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Short communication

# Comparative analysis of transcriptional activities of heterologous promoters in the rare actinomycete *Actinoplanes friuliensis*

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ABSTRACT

Manipulation of secondary metabolite production in the rare actinomycete *Actinoplanes friuliensis*, the producer of the lipopeptide antibiotic friulimicin, is hampered by the lack of sophisticated genetic tools. Since no expression vectors have been developed from endogenous *Actinoplanes* plasmids and expression signals, engineering of antibiotic biosynthesis relies on the use of vector systems derived from *Streptomyces*. While ΦC31 derived vectors were shown to integrate efficiently into the chromosome of *Actinoplanes*, information on promoter activity is missing. The manuscript describes the investigation of several different promoter systems which are widely used in *Streptomyces* in *A. friuliensis* by promoter probe experiments using eGFP as a reporter. These experiments indicated that promoter strength in *A. friuliensis* did not correlate to activity in *Streptomyces lividans*. The *ermE** promoter regarded as one of the strongest promoter in *Streptomyces* has only low activity in *A. friuliensis*. In contrast, the promoter of the apramycin resistance gene *aac(3)IV*, originating from the Gram-negative *Escherichia coli* had the highest activity. By real-time RT-PCR experiments the transcription activity of *ermE** promoter in comparison to a native promoter of the friulimicin biosynthetic gene cluster was analysed. This confirmed the results of the promoter probe experiments that indicated quite weak promoter activity of P-*ermE** in *Actinoplanes*.


## 1. Introduction

Actinomycetes are mycelial soil bacteria renowned for their ability to produce a variety of bioactive compounds such as antibiotics. Especially, rare actinomycetes are promising sources in search for new drugs (Bredholdt et al., 2007; Lazzarini et al., 2001). Therefore, genetic manipulations of these microorganisms are crucial for drug discovery and development. Whereas in "standard" actinomycetes such as *Streptomyces lividans*, *Streptomyces coelicolor* or *Streptomyces griseus* a large number of genetic tools such as small replicative multicopy plasmids (Baltz, 1998) or suitable strong promoters is known, the situation in rare actinomycetes, e.g. *Actinoplanes friuliensis* (the producer of the lipopeptide antibiotic friulimicin) is quite different. Only a single replicative plasmid has been characterized in *Actinoplanes* yet. pAM1 is a large linear plasmid from *Actinoplanes missouriensis* DSM 43046 (Rose and Fetzner, 2006), but it has not been used to construct cloning or expression vectors. So far genetic manipulations in these microorganisms were successfully performed with integrative plasmids on the basis of phage ΦC31 (Bierman et al., 1992; Müller et al., 2007; Ha et al., 2008). In order to improve secondary metabolite biosynthesis by prospective overexpression of regulatory or bottleneck genes using such plasmids, strong heterologous promoters and the analysis of their transcriptional activity are of particular importance.

Promoter strength can be analysed either by measuring the transcript rate with real-time RT-PCR or by promoter probe systems. In streptomycetes, antibiotic resistance proteins, the catechol dioxygenase XylE, or the enhanced green fluorescent protein (eGFP) were commonly used in such experiments (Kieser et al., 2000).

The use of eGFP as a reporter protein in *S. coelicolor* was first described in 1999 (Sun et al., 1999) and rapidly became a mainstay in the field, finding applications in studies of cell division (Flärdh, 2003; Grantcharova et al., 2005), chromosome segregation (Jakimowicz et al., 2006), cell type specific gene expression (Kelemen et al., 2001) and promoter probe experiments (Sun et al., 1999; Chevillotte et al., 2008).

Here we describe the analysis of the strength of different heterologous promoters in *A. friuliensis* by both real-time RT-PCR and eGFP based promoter probe experiments.

### 1.1. Analysis of promoter activity by promoter probe experiments using the eGFP reporter system

Fluorescence arising due to eGFP expression was used as a reporter to monitor transcription driven from the different

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**Table 1**
Primers used for the amplification of promoter regions.

| Promoter region | Primer | Primer sequence 5′ → 3′[a] | Corresponding genes/phages [accession number] |
|---|---|---|---|
| P-*aphII* | aphII for<br>aphII rev | GGATCC GTA GAA AGC CAG TCC GCA GAA<br>ATGCAT CAT GCG AAA CGA TCC TCA TCC | Neomycin resistance gene *aphII* [U00004] |
| P-*aac(3)IV* | apra for<br>apra rev | GGATCC TCG AAA TCC AGA TCC TTG ACC<br>ATGCAT CAG TCG ATC ATA GCA CGA TCA | Apramycin resistance gene *aac(3)IV* [X99313] |
| P-*ermE** | ermE for<br>ermE rev | GGATCC TAC CAT GCG AGT GTC CGT TCG A<br>ATGCAT ATC CTA CCA ACC GGC ACG ATT | Mutated variant of the promoter of the erythromycin resistance gene *ermE* (Bibb and Janssen, 1986) |
| P-*hyg* | hyg for<br>hyg rev | GGATCC AGA ACC AGG CGG TGG CGT ACA<br>ATGCAT AGC GGA CCT CTA TTC ACA GGG | Hygromycin resistance gene *hygB* [X99315] |
| P-*tsr* | tsr for<br>tsr rev | GGATCC CTA CGC GAA GAT CAA GGC GAA T<br>ATGCAT GTC ATG GTC GTC CTA CCG GCT | Thiostrepton resistance gene tsr [X54219] |
| P-Φ*C31* | ΦC31for<br>ΦC31 rev | GGATCCCCCCGTGCCGGAGCAATCGC<br>ATGCAT CATGTCGGCGACCCTACGCC | Integrase gene of the phage ΦC31 [AJ006589] |
| P-*SF14* | SF14 for<br>SF14 rev | GGATCCGGCATGCAAGCTTCCTATCCA<br>ATG CAT CTCCCTCATCACCTGACTACC | Promoter from phage SF14 (Labes et al., 1997) |

[a] Restriction sites used for cloning are underlined.

promoters. Promoter regions of the following genes were selected: the apramycin resistance gene *aac(3)IV* from *Klebsiella pneumoniae* (Bierman et al., 1992), the *aphII*-gene of transposon *Tn5* (Beck et al., 1982), the hygromycin-resistance gene *hygB* of *Streptomyces hygroscopicus* (Malpartida et al., 1983) and the thiostrepton resistance gene *tsr* from *Streptomyces azureus* (Thompson et al., 1980), the integrase promoters of the *Streptomyces* phages ΦC31 (Bierman et al., 1992) as well as SF14 promoter from the *Streptomyces ghanaensis* phage I19 (Labes et al., 1997). Furthermore the mutated variant of the promoter of the erythromycin resistance gene from *Saccharopolyspora erythrea* (*ermE**) (Bibb and Janssen, 1986) was utilized. The corresponding regions were amplified by PCR using the primers listed in Table 1. For amplification of the fragments, the following PCR procedure was used: initial denaturation (94 °C, 5 min), 30 cycles of denaturation (94 °C, 30 s), annealing (60 °C, 30 s), elongation (72 °C, 30 s) and final elongation (72 °C, 10 min). The PCR fragments were subcloned as BamHI/NsiI fragments in pIJ8660 (Sun et al., 1999) in order to achieve a transcriptional fusion with the *egfp* gene. pIJ8660 represents a derivative of the conjugative and integrative plasmid pSET152 (Bierman et al., 1992), It contains the *int* gene and *attP* site of phage ΦC31 allowing insertion of the plasmid at chromosomal ΦC31 attachment sites and the apramycin resistance gene *aac3(IV)* for selection. The promoter probe cassette (multiple cloning sites and promoter less *egfp* gene) is flanked by the major transcription terminator of phage fd (tfd) and by the transcription terminator to of phage λ, respectively.

The pIJ8660 derived plasmids carrying different PCR-generated promoter regions were transferred in *A. friuliensis* by intergeneric conjugation following the procedure described by Heinzelmann et al. (2003). In order to detect possible differences in transcription activities to the situation in "standard" actinomycete, *S. lividans* TK23 was also transformed with the generated plasmids by PEG mediated protoplast transformation (Bibb et al., 1978). The generated strains, carrying the integrated plasmids, are listed in Table 2. Integration of the reporter plasmids into the chromosome was confirmed by Southern hybridization and PCR, respectively (data not shown). For the analysis of eGFP-expression 30 ml Tryptic soy broth (TSB)-medium (Becton Dickinson, MD, USA) supplemented with 100 μg/ml apramycin and 10 mM $CaCl_2$ (to improve spore germination of *S. lividans* strains) were inoculated with 3 ml of a 4–5-old preculture ($OD_{600}$ = 2.6) of *A. friuliensis* strains and with $3 \times 10^6$ spores of *S. lividans*, respectively. Cells were grown up to the early exponential growth phase, harvested by centrifugation (12,000 × *g*, 10 min, 4 °C), resuspended in 3 ml lysis buffer [20 mM $Na_2HPO_4$, 20 mM $NaH_2PO_4$, 50 mM NaCl, 1 mg/ml chicken egg lysozyme (Serva, Heidelberg, Germany), 1 mg/ml Pefabloc®, pH 7.7] and disrupted by sonication under ice cooling using Sonoplus HD 2200 (Bandelin, Berlin, Ger-

**Table 2**
Bacterial strains used in this study.

| Bacterial strains | Relevant features | Source/reference |
|---|---|---|
| *Actinoplanes friuliensis* HAG010964 (=AF) | Wild-type, friulimicin-producer | Aretz et al. (2000) |
| AF O4 23 | *orf4*-nullmutant, '*orf4*/*orfC*/*pstA* regulated by P-*ermE**, Apr$^r$, | Müller et al. (2007) |
| AF-P-aph | AF with integrated pNW_aph | This study |
| AF-P-apra | AF with integrated pNW_apra | This study |
| AF-P-ermE | AFwith integrated pNW_ermE | This study |
| AF-P-hyg | AFwith integrated pNW_hyg | This study |
| AF-P-tsr | AF with integrated pNW_tsr | This study |
| AF-P-SF14 | AF with integrated pNW_SF14 | This study |
| AF-P-ΦC31 | AF with integrated pNW_ΦC31 | This study |
| *Streptomyces lividans* TK 23 (=SL) | *spc-1* | Kieser et al. (2000) |
| SL-P-aph | SL with integrated pNW_aph | This study |
| SL-P-apra | SL with integrated pNW_apra | This study |
| SL-P-ermE* | SL with integrated pNW_ermE | This study |
| SL-P-hyg | SL with integrated pNW_hyg | This study |
| SL-P-tsr | SL with integrated pNW_tsr | This study |
| SL-P-SF14 | SL with integrated pNW_SF14 | This study |
| SL-P-ΦC31 | SL with integrated pNW_ΦC31 | This study |