J. Mol. Microbiol. Biotechnol. (2002) 4(4): 417–426.

JMMB Research Article

Increasing the Efficiency of Heterologous Promoters in Actinomycetes

Christopher J. Wilkinson[1,3], Zoë A. Hughes-Thomas[1], Christine J. Martin (née Rowe)[1,4], Ines Böhm[1], Tatiana Mironenko[1], Matthew Deacon[1*], Michael Wheatcroft[1], Gabriele Wirtz[1], James Staunton[2], and Peter F. Leadlay[1,*]

[1]Cambridge Centre for Molecular Recognition and Department of Biochemistry, University of Cambridge, 80 Tennis Court Road, Cambridge, CB2 1GA, UK
[2]Cambridge Centre for Molecular Recognition and Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge, CB2 1EW, UK
[3]Present address: Max Planck Institut für Biochemie, Am Klopferspitz 18a, D-82152 Martinsreid, Munich, Germany
[4]Present address: Biotica Technology Ltd., 181a Huntingdon Rd., Cambridge, CB3 0DJ, UK

Abstract

An *Escherichia coli* – actinomycete shuttle vector, pCJW93, was constructed which places cloned genes under the control of the thiostrepton-inducible *tip* promoter from *Streptomyces lividans*. We also constructed expression vectors bearing the *actII-ORF4/PactI* activator-promoter system of the actinorhodin biosynthetic pathway of *Streptomyces coelicolor*. With both types of vector, levels of expression varied widely in different actinomycete strains, indicating different levels of the host factors needed for optimal expression. Deletion of the *actII-ORF4* activator gene from one such plasmid in *Saccharopolyspora erythraea* drastically reduced expression from the cognate *actI* promoter, showing that host factors are required for optimal production of the activator protein itself. However, a low copy number expression vector pWIZ1 for the polyketide synthase DEBS1-TE, in which the promoter for the activator gene was replaced by the strong heterologous *ermE promoter of *S. erythraea* directed highly efficient production of polyketide synthase protein in *Streptomyces cinnamonensis*; and the levels of triketide lactone product found were up to 100-fold greater than were produced by the same plasmid in which *actII*-ORF4 was expressed from its own promoter. Ensuring appropriate expression of a specific activator protein should enable more convenient and consistent heterologous expression of genes in a broad range of actinomycete hosts.**

*For correspondence. Email pfl10@mole.bio.cam.ac.uk; Tel. (+44) 1223 333656; Fax. (+44) 1223 766091.

Introduction

The regulation of gene expression in *Streptomyces* and related filamentous bacteria is complex and remains poorly-understood. Environmental and nutritional signals are transduced through complex pathways involving pleiotropic regulatory proteins which control *inter alia* formation of aerial mycelium, sporulation, and antibiotic production. These proteins in turn often appear to control pathway-specific regulatory proteins (for a review, see Chater and Bibb, 1997) which govern, for example, the transcription of gene clusters for antibiotic biosynthesis. Our limited understanding of these regulatory circuits has handicapped the use of individual actinomycete promoters to achieve the high-level expression of heterologous genes: most studies so far have used a very limited range of promoters and of host strains. In particular, when the promoter being used is heterologous to the host strain, expression may be feeble or even non-existent, for unknown reasons.

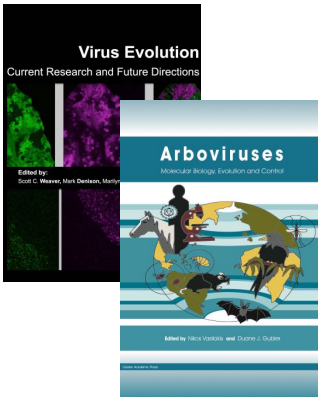
The *ermE* gene of the erythromycin-producing strain of *Saccharopolyspora erythraea* encodes a specific 16S rRNA methyltransferase. Partly inducible in its native context, the *ermE* promoter (usually as its upregulated variant *ermE**) (Bibb *et al*., 1985) has been frequently deployed as a strong constitutive promoter for native and heterologous genes in *Streptomyces* and related bacteria. The *tipA* gene of *Streptomyces lividans* encodes a protein induced by the presence of the antibiotic thiostrepton (Murakami *et al*., 1989), and the *tipA* promoter therefore provides a useful inducible promoter system for heterologous gene expression. However, the *tipA* gene encoding the protein needed for expression from P_{tipA} is reportedly not present in all *Streptomyces* (Takano *et al*., 1995; Yun *et al*., 2001).

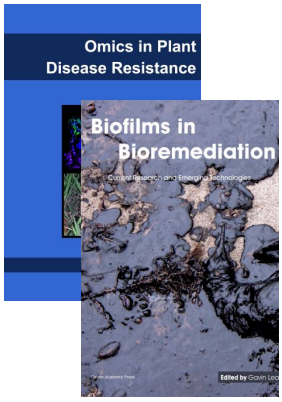
In common with the *ermE** and the *tipA* systems, and the more-recently characterised promoter for pristinamycin resistance from *Streptomyces pristinaespiralis* (Salahbey *et al*., 1995; Salahbey & Thompson 1995) which appears to be inducible by an extraordinarily wide range of stress conditions, other useful promoters are linked to nutritional and environmental signals. In particular, the activator-promoter system *actII-ORF4/*P*actI* from the actinorhodin biosynthetic gene cluster of *S. coelicolor* A3(2) (Fernández-Moreno *et al*., 1991) has been used to ensure overexpression of both native and heterologous antibiotic biosynthesis genes at the same late stage of mycelial development at which the native actinorhodin genes are normally expressed (McDaniel *et al*., 1993). This link to normal development in *S. coelicolor* was thought to limit the use of *actII-ORF4/*P*actI* to *S. coelicolor*, until it was demonstrated that the activated promoter also functions well in the distantly-related actinomycete

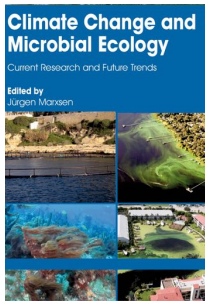
Further Reading

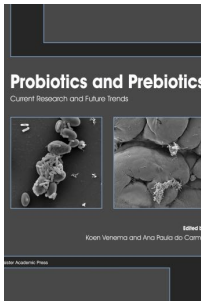
Caister Academic Press is a leading academic publisher of advanced texts in microbiology, molecular biology and medical research. Full details of all our publications at **caister.com**

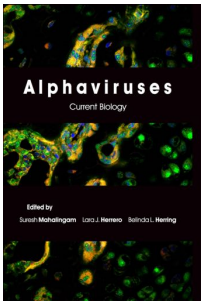
- **MALDI-TOF Mass Spectrometry in Microbiology**
  Edited by: M Kostrzewa, S Schubert (2016)
  www.caister.com/malditof
- ***Aspergillus* and *Penicillium* in the Post-genomic Era**
  Edited by: RP Vries, IB Gelber, MR Andersen (2016)
  www.caister.com/aspergillus2
- **The Bacteriocins: Current Knowledge and Future Prospects**
  Edited by: RL Dorit, SM Roy, MA Riley (2016)
  www.caister.com/bacteriocins
- **Omics in Plant Disease Resistance**
  Edited by: V Bhadauria (2016)
  www.caister.com/opdr
- **Acidophiles: Life in Extremely Acidic Environments**
  Edited by: R Quatrini, DB Johnson (2016)
  www.caister.com/acidophiles
- **Climate Change and Microbial Ecology: Current Research and Future Trends**
  Edited by: J Marxsen (2016)
  www.caister.com/climate
- **Biofilms in Bioremediation: Current Research and Emerging Technologies**
  Edited by: G Lear (2016)
  www.caister.com/biorem
- **Microalgae: Current Research and Applications**
  Edited by: MN Tsaloglou (2016)
  www.caister.com/microalgae
- **Gas Plasma Sterilization in Microbiology: Theory, Applications, Pitfalls and New Perspectives**
  Edited by: H Shintani, A Sakudo (2016)
  www.caister.com/gasplasma
- **Virus Evolution: Current Research and Future Directions**
  Edited by: SC Weaver, M Denison, M Roossinck, et al. (2016)
  www.caister.com/virusevol
- **Arboviruses: Molecular Biology, Evolution and Control**
  Edited by: N Vasilakis, DJ Gubler (2016)
  www.caister.com/arbo
- ***Shigella*: Molecular and Cellular Biology**
  Edited by: WD Picking, WL Picking (2016)
  www.caister.com/shigella
- **Aquatic Biofilms: Ecology, Water Quality and Wastewater Treatment**
  Edited by: AM Romaní, H Guasch, MD Balaguer (2016)
  www.caister.com/aquaticbiofilms
- **Alphaviruses: Current Biology**
  Edited by: S Mahalingam, L Herrero, B Herring (2016)
  www.caister.com/alpha
- **Thermophilic Microorganisms**
  Edited by: F Li (2015)
  www.caister.com/thermophile

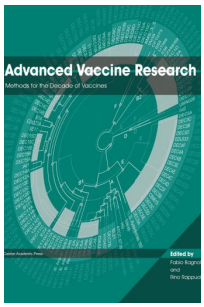


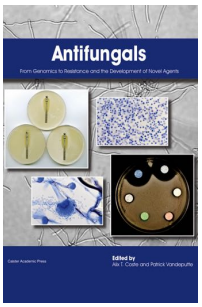












- **Flow Cytometry in Microbiology: Technology and Applications**
  Edited by: MG Wilkinson (2015)
  www.caister.com/flow
- **Probiotics and Prebiotics: Current Research and Future Trends**
  Edited by: K Venema, AP Carmo (2015)
  www.caister.com/probiotics
- **Epigenetics: Current Research and Emerging Trends**
  Edited by: BP Chadwick (2015)
  www.caister.com/epigenetics2015
- ***Corynebacterium glutamicum*: From Systems Biology to Biotechnological Applications**
  Edited by: A Burkovski (2015)
  www.caister.com/cory2
- **Advanced Vaccine Research Methods for the Decade of Vaccines**
  Edited by: F Bagnoli, R Rappuoli (2015)
  www.caister.com/vaccines
- **Antifungals: From Genomics to Resistance and the Development of Novel Agents**
  Edited by: AT Coste, P Vandeputte (2015)
  www.caister.com/antifungals
- **Bacteria-Plant Interactions: Advanced Research and Future Trends**
  Edited by: J Murillo, BA Vinatzer, RW Jackson, et al. (2015)
  www.caister.com/bacteria-plant
- ***Aeromonas***
  Edited by: J Graf (2015)
  www.caister.com/aeromonas
- **Antibiotics: Current Innovations and Future Trends**
  Edited by: S Sánchez, AL Demain (2015)
  www.caister.com/antibiotics
- ***Leishmania*: Current Biology and Control**
  Edited by: S Adak, R Datta (2015)
  www.caister.com/leish2
- ***Acanthamoeba*: Biology and Pathogenesis (2nd edition)**
  Author: NA Khan (2015)
  www.caister.com/acanthamoeba2
- **Microarrays: Current Technology, Innovations and Applications**
  Edited by: Z He (2014)
  www.caister.com/microarrays2
- **Metagenomics of the Microbial Nitrogen Cycle: Theory, Methods and Applications**
  Edited by: D Marco (2014)
  www.caister.com/n2

Order from caister.com/order

S. erythraea (see, for example Rowe *et al.*, 1998). Numerous high copy number, low copy number plasmids and vectors that integrate into the genome by homologous recombination or site-specific integration are now available that incorporate the *tipA*, *ermE** or *actII*-ORF4/P*actI* promoters (see Kieser *et al.*, 2000). Since these replication mechanisms give these plasmids a broad-host range, the dependence of the promoters on host factors has become a limiting step in their exploitation, particularly for *actII-ORF4*/P*actI*.

The control of P*actI* by *actII-ORF4* in *S. coelicolor* has been intensively studied. A homologue of this gene (*redD*) is required for expression of the biosynthetic genes for the red pigment undecylprodigiosin. Deletion of both these genes abolishes production of both actinorhodin and undecylprodigiosin (Floriano & Bibb, 1996). Both these proteins belong to a growing class of *Streptomyces* activators called SARPs (*Streptomyces* antibiotic regulatory protein) which share a weak structural similarity with the OmpR family of regulatory DNA binding proteins (Wietzorrek & Bibb 1997). Two SARPS have been shown to bind to their proposed target promoters (Arias *et al.*, 1999; Tang *et al.*, 1996). Expression of RedD and ActII-ORF4 are naturally limited to the stationary phase of the *S. coelicolor* life cycle (Gramajo *et al.*, 1993; Takano *et al.*, 1992). However, precocious production of these pigments can be elicited by expression of *redD* or *actII-ORF4* gene *in trans* from a multi-copy plasmid. Pleiotropic effector proteins in *S. coelicolor* such as the *bldA* and *afsR* gene products have been shown to affect secondary metabolite production through SARPs, but no single gene is both necessary and sufficient for SARP activity (Floriano & Bibb 1996; White & Bibb 1997) and the molecular mechanisms of the interactions remain obscure.

The fact that the *actII-ORF4*/P*actI* activator-promoter system from *S. coelicolor* functions very efficiently to overexpress polyketide synthase genes in *S. erythraea* NRRL 2338 (Rowe *et al.*, 1998) might imply that *S. erythraea* possesses a SARP that can replace ActII-ORF4, since SARPs from other *Streptomyces* have been found to complement *S. coelicolor* lacking *redD* or *actII*-ORF4 (Lombo *et al.*, 1999; Stutzman-Engwall *et al.*, 1992). Alternatively the host factor(s) in *S. erythraea* might be necessary for expression of the *actII-ORF4* itself. We describe here the results of experiments to explore this question, and the use of both *actII*-ORF4-activated and *tipA* promoter systems in several different actinomycetes. As a result, we have devised a strategy to boost the efficiency of the *actII*-ORF4-activated promoter in heterologous gene expression. A number of convenient *E. coli-Streptomyces* suicide and shuttle vectors were also constructed and their DNA sequences determined.

Results

DNA Sequence of pSET152, pIJ6021/4123 and the SCP2* Origin of Replication

We wished to use in this work plasmids based on pSET152, pIJ6021/4123 and the SCP2* (Figure 1 and Table 1) origin of replication, which have been used extensively in *Streptomyces* microbiology. Surprisingly, the SCP2* origin of replication had not been sequenced and the complete sequence of pSET152 had not been deposited. In order to facilitate the manipulation of these plasmids, we determined their sequences in full using a mixture of shotgun cloning and primer walking; those parts of these plasmids which are already in the public databases under separate accession numbers are listed in Table 2.

The SCP2* origin of replication contains six open reading frames (ORFs). ORFs 1, 4 and 5 do not closely match any previously sequenced genes so there are no obvious clues as to their function. A 34 amino acid portion of ORF1 resembles part of the tetracycline repressor, but the significance of this is hard to judge. ORF3 is a member of the GntR (*gntR*, regulator gene for *E. coli* gluconate metabolism pathway) family of transcriptional regulators. Homologues are found in other *Streptomyces* plasmids and include KorA from *Streptomyces natalensis* plasmid pSNA1 (Mendes *et al.*, 2000), KorsA from *Streptomyces ambofaciens* plasmid pSAM2 (Hagege *et al.*, 1993) and KorA from *S. lividans* plasmid pIJ101 (Stein & Cohen 1990). These genes repress expression of the intermycelial transfer functions of these plasmids (Hagege *et al.*, 1993; Stein & Cohen 1990). Similarly, genes that closely match ORF6 have been found in the *S. coelicolor* genome (accession number Q9X894) and the *S. coelicolor* plasmid SCP1 (accession number CAC36661). Both are putative integral membrane proteins. The SCP2 origin therefore shares two genes with other *Streptomyces* plasmids, whereas some features are novel.

The sequence of pIJ6021/4123 revealed two novel ORFs from the *kan* locus. One is the *Micromonospora echinospora* kanamycin resistance gene (kan_{Mec}), which closely resembles those of other *Micromonopora* species (Kelemen *et al.*, 1991). Downstream of this gene is another ORF whose gene product closely resembles glycosyltransferases. Presumably, kan_{Mec} and this gene are part of a *Micromonospora echinospora* aminoglycoside biosynthetic cluster, resistance to which is conferred by the kan_{Mec} gene. During initial cloning, the glycosyltransferase gene has been fused in frame with part of the *E. coli lacZ*α gene and additional, redundant sequence. The effect of this unintended and presumably non-functional, hybrid protein is unknown.

Construction of *E. coli*-Streptomyces Shuttle Vectors Incorporating The tipA Promoter

pIJ6021 and pIJ4123 have successfully been used to overexpress genes in *S. coelicolor* and *S. lividans* (Matharu *et al.*, 1998; Takano *et al.*, 1995). However, expression cloning using these vectors requires cloning in *Streptomyces* rather than *E. coli*. Using the knowledge of the sequence of these plasmids and of pSET152, we therefore sought to make a more convenient derivative, called pCJW93 (Figure 2). This was used to overexpress the actinorhodin KSαKSβ in *S. coelicolor* CH999. This protein complex was expressed well in its native host and was obtained at a high degree of purity after nickel-affinity

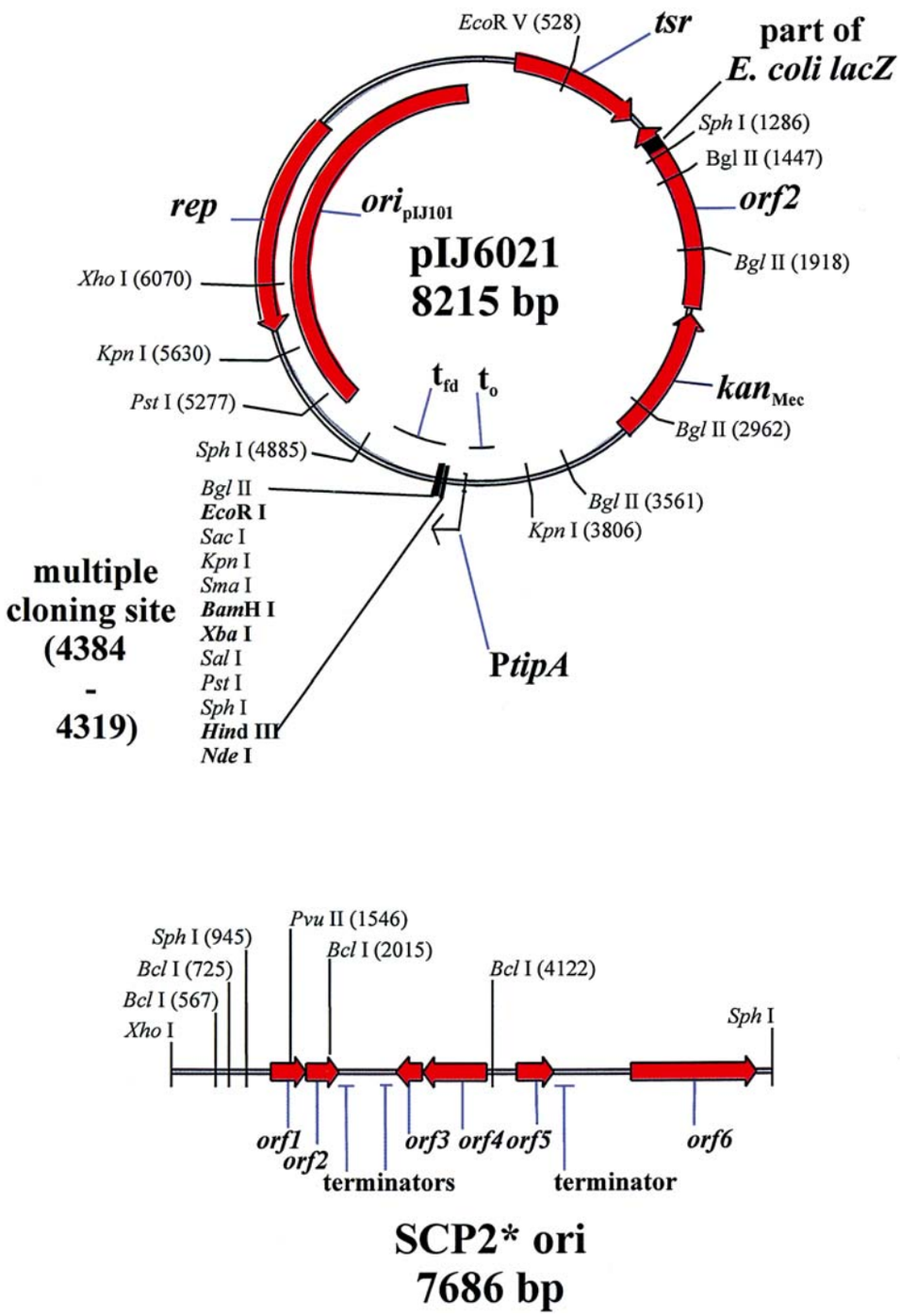


Figure 1. Maps of pIJ6021/4123 and the SCP2* origin of replication. *tsr*, *S. azureus* thiostrepton resistance gene; kan_{Mcc}, *Micromonospora echinospora* kanamycin/gentamicin resistance gene; t_o and t_{fd}, terminators; P_{tipA}, *tipA* promoter; ori_{pIJ101}, origin of replication from pIJ101.

chromatography (Figure 3). Plasmid pCJW93 also functioned just as well in *S. cinnamonensis* (data not shown).

Expression From The *actII-ORF4/P*$_{actI}$ Activator-Promoter System In Heterologous Hosts

The *actII-ORF4*/P_{actI} activator-promoter system functions well in *S. erythraea* (Rowe *et al.*, 1998). To test whether this was due to activation of the *actI* promoter by ActII-ORF4 or whether *S. erythraea* itself possesses homologues of ActII-ORF4 that can substitute for this protein, we deleted the *actII–ORF4* gene from the expression vector pCJR24. The DEBS1-TE modular PKS (Cortés *et al.*, 1995) was then placed under the control of the P_{actI} promoter. When *S. erythraea* JC2 (Δ*eryA eryTEI*$^+$) cells were transformed with this

Table 1. Plasmids and strains used in this study.

	Characteristics	Source
Strains		
S. erythraea JC2	Δ*eryA*	(Rowe *et al.*, 1998)
S. coelicolor CH999	Δact *redE*$^-$ *pro arg*	(McDaniel *et al.*, 1993)
S. coelicolor M512	Δ*actII-ORF4* Δ*redD*	(Floriano & Bibb 1996)
S. cinnamonensis	NRRL 15413, monensin producer	NRRL
S. venezuelae	ATCC 15439; pikromycin / methymycin producer	ATCC
Plasmids		
pSET152	ϕC31 *int* and *attP*; *aac(3)IV* (apramycin) RP4 *mob*	(Bierman *et al.*, 1992)
pCJR24	*actII-ORF4*/P$_{act}$ *tsr*	(Rowe *et al.*, 1998)
pCJR29	*actII-ORF4*/P$_{act}$ *tsr ori*$_{SCP2*}$	(Rowe *et al.*, 1998)
pIJ6021	P$_{tipA}$ *tsr kan*$_{Mec}$ *ori*$_{pIJ101}$	(Takano *et al.*, 1995)
pIJ4123	P$_{tipA}$ *tsr kan*$_{Mec}$ *ori*$_{pIJ101}$ *redD*	(Takano *et al.*, 1995)
pUWL201	P$_{ermE*}$	(Doumith *et al.*, 2000)

construct, fermentation of the recombinant strain yielded less than 0.5% of the triketide lactones produced by the control strain, *S. erythraea* JC2::pCJR65, which produced 50 mg/L of triketide lactones under similar culture conditions. When neither *actII–ORF4* nor P$_{act}$ were upstream of the DEBS1-TE gene, there was no production of triketide lactone, within the limits of detection. Therefore, in *S. erythraea* there is no SARP capable of replacing *actII–ORF4*; and *S. erythraea* must possess factors needed to activate ActII-ORF4 itself, or the transcription of its gene.

In order to test the *actII-ORF4*/P*actI* activator-promoter system in other hosts we made a series of plasmid vectors which incorporated this promoter together with the replication origin of the low copy number *Streptomyces* plasmid SCP2* (pWIT2) (Bibb & Hopwood 1981) and the site-specific integration functions of bacteriophage ϕC31 (pCJR133). To aid expression cloning, the pUC18/19 origin of replication, antibiotic resistance markers for selection in *E. coli*, and the RP4 mobilisation region to enable transfer of the plasmids from *E. coli* to *Streptomyces* (Bierman *et al*. 1992; Mazodier *et al.*, 1989) were also included. The donor *E. coli* strain used for conjugation was ET12567 (MacNeil *et al.*, 1992) containing plasmid pUB307 (Piffaretti *et al.*, 1988). However, strain S17-1 (Simon *et al*., 1983) could also be used.

The properties of these vectors are summarised in Table 3. Initially, these vectors were tested in *S. coelicolor*. As expected, the vectors incorporating the *actII-ORF4*/PactI activator-promoter system, whether self-replicating (pWIT2) or integrating (pCJR133), transformed *S. coelicolor* with high efficiency and led to high levels of expression (20–30 mg/L of triketide lactones present in culture broths). Qualitatively, expression using the constitutive *ermE** promoter was not as high (2 mg/L of triketide lactones from culture broths). We then tested these vectors in a number of other *Streptomyces* hosts. The *actII-ORF4*/P *actI* activator-promoter system did function in both *Streptomyces venezuelae* or in *S. cinnamonensis*, but the level of expression was approximately 1% of that observed in *S. coelicolor*. Similar results were obtained when the *ermE** promoter (pIB139/pIB140) substituted for the *actII-ORF4*/P*actI* activator-promoter system.

Table 2. Features of pSET152, pIJ6021/4123 and the SCP2* origin of replication.

Plasmid	Locus	Function	Best Match	Accession
SCP2*	ORFs 1 4 5	unknown	none	
	ORF2	unknown	*E. coli* tet repressor	
	ORF3	transcriptional regulator	KorA; GntR-family	CAC36612
	ORF6	putative transmembrane protein	SCP1 protein	CAC36661
pIJ6021	1..1065	*tsr* thiostrepton resistance gene		X02392
	3998..4187	bacteriophage lambda t$_o$ terminator		V00635
	4202..4316	*Streptomyces lividans tipA* promoter		M24524
	4389..4763	bacteriophage fd t$_{fd}$ terminator		E03514
	5146..8115	pIJ101 origin		M21778
	1078..2280	none; part glycosyltransferase	butirosin cluster	Q9F204
	1186..1284	none; part of *E. coli lacZ*		
	2317..3141	*kan* kanamycin resistance	gentamicin resistance gene	P24618
pSET152	230..1608	pUC18 *lacZ* and *ori*		
	1609..2761	*aac(3)IV* apramycin resistance gene		X01385
	2810..3583	RP4 *mob* region		X54459
	3624..5715	bacteriophage ϕC31 *int* and *attP*		X59938

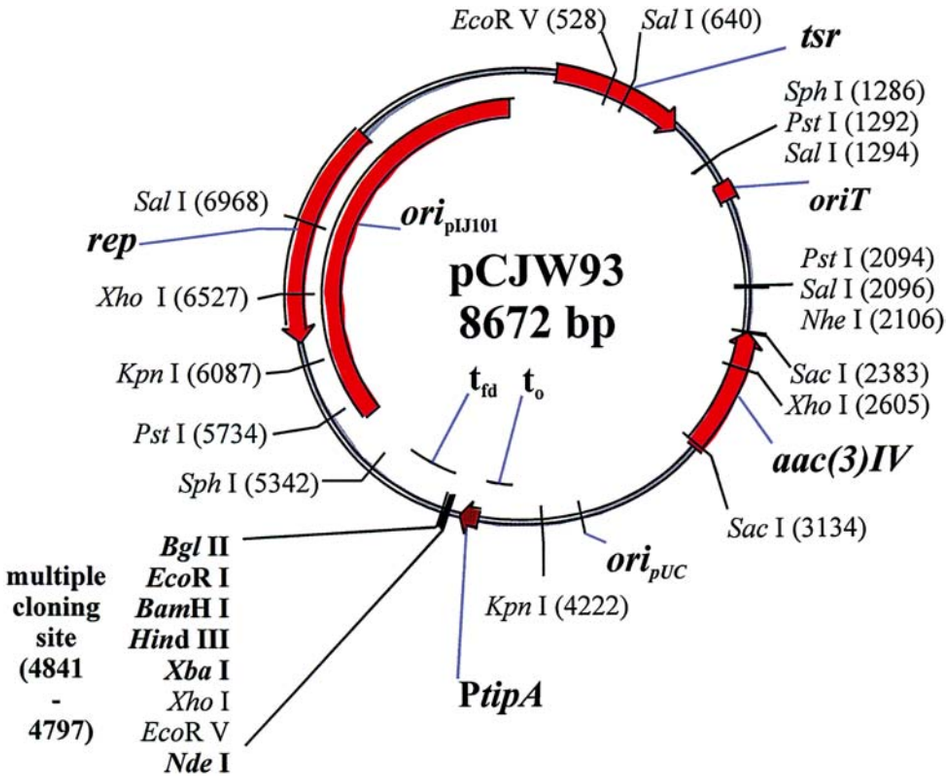


Figure 2. Map of pCJW93. *tsr*, *S. azureus* thiostrepton resistance gene; *oriT*, origin of transfer for single-stranded DNA from RK2; *aac(3)IV* apramycin resistance gene; ori_{pUC}, pUC18.19 origin of replication; t_o and t_{fd}, terminators; P_{tipA}, *tipA* promoter; ori_{pIJ101}, origin of replication from pIJ101.

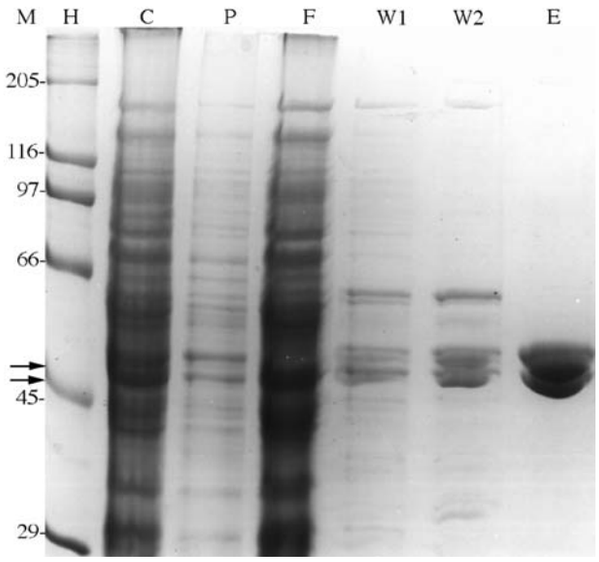


Figure 3. Purification of the Act KSαKSβ from *S. coelicolor* CH999[PCJW93]. M = molecular weight standards/kDa; H = Sgma High Molecular Weight Markers; P = pellet fraction; C = clarified cell extract; W1 = 5 mM imidazole wash fraction; W2 = 50 mM imidazole was fraction; E = eluate from Ni resin column. The Act KSα and KSβ have molecular weights of 45 and 43 kDa respectively. Yield of protein was approximately 10 mg/L.

Engineering the actII-ORF4/pactI Activator-Promoter System

We reasoned that it might be possible to bypass the normal regulation of *actII-ORF4* by using a heterologous promoter to drive transcription of this activator-promoter system, and overcome a relative lack of host factors needed for optimal production and activity of the activator protein. The *ermE** promoter functions in both *S. erythraea*, *S. fradiae* and *S. coelicolor* (see above; Z. A. Hughes-Thomas, unpublished data) so we chose this promoter to test whether such a strategy would work.

In control experiments, *S. coelicolor* M512 (Δ*actII-ORF4*) was complemented as expected by *actII-ORF4* placed under the control of the *ermE* promoter (Figure 4). A more dramatic effect was observed when this cassette was introduced into *S. coelicolor* M512 on a high-copy number plasmid. Exconjugants transformed with this plasmid, pCJW161, produced the blue actinorhodin pigment as soon as colonies were visible (Figure 4).

We then constructed an expression system in which the *ermE** promoter drives transcription of *actII-ORF4* and ActII-ORF4 then drives transcription from the *actI* promoter (Figure 5). We attempted to express the DEBS1-TE triketide lactone synthase in several species using this system cloned on an integrative plasmid (pWIZ1) based on pSET152. In a control experiment, levels of production of the product triketide lactones in *S. coelicolor* were

Table 3. Properties of the expression vectors constructed in this study. amp, ampicillin; apr, apramycin; thio, thiostrepton; hcn, high copy number; lcn, low copy number; ssi, site-specific integration.

Plasmid	Replicon	Antibiotic	Promoter	Other
pCJR133	ssi	apr	*actII-ORF4*/P*actI* activator-promoter	DEBS1-TE
pIB139	ssi	apr	*ermE** promoter	
pIB140	ssi	apr	*ermE** promoter	DEBS1-TE
pWIT2	lcn	amp thio	*actII-ORF4*/P*actI* activator promoter	
pWIT9	lcn	amp thio	*actII-ORF4*/P*actI* activator promoter	DEBS1-TE
pCJW93	hcn	apr	*tipA* promoter	
pCJW94	hcn	apr	*tipA* promoter	*act* KS-CLF
pWIZ1	ssi	apr	*ermE*/actII-ORF4*/P*actI* activator-promoter	DEBS1-TE
pCJW161	hcn	apr	*ermE** promoter	*actII-ORF4*
pCJW5	ssi	apr	*actII* promoter	*actII-ORF4*
pCJW50	ssi	apr	*ermE** promoter	
pCJW52	ssi	apr	*ermE** promoter	*actII-ORF4*

found to be similar to those obtained from *S. coelicolor* CH999::pCJR133. In *S. cinnamonensis*, similar yields were also obtained, in marked contrast to the trace amounts observed in *S. cinnamonensis*::pCJR133 and *S. cinnamonensis*::pIB140 culture broths.

Discussion

There is increasing interest in achieving consistent and efficient heterologous gene expression in a wide range of actinomycete hosts. We have shown here that for the *actII–ORF4*/P*actI* activator-promoter system from *S. coelicolor* to function effectively in the distantly-related actinomycete *S. erythraea*, it is not necessary for that host to contain a SARP activator which replaces the action of ActII-ORF4. Rather, the strain supports transcription of the *actII–ORF4* gene from its own promoter, P*actII*, and the ActII-ORF4 protein then activates the P*actI* promoter. Placing the activator gene under the control of a different promoter (*ermE**) gives equally good expression.

This suggested a strategy for improving the feeble expression from the *actII–ORF4*/P*actI* activator-promoter system in *S. cinnamonensis*, which is otherwise an excellent candidate host strain for the heterologous expression of actinomycete genes. It is robust, grows and sporulates quickly, is transformable at high efficiency, by a wide range of vectors, and is known as an outstandingly efficient producer of polyketides at all scales of fermentation.

The results shown have demonstrated this indeed can be achieved by placing the activator gene under the control of P*ermE**. The *ermE** promoter could obviously be replaced by another promoter known to function in the target strain. As in *S. coelicolor*, the nature of the host factors in *S. erythraea* that promote the transcription of ActII-ORF4 remains elusive. However, the message from these results is that a decisive increase in polyketide production in a heteroglogous host strain has been achieved by replacing the native *actII-ORF4* promoter by P*ermE**.

In contrast, the activation of P*tipA* by the TipA protein is quite well described (Chiu *et al.*, 1996; Chiu *et al.* 1999; Holmes *et al.*, 1993; Murakami *et al.*, 1989). This suggests a similar way of overcoming the restricted host range of the *tipA* promoter (Takano *et al.* 1995; Yun *et al.*, 2001), by providing a copy of the *tip* operon *in trans* to the *tipA* promoter present on the expression vector. However, this strategy might be flawed by the autoregulation of the *tip* operon. Thompson and his colleagues have shown that the *tipA* gene contains an alternative start codon for a protein, TipAS, consisting of the C-terminal domain of the full-length TipA protein, TipAL (Chiu *et al.*, 1999; Holmes *et al.*, 1993). As part of TipAL, this C-terminal domain binds thiostrepton irreversibly and activates the N-terminal transcriptional regulator domain of the TipA protein (Chiu *et al.*, 1999). When expressed as separate protein, this C-terminal domain acts to remove the inducing signal without activating transcription. Translation of TipAS is greater than TipAL (Holmes *et al.*, 1993). It may therefore not in itself be sufficient, for effective expression from the *tip* promoter in a heterologous actinomycete strain, to supply the *tip*operon of *S. lividans* on the expression plasmid. However, it might be possible selectively to interfere with the production of the inhibitory protein (TipAS) by appropriate mutagenesis of the *tipA* gene.

Experimental Procedures

Strains

All DNA manipulations were performed in *E. coli* DH10B (GibcoBRL) using standard culture conditions (Sambrook *et al.*, 1989). *E. coli* cells were transformed by electroporation (Dower *et al.*, 1988). *S. coelicolor* CH999 (McDaniel *et al.*, 1993), *S. coelicolor* M512 (Floriano & Bibb 1996), *S. cinnamonensis* NRRL 15413, *S. venezuelae* ATCC 15439, *S. erythraea* JC2 (Rowe *et al.*, 1998) were used as hosts for expression of PKS genes.

Plasmids

Cloning and expression vectors pUC18 (Yanisch-Perron *et al.*, 1985), pSET152 (Bierman *et al.*, 1992) and pCJR24 / pCJR29 (Rowe *et al.*, 1998) and pIJ6021 /4123 (Takano *et al.*, 1995) have been previously described.

Figure 4. Complementation of the deletion of the actinorhodin positive regulator in *S. coelicolor* M512 by expression of the *actII-ORF4* gene *in trans* under the control of the *ermE** promoter. a) (1) *S. coelicolor* M512::pCJW52, *ermE*/actII-ORF4* cassette on the integrative plasmid pSET152; (2) *S. coelicolor* M512::pCJW5, *actII-ORF4* under the control of its native promoter on the integrative plasmid pSET152; (3) *S. coelicolor* M512. b) *S. coelicolor* M512[pCJW161], *ermE*/actII-ORF4* cassette on a high copy number plasmid derived from pIJ101.

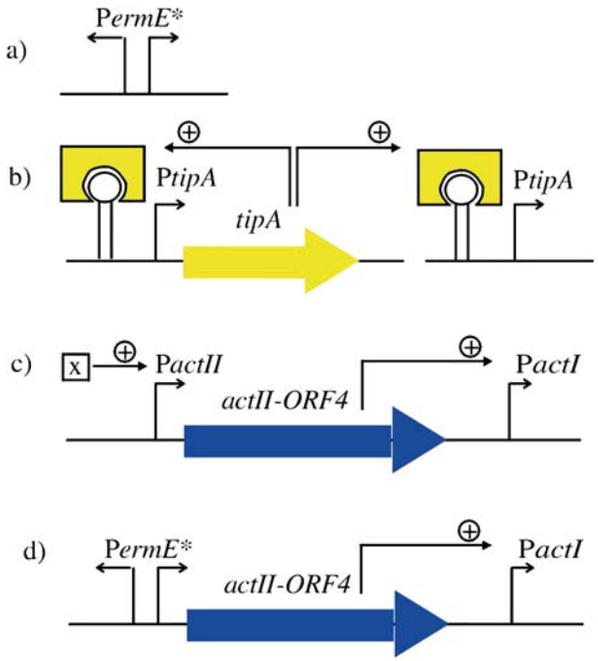


Figure 5. Promoter cassettes constructed during this study. a) *ermE** promoter is constitutive, whereas the *tipA* promoter (b) is inducible, activated by TipA when the protein has bound thiostrepton. The P*actI* promoter (c) is a regulated promoter; the necessary transcription factor, ActII-ORF4, is expressed after *S. coelicolor* has entered stationary phase. We have placed the *actII-ORF4* gene under the control of the *ermE** promoter (d).

pUWL201 was from (Doumith *et al.*, 2000) and plasmid pCB84a was derived from pRJC8006 (Matharu *et al.*, 1998) by insertion of pUC18 between the *tipA* promoter and the fd terminator (C. P. Bisang, unpublished data). The construction of other plasmids used in this study was as follows:

pCJR65: the gene for DEBS1-TE was inserted into pCJR24 as an *Nde* I-*Xba* I fragment.

pCJR89: pCJR65 was digested with *Spe* I and *Nde* I, treated with Klenow fragment of *E. coli* DNA polymerase I to remove the 5′ overhangs and the vector backbone was self-ligated.

pCJR90: pCJR65 was digested with *Spe* I and *Afl* II, treated with Klenow fragment of *E. coli* DNA polymerase I to remove the 5′ overhangs and the vector backbone was self-ligated.

pCJR124: pCJR24 was digested with *Spe* I and *Sal* I, treated with Klenow fragment of *E. coli* DNA polymerase I to remove the 5′ overhangs. The fragment encoding the *actII-ORF4*/PactI activator-promoter system was then ligated to pSET152 vector backbone created by digestion with *Pvu* II and treatment with SAP.

pCJR133: pCJR124 was digested with *Nde* I and *Xba* I and the vector backbone ligated to an *Nde* I-*Xba* I fragment encoding DEBS1-TE obtained from pCJR65.

pCJR135: two fragments were ligated together: a linear pCJR65 made by digestion with *Hin*d III, treatment with Klenow fragment of *E. coli* DNA polymerase I to remove the 5′ overhangs and dephosphorylation with SAP; and a 0.8 kb *Sal* I fragment of pSET152 that had also been treated with Klenow fragment of *E. coli* DNA polymerase I to remove the 5′ overhangs. When these fragments are ligated another *Hin*d III restriction site is created either side of the insert.

For pCJW1, *actII-ORF4* was amplified by PCR using oligonucleotides oCJW2.3 (5′-TACATATGA-GATTCAACTTATTGGGACGTG-3′) and oCJW2.4

(5′-AATCTAGAAGGGGCTCCACCGCGTTCACG-GACCG-3′) as primers and pCJR24 as template. The 800 bp fragment generated was 5′ phosphorylated with T4 polynucleotide kinase then ligated to pUC18 treated with *Sma* I and SAP. pCJW2: the *actII-ORF4* promoter was amplified by PCR using oligonucleotides oCJW2.1 (5′-TTGATATCC-CACTGCCTCTCGGTAAAATCCAGC-3′) and oCJW2.2 (5′-AACATATGCGCCCCCGTCGAGATTCTCCGTCTC-CT-3′) as primers and pCJR24 as template. The 300 bp fragment generated was 5′ phosphorylated with T4 polynucleotide kinase then ligated to pUC18 treated with *Sma* I and SAP.

pCJW5: *actII-ORF4* was excised from pCJW2 as an *Nde* I-*Xba* I fragment and ligated to pCJW8 vector backbone generated with the same enzymes.

pCJW8: *actII-ORF4* promoter was excised from pCJW2 as an *Nde* I-*Eco*R V fragment and ligated to pSET152 vector backbone generated with the same enzymes.

pCJW52: pIB139 and pCJW1 were both digested with *Nde* I and *Xba* I and the fragment encoding ActII-ORF4 was ligated to pIB139 backbone.

pCJW91: pCB84 was digested with *Eco*R I and *Sph*I and the fragment containing t_{fd} ligated with an *Eco*R I-*Sph*I fragment of pSET152 containing ori_{pUC}, *aac(3)IV* and *mob*.

pCJW92: pCJW91 was digested with *Eco*R I and *Pvu*II and ligated to an *Ecl*136II-*Eco*RI fragment of pCB84a containing t_o.

pCJW93: pCJW92 was digested with *Sph*I, dephosphorylated with SAP and ligated to an *Sph*I fragment of pCB84a containing $^{hcn}oriV_{Strep.}$ and *tsr*. The plasmid that was produced with the correct orientation of this fragment was designated pCJW93.

pCJW94: an *Nde*I-*Hin*d III fragment encoding the Act KS-CLF Cys-Ala Gln-Ala mutant (a kind gift of Dr. C. Bisang) was ligated to *Nde*I- and *Hin*d III-cut pCJW93.

pCJW161: pCJW52 and pCJW93 were both digested with *Hin*d III and *Nhe* I. The fragment encoding P_{ermE*}::*actII-ORF4*, ori_{pUC} and *aac(3)IV* was ligated to the fragment encoding *mob*, *tsr* and $^{hcn}oriV_{Strep.}$.

pIB135: the *ermE** promoter was amplified by PCR using oligonucleotides oIB10 (5′-CCACTAG-TATGCATGCGAGTGTCCGTTCGAGTG-3′) and oIB11 (5′-AACATATGTGGATCCTACCAACCGG-CACG-3′) as primers and pUWL201 as template. The 300 bp fragment generated was 5′ phosphorylated with T4 polynucleotide kinase then ligated to pUC18 treated with *Sma* I and SAP. pIB139: the *ermE** promoter was excised from pIB135 as a *Spe* I-*Xba* I fragment and ligated to pSET152 treated with *Xba* I and SAP.

pIB140: pIB139 was digested with *Nde*I and *Xba*I and ligated with an *Nde*I-*Xba*I fragment encoding DEBS1-TE that was obtained by digesting pCJR65 with the same enzymes.

pWIT2: the 0.8kb fragment containing the RP4 *mob* region was excised from pCJR135 by digesting with *Hin*d III. It was then ligated to *Hin*d III-digested and SAP treated pCJR29.

pWIT9: the DEBS1-TE gene was inserted into pWIT2 by ligating *Nde*I-*Xba*I backbone of pWIT2 with an *Nde*I-*Xba*I fragment of the DEBS1-TE gene obtained from pCJR65.

pWIZ1: pCJW1 was digested with *Nde* I and *Pst* I and part of *actII-ORF4* isolated; pCJR133 was digested with *Nde* I and *Nhe* I and the DEBS1-TE gene isolated; and pIB139 was digested with *Nde* I and *Nhe* I and the vector backbone isolated. These three fragments were then ligated to give pWIZ1.

Manipulation of DNA

Routine cloning and transformation procedures for *E. coli* were as previously described (Sambrook *et al.*, 1989). PCR was performed using *Pfu* polymerase (Promega) according to the manufacturer's instructions using a programmable Robocycler Gradient 40 (Stratagene, USA).

DNA Sequencing

Automated DNA sequencing was carried out on double-stranded DNA using an automated ABI 373A sequencer (Applied Biosystems). Parts of the DNA sequence of plasmid pSET152 are already in the EMBL database (accession numbers: ϕC31 *attP* and *int*, x59938; RP4 *mob*, x54459; *aac(3)IV*, x01385). The rest was sequenced by primer walking and submitted to the EMBL sequence database with accession number AJ414670. pWIT2, which contains the SCP2* origin of replication; and pCB84a (C. P. Bisang, unpublished data), which contains pIJ6021/4123, are both pUC-derived; the remaining sequence was determined by a combination of shotgun cloning of 1.5 kb *Sau*3A I fragments followed by end sequencing and primer walking to join the contigs obtained. The sequences of the SCP2* origin and of pIJ6021/4123 have been submitted to the EMBL database with accession numbers AJ414671 and AJ414669 respectively. Part of the *ori* region of SCP2* has been previously studied by Larson and Hershberger (Larson & Hershberger 1990).

Actinomycete Microbiology

Transformation and propagation of *S. coelicolor, S. venezuelae* and *S. cinnamonensis* used standard methods described by Kieser *et al.* (2000). SFM, SY and AM were used, respectively, when generating exconjugant strains. YEME, SCM and SM16 respectively were used as media for these strains for polyketide production. Transformation of *S. erythraea* was carried out using standard methods (Gaisser *et al.*, 1997; Yamamoto *et al.*, 1986) and the strain was propagated on TWM agar. SSDM (Caffrey *et al.*, 1992) or SM3 was used as liquid medium for *S. erythraea* for polyketide production.

Media

AM: Sigma wheat starch 10 g, corn steep powder 2.5 g, yeast extract 3 g,, $CaCO_3$ 3 g, $FeSO_4$ 12 mg, Difco agar 20 g, Milli-Q™ water to 1 l and pH adjusted to 7.0. SCM: soluble starch 15 g, Difco soytone 20 g, $CaCl_2$ 0.1 g, yeast extract 1.5 g, MOPS 10.5 g. SM3: glucose 5 g,

MD30E/glucidex 50 g, soya bean flour 25 g, beet molasses 3 g, K_2HPO_4 0.25 g, $CaCO_3$ 2.5 g, Milli-Q™ water to 1 l and pH adjusted to 7.0 with KOH. TWM: glucose 5 g, sucrose 10 g, tryptone, yeast extract 2.5 g, EDTA 36 mg, tap water to 1 l and pH adjusted to 7.0 with KOH.

Analysis Of Hybrid Triketide Lactone Synthases in vivo

Seed cultures of actinomycete strains were grown at 30°C in TSB (Difco) or YEME for *S. coelicolor*. These cultures were used to inoculate 50 ml of the appropriate production medium which was then incubated for 5 days. 10 ml of this fermentation broth was then used for analysis. The broth was first acidified to pH 3 with formic acid before being extracted three times with an equal volume of ethyl acetate. These crude extracts were then analysed by GC-MS on a GCQ instrument (Finnigan, MAT). A Restek Corporation Crossbond™ 5% diphenyl-, 95% dimethyl-polysiloxane column was used with helium as carrier gas. The constant gas velocity was 50 cm/s and the injector temperature was 250°C. The GC-MS was used in CI (collision ionisation) mode with NH_3 as reagent gas.

Protein Purification

One colony of the relevant strain was selected and a 10 l culture grown in a FT-Applikon fermenter with the appropriate production medium and a 5% inoculum. After 48 h growth, protein expression was induced by addition of thiostrepton to 5 μg/ml. Growth was then continued for another 24 h. Proteins were then purified by nickel affinity chromatography. 20-30 g of cells were resuspended in buffer A (300 mM KH_2PO_4/K_2HPO_4, 200 mM NaCl, 1 mM PMSF, pH 7.4) and by two passes through a French™ Press. The suspension was then clarified by centrifugation at 48000 x *g* for 30 minutes in a Beckman Avanti centrifuge with a JA-20 rotor. 8 ml Ni-NTA resin (Qiagen) were then added to the supernatant and incubated for 1 hour at 4°C.

For purification by FPLC the resin was then allowed to drain in a Pharmacia XK16 column and connected to a Pharmacia AKTA FPLC. The column was then washed with buffer A containing 5 mM imidazole until the A_{280} of the eluate reached a constant low level. The column was then washed with buffer A containing 50 mM imidazole until the A_{280} of the eluate again reached a constant low level. Bound protein was eluted with buffer A using a gradient of imidazole from 50 mM to 250 mM over 3 column volumes.

For batch purification the resin was then washed twice with equal volumes of buffer A containing 5 mM imidazole, then three times with buffer A containing 50 mM imidazole. Resin was then allowed to drain in a gravity-flow column (Qiagen). The target protein was eluted with buffer A containing 250 mM imidazole and 1 ml fractions collected.

Acknowledgements

This work was supported by grants from GlaxoSmithKline (G1038) and from the Biotechnology and Biological Sciences Research Council (BBSRC) (8/B11754) and by a Postdoctoral Fellowship from the Schweizeriche Nationalfonds (to G.W.) and postgraduate studentships from the BBSRC (to C.J.W. and Z.A.H.-T.). We are grateful to Christian Bisang, Russell Cox, Iain Thomas and Sarah Coulthurst for gifts of plasmids; and to John Lester for DNA sequencing.

Supplementary material

Details on plasmid construction and oligonucleotide sequence can be found on the web at http://www.horizonpress.com/jmmb/supplementary/.

References

Arias, P., Fernandez-Moreno, M.A., and Malpartida, F. 1999. Characterization of the pathway-specific positive transcriptional regulator for actinorhodin biosynthesis in *Streptomyces coelicolor* A3(2) as a DNA-binding protein. J. Bacteriol. 181: 6958–6968.

Bibb, M.J., and Hopwood, D.A. 1981. Genetic studies of the fertility plasmid SCP2 and its SCP2* variants in *Streptomyces coelicolor* A3(2). J. Gen. Microbiol. 126: 427–442.

Bibb, M.J., Janssen, G.R., and Ward, J.M. 1985. Cloning and analysis of the promoter region of the erythromycin resistance gene (*ermE*) of *Streptomyces erythraeus*. Gene. 38: 215–226.

Bierman, M., Logan, R., O'Brien, K., Seno, E.T., Nagaraja Rao, R., and Schoner, B.E. 1992. Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. Gene. 116: 43–49.

Caffrey, P., Bevitt, D.J., Staunton, J., and Leadlay, P.F. 1992. Identification of DEBS 1, DEBS 2 and DEBS 3, the multienzyme polypeptides of the erythromycin-producing polyketide synthase from *Saccharopolyspora erythraea*. FEBS. Lett. 304: 225–228.

Chiu, M.L., Folcher, M., Griffin, P., Holt, T., Klatt, T., and Thompson, C.J. 1996. Characterization of the covalent binding of thiostrepton to a thiostrepton-induced protein from *Streptomyces lividan*s. Biochemistry. 35: 2332–2341.

Chiu, M.L., Folcher, M., Katoh, T., Puglia, A.M., Vohradsky, J., Yun, B.S., Seto, H., and Thompson, C.J. 1999. Broad spectrum thiopeptide recognition specificity of the *Streptomyces lividans* TipAL protein and its role in regulating gene expression. J. Biol. Chem. 274: 20578–20586.

Cortés, J., Wiesmann, K.E.H., Roberts, G.A., Brown, M.J.B., Staunton, J., and Leadlay, P.F. 1995. Repositioning of a domain in a modular polyketide synthase to promote specific chain cleavage. Science. 268: 1487–1489.

Doumith, M., Weingarten, P., Wehmeier, U.F., Salah-Bey, K., Benhamou, B., Capdevila, C., Michel, J.M., Piepersberg, W., and Raynal, M.C. 2000. Analysis of genes involved in 6-deoxyhexose biosynthesis and transfer in *Saccharopolyspora erythraea*. Mol. Gen. Genet. 264: 477–485.

Dower, W.J., Miller, J.F., and Ragsdale, C.W. 1988. High efficiency transformation of *E. coli* by high voltage electroporation. Nucl. Acids Res. 16: 6127–6145.

Fernández-Moreno, M.A., Caballero, J.L., Hopwood, D.A., and Malpartida, F. 1991. The *act* cluster contains regulatory and antibiotic export genes, direct targets for translational control by the *bldA* tRNA gene of *Streptomyces*. Cell 66: 769–780.

Floriano, B., and Bibb, M. 1996. *afsR* is a pleiotropic but conditionally required regulatory gene for antibiotic production in *Streptomyces coelicolor* A3(2). Mol. Microbiol. 21: 385–396.

Gaisser, S., Böhm, G.A., Cortés, J., and Leadlay, P.F. 1997. Analysis of seven genes from the *eryAI*/*eryK* region of the erythromycin biosynthetic gene cluster in *Saccharopolyspora erythraea*. Mol. Gen. Genet. 256: 239–251.

Gramajo, H.C., Takano, E., and Bibb, M.J. 1993. Stationary-phase production of the antibiotic actinorhodin in *Streptomyces coelicolor* A3(2) is transcriptionally regulated. Mol. Microbiol. 7: 837–845.

Hagege, J., Pernodet, J.L., Sezonov, G., Gerbaud, C., Friedmann, A., and Guerineau, M. 1993. Transfer-Functions of the Conjugative Integrating Element pSAM2 From *Streptomyces ambofaciens* - characterization of a kil-kor system associated with transfer. J. Bacteriol. 175: 5529–5538.

Holmes, D.J., Caso, J.L., and Thompson, C.J. 1993. Autogenous transcriptional activation of a thiostrepton-induced gene in *Streptomyces lividans*. EMBO J. 12: 3183–3191.

Kelemen, G.H., Cundliffe, E, and Financsek, I. 1991. Cloning and characterization of gentamicin resistance genes from *Micromonospora purpurea* and *Micromonospora rosea*. Gene. 98: 53–60.

Larson, J.L., and Hershberger, C.L. 1990. Insertion of plasmids into the chromosome of *Streptomyces griseofuscus*. Plasmid. 23: 252–256.

Lombo, F., Brana, A.F., Mendez, C., and Salas, J.A. 1999. The mithramycin gene cluster of *Streptomyces argillaceus* contains a positive regulatory gene and two repeated DNA sequences that are located at both ends of the cluster. J. Bacteriol. 181: 642–647.

MacNeil, D.J., Gewain, K.M., Ruby, C.L., Dezeny, G., Gibbons, P.H., and MacNeil, T. 1992. Analysis of *Streptomyces avermitilis* genes required for avermectin biosynthesis utilizing a novel integration vector. Gene 111: 61–68.

Matharu, A.L., Cox, R.J., Crosby, J., Byrom, K.J., and Simpson, T.J. 1998. MCAT is not required for *in vitro* polyketide synthesis in a minimal actinorhodin polyketide synthase from *Streptomyces coelicolor*. Chem. Biol. 5: 699–711.

Mazodier, P., Petter, R., and Thompson, C. 1989. Intergeneric conjugation between *Escherichia coli* and *Streptomyces* species. J. Bacteriol. 171: 3583–3585.

McDaniel, R., Ebert-Khosla, S., Hopwood, D.A., and Khosla, C. 1993. Engineered biosynthesis of novel polyketides. Science. 262: 1546–1550.

Mendes, M.V., Aparicio, J.F., and Martin, J.F. 2000. Complete nucleotide sequence and characterization of pSNA1 from pimaricin-producing *Streptomyces natalensis* that replicates by a rolling circle mechanism. Plasmid. 43: 159–165.

Murakami, T., Holt, T.G., and Thompson, C.J. 1989. Thiostrepton-induced gene expression in *Streptomyces lividans*. J. Bacteriol. 171: 1459–1466.

Piffaretti, J.C., Arini, A., and Frey, J. 1988. pUB307 mobilizes resistance plasmids from *Escherichia coli* into *Neisseria gonorrhoeae*. 212: 215–218.

Rowe, C.J., Cortés, J., Gaisser, S., Staunton, J., and Leadlay, P.F. 1998. Construction of new vectors for high-level expression in Actinomycetes. Gene. 216: 215–223.

Salahbey, K., Blanc, V., and Thompson, C.J. 1995. Stress-activated expression of a *Streptomyces pristinaespiralis* multidrug-resistance gene (Ptr) in various *Streptomyces* Spp and *Escherichia coli*. Mol. Microbiol. 17: 1001–1012.

Salahbey, K., and Thompson, C.J. 1995. Unusual regulatory mechanism for a *Streptomyces* multidrug- resistance gene, Ptr, involving 3 homologous protein-binding sites overlapping the promoter region. Mol. Microbiol. 17: 1109–1119.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Simon, R., Priefer, U., and Pühler, A. 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram-negative bacteria. BioTechnology 1: 784–791.

Stein, D.S., and Cohen, S.N. 1990. Mutational and functional analysis of the KorA and KorB gene products of *Streptomyces* plasmid pIJ101. Mol. Gen. Genet. 222: 337–344.

Stutzman-Engwall, K.J., Otten, S.L., and Hutchinson, C.R. 1992. Regulation of secondary metabolism in *Streptomyces*spp. and overproduction of daunorubicin in *Streptomyces peucetius*. J. Bacteriol. 174: 144–154.

Takano, E., Gramajo, H.C., Strauch, E., Andres, N., White, J., and Bibb, M.J. 1992. Transcriptional regulation of the *redD* transcriptional activator gene accounts for growth phase-dependent production of the antibiotic undecylprodigiosin in *Streptomyces coelicolor* A3(2). Mol. Microbiol. 6: 2797–2804.

Takano, E., White, J., Thompson, C.J., and Bibb, M.J. 1995. Construction of thiostrepton-inducible, high-copy-number expression vectors for use in *Streptomyces* spp. Gene. 166: 133–137.

Tang, L., Grimm, A., Zhang, Y.X., and Hutchinson, C.R. 1996. Purification and characterization of the DNA-binding protein DnrI, a transcriptional factor of daunorubicin biosynthesis in *Streptomyces peucetius*. Mol. Microbiol. 22: 801–813.

White, J., and Bibb, M. 1997. *bldA* dependence of undecylprodigiosin production in *Streptomyces coelicolor* A3(2) involves a pathway-specific regulatory cascade. J. Bacteriol. 179: 627–633.

Wietzorrek, A., and Bibb, M. 1997. A novel family of proteins that regulates antibiotic production in *Streptomycetes* appears to contain an OmpR-like DNA-binding fold. Mol. Microbiol. 25: 1181–1184.

Yamamoto, H., Maurer, K.H., and Hutchinson, C.R. 1986. Transformation of *Streptomyces erythraeus*. J. Antibiot. 39: 1304–1313.

Yanisch-Perron, C., Vieira, J., and Messing, J. 1985. Improved M13 phage cloning vectors and host strains - nucleotide sequences of the M13MP18 and pUC19 ectors. Gene 33: 103–109.

Yun, B.S., Hidaka, T., Kuzuyama, T., and Seto, H. 2001. Thiopeptide non-producing *Streptomyces* species carry the *tipA* gene: a clue to its function. J. Antibiot. 54: 375–378.