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Robust reporter system based on chalcone synthase *rppA* gene from *Saccharopolyspora erythraea*

V. Magdevska [a,b], R. Gaber [b], D. Goranovič [a,b], E. Kuščer [a], S. Boakes [d], M.B. Durán Alonso [a], R.I. Santamaría [c], P. Raspor [b], P.F. Leadlay [d], Š. Fujs [a], H. Petković [a,b,*]

[a] *Acies Bio d.o.o., Tehnološki Park 21, SI-1000 Ljubljana, Slovenia*
[b] *University of Ljubljana, Biotechnical Faculty, Department of Food Science and Technology, Jamnikarjeva 101, SI-1000 Ljubljana, Slovenia*
[c] *Instituto de Microbiología Bioquímica/Departamento de Microbiología y Genética, Consejo Superior de Investigaciones Científicas (CSIC)/Universidad de Salamanca, Edificio Departamental, Campus Miguel de Unamuno, 37007 Salamanca, Spain*
[d] *University of Cambridge, Department of Biochemistry, Cambridge CB2 1GA, UK*

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ABSTRACT

Industrial overproducing strains present unique hosts for expression of heterologous gene clusters encoding secondary metabolite biosynthesis. For this purpose, efficient gene expression tools and methods are needed. A robust and versatile reporter system based on the *rppA* gene from *Saccharopolyspora erythraea* is presented as the method of choice when studying gene expression in actinomycete hosts. The method is easily scalable to accommodate high-throughput procedure, and collected samples can be easily stored and re-tested when needed. The product of RppA is an inert 1,3,6,8-tetrahydroxynaphthalene which spontaneously oxidises to a dark-red quinone flaviolin providing a qualitative visual assessment of gene expression on an agar plate as well as a quantitative spectrophotometric measurement in liquid broth without the need for invasive procedures or external substrate addition. The applicability of the reporter system has been demonstrated by expressing the *rppA* gene under the control of the heterologous promoters *actII*-ORF4/P_{actI}, *ermE* and its upregulated variant *ermE**. The model streptomycete *Streptomyces coelicolor*, and three industrially important species, *Streptomyces tsukubaensis* (FK506), *Streptomyces cinnamonensis* (monensin) and *Streptomyces rimosus* (oxytetracycline) were used as hosts. The reporter system has shown its utility independently of cultivation conditions or composition of growth medium, from simple laboratory to complex industrial media. The simplicity and robustness of the system, demonstrated even in industrial settings, shows great potential for wider use in different microbial hosts and applications, and may thus represent a new generic and versatile tool useful to a wider scientific community.

1. Introduction

Streptomycetes, a member of order *Actinomycetales* are Gram-positive bacteria producing a multitude of primary metabolites of industrial importance, such as enzymes, as well as secondary metabolites with diverse chemical structures and a broad range of biological functions, such as antibacterial (erythromycin and tetracycline), antifungal (amphotericin), immunosuppressive (rapamycin and FK506), anticancer (doxorubicin and epothilone) and other activities of medical and industrial importance (Menzella and Reeves, 2007). Industrial overproducing streptomycete strains which underwent years of simultaneous intensive strain improvement and medium/process development, today often exceeding yields of over 20 g/l of the target product(s), present unique hosts for expression of heterologous gene clusters encoding secondary metabolite biosynthesis. These may be from gene clusters with known origin, or unknown clusters from metagenome source(s). To achieve the full potential of industrial high-producing hosts, a number of gene tools and methods have to be significantly improved. In order to study gene expression and its regulation, a reliable and convenient reporter system that allows for fast and sensitive detection of promoter activities is essential. While reporter systems are available for use in *Streptomyces*, significant compromises often have to be made with regard to their application. For example, some reporter systems require the addition of an active substrate and most often include laborious invasive procedure while others require rate-limiting metabolites. Commonly used reporter systems include the catechol dioxygenase-coding *xylE* (Ingram et al., 1989), the light-emitting *luxCDABE* and its enhanced version optimised for GC-rich bacteria (Craney et al., 2007), and the enhanced green fluorescent protein EGFP also with an optimised codon usage for GC-rich bacteria (Cormack et al., 1996; Sun et al., 1999). Less commonly used reporter

* Corresponding author. Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, SI-1000 Ljubljana, Slovenia. Tel.: +386 1 423 11 61; fax: +386 1 257 40 92.
E-mail address: hrvoje.petkovic@bf.uni-lj.si (H. Petković).

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systems such as *redD* transcriptional activator (van Wezel et al., 2000), *melC* operon (Rodriguez et al., 2005), genes *whiE* (Yu and Hopwood, 1995), *ampC* (Forsman and Jaurin, 1987) and others, find their use for limited and specific purposes.

Most of the reporter systems developed so far, however, have their limitations and do not offer a simple quantitative, high-throughput and non-invasive procedure for gene expression studies. The existing reporter systems are particularly inconvenient in industrial settings where complex and ingredient-rich media with non-soluble components, as well as extremely high cell densities, are greatly limiting their use and applicability. Clearly, none of the existing reporter systems comes close to the reliability, convenience and range of applications as the *lacZ* system (Sambrook and Russell, 2001). Unfortunately, *lacZ* finds limited use in *Streptomyces* species which most often encode extracellular enzymes with β-galactosidase activity (Eckhardt et al., 1987).

The potential applicability of chalcone synthases as a reporter system was initially indicated by the successful expression of the *rppA* gene homologue *from Streptomyces griseus* and the production of the dark-red pigment in *E. coli* and in *S. lividans* (Ueda et al., 1995). The nature of the inert red–brown diffusible pigment produced by RppA was first described by Horinouchi and co-workers when they reported a new pathway for polyketide synthesis in microorganisms (Funa et al., 1999; Ueda et al., 1995). The pigment produced by RppA was shown to be 2,5,7-trihydroxy-1,4-naphtoquinone (flaviolin), which is a spontaneous oxidation product of 1,3,6,8-tetrahydroxynaphthalene (THN), the actual product of RppA (Fig. 1). RppA-homologue producing analogous flaviolin molecules with a 69% sequence identity to the RppA of *S. griseus* was also discovered in *Saccharopolyspora erythraea*, the producer of the medically important polyketide antibiotic erythromycin A (Cortes et al., 2002).

The potential use of dark red–brown pigment producing chalcone synthase *rppA* gene from *S. erythraea* as a reporter system was initially demonstrated by Kuscer et al. (2007). In that work, the *rppA* gene was used as a reporter system in *Streptomyces hygroscopicus* to evaluate the expression of studied regulatory genes and the activity of a promoter involved in the transcription of a large polyketide synthase which catalyses the biosynthesis of immunosuppressant and anticancer drug rapamycin. The gene *rppA* indicated a great potential as a small and convenient reporter system for wider use in *Actinomycetes* hosts on both solid as well as liquid media. However, the applicability and robustness of the method was not tested and optimised for a wider use in a broad spectrum of host backgrounds.

To demonstrate the broader applicability and reliability of the *rppA*-based reporter system for use in *Streptomyces* strains, we have in this paper further optimised the method and carried out an in-depth comparative analysis of three well-known *Streptomyces* promoters, the *actII*-ORF4/P_{actI} activator/promoter system from the actinorhodin biosynthetic gene cluster (Fernandez-Moreno et al., 1991), *ermE* promoter from *S. erythraea* and its upregulated variant *ermE**, which is considered to be one of the strongest constitutive promoters for native and heterologous gene expression in *Streptomyces* and taxonomically related bacteria (Bibb et al., 1985; Wilkinson et al., 2002). Four *Streptomyces* strains, all producing polyketide-derived metabolites, including the model streptomycete *Streptomyces coelicolor* were tested as suitable hosts. The *rppA* expression was studied in the laboratory medium tryptic soy broth (TSB) and industrial production media were used for production of FK506 and monensin by streptomycete hosts *S. tsukubaensis* and *S. cinnamonensis*, respectively. Following the initial work by Kuscer et al. (2007), the aim of this work was, thus, to develop a simple and robust reporter system based on the *rpp*A gene using a method which will enable a quantitative, non-invasive and high-throughput analysis for expression studies in a broad range of actinomycete hosts regardless of the complexity of the media used, and which can be easily extended to other hosts/microorganisms of wider scientific and industrial relevance.

2. Materials and methods

2.1. Bacterial strains

Four streptomycete strains were selected in order to test the *rppA*-based reporter system. The model streptomycete *Streptomyces coelicolor* M145 (Bentley et al., 2002; Kieser et al., 2000) which produces antibacterial actinorhodin, and three industrially important strains *Streptomyces tsukubaensis* NRRL 18488 (Fehr, 1990), producing immunosuppressant FK506, *Streptomyces cinnamonensis* AB003 (Acies Bio), producing coccidiostatic monensin, and *Streptomyces rimosus* M4018 (Rhodes et al., 1984), producing antibacterial oxytetracycline, were used as host strains (Table 1).

2.2. Standard microbiological and recombinant DNA methods

The *Streptomyces* strains, *S. coelicolor* and *S. rimosus* were propagated on soya–mannitol (SM) agar medium (Kieser et al., 2000), *S. cinnamonensis* on sporulation agar medium (SAM) as described by Benicki et al. (1991) and *S. tsukubaensis* on ISP4 agar plates (Shirling and Gottlieb, 1968). All *Streptomyces* strains were routinely grown in tryptic soy broth (TSB) liquid medium (Kieser

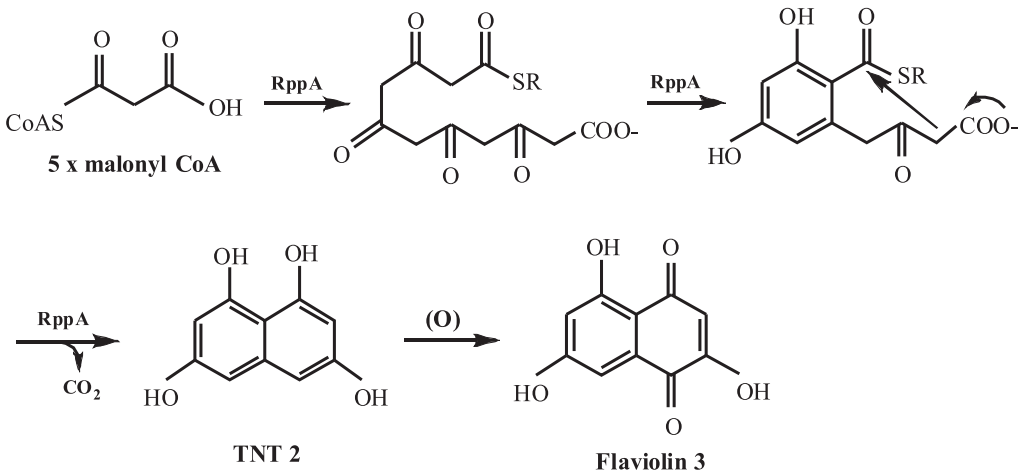

Fig. 1. Biosynthesis of THN (2) and flaviolin (3) by RppA in *Streptomyces griseus* (16) *R* = co-enzyme A (CoA) or the enzyme active site cysteine thiol group.