

# Application of *redD*, the Transcriptional Activator Gene of the Undecylprodigiosin Biosynthetic Pathway, as a Reporter for Transcriptional Activity in *Streptomyces coelicolor* A3(2) and *Streptomyces lividans*

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## Abstract

*redD* encodes the transcriptional activator of the biosynthetic pathway for undecylprodigiosin, a red-pigmented, mycelium-bound antibiotic made by *Streptomyces coelicolor* A3(2) and *Streptomyces lividans*. A promoterless version of *redD* preceded by the efficiently used *tuf1* ribosome binding site was inserted into two different plasmid vectors, providing a convenient reporter of transcriptional activity in both species. One plasmid, pIJ2587, replicates autonomously in both *Escherichia coli* and streptomycetes, while the other, pIJ2585, replicates in *E. coli* and can be transferred to streptomycetes by conjugation or transformation, whereupon it integrates stably at the chromosomal attachment site for the temperate phage  $\phi$ C31. The utility of the plasmids in detecting not only transcriptional activity, but also its regulation, was confirmed using the *rrnAp*, *ermEp*<sup>\*</sup>, and *glnRp* promoters. The ability to screen visually and spectrophotometrically for red pigmentation should make the vectors particularly attractive for analysing the regulation of gene expression, and for the isolation of mutants, in both *S. coelicolor* and *S. lividans*.

## Introduction

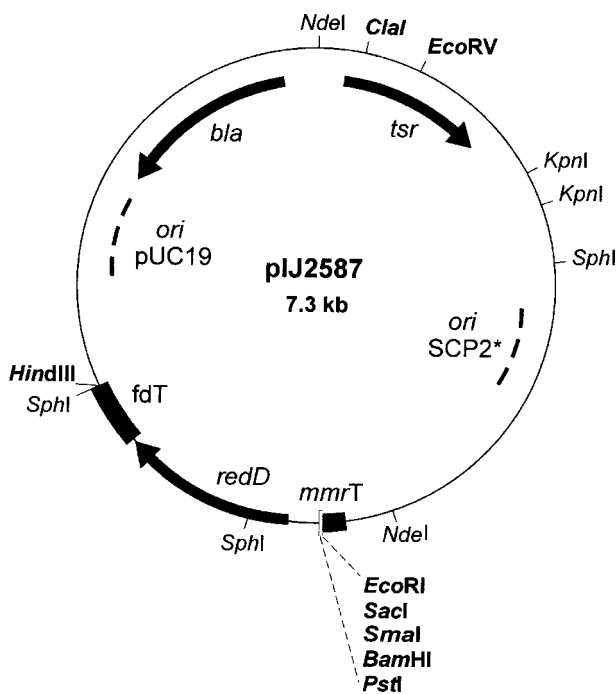
Reporter genes, such as *lacZ* of *Escherichia coli* (Silhavy *et al.*, 1984), have proven invaluable for genetic analysis in a wide range of microorganisms, facilitating the isolation of mutants and frequently allowing convenient and simple monitoring of transcriptional and translational activity. Members of the genus *Streptomyces* are Gram-positive, mycelial soil bacteria that make many important secondary metabolites; they also undergo a complex process of morphological development that typically results in sporulation (Chater and Losick, 1997). Several reporter

systems have been developed to enable or simplify transcriptional analysis of these and other processes in streptomycetes. These include *xylE* of *Pseudomonas putida*, encoding catechol dioxygenase (Ingram *et al.*, 1989; Clayton and Bibb, 1990), *neo* from the transposon Tn5 (Beck *et al.*, 1982), which confers resistance to kanamycin and neomycin (Ward *et al.*, 1986), the *luxAB* operon of *Vibrio harveyi*, which confers light emission (Schauer *et al.*, 1988), *melC* of *Streptomyces glaucescens*, which encodes tyrosinase (Paget *et al.*, 1994), and the EGFP gene for green-fluorescent protein (Sun *et al.*, 1999). While these reporter genes have been applied successfully to individual studies, none has so far gained wide-spread use. The most often used reporter gene is probably *xylE*. Although successful in some studies (e.g. Ingram *et al.*, 1989; Delic *et al.*, 1992), XylE activity can be difficult to assess at low levels of expression. Furthermore, detection on agar plates requires spraying the colonies with catechol, preventing the subsequent temporal analysis of gene expression. While the EGFP gene should prove particularly useful when analysing the spatial and temporal regulation of gene expression during development, it is less suitable for screening promoter libraries, and requires additional equipment for detection of fluorescence.

*Streptomyces coelicolor* A3(2) is by far the most genetically characterised streptomycete, and the recognised model species for the genus. It possesses a large genome of approximately 8 Mb that is currently the subject of a genome sequencing project ([www.sanger.ac.uk/Projects/S\\_coelicolor/](http://www.sanger.ac.uk/Projects/S_coelicolor/)). Efficient exploitation of this sequence data will undoubtedly require the development of new and improved reporter systems. *redD* encodes the transcriptional activator of the biosynthetic genes for the red-pigmented tripyrrole antibiotic undecylprodigiosin (Red) (Narva and Feitelson, 1990; Takano *et al.*, 1992). RedD belongs to the SARP family of antibiotic regulatory proteins (Wietzorrek and Bibb, 1997), and is the final protein in a pathway-specific regulatory cascade for activation of Red synthesis (White and Bibb, 1997). While many genes influence Red production (reviewed in Bibb, 1996), the only limitation to Red synthesis appears to be the availability of sufficient RedD to activate transcription of the Red biosynthetic structural genes. Moreover, the level of Red synthesis appears to reflect the intracellular concentration of RedD (Takano *et al.*, 1992; White and Bibb, unpublished). Furthermore, Red remains in the mycelium, unlike the second pigmented antibiotic made by *S. coelicolor* (actinorhodin, Act), greatly facilitating the simple identification of Red producing or non-producing variants at high colony densities.

In this work, the use of *redD* as a reporter gene for transcriptional activity in *S. coelicolor* A3(2) was assessed.

Received January 17, 2000; revised April 1, 2000; accepted April 1, 2000.  
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GAATTGGAGCTCCCGGATCTGCAGGGCTAACCTGATCCTCTAGGGGACTTAACAGCAAAGATC  
 EcoRI SacI SmaI BamHI PstI  
 ...  
 ACCTGGCGCCGATTGAACCAAGCGCTACAGAACCCTCCACAGGAGGAATTTATGGAAATCAAC  
 RBS M E I N  
 ATATTGGGACCCGATTCGATCGACACGTCGCACAGCGGGCGGGCGG  
 I L G P V S I D T S H S G G G

Figure 1. Restriction map of plJ2587, and the sequence of the multiple cloning site (MCS) and start of *redD*. (A) Map of plJ2587. Filled arrows indicate direction of transcription. Unique restriction sites are shown in bold face. (B) Sequence of the MCS and of the start of *redD*. The start of the *redD* coding sequence is shown in italics, with the aa translation shown under the sequence. Translation stop codons preceding the *redD* coding sequence and present in each of the three possible reading frames are indicated by dots above the nt sequence; the *tuf1* RBS (AGGAGG) is underlined. Enzymes that cut within the MCS are given below the DNA sequence, and their recognition sequences are indicated by lines above or below the nt sequence.

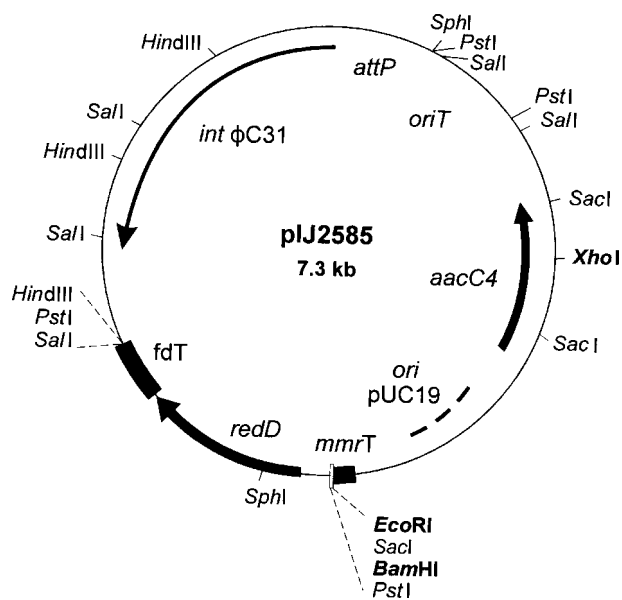


Figure 2. Restriction map of plJ2585. Filled arrows indicate the direction of transcription. Unique restriction sites are shown in bold face. The nt sequence of the start of the coding region of *redD* and of the upstream region is shown in Figure 1B.

For most of the experiments *S. coelicolor* strain M512 was used (Floriano and Bibb, 1996); it contains deletions in *actII-ORF4* (encoding the transcriptional activator of the *act* gene cluster) and *redD*, rendering the cells essentially colourless. Previous unpublished experiments indicated that restoration of a low level of RedD production in M512 would restore Red biosynthesis, resulting in easily detected red colonies. The closely related species *Streptomyces lividans* was also used. While *S. coelicolor* is the preferred streptomycete for most genetic studies, *S. lividans* has gained widespread use as a general *Streptomyces* cloning host, and is frequently used for the expression of heterologous genes (Binnie *et al.*, 1997). Although *S. lividans* possesses an intact *red* gene cluster, on most laboratory media it is either not expressed at all or at only low levels; however, its expression can be activated by introduction of *redD* from *S. coelicolor*.

Table 1. Plasmids Used and Constructed

Plasmid	Description	Reference
pHJL401	<i>E. coli-Streptomyces</i> shuttle vector with pUC18 and SCP2* origins of replication ( <i>ori</i> )	Larson and Herschberger, 1986
pSET152	<i>E. coli-Streptomyces</i> shuttle vector with pUC18 <i>ori</i> and <i>oriT</i> . Integrates at the $\phi$ C31 attachment site in streptomycetes.	Biermann <i>et al.</i> , 1992
pMT3002	Promoter-probe vector containing <i>melC</i> flanked by transcriptional terminators	Paget <i>et al.</i> , 1994
pJ4114	pJ2925 containing 900 bp <i>S. coelicolor redD</i> gene with an <i>NdeI</i> site overlapping the translational start codon	This work
pUSRT3-3	pUC18 containing the <i>S. ramocissimus tuf1</i> gene and 300 bp of upstream region, with an <i>EcoRI</i> site immediately downstream of the RBS	Vijgenboom <i>et al.</i> , 1994
pJ2582	pUC18 containing <i>redD</i> fused to the RBS of <i>S. ramocissimus tuf1</i>	This work
pJ2583	pMT3002 containing <i>redD</i> cassette, consisting of promoterless <i>redD</i> gene preceded by RBS of <i>S. ramocissimus tuf1</i> , and flanked by the <i>mmrT</i> and <i>fdT</i> terminator sequences	This work
pJ2586	pHJL401 containing insert of pJ2583	This work
pJ2587 (Figure 1)	pJ2586 with modified MCS	This work
pJ2587- <i>ermE</i> *p	pJ2587 containing the <i>Sac. erythraea ermE</i> * in front of <i>redD</i>	This work
pJ2587- <i>glnRp</i>	pJ2587 with <i>S. coelicolor glnRp</i> in front of <i>redD</i>	This work
pJ2587- <i>rrnAp</i>	pJ2585 with <i>rrnAp</i> in front of <i>redD</i>	This work
pJ2585 (Figure 2)	pSET152 containing <i>redD</i> cassette from pJ2587	This work
pJ2585- <i>ermE</i> *p	pJ2585 containing the <i>Sac. erythraea ermE</i> * in front of <i>redD</i>	This work
pJ2585- <i>glnRp</i>	pJ2585 with <i>S. coelicolor glnRp</i> in front of <i>redD</i>	This work
pJ2585- <i>rrnAp</i>	pJ2585 with <i>S. coelicolor rrnAp</i> in front of <i>redD</i>	This work

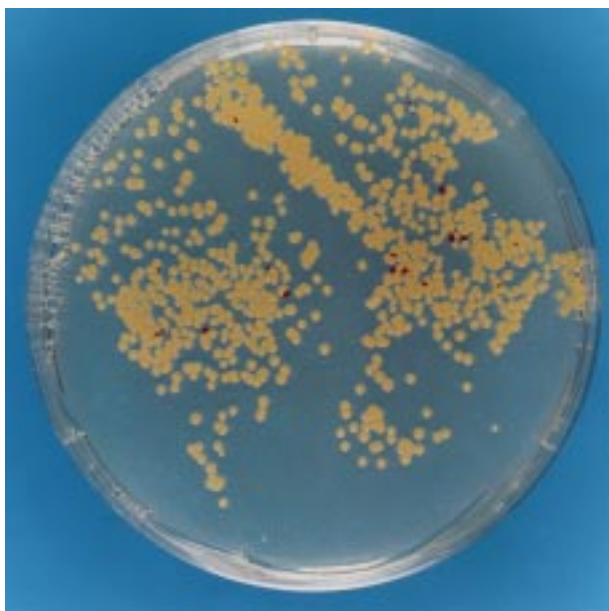


Figure 3. Detection of Red-producing transformants among control colonies. Colonies of *S. coelicolor* strain M512 harbouring pIJ2587 were mixed with the same strain harbouring pIJ2587-*glnRp*, which allows undecylprodigiosin production, and plated on R2YE with 50 mM thiostrepton. The Red-producing colonies clearly stand out among the yellowish control transformants.

This paper describes the construction of a reporter cassette based on a promoterless *redD* gene, and its use in integrative and low-copy-number shuttle vectors to assess the activity of both constitutively expressed and regulated promoters.

## Results and Discussion

### Utility of the *redD* Promoter-Probe Vectors pIJ2585 and pIJ2587

Two *redD* promoter-probe vectors were constructed (see Experimental Procedures). pIJ2587 (Figure 1) is an *E. coli*-*Streptomyces* shuttle vector derived from pHJL401 (Larson and Herschberger, 1986) with pUC19 and SCP2\* origins of replication; it possesses a copy number of approximately 10 per chromosome in streptomycetes. pIJ2585 (Figure 2) is a derivative of the conjugative pSET152 (Bierman *et al.*, 1992) and integrates at single copy into the chromosomal attachment site of the temperate phage  $\phi$ C31.

To assess initially the utility of pIJ2587, two highly expressed promoter elements, the *ermE*\* promoter of the related actinomycete *Saccharopolyspora erythraea* and part of the promoter region of the ribosomal RNA operon *rrnA* of *S. coelicolor*, were used. A 0.3 kb *EcoRI*-*Bam*HI fragment from pIJ4090 containing the *ermE*\* promoter (Bibb *et al.*, 1994), and a 0.5 kb *EcoRI*-*Bam*HI fragment from pUSCRA-U1 containing the *rrnA* P2, P3 and P4 promoters (van Wezel *et al.*, 1995), were cloned in *EcoRI* + *Bam*HI-digested pIJ2587, resulting in pIJ2587-*ermEp*\* and pIJ2587-*rrnAp*, respectively (Table 1).

Introduction of pIJ2587-*ermEp*\* or pIJ2587-*rrnAp* into M512 (M145  $\Delta$ *redD*,  $\Delta$ *actII*-ORF4) led to strong Red production on R2YE and MM agar plates, with the red pigment clearly apparent as soon as the colonies were visible, while control transformants (pIJ2587 without an insert) remained white, with no hint of Red production even after prolonged incubation. We also assessed Red production in *S. lividans* 1326 using the same three plasmids, and in addition the parental vector pHJL401. *S. lividans* derivatives containing pIJ2587 or pHJL401 showed no difference in Red production, which occurred at a low level after approximately three days on R2YE, consistent with the absence of promoter activity in pIJ2587. However, transformants containing pIJ2587-*ermEp*\* or pIJ2587-*rrnAp* produced large amounts of Red pigment as soon as the colonies were visible, indicating that the *redD* cassette can also be used to assess promoter activity in *S. lividans*.

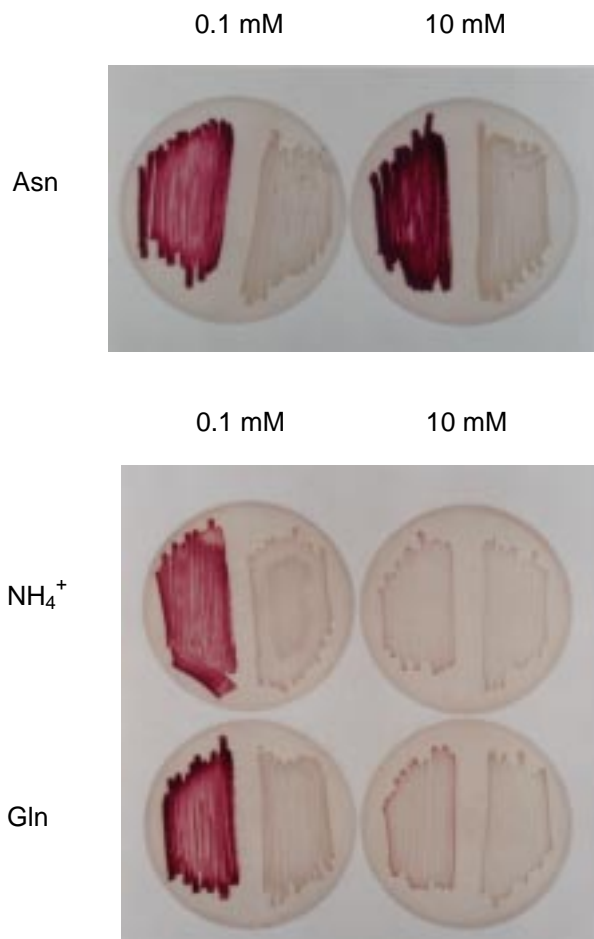


Figure 4. Dependence of *glnR* promoter activity on nitrogen source. Transformants were grown on MM plates with 1% glucose as carbon source, and incubated for 4 days at 30°C. On each plate, M512/pIJ2587-*glnRp* is shown on the left, and M512/pIJ2587 (control) on the right. Nitrogen sources and concentrations used in the plates were: Top panel: left, 0.1 mM Asn; right, 10 mM Asn. Bottom panel: Top left, 0.1 mM NH<sub>4</sub>Cl; top right, 10 mM NH<sub>4</sub>Cl; bottom left, 0.1 mM Gln; bottom right, 10 mM Gln.

### Use of the *redD* Cassette to Analyse the Regulation of the *glnR* Promoter

The *S. coelicolor* *glnR* gene encodes a transcriptional activator of *glnA*. *glnA* encodes glutamine synthetase I (GSI) (Wray *et al.*, 1991; Wray and Fisher, 1993), which converts glutamate and ammonia into glutamine in an ATP-dependent manner (Reitzer, 1996). No *glnA* transcription is observed in a *glnR* null mutant (Wray *et al.*, 1991). In the wild-type strain, a high level of ammonia (which leads to the rapid depletion of glutamate) or glutamine cause negative feedback regulation of GSI production, and hence of *glnR*-dependent activation of *glnA* transcription. Transcription of *glnR* occurs from three promoters, P1-P3, with P1 located closest to the start of the gene, and does not appear to be autoregulated (Wray and Fisher, 1993).

To analyse the transcriptional regulation of *glnR*, and to assess the utility of pIJ2587 as a reporter for regulated transcription, the ability of the *glnR* promoters to transcribe *redD* in the presence of various nitrogen sources was tested. A 1 kb *EcoRI*-*BglII* fragment harbouring the *glnR* promoter region was cloned in *EcoRI* + *Bam*HI-digested pIJ2587, resulting in pIJ2587-*glnRp* (Table 1). pIJ2587-*glnRp* was introduced into *S. coelicolor* M512, and the resulting transformants assayed for Red production. On the rich medium R2YE, M512/pIJ2587-*glnRp* produced large amounts of Red, while the control strain M512/pIJ2587 remained colourless. A few pIJ2587-*glnRp* transformants expressing *redD* were readily detected among many control pIJ2587 transformants, confirming the possible use of the *redD* cassette in screening promoter libraries (Figure 3; the detection of Red non-producing derivatives in an almost confluent lawn of red colonies containing pIJ2587-*glnRp* proved equally facile). To test the influence of different nitrogen sources on *glnR* promoter activity, M512/pIJ2587-*glnRp* was grown on MM plates containing either 1% glucose or 1% mannitol as carbon sources, and increasing concentrations of ammonium chloride, asparagine, or glutamine (10  $\mu$ M, 0.1 mM, 1 mM, and 10 mM) as nitrogen sources. At these concentrations, neither nitrogen source had a negative influence on Red production by *S. coelicolor* M145 or M512/pIJ2587-*ermEp*\*, indicating the lack of any significant level of repression or inhibition of the *red* biosynthetic genes or enzymes, respectively. Consequently, any effect on Red production in M512/pIJ2587-*glnRp* should reflect alterations in *glnR* promoter activity. M512/pIJ2587-*glnRp* produced large amounts of Red when grown on MM plates containing asparagine, irrespective of the carbon source or the asparagine concentration used, indicating that asparagine had no effect on *glnR* transcription (Figure 4A). However, both ammonium chloride and glutamine had a strong repressive effect on *glnR* promoter activity, as shown by the complete absence of Red production on plates containing either nitrogen source at concentrations of 1 mM or higher (Figure 4B). Using a narrower range of concentrations, the pivotal point for *glnR* repression lay around 0.7 mM for both nitrogen sources. This is consistent with observations in *E. coli* where, at concentrations below 0.1 mM, ammonium ions are converted into glutamine, and glutamine synthetase expression is high, while at concentrations above 1 mM, ammonium ions are largely incorporated into other molecules (Reitzer, 1996). These results suggest that transcription of *redD* from the *glnR* promoter in pIJ2587-*glnRp* truly reflects the regulation of

the chromosomal *glnR* gene, underlining the suitability of pIJ2587 for studying the regulation of gene expression in *S. coelicolor*.

Transformants harbouring pIJ2587-*glnRp* were grown in liquid minimal medium (NMMP) with either asparagine, glutamine or ammonium chloride (20 mM) as nitrogen sources. Cultures grown in NMMP + Asn produced a large amount of Red, while those grown in NMMP + ammonium chloride or NMMP + Gln showed no visible pigmentation, confirming the data obtained with agar-grown cultures, namely that *glnR* promoter-activity is repressed efficiently by ammonium ions and glutamine, but not by asparagine. Since undecylprodigiosin can be extracted from liquid-grown mycelium (Tsao *et al.*, 1985), and its concentration determined spectrophotometrically ( $\lambda_{max}$  = 533 nm; extinction coefficient ( $\epsilon$ ) =  $10^5$ ; MW = 393), in principle the *redD* cassette can also be used for quantitative assessment of transcriptional activity. However, since Red is the product of a complex enzymatic pathway that may be subject to a variety of physiological influences, it is likely that the relationship between the level of *redD* transcription and Red production would have to be determined empirically for each growth condition used.

### Integration of the *redD* Cassette into the Chromosome

To test the utility of pIJ2585, we inserted the *ermE*\*, *rrnA* and *glnR* promoters into the vector, using the same cloning strategy as for pIJ2587. The resulting constructs pIJ2585-*ermEp*\*, pIJ2585-*rrnAp*, and pIJ2585-*glnRp* (Table 1) were tested for Red production on agar plates. The results were similar to those obtained with pIJ2587; *S. coelicolor* M512 transformants harbouring pIJ2585-*ermEp*\* or pIJ2585-*rrnAp* showed strong Red production early in growth; transformants containing pIJ2585-*glnRp* produced high levels of Red at glutamine concentrations of 0.5 mM or lower, while Red production was strongly repressed by high glutamine levels.

### Conclusions

We have shown the promoterless *redD* gene to be a useful reporter of transcriptional activity in *S. coelicolor* and *S. lividans*. Analysis of a *glnRp-redD* fusion illustrated that the system can be applied efficiently to analyse the regulation of transcription. While the vectors can be used in principle to quantify transcriptional activity, they should prove particularly effective when screening promoter libraries, and perhaps more importantly, for the isolation of mutants, where the ability to screen large numbers of mutagenised colonies is desirable. The system described should add significantly to the armoury of genetic tools available for the study of the biology of both *S. coelicolor* and *S. lividans*.

### Experimental Procedures

#### Bacteria and Growth Conditions

*E. coli* K-12 strain JM109 (Messing *et al.*, 1981) was used for routine sub-cloning, and was grown and transformed by standard procedures (Sambrook *et al.*, 1989). *S. coelicolor* A3(2) strains M145 (Hopwood *et al.*, 1985) and M512 (Floriano and Bibb, 1996), and *S. lividans* 1326 (Hopwood *et al.*, 1985) were used for transformation and propagation of *Streptomyces* plasmids. Protoplast preparation and transformation were performed as described by Hopwood *et al.* (1985). The rich solid medium R2YE was used for regenerating protoplasts; R2YE and the minimal medium (MM) plates, containing the appropriate antibiotic, were used for screening recombinants. For submerged cultures we used minimal medium (NMMP)

with 1% mannitol as carbon source and either ammonium chloride or asparagine (20 mM) as nitrogen source. Plasmids used and constructed in this paper are summarised in Table 1.

#### Construction of pIJ2585 and pIJ2587

A promoterless version of *redD* of *S. coelicolor* strain M145 with an *NdeI* site overlapping the translational start codon was obtained from pIJ4114 (unpublished construct). To provide a ribosome binding site (RBS) for *redD*, the upstream region of the *tuf1* gene of *Streptomyces ramocissimus* was used. *tuf1* encodes the translation elongation factor EF-Tu, which is expressed at high levels in rapidly growing cells, and is likely to possess an efficient RBS (Vijgenboom *et al.*, 1994; Motamedi *et al.*, 1995). The 0.9 kb *NdeI* fragment from pIJ4114 harbouring the promoterless *redD* was cloned in *EcoRI*-digested pURT3-3 containing the *tuf1* RBS (Vijgenboom *et al.*, 1994). To achieve this, the 5' protruding ends of the *NdeI* and *EcoRI* sites were filled-in using the Klenow fragment of DNA polymerase I and dNTPs using standard procedures (Sambrook *et al.*, 1989). The 1 kb *SmaI* fragment of the resulting plasmid pIJ2582 (containing *redD* fused to the ribosome binding site of *tuf1*) was cloned in *XbaI*-digested and filled-in pMT3002 (Paget *et al.*, 1994), resulting in pIJ2583, a high-copy number *E. coli* vector containing the promoterless *redD* flanked by transcriptional termination signals.

To introduce the *redD* cassette into *S. coelicolor*, pHJL401, an *E. coli*-*Streptomyces* shuttle vector containing the *E. coli* pUC19 and *Streptomyces* SCP2\* (Lydiat *et al.*, 1985) origins of replication (Larson and Herschberger, 1986) was used, giving 100-200 copies per cell in *E. coli* and approximately 10 copies per chromosome in *Streptomyces*. To achieve this, the *EcoRI*-*SmaI* segment of the multiple cloning site of pHJL401 was removed, and the *BglII* insert from pIJ2583 was ligated into the newly created vector pHJL401- $\Delta$ ESm, resulting in pIJ2586. The *EcoRI*-*BamHI* segment of pIJ2586 was subsequently replaced by a double-stranded oligonucleotide, containing unique *BamHI*, *EcoRI*, *PstI*, *SacI*, and *SmaI* sites, resulting in pIJ2587. A restriction map of pIJ2587 is shown in Figure 1A. The sequence of the multiple cloning site and of the start of *redD* is shown in Figure 1B; the *redD* coding sequence is preceded by translational stop codons in each of the three possible reading frames, preventing the formation of potentially deleterious RedD translational fusions.

To enable insertion of the promoterless *redD* gene into the *Streptomyces* chromosome at single copy, the *redD* cassette was cloned in the conjugative vector pSET152 (Bierman *et al.*, 1992), which integrates at the chromosomal attachment site of the temperate phage  $\phi$ C31. To accomplish this, the *NdeI*-*HindIII* fragment of pIJ2587 harbouring the *redD* cassette was inserted into *EcoRI* + *XbaI*-digested pSET152, after filling in the 5' protruding ends with the Klenow fragment of DNA polymerase I and dNTPs, resulting in pIJ2585 (Figure 2).

#### Isolation and Manipulation of the *glnR* Promoter Region

DNA from cosmid D84 (Redenbach *et al.*, 1994) containing *glnR* was digested with *NcoI*, and the 1 kb fragment containing the promoter region and 5' end of *glnR* was cloned into *SmaI*-digested pIJ2925 (Janssen and Bibb, 1993) after filling in the 5' protruding ends of the *NcoI* fragment with the Klenow fragment of DNA polymerase I and dNTPs. A clone with *glnR* reading towards the *HindIII* site of the pIJ2925 multiple cloning site was selected, and the 1 kb *EcoRI*-*BglII* fragment harbouring the *glnR* promoter region was cloned in *EcoRI* + *BamHI*-digested pIJ2587, resulting in pIJ2587-*glnRp* (Table 1).

#### Acknowledgements

We thank Belén Floriano for helpful discussions, and Keith Chater for comments on the manuscript. This work was supported by a grant from the Biotechnology and Biological Sciences Research Council to the John Innes Centre, and by the European Community (HCM programme) to GVW.

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