Adaptation of the Highly Productive T7-Expression System to *Streptomyces lividans*

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**ABSTRACT**

*Streptomyces lividans* is a Gram-positive bacterium known for its remarkable secretion efficiency and low extracellular protease activity. In the present work, we adapted the highly productive T7-expression system to *S. lividans*. A codon-optimized T7 RNA polymerase gene was chromosomally integrated and a bifunctional T7-expression vector was constructed.
The *Escherichia coli* T7 RNA polymerase-based expression system, developed by Studier and Moffatt (35), is currently used in many laboratories for heterologous protein production. The system is based on the T7 bacteriophage RNA polymerase (T7 RNApol), which directs selective transcription of genes cloned downstream of the major T7 late promoter. T7 RNApol is characterized by a very high activity, elongating chains about five times faster than *E. coli* RNA polymerase, and can generate very long mRNAs (19, 35). Although *E. coli* has proved to be useful for gene overexpression, different problems can occur and limit the productivity (38). As such, the use of an alternative expression host is often desirable to obtain adequate protein production.

Streptomyces are Gram-positive G+C-rich bacteria known for their high secretion capacity and have been used extensively in commercial settings for antibiotic production in very large-scale fermentation systems (6). Among the streptomycetes, the readily transformable *Streptomyces lividans* has been used for the expression of a wide variety of genes from diverse sources (4). This host can secrete directly in the culture medium large quantities of proteins in mature conformation, and given that few endogenous proteins are present in the media, downstream purification processes are simplified. *S. lividans* also displays a very low level of endogenous extracellular protease activity, making it a suitable host for heterologous protein production (26). Most of the *S. lividans* expression systems are based on strong constitutive promoters (4, 34). Few inducible promoters are also used, the thiostrepton-inducible *tipA* promoter (*tipAp*) being the most popular (37).

Since the original publication of the *E. coli* T7-expression system, it has been adapted to mammalian cells and several bacteria (2, 9, 13, 16, 18, 22, 25). In this report, the T7-expression system was adapted to *S. lividans* to combine the T7 RNApol
efficiency to the great features of this host. During the revision process of this paper, we
learned that a similar system had been developed and published in a thesis at the
University of Stuttgart (17).

**T7 RNA polymerase production in *S. lividans***. To efficiently express the T7
RNApol gene in *S. lividans*, the four rare TTA<sup>leu</sup> codons were replaced by CTC<sup>leu</sup> codons
by overlap extension PCR (20) (Supplementary material). The production of the T7
RNApol was evaluated with the codon-modified (CM) and the wild-type (W) genes.
They were cloned into the pIJ702-derived (24) multicopy expression vector pIAFC109
(François Shareck, personal communication), under the control of the constitutive
promoter C109, resulting in pIAFC109_T7CM and pIAFC109_T7W. Both constructions
were introduced in *S. lividans* 10-164 (21) by protoplasts transformation according to
Kieser _et al._ (26). Protein production and mycelium disruption was conducted as
described by Nisole _et al._ (31). The intracellular protein fractions were analyzed by
Coomassie-stained SDS-PAGE and Western Blot (Figure 1). The T7 RNApol producing
strain _E. coli_ BL21/pAR1219 (10) was used as positive control. Western blot was
performed with anti-T7 RNA polymerase mouse monoclonal antibody (Novagen) and
alkaline phosphatase-conjugated goat anti-mouse antibody (GE Healthcare).
Colorimetric-detection was performed with the substrates Nitroblue Tetrazolium and 5-
bromo-4-chloro-3-indolyl phosphate (Bio-Rad). On the SDS-PAGE, a band of about 100
kDa appeared in the intracellular fraction of 10-164/pIAFC109_T7CM, but was
undetectable in 10-164/pIAFC109_T7W. Western blot analysis confirmed that T7
RNApol was produced with the codon-modified gene, but not detected with the wild-type
version.
In *Streptomyces lividans*, TTA<sub>leu</sub> is the rarest codon (Codon Usage Database, www.kazusa.or.jp/codon/) and can be efficiently transcribed by only one tRNA encoded by *bldA* (28, 39). The *bldA* gene is constitutively transcribed, but the mature form of the tRNA seems to be present only near the end of the logarithmic growth phase (29), when the secondary metabolism is activated (7). Different papers have shown that the presence of a TTA<sub>leu</sub> codon in a gene causes temporal expression delay or prevents translation in a *bldA*<sup>-</sup> strain (23, 29, 32, 33). Since the T7 RNAPol gene contains four TTA<sub>leu</sub>, it was not surprising that its expression was impaired in *S. lividans*. By mutating these four codons, the T7 RNAPol gene was over-expressed for the first time in *S. lividans*.

*S. lividans* T7-expression strain. The codon-modified T7 RNAPol gene was cloned into a derivative of pSET152 (3) under the control of the thiostrepton-inducible promoter *tipAp* (30), resulting in pFXPtipAT7 (Figure 2) (detailed construction in supplementary material). This construction was integrated into the chromosome of *S. lividans* 10-164 via the bacteriophage φC31 att/int system (3), giving rise to the apramycin and thiostrepton resistant *S. lividans* 10T7 strain. Integration at the chromosomal *attB* site was confirmed by PCR and DNA sequencing, but T7 RNAPol production by *S. lividans* 10T7 under thiostrepton-induced condition could not be detected by SDS-PAGE or Western blot (results not shown). The strain 10T7 has not shown any growth retardation in presence of thiostrepton compared to that of a non-induced culture (results not shown). It has to be noted that pSET152-derived plasmids can sometimes integrate as tandem repeat and into at least three pseudo-*attB* sites with a 300-fold lower efficiency (8). The plasmid pFXPtipAT7 can be used to create T7-
expression strains in *S. lividans* and *Streptomyces coelicolor* with one simple transformation step (8, 37).

**Bifunctional T7-expression vector.** Construction of pFX583 was realized by using the *E. coli* T7-expression vector pET-9a (Novagen) (36) as backbone (Figure 3). Detailed construction is presented in the supplementary material. The pFX583 vector contains pMB1 (5) and pJV1 (1) replicons, allowing replication in *E. coli* and *S. lividans* with a high-copy number. Kanamycin or neomycin selection can also be used with both bacteria due to the FD Neo-S cassette (11). The vector pFX583 is compatible with the widely used *Streptomyces* pIJ101 replicon (1). Shuttle vectors are very attractive because they allow to do all the DNA manipulation in *E. coli*, but are sometimes structurally unstable in *Streptomyces* for unknown reasons (26). Here, pFX583 have been maintained in *E. coli* and *S. lividans* in presence of selection without notable structure instability. Since pFX583 harbours an oriT sequence, it can be transferred by conjugation from *E. coli* to *Streptomyces* strains that are difficult to transform. The presence of a λ cos sequence allows the use of pFX583 as a cosmid vector for large DNA fragment cloning.

**T7 RNA polymerase-directed xylanase production.** The efficacy of the *S. lividans* T7-expression system was demonstrated by over-expressing the gene encoding a truncated version of the *S. lividans* xylanase A (*xlnA2*) (12). The *xlnA2* gene was amplified by PCR and cloned into the NdeI and BamHI sites of pFX583. The resulting construction, pFX583xlnA2, was introduced into the *S. lividans* T7-expression strain 10T7 by protoplast transformation. Transformants were picked from R5 medium (26) and streaked onto Bennett agar (26) containing 50 µg/mL apramycin and 50 µg/mL kanamycin. After 3-4 days of incubation at 34 °C, the sporulated mycelium was used to
inoculate Tryptic Soy Broth medium (Difco) and cultured in Erlenmeyer flask. Incubation was carried out at 34 °C on a rotary shaker at 240 rpm for 48 h. Recombinant expression of \( xlnA2 \) was induced by addition of thiostrepton to the culture medium. Different concentrations of thiostrepton were tested and 25 µg/mL allowed the highest XlnA2 production (Figure S1). Maximal enzyme production was obtained when thiostrepton was added at the beginning of the incubation period and significant activity increase stopped after 48 h (Figure S2).

Equal volumes of culture supernatants were analyzed by Coomassie-stained SDS-PAGE and xylanase activity was measured as described by Ebanks et al. (15) (Figure. 4). Xylanase A2 was absent in non-induced cultures, while readily detected as a 31 kDa band by SDS-PAGE in the presence of thiostrepton. As for all tipAp-based expression system, thiostrepton also induced the production of the TipAL protein that can be seen on the SDS-PAGE around 20 kDa (30). Xylanase activity assays were consistent with SDS-PAGE analysis. After 48h, no xylanase activity was measured in non-induced cultures, while 13.8 U/mL (30.2 U/mg) were detected under induced conditions, clearly demonstrating inducible \( xlnA2 \) expression in \( S. \) lividans 10T7. Based on the specific activity of the purified XlnA2 (286 U/mg) (15), the concentration can be estimated to 48 mg/L.

To confirm that pFX583 is also functional in \( E. \) coli, pFX582xlnA2 was introduced into the T7-expression strain BL21(DE3) (Novagen). Protein production was induced with 0.025 mM isopropyl \( \beta \)-D-1-thiogalactopyranoside (IPTG) and conducted for 20 h at 37 °C. Supernatants of induced and non-induced cultures were analyzed by Coomassie-
stained SDS-PAGE (Figure S3). As for \textit{S. lividans}, the Xylanase A2 was produced under
induced condition without obvious expression leaking in absence of IPTG.

**Conclusion.** Combined with the bifunctional T7-expression vector pFX583, \textit{S. lividans} 10T7 allowed inducible T7 RNApol-directed overproduction of the xylanase A2 without detectable expression leaking in absence of inducer. Although the amount of protein produced was relatively low compared to what can be obtain with non-inducible \textit{Streptomyces} expression systems (14, 27, 31), the T7-expression system developed here presents interesting features. It is well regulated, has the potential to transcribe very large DNA fragments and can be used in combination with pIJ101-derived plasmids. The vector pFX583 is functional in \textit{E. coli} and \textit{Streptomyces} strains producing T7 RNApol.

With a single construction it is therefore possible to compare the expression of a gene in two kinds of host and determine which one is the most appropriate based on the productivity and requirements of the study.

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FIGURE LEGENDS

**FIG 1.** Structure of the integrative plasmid pFXPtipAT7. T7 RNApol*, codon-modified gene of the T7 RNA polymerase; *tipAp*, thiostrepton-inducible promoter; *aac(3)IV*, apramycin acetyltransferase, apramycin resistance; *tsr*, 23S A1067 rRNA methylase, thiostrepton resistance; *int*, φC31 integrase; *rrnB1t2*, transcription terminators T1 and T2 from *E. coli rnrB* gene; *tO*, λ transcription terminator; *ori* pUC, replication origin of pUC18; *oriT*, RK2 origin of transfer; *attP*, φC31 phage attachment site.

**FIG 2.** Structure of the bifunctional T7-expression vector pFX583. PT7, T7 gene φ10 promoter; T7ter, T7 transcription terminator φt; *neo*, Tn903 aminoglycoside phosphotransferase, kanamycin and neomycin resistance; *ori* pMB1, replication origin of pMB1; *ori* pJV1, replication origin of pJV1; pJV1 *rep*, pJV1 Rep protein; *oriT*, RK2 origin of transfer; *cos*, λ cohesive end site; SD, Shine-Dalgarno sequence.

**FIG 3.** Analysis of the T7 RNApol production by (A) Coomassie-stained SDS-PAGE and (B) Western blot. M, molecular mass standard; lane 1, positive control; lane 2, *S. lividans* 10-164/pIAFC109_T7W; lane 3, *S. lividans* 10-164/pIAFC109_T7CM.

**FIG 4.** (A) Coomassie-stained SDS-PAGE showing the extracellular production of XlnA2 in non-induced (−) and induced (+) cultures. M, molecular mass standard. (B) Xylanase activity in non-induced (− Thio.) and induced (+ Thio.) cultures.
pFX583
5839 bp

___T7 promoter___
CCGCGAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGGTTAATCTT

___SD___
TAAGAAGGAGATATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGGTTAATCTT

___NdeI___
GGCTGCTAACA

___BamHI___
AAGCCCGAAGGAACGGAGGACTGATTCGGCTGGCGACCGGACTGAGCAATAACTAGCAAACTCCCCAGGGCCTCT

___T7 terminator___
AAACGGGTTCGAGGGGTTTTTGCTGAAAGGAGGAA
Xylanase activity

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<td></td>
<td>ND*</td>
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* Not detected