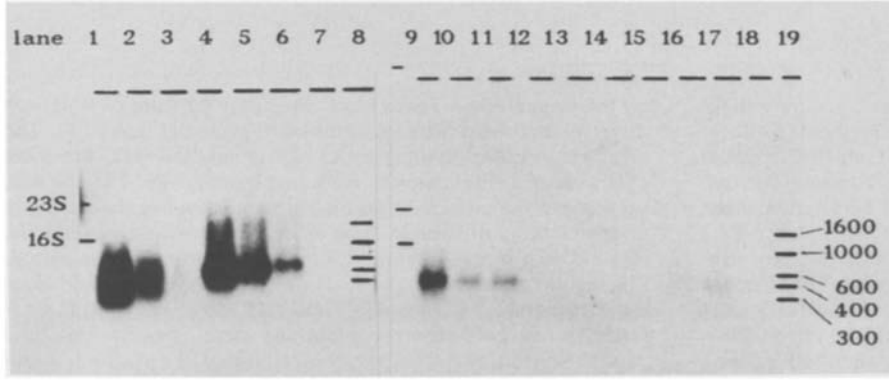

Fig. 4. Northern blots to view the transcripts of Tendamistat-producing *S. lividans* clones: *lanes 1–3*, the RNA of the *erm*E-up promoter construction (pTP11, 10 µg, 3 µg, 1 µg total RNA); *lanes 4–6*, the RNA of the *mel*C promoter clone (pTP13, 10, 3 and 1 µg); *lane 7*, the pTT1 plasmid carrying strain (12 µg) for a control; *lane 8*, RNA molecular mass (kilodaltons) marker III; *lanes 9–11*, the wild-type Tendamistat clone (pTP1, 12, 3 and 3 µg); *lanes 12–14* the RNA of the *aph*I promoter clone (pTP5, 12, 10 and 7 µg); *lanes 15–17* the synthetic promoter strain (pTP9, 7, 10 and 12 µg); *lane 18*, a second control (pTT1, 12 µg); *lane 19*, a second RNA marker

Table 1. Tendamistat production

Promoter	Tend.[a] (pTP1)	*aph*I[b] (pTP5)	Synth.[c] (pTP9)	*erm*E[d] (pTP11)	*mel*C[e] (pTP13)	*tip*A[f] (pTP17)
−thio	36	10	9	475	275	0.5
+thio						38

The table shows the maximum Tendamistat secretion (mg/l) of the different Tendamistat promoter constructions, normally reached at the 6th or 7th day in liquid culture
[a] The original Tendamistat promoter (pTP1)
[b] The *aph*I promoter (pTP5)
[c] The synthetic promoter (pTP9)
[d] The *erm*E-up promoter (pTP11)
[e] The *mel*C promoter (pTP13)
[f] The *tip*A promoter (pTP17) in the absence and in the presence of thiostrepton (thio)

one consensus sequence for *E. coli*-like promoters must be proposed.

The *aph*I promoter construction (pTP5 and pTP6) secreted about 10 mg/l of inhibitor. To detect the inhibitor on SDS gels, silver staining or western blot analysis must be used or supernatants must be concentrated. By Northern blot no signals of the *aph*I-transcripts were detected. In our hands the *aph*I-promoter region proved to be a poor promoter, diminishing secretion by a factor of three compared to the wild-type Tendamistat construct.

Much more Tendamistat - more than 400 mg/l - was produced in the case of the *erm*E-up promoter construction (pTP11). In SDS-PAGE the major band is due to Tendamistat and on Northern blot very strong signals from both promoters, the mutated *erm*E P1 and the P2 promoter were detected. This *E. coli*-like promoter gives the best yield of secreted Tendamistat. Bibb and Janssen (1986) characterized their promoter mutants by expression of the *aph*II gene derived from TN5. The resulting *erm*E-up mutant that we dealt with consists of a 3-bp deletion in the −35 region of the P1 promoter. This deletion alters the promoter towards being less consensus to *E. coli*. Other mutants (Bibb and Janssen 1986) also brought into question the importance of the −35 region. Chang and Chang (1988), when testing this *erm*E-up promoter for the TNF expression, also reached an enhanced expression by a factor of two. We tested this promoter region exclusively as a tandem promoter and observed signals of both transcripts on RNA blotting. Unexpectedly there was no significant preference of the mutated P1 promoter visible (Fig. 4).

The *mel*C promoter fragment in front of the promoterless Tendamistat gene (pTP13) mediated production up to 300 mg/l. This result is in contrast to the apparent activity of this promoter in constructions containing other promoters in addition, where no *mel*C promoter activity is detectable (pTP1, 3 or 5 (Fig. 2, IX)). In the case of pTP13, on SDS-PAGE Tendamistat was the major band (Fig. 3). The pTP13 plasmid caused a decrease in cell growth, resulting in very low protein background on SDS-PAGE. On Northern blotting we detected a very strong signal for one promoter (Fig. 4). The *mel*C-promoter transcript seemed actually to mediate the strongest signal. In contrast to the secretion data the Northern blot analysis is nearly independent of cell mass. This means that the *mel*C promoter might be the strongest promoter tested. Whereas in none of the other cases, pTP1, 3, or 5 (Fig. 2, IX), could any transcript be detected corresponding to the *mel*C promoter region, the promoter is inactive. In the active pTP13 construction between the *mel*C promoter and the Tendamistat gene only restriction recognition sites of a synthetic linker (Fig. 2, II) are located. Thus we did not create an additional promoter based on the mRNA size defined by Northern blot analysis. The length of these transcripts corresponds well to the promoter region proposed by Bernan et al. (1985) (Fig. 1). Possibly additional sequences play an important role for promoter activity in Streptomycetes besides the −35 and −10 region, maybe even downstream. Furthermore, we do not know enough about spacing between the promoter and structural genes, or regulation of *Streptomyces* promoters.

All promoters described so far express Tendamistat constitutively. The *tip*A promoter (pTP17) mediated comparable amounts of inhibitor secretion as the original Tendamistat promoter of about 30 to 40 mg/l in the presence of thiostrepton, whereas we measured less than 0.5 mg/l in the absence of thiostrepton. The *tip*A promoter enabled us to test an inducible secretion expression. The induction of a factor of about 100 will be a valuable tool for the expression of heterologous genes.

In conclusion, we constructed several secretion expression systems based on the pIJ702 plasmid, the *aph* terminator, several *Streptomyces* secondary metabolite promoters, the Tendamistat ribosome-binding site and its signal sequence. Thus the limiting factor seemes to be the efficiency of transcription. As a result the very potent promoters like *erm*E-up or *mel*C in addition to the inducible *tip*A promoter will be tested for the expression of heterologous genes such as IL-2 and hirudin in order to test the generality of these results.

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