The *dnrO* gene encodes a DNA-binding protein that regulates daunorubicin production in *Streptomyces peucetius* by controlling expression of the *dnrN* pseudo response regulator gene

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The *dnrO* gene is located adjacent to and divergently transcribed from the response regulator gene, *dnrN*, that activates the transcription of the *dnrI* gene, which in turn activates transcription of the daunorubicin biosynthesis genes in *Streptomyces peucetius*. Gene disruption and replacement of *dnrO* produced the *dnrO::aphII* mutant strain and resulted in the complete loss of daunorubicin biosynthesis. Suppression of the *dnrO::aphII* mutation by the introduction of *dnrN* or *dnrI* on a plasmid suggested that DnrO is required for the transcription of *dnrN*, whose product is known to be required for *dnrI* expression. These conclusions were supported by the effects of the *dnrO* mutation on expression of *dnrO*, *dnrN* and *dnrI*, as viewed by *melC* fusions to each of these regulatory genes. DnrO was overexpressed in *Escherichia coli* and the cell-free extract was used to conduct mobility shift DNA-binding assays. The results showed that DnrO binds specifically to the overlapping *dnrN/dnrO*_{p1} promoter region. Thus, *DnrO* may regulate the expression of both the *dnrN* and *dnrO* genes.

Keywords: anthracycline antitumour antibiotic, biosynthesis, melanin gene fusions, transcriptional mapping

INTRODUCTION

The filamentous soil bacteria that make up the genus *Streptomyces* undergo a complex life cycle that is characterized by morphological and physiological differentiation (Chater, 1993). An intricate array of regulatory mechanisms is needed to control and co-ordinate the developmental processes involved in aerial mycelium formation and sporulation as well as the production of antibiotics and other secondary metabolites. Since many of the wide variety of secondary metabolites produced by *Streptomyces* are useful chemotherapeutic agents, there is considerable interest in

understanding the molecular mechanisms that regulate the biosynthesis of these compounds (Chater & Bibb, 1997). Such regulation can occur through different mechanisms and at different levels; the *Streptomyces coelicolor bldA* gene (Lawlor *et al.*, 1987), for example, encodes a tRNA for the rare leucine codon UUA and regulates both aerial mycelium formation and antibiotic production. Genes such as *absA* and *absB* (Aceti & Champness, 1998) and *afsR* (Floriano & Bibb, 1996; Horinouchi *et al.*, 1990) in *S. coelicolor* control more than one antibiotic pathway, whereas other regulatory genes are pathway-specific and are directly linked to their cognate biosynthetic gene clusters. These include *actII-Orf4* (Fernandez-Moreno *et al.*, 1991) and *redD* (Narva & Feitelson, 1990), which act as transcriptional activators of the actinorhodin and undecylprodigiosin biosynthetic pathways, respectively, in *S. coelicolor*.

Our studies of the molecular biology of daunorubicin (DNR) and doxorubicin (DXR) biosynthesis and regulation in *Streptomyces peucetius* ATCC 29050 have

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Abbreviations: DNR, daunorubicin; DXR, doxorubicin; HTH, helix–turn–helix; RHO, ϵ -rhodomycinone.

Fig. 1. (a) Physical map of the DNR and DXR production genes. The restriction sites *Bam*HI (B), *Bgl*II (Bg) and *Eco*RI (E) are shown above pointed blocks oriented in the direction of transcription and shaded according to the functions shown beneath the map. (b) Nucleotide sequence of the *dnrN/dnrO* intergenic region (Otten *et al.*, 1995), showing the location of the apparent transcription start sites and assigned $-10/-35$ promoter regions and a small portion of the two ORFs. Only the restriction sites mentioned in the text are indicated. The dashed arrows show the locations of direct or inverted repeats.

demonstrated that DNR and DXR biosynthesis are regulated by the pathway-specific transcriptional activators *dnrN* (Otten *et al.*, 1995) and *dnrI* (Madduri & Hutchinson, 1995; Stutzman-Engwall *et al.*, 1992) (Fig. 1a). DnrI is related to other *Streptomyces* regulatory proteins such as *actII-Orf4* and *redD*, all of which belong to the superfamily of SARP proteins (Wietzorrek

& Bibb, 1997), and activates the transcription of the structural and resistance genes of the DNR and DXR gene cluster (Madduri & Hutchinson, 1995; Stutzman-Engwall *et al.*, 1992). DNA-binding studies have shown that DnrI binds near the -35 region of promoters controlling early- and late-acting genes of the DNR biosynthetic pathway (Tang *et al.*, 1996). The *dnrI* gene

is in turn regulated by the pseudo response regulator protein DnrN, which activates the transcription of *dnrI* (Furuya & Hutchinson, 1996; Otten *et al.*, 1995). DnrN has been shown to bind specifically to the *dnrI* promoter region, but not to the promoter regions of three biosynthetic genes nor to its own promoter (Furuya & Hutchinson, 1996).

In contrast to typical bacterial response regulator proteins (Gross *et al.*, 1989), DnrN does not appear to require phosphorylation for its activity even though it possesses a putative phosphorylation site (D55) and other highly conserved amino acid residues characteristic of response regulator proteins. In this regard, DnrN resembles the *S. coelicolor* RedZ protein that regulates *redD* transcription (Guthrie *et al.*, 1998; White & Bibb, 1997). Mutants of *dnrN* containing substitutions (D55E and D55N) at the putative site of phosphorylation were still able to restore antibiotic production to the non-producing *dnrN::aphII* mutant strain, although at lower levels than the wild-type *dnrN* gene (Otten *et al.*, 1995). No phosphorylation of DnrN could be detected *in vivo* or *in vitro*, and the DnrN D55N mutant protein that contains a nonphosphorylatable residue at the putative phosphorylation site exhibited the same binding affinity for the *dnrI* promoter as the wild-type DnrN protein in the presence of phospho donors (Furuya & Hutchinson, 1996). Since the activities of response regulator proteins are typically regulated by phosphorylation, these results raised the interesting question of how the activity of DnrN might be modulated. The *dnrO* gene provided a likely candidate since the N terminus of DnrO possesses high similarity to the N-terminal helix–turn–helix (HTH) DNA-binding domain of the biotin operon repressor, BirA (Buoncrisiani *et al.*, 1986), and also conforms to the consensus sequence encompassing the DNA-binding domain that characterizes the DeoR family (von Bodman *et al.*, 1992) of transcriptional regulators. Furthermore, *dnrO* is located adjacent to and is divergently transcribed from *dnrN*, as is often the case for repressor genes and their targets (Fig. 1a). These observations led to the suggestion that DnrO might regulate DNR biosynthesis by repression of the *dnrN* gene (Otten *et al.*, 1995).

In this report we show that *dnrO* represents a third pathway-specific regulator of DNR biosynthesis and that DnrO acts, not like a classical repressor of *dnrN*, but rather epistatically to the *dnrN* regulator of the DNR biosynthetic pathway. As a consequence, the DnrO protein modulates the expression of *dnrN* and *dnrI*. Further, we show that DnrO binds specifically to the *dnrNO* promoter region as part of the mechanism controlling the transcription of *dnrN*.

METHODS

Biochemicals and chemicals. DNR, carminomycin and ϵ -rhodomycinone (RHO) were obtained from Pharmacia & Upjohn. Thiostrepton was obtained from Sal Lucania, Bristol-Myers-Squibb, Princeton, NJ, USA. Restriction enzymes and

other molecular biology reagents were obtained from standard commercial sources.

Bacterial strains and plasmids. *S. peucetius* ATCC 29050 was obtained from the American Type Culture Collection (Manassas, VA, USA); and *S. peucetius* mutant strains WMH1677 *dnrO::aphII*, WMH1530 *dnrN::aphII* (Otten *et al.*, 1995) and WMH1445 *dnrI::aphII* (Stutzman-Engwall *et al.*, 1992) were used as hosts for the expression of the *melC* operon under the control of different promoters. *Escherichia coli* DH5 α and JM105 (Sambrook *et al.*, 1989) were used for subcloning and ssDNA preparations. *E. coli* BL21(DE3) (Novagen) was used as the expression host, and *E. coli* pET-16b and pET-26b (Novagen) were used as the expression vectors. The high-copy-number *Streptomyces* shuttle vector pWHM3 was from Vara *et al.* (1989), and the low-copy-number shuttle vector pWHM601 was from Guilfoile & Hutchinson (1991). pWHM219 was from Otten *et al.* (1997), pWHM533 was from Otten *et al.* (1995), pWHM1104 was from Tang *et al.* (1996), and pWHM289 and pWHM298 were from Lomovskaya *et al.* (1999). pMT3000 and the promoter probe vector pMT3003.1 (Paget *et al.*, 1994) were provided by C. P. Smith (University of Manchester, UK). The pUC4-KIXX plasmid containing the neomycin resistance gene (*aphII*) was obtained from Pharmacia, and the ssDNA integration vector pDH5 was obtained from W. Wohlleben (Universität Bielefeld, Germany).

Media and growth conditions. *E. coli* strains were grown in LB medium or 2 $\times$ YT medium (Sambrook *et al.*, 1989) at 37 °C for preparation of ssDNA from strains containing M13. *E. coli* transformants were selected with 100 μ g ampicillin ml⁻¹, 100 μ g apramycin (Fluka Chemicals) ml⁻¹ or 50 μ g neomycin ml⁻¹. *S. peucetius* strains were grown for 7–10 d at 30 °C on ISP medium 4 (Difco) for spore preparation and at 30 °C in R2YE medium (Hopwood *et al.*, 1985) for protoplast preparation and the isolation of chromosomal DNA. Plasmid-containing *Streptomyces* strains were selected with 10 μ g thiostrepton ml⁻¹, 25 μ g apramycin ml⁻¹ or 25 μ g kanamycin ml⁻¹. Cultures used for the determination of anthracycline production were grown in GPS medium (Dekleva *et al.*, 1985), extracted with chloroform and analysed by HPLC as previously described (Otten *et al.*, 1990; Stutzman-Engwall & Hutchinson, 1989).

APM seed and production media in liquid versions (Guilfoile & Hutchinson, 1991; Lomovskaya *et al.*, 1999) were used to carry out *melC* expression experiments and quantification of anthracycline metabolite production in *S. peucetius*. Production of melanin was achieved by supplementing the growth medium with 0.0375% (w/v) L-tyrosine, 5 mg CuSO₄·5H₂O l⁻¹ and 0.2 mM FeCl₃ (Paget *et al.*, 1994). Spores from *S. peucetius* strains were incubated in 150 $\times$ 15 mm test tubes containing 5 ml seed medium for 3 d at 30 °C with shaking at 250 r.p.m. The cultures were transferred to 250 ml baffled flasks containing 25 ml seed medium and incubated for a further 3 d at 30 °C with shaking at 250 r.p.m. The resulting seed cultures were centrifuged, and the mycelial cells were washed three times with 50 ml portions of 10.3% sucrose and stored at –20 °C for not longer than 1 month. This method provided sufficient inoculum to conduct a large number of experiments from the same batch of cells. APM liquid medium (5 ml) in 150 $\times$ 15 mm test tubes was inoculated with seed cultures to OD₄₅₀ 0.4, which amounted to the addition of 0.1–0.3 ml seed culture. In the *melC* operon expression experiments, cell growth was measured as OD₄₅₀. The pattern of growth sometimes showed a lag period during the first 24 h, followed by an exponential phase over the next 24 h period, and usually reached stationary phase by 48 h. Samples were

removed every 24 h during a 5 d period. Data from triplicate cultures were used to calculate the means and standard errors shown in Fig. 2.

Analysis of melanin production. Melanin production was estimated following the procedure of Della-Cioppa *et al.* (1990) using 0.3 ml culture and mixing it with 0.6 ml concentrated aqueous HCl before spectrophotometric determination at 670 nm. Data from triplicate cultures were used to calculate the means and standard errors shown in Fig. 3.

Construction of *melC* transcriptional fusions. A control plasmid for the expression of the *melC* operon in *S. peucetius* was generated by cloning a *Bgl*II fragment containing the *melC* operon (Hintermann *et al.*, 1985) and the transcription terminators *mmr*_I (Neal & Chater, 1987) and *fd*_I (Ward *et al.*, 1986) flanking the coding region from pMT3003.1 (Paget *et al.*, 1994) into pWHM601 digested with *Bam*HI, resulting in pWHM2000.

To generate the *dnrO*_p::*melC* and *dnrN*_p::*melC* transcriptional fusions, a 450 bp *Sph*I–*Nsi*I fragment from pWHM533 (Otten *et al.*, 1995) containing the intergenic region between *dnrN* and *dnrO* (Fig. 1b) was cloned into *Sph*I/*Pst*I-digested pMT3000 (Paget *et al.*, 1994), resulting in pWHM2001. From pWHM2001, a *Bam*HI–*Bgl*II fragment was cloned in both orientations in *Bam*HI-digested pMT3003.1, leading to the constructions pWHM2002 (*dnrO*_p::*melC*) and pWHM2003 (*dnrN*_p::*melC*). From these constructions, *Bgl*II fragments containing the fusions were removed and ligated into the *Bam*HI site of pWHM601 to obtain pWHM2004 and pWHM2005, respectively. The *dnrI*_p::*melC* transcriptional fusion was generated by cloning a 347 bp *Bam*HI–*Sac*I fragment from pWHM1104 (Tang *et al.*, 1996) containing the *dnrI* promoter region and 147 bp of the *dnrI* coding region into pMT3000 to give pWHM2006, from which a *Bam*HI–*Bgl*II fragment was cloned into *Bam*HI-digested pWHM2000 to give pWHM2007.

All of these pWHM601-borne transcriptional fusions were introduced by transformation into *S. peucetius* ATCC 29050 to give the strains WMH2001–WMH2003 containing the *dnrO*::*melC*, *dnrN*::*melC* and *dnrI*::*melC* expression plasmids, respectively. In addition, pWHM2000 (*melC* operon only) and the gene fusions, pWHM2004 (*dnrO*_p::*melC*), pWHM2005 (*dnrN*_p::*melC*) and pWHM2007 (*dnrI*_p::*melC*) were introduced into the mutant strains WMH1677 *dnrO*::*aphII*, WMH1530 *dnrN*::*aphII* (Otten *et al.*, 1995) and WMH1445 *dnrI*::*aphII* (Stutzman-Engwall *et al.*, 1992) to give strains WMH2041, WMH2051 and WMH2061, respectively, containing the *dnrO*::*melC* fusion; WMH2042, WMH2052 and WMH2062, respectively, containing the *dnrN*::*melC* fusion; and WMH2043, WMH2053 and WMH2063, respectively, containing the *dnrI*::*melC* fusion.

Southern analysis. Chromosomal DNA was digested for 4 h with *Sal*I, electrophoresed overnight in a 0.7% agarose gel and blotted to Hybond N (Amersham) by capillary transfer (Sambrook *et al.*, 1989). Labelling, hybridization and detection were carried out with the Genius I DNA labelling kit (Boehringer Mannheim) under standard conditions and according to the manufacturer's directions.

RNA isolation. RNA was isolated using guanidinium thiocyanate and CsCl centrifugation (Sambrook *et al.*, 1989). The cells from two 25 ml cultures grown for 48 h in APM medium were harvested by vacuum filtration through Whatman no.1 paper. The cells were lysed by grinding in liquid nitrogen with a mortar and pestle and then were resuspended in 20 ml disruption buffer (Fisher & Wray, 1989). After shearing the

DNA by passage through a 20-gauge needle several times, the solution was centrifuged at 16000 g at 4 °C for 10 min. Aliquots (9.7 ml) of the clear supernatant were layered over 3.5 ml cushions of 5.7 M CsCl, 0.1 M EDTA (pH 8) and centrifuged in a Beckman SW41Ti rotor at 32000 r.p.m. at 20 °C for 24 h.

Primer extension and S1 nuclease mapping. Primer extension reactions were performed as previously described (Guilfoile & Hutchinson, 1992) except that the reaction was incubated at 42 °C. Synthetic oligodeoxynucleotides used as primers included: for the *dnrN* gene (a) 5'-GGCCCGCGACCGGT-GCTACTCATCGACTG-3' and (b) 5'-CGGGTGCTACTC-ATCGACTCCCCGTCTTC-3', beginning 242 bp and 252 bp, respectively, upstream of the predicted *dnrN* ATG start codon; and for the *dnrO* gene (c) 5'-CGCCTGCCCG-TTCACTCCCCGGGAGAGTCCG-3' and (d) 5'-GCCCCGTT-CACTCCCCGGGAGAGTCCCGCTGG-3', beginning 206 bp and 211 bp, respectively, upstream of the predicted *dnrO* ATG start codon, and (e) 5'-TGCGCCCTGGCCAGTCCCC-GGCTGTTCTGGA-3' and (f) 5'-CCAGTCCCCGGCTGTT-CTGGAGGAGGGACAG-3', beginning 72 bp and 62 bp, respectively downstream of the *dnrO* ATG start codon.

Low-resolution S1 mapping was conducted essentially as described by Neal & Chater (1987) using approximately 1 µg M13 ssDNA and 40 µg RNA. The samples were denatured at 80 °C for 10 min, hybridized at 50 °C overnight and treated with 130 units S1 nuclease at 37 °C for 45 min. The protected fragments were fractionated on a 8 M urea/4% polyacrylamide gel and electroblotted to Hybond N. The probe, a 705 bp *Sal*I–*Sac*II DNA fragment containing the *dnrNO* intergenic region and the N-terminal ends of the *dnrN* and *dnrO* genes, was labelled with [α -³²P]dCTP using the Multi-prime DNA labelling system (Amersham) in accordance with the manufacturer's rapid protocol.

Expression of *dnrO* in *E. coli*. The *dnrO* gene was introduced into pET expression vectors by means of a linker prepared by annealing the synthetic oligodeoxynucleotides 5'-TATGAC-CGAAACCCCGTCCCGCATG-3' and 5'-ACTGGCTTTG-GGGCAGGGC-3'. The linker spanned the first eight codons of the *dnrO* gene from the predicted translational start codon (ATG) to the *Sph*I site and introduced an *Nde*I site at the ATG start codon. In addition, the linker introduced nucleotide changes at the third codon position in order to produce the preferred codons in *E. coli* (the altered nucleotides are shown in bold in the oligodeoxynucleotides above). The *dnrO* gene was reassembled by three-piece ligation of the linker fragment, the remainder of the *dnrO* gene as a 1.2 kb *Sph*I–*Not*I fragment and the *Nde*I–*Not*I fragment of pET-26b to produce pWHM562. The *dnrO* gene was transferred from pWHM562 as a *Nde*I–*Xho*I fragment into pET-16b to produce pWHM563.

Transformants of *E. coli* BL21(DE3) containing pWHM562 or pWHM563 were grown in LB medium containing kanamycin (25 µg ml⁻¹) or ampicillin (150 µg ml⁻¹), respectively, at 25, 29 or 37 °C until the OD₆₀₀ reached 0.6, and expression of *dnrO* was then induced by addition of IPTG to a final concentration of 1 mM. After incubation for an additional 3 h, the cultures were chilled on ice for 5 min and then centrifuged at 5000 g for 5 min. The cell pellet was washed with 0.25 culture vols ice-cold TEGT buffer (50 mM Tris/HCl pH 8.0, 2 mM EDTA, 10% glycerol and 0.1% Triton) and resuspended in 0.1 culture vol. TEDP buffer (TEGT buffer containing 1 mM DTT and 1 mM PMSF). Lysozyme was added to a final concentration of 100 µg ml⁻¹, and after incubation for 15 min at 30 °C, the DNA was sheared by sonication on ice with a microtip for

three 10 s pulses. The lysate was centrifuged at 12000 g for 15 min at 4 °C, the supernatant containing the soluble protein was decanted and the insoluble pellet was resuspended in 0.1 culture vol. TEDP buffer.

Partial purification of native DnrO protein. The cell-free extract from *E. coli* BL21(DE3)/pWHM562 transformants was adjusted to 50 mM NaCl by addition of 5 M NaCl, and the DnrO protein was precipitated on ice by addition of 10% polyethylenimine to a final concentration of 0.7%. After stirring on ice for 1 h, the mixture was centrifuged at 12000 g for 15 min at 4 °C and the pellet was washed with 0.5 vol. TEGT buffer containing 50 mM NaCl. The pellet was resuspended in 1 vol. TEDP buffer containing 1 M NaCl and the DnrO protein was eluted by stirring on ice for 10 min. Insoluble material was removed by centrifugation at 12000 g for 15 min, and the DnrO protein was precipitated from the supernatant with 25–45% saturated ammonium sulfate and collected by centrifugation. The partially purified DnrO protein was dissolved in 0.1 vol. TEDP buffer and stored at –80 °C.

Protein concentrations were determined by the Bradford (1976) method using BSA as the standard. SDS-PAGE was performed according to the method of Laemmli (1970) and the gels were stained with Coomassie blue R.

Mobility shift DNA-binding assays. Mobility shift assays were conducted according to the method of Burotowski & Chodosh (1996). A 136 bp *SumI-SphI* DNA fragment containing *dnrN* and the *dnrO*_{p1} promoter region and a 212 bp *NsiI-SmaI* DNA fragment containing the *dnrO*_{p2} and *dnrO*_{p3} promoter regions were end-labelled with [α -³²P]dCTP (Amersham) and Klenow polymerase. The binding reaction contained the ³²P-labelled DNA fragment (1 ng; 5000–12000 c.p.m.), the DnrO cell-free extract and 1.5 µg poly(dI-dC)·(dI-dC) in a total volume of 20 µl in a buffer composed of 20 mM Tris/HCl pH 8, 1 mM EDTA, 100 mM KCl, 1 mM DTT, 15% glycerol and 50 µg BSA ml⁻¹. After incubation for 20 min at 30 °C, the protein-bound and free DNA were separated by electrophoresis at 4 °C on a 5% nondenaturing polyacrylamide gel in 2 × Tris/glycine running buffer (50 mM Tris, 380 mM glycine, 2 mM EDTA, pH 8.3). The gels were analysed with a Phosphor-Imager SI (Molecular Dynamics) using the ImageQuant program.

RESULTS

Inactivation of *dnrO* blocks antibiotic production in *S. peucetius*

The *dnrO* gene was subcloned as a 3.1 kb *BglII-StuI* fragment in pUC19, and a 253 bp *MscI* fragment near the N-terminal end of *dnrO* was deleted and replaced with the kanamycin resistance gene as a 1.2 kb *SmaI* fragment from pUC4-KIXX. The disrupted *dnrO* gene was subcloned into the ssDNA vector pDH5, and single-stranded plasmid prepared from this construct was used to transform *S. peucetius* ATCC 29050. Representative transformants containing the integrated vector and disrupted *dnrO* gene were subjected to several rounds of sporulation and screened for the required kanamycin-resistant, thiostrepton-sensitive phenotype resulting from gene replacement by a double crossover. The four putative *dnrO*::*aphII* mutants that were obtained were analysed by Southern hybridization to confirm the inactivation of *dnrO*. Chromosomal DNA from the

putative mutant strains and the wild-type strain was digested with *SalI* and probed with a 0.7 kb *BamHI-SphI* fragment containing the *dnrO* gene. The probe hybridized to the expected 1.4 kb *SalI* fragment for the wild-type strain and to the predicted 2.3 kb *SalI* fragment for the *dnrO*::*aphII* mutant strains (data not shown), confirming the mutation in the *dnrO* gene.

The four *dnrO*::*aphII* mutant strains were analysed for anthracycline production and found to produce no DNR nor any of its biosynthetic intermediates. One of the *dnrO*::*aphII* mutant strains, WMH1677, was selected for further study, and as expected the *dnrO*::*aphII* mutation in WMH1677 could be complemented by a 1.6 kb *NsiI-NotI* fragment containing the *dnrO* gene cloned on the low-copy-number vector pWHM601 as pWHM557 (Fig. 2a), and the high-copy-number vector pWHM3 as pWHM558 (Fig. 2b). With the low-copy-number plasmid pWHM557, the levels of RHO production (14.9 ± 4.99 µg ml⁻¹) and DNR production (2.4 ± 0.69 µg ml⁻¹) were approximately twofold less than those obtained with the wild-type strain transformed with the vector alone. Likewise, transformants of WMH1677 containing the high-copy-number plasmid pWHM558 produced RHO (32.4 ± 5.07 µg ml⁻¹) and DNR (3.4 ± 0.41 µg ml⁻¹) at levels which were approximately equivalent to and twofold less, respectively, than those obtained with the wild-type strain containing the vector alone.

Bioconversion experiments (Madduri & Hutchinson, 1995; Otten *et al.*, 1995) were conducted with the WMH1677 strain to probe the nature of the *dnrO*::*aphII* mutation. The addition of RHO, carminomycin or DNR to WMH1677 cultures resulted in no conversion of these substrates to anthracycline products, indicating that the antibiotic non-producing phenotype of WMH1677 did not result from a simple block in an early step of the DNR biosynthetic pathway. Rather, these results implied the loss of a number of enzymic activities involved in DNR biosynthesis, suggesting that DnrO functions as a positive regulator of DNR biosynthesis.

The *dnrO*::*aphII* mutation is suppressed by *dnrN* and *dnrI*

The response regulator protein DnrN positively regulates DNR biosynthesis by activating the transcription of DnrI (Madduri & Hutchinson, 1995), which in turn activates the transcription of the structural and resistance genes of the DNR pathway (Furuya & Hutchinson, 1998; Madduri & Hutchinson, 1995). Since *dnrN* is located adjacent to and is divergently transcribed from the *dnrO* gene, it seemed a likely target for regulation by DnrO. To explore this possibility, the *dnrO*::*aphII* strain was transformed with the *dnrN* gene subcloned in pWHM601 and pWHM3 as pWHM533 and pWHM534, respectively (Otten *et al.*, 1995) (Fig. 2a, b). Although the *BamHI-SphI* segment used in these experiments contains the *dnrN*_p promoter and DnrO-binding region identified below, we assume *dnrN* was

Fig. 2. Production of RHO (■) and DNR (□) by the *S. peuceitius* 29050 wild-type strain, and the *dnrO::aphII* mutant strain and its *dnrO*, *dnrN*, *dnrNO* and *dnrI* transformants. (a) 29050/pWHM601 and pWHM601 are wild-type and mutant strain, respectively, transformed with low-copy-number pWHM601 vector only; pWHM557 (*dnrO*), pWHM533 (*dnrN*), pWHM567 (*dnrNO*) and pWHM564 (*dnrI*) are the mutant strain transformed with wild-type gene(s) indicated. (b) 29050/pWHM3 and pWHM3 are wild-type and mutant strain, respectively, transformed with high-copy-number pWHM3 vector only; pWHM558 (*dnrO*), pWHM534 (*dnrN*) and pWHM568 (*dnrNO*) are the mutant strain transformed with wild-type gene(s) indicated. Data are means $\pm$ SE of triplicate experiments.

expressed from some promoter in the vector so as to relieve its dependence on *dnrO*. Analysis of the anthracyclines produced by these transformants (Fig. 2) showed that the *dnrO::aphII* mutation was suppressed by the *dnrN* gene to give RHO and DNR in an approximately 1:1 ratio with only a small copy number effect. The yields of RHO were not restored to the control level in these experiments, but introduction of the *dnrNO* genes together, as pWHM567 (low copy) or pWHM568 (high copy), resulted in restoration to the control level (pWHM567) or to five times more RHO and four times more DNR (pWHM568) than in the control culture (Fig. 2). The suppression of the *dnrO::aphII* mutation by the *dnrN* gene suggested that DnrO may be required for expression of *dnrN*. In this event, *dnrI* would be expected to also suppress the *dnrO::aphII* mutation since we have previously shown that *dnrN* functions by activating the transcription of *dnrI* (Madduri & Hutchinson, 1995). To address this possibility, WMH1677 was transformed with the *dnrI* gene as a 1.2 kb *PstI*–*SmaI* fragment under the control of the strong, constitutive *ermE**_p promoter (Bibb *et al.*, 1994) in pWHM601 as pWHM564. The *dnrI* gene suppressed the *dnrO::aphII* mutation in these transformants and resulted in the accumulation of wild-type levels of RHO ($26.3 \pm 1.19 \mu\text{g ml}^{-1}$), and DNR levels ($3.3 \pm 1.23 \mu\text{g ml}^{-1}$) that were twofold less than wild-type. The suppression of the *dnrO::aphII* mutation by *dnrN* and *dnrI* suggests that DnrO is required for the transcription of *dnrN* and that the antibiotic non-producing phenotype of the *dnrO::aphII* mutant WMH1677 ultimately results from the absence of DnrI.

The *dnrO::aphII* mutation affects expression of the *dnrI* and *dnrN* regulatory genes as well as *dnrO* itself

We monitored expression of *dnrO* and the two known regulatory genes, *dnrI* and *dnrN*, by measuring the production of melanin formed as a result of fusing the *Streptomyces glaucescens melC* operon (Hintermann *et*

al., 1985) to the promoter region of each *S. peuceitius* gene. The *melC* cassette described by Paget *et al.* (1994) was used in these experiments because the cloning site and *melC* genes are flanked by strong transcriptional terminators to insulate *melC* expression from any effect of the plasmid vector promoters. Since the tyrosinase produced occurs as both cell-associated and extracellular forms (Huber *et al.*, 1985), both the bacterial cells and medium were solubilized with acid prior to spectrophotometric assays, to be sure that all of the melanin was measured.

Expression of the *melC* operon under the control of each promoter, carried on the low-copy SCP2*-derived plasmid pWHM601 (Guilfoile & Hutchinson, 1991), was monitored in liquid APM production medium, using the wild-type WMH2000 strain (*melC* operon alone in pWHM601) as the control. The absorbance of the WMH2000 culture was used to equilibrate the spectrophotometer to an A_{670} of zero, to remove the background absorbance of the solubilized culture from that due to melanin. Since the mycelial cells grew diffusely in the APM medium, cell growth was also measured spectrophotometrically (OD_{450}) to allow the growth rate and anthracycline production to be correlated to melanin production.

Expression of all three regulatory genes began at approximately 24 h during the exponential phase of growth and reached the maximum at the beginning of the stationary phase (Fig. 3). Production of total anthracyclines in the wild-type strain began between 24 and 48 h in the APM medium and steadily rose to the maximum value by 168 h (data not shown). Since the growth rate of the WMH2001 wild-type strain lagged by about 1 d behind that of the three regulatory gene mutants (Fig. 3d), the melanin productivity data recorded for the regulatory gene::*melC* fusion experiments were shifted by +24 h so that these data could be compared with those from the WMH2001 strain. In the wild-type strain at 72 h, *dnrO* was expressed at a two-to

Fig. 3. (a–c) Melanin production resulting from expression of the *dnrO_p::melC* (a), *dnrN_p::melC* (b) and *dnrI_p::melC* (c) transcriptional fusions in the wild-type WMH2001 ($\diamond$) and non-DNR-producing regulatory gene mutant strains WMH1677 *dnrO::aphII* ($\square$), WMH1530 *dnrN::aphII* ($\triangle$) and WMH1445 *dnrI::aphII* ($\circ$). (d) Cell growth of strains ATCC 29050 ($\diamond$), WMH1677 *dnrO::aphII* ($\square$), WMH1530 *dnrN::aphII* ($\triangle$) and WMH1445 *dnrI::aphII* ($\circ$). Data are means $\pm$ SE of triplicate experiments.

threefold higher level than that of *dnrN* and *dnrI*, respectively, but the timing of its expression was similar to that of *dnrN* and *dnrI* (Fig. 3a vs 3b, c). Its expression decreased considerably in the *dnrO* null mutant, to about one-eighth that seen in the wild-type strain, and was almost negligible in the *dnrI* and *dnrN* null mutants (Fig. 3a). The expression of *dnrN* also decreased in the *dnrO* null mutant, but only by about fourfold, whereas its expression was essentially negligible in the *dnrI* and *dnrN* null backgrounds (Fig. 3b). Curiously, expression of *dnrI* was nearly zero in all three regulatory gene mutants (Fig. 3c). These results suggest that the expression of *dnrO* and *dnrN* is influenced by both of their products, but create an enigma about the regulation of *dnrI* expression, even though its lack of significant

expression in the *dnrO* and *dnrN* null mutants is expected.

Identification of the apparent transcript start sites for the *dnrO* and *dnrN* genes

The *dnrO* gene and divergently transcribed *dnrN* gene are separated by a 433 nt intergenic region that was subcloned along with the N-terminal ends of *dnrN* and *dnrO* as a 705 bp *SalI*–*SacII* fragment and transferred as a *SalI*–*HindIII* fragment to M13mp18 and M13mp19 (Sambrook *et al.*, 1989) to create pWHM565 and pWHM566, respectively. The ssDNAs prepared from these plasmids were annealed to total RNA from *S. peucetius* 29050 and treated with S1 nuclease. The

Fig. 4. Mapping the apparent transcription start sites of the *dnrO* and *dnrN* genes. (a) Low-resolution S1 nuclease protection experiment showing the relative sizes of the four protected DNA fragments. The sizes (bp) of molecular mass markers are shown in the left hand panel. (b) Primer extension experiment showing the sizes of the three products made with the primers described in the text adjacent to a sequencing ladder.

protected DNA fragments were fractionated by PAGE, transferred to Hybond N and analysed by hybridization using the 705 bp *SalI*–*SacII* DNA fragment as the ^{32}P -labelled probe (Fig. 4a). The leftward *dnrN* transcript was found to protect approximately 490 nt of the pWHM565 ssDNA indicating that the 5' end of the *dnrN* transcript was approximately 320 bp upstream of the predicted *dnrN* translational start codon. The rightward *dnrO* transcript protected three fragments of the pWHM566 ssDNA that were approximately 110, 430 and 490 nt in length, indicating apparent transcriptional start sites p1, p2 and p3 at approximately 4, 317 and 386 nt, respectively, upstream of the predicted *dnrO* translation start codon (Fig. 4a).

Primer extension experiments using synthetic oligodeoxynucleotide primers (Methods) were performed to further localize the *dnrN* and the *dnrO*_{p1} and *dnrO*_{p2} transcriptional start points. The primer extension products were electrophoresed beside dideoxy sequencing ladders generated with the same primers (Fig. 4b), and the results indicate that the apparent transcriptional start point of *dnrN* is located at an A 319 nt upstream of the predicted ATG start codon. This

promoter has a –10 region that conforms to the those observed for many other *Streptomyces* genes (Strohl, 1992) but its –35 region diverges more from the consensus sequence (Fig. 1b), as has often been seen (Strohl, 1992). On the other hand, neither of the apparent *dnrO*_{p1} nor the *dnrO*_{p2} promoters display typical –10 or –35 regions (Fig. 1b) when their apparent transcriptional start points were localized by primer extension at an A 4 nt upstream and at a C 317 nt upstream, respectively, of the predicted ATG start codon. These data together provide strong support for a single apparent transcriptional start site for *dnrN*. On the other hand, it is less certain that *dnrO* has two, or perhaps three, transcriptional start sites from the available data, suggesting that this matter should be clarified by later work.

Overexpression of the DnrO protein in *E. coli*

The *dnrO* gene was overexpressed in *E. coli* using pET vectors containing the strong T7-*lac* promoter. For expression of native protein, the *dnrO* gene was subcloned by means of a synthetic linker as described in

Fig. 5. Gel mobility shift experiments. (a) Binding between DnrO in the *E. coli*/pWHM562 cell lysate and the *dnrN_p/dnrO_{p1}* segment. The amount of lysate (ng) containing DnrO is indicated beneath each lane; the last three lanes show the effect of adding unlabelled template DNA to the reaction. (b) Apparent binding between DnrO in the *E. coli*/pWHM562 cell lysate and the *dnrO_{p2}* and *dnrO_{p3}* segment. Lanes: 0, no protein; v, cell lysate (10 µg) from *E. coli*/pET-26b vector only; 25, 50, 250 and 2500, cell lysate (25, 50, 250 and 2000 ng, respectively) from *E. coli*/pWHM562.

Methods into pET-26b to give pWHM562. The *dnrO* gene was transferred as a *NdeI*–*XbaI* fragment from pWHM562 into pET-16b to give pWHM563 for expression of His-tagged DnrO protein. Transformants of *E. coli* BL21(DE3) containing pWHM562 or pWHM563 grown at 37 °C were induced with IPTG, and the soluble and insoluble fractions were analysed by SDS-PAGE. Most of the DnrO was found in the soluble fraction when it was expressed as the native protein from pWHM562; however nearly all of the His-tagged DnrO expressed from pWHM563 was found in the insoluble fraction (data not shown). Growth and induction at 25 or 29 °C did not noticeably increase the expression of soluble DnrO protein from either pWHM562 or pWHM563.

Native DnrO could be efficiently precipitated from the cell-free extract with 0.7% polyethylenimine (Jendrisak & Burgess, 1975), as determined by SDS-PAGE. Subsequent precipitation of the polyethylenimine-purified DnrO with 25–45% saturated ammonium sulfate resulted in an approximately 10-fold purification of DnrO.

The DnrO protein binds specifically to the *dnrN/dnrO_{p1}* promoter region

The phenotype of the *dnrO::aphII* mutant strain and the suppression of this mutation by the pathway-specific transcriptional activators *dnrN* and *dnrI*, together with the large decrease in *dnrN_p::melC* expression in the *dnrO* null mutant, indicated that DnrO functions by activating the transcription of the *dnrN* gene. This led us to conduct mobility shift DNA-binding assays to determine if the DnrO protein binds specifically to the

dnrNO promoter region. Cell-free extracts from *E. coli* BL21(DE3)/pWHM562 showed concentration-dependent binding of DnrO throughout a range from 1 to 125 ng total cellular protein to the *dnrN/dnrO_{p1}* promoter region as evidenced by the reduced mobility of the end-labelled 136 bp *SunI*–*SphI* promoter fragment on 5% nondenaturing gels (Fig. 5a), whereas 10 µg of cell-free extract of BL21(DE3) containing vector alone had no detectable binding activity (data not shown). Hence it is very likely that the mobility shift is due to DnrO and not some DNA-binding protein of *E. coli*. Specific competition of DnrO binding by the addition of a 100-fold excess of unlabelled *dnrN/dnrO_{p1}* promoter fragment resulted in the loss of DnrO binding, indicating that the DnrO protein recognizes the *dnrN/dnrO_{p1}* promoter DNA in a sequence-specific manner (Fig. 5a). However, when the *dnrO_{p2}* and *dnrO_{p3}* promoter region as a 212 bp *NsiI*–*SmaI* DNA fragment was analysed by mobility shift assay, the protein concentration required for DNA binding increased 250-fold relative to the *dnrN/dnrO_{p1}* promoter fragment, and only non-specific DNA binding was observed (Fig. 5b). Similar DNA-binding activity to the *dnrN/dnrO_{p1}* promoter region was observed with partially purified DnrO protein except that the protein concentration required for binding was reduced approximately 10-fold (data not shown).

DISCUSSION

The properties of *dnrO* described above show that three genes regulate DNR and DXR production in *S. peucetius*. Each of these genes has a distinct role and appears to function at a different time in the hierarchy of events preceding the onset of antibiotic production.

Because a functional *dnrO* gene is required for optimal expression of *dnrN*, as shown by the results of the complementation (Fig. 2) and gene fusion (Fig. 3b) experiments, we assume that the *dnrO* gene is likely to be expressed before *dnrN*. The fact that the DnrO protein can bind to the *dnrN/dnrO*_{p1} promoter region suggests that DnrO may either coordinate or interfere with binding of an RNA polymerase holoenzyme to this region and thereby regulate the timing or amount of *dnrN* expression. Autoregulation of the expression of *dnrO* itself by DnrO is also a possibility since the apparent expression of the *dnrO* gene decreased significantly in the *dnrO::aphII* mutant (Fig. 3a). However, we cannot decipher the interplay between the *dnrO* and *dnrN* genes in greater detail and how this influences the expression of *dnrI* until further work is done.

The results of preliminary studies using promoter fusions to monitor the expression of *dnrO*, *dnrN* and *dnrI* support the basic idea that expression of *dnrN* and *dnrO* are interconnected. This idea is also supported by the complementation studies wherein it was seen that the *dnrO::aphII* mutation is only partially complemented *in trans* by *dnrO* or *dnrN* alone (Fig. 2). It is likely that *dnrO* acts epistatically to *dnrN* (Fig. 3b) like the case for *dnrI* whose expression is highly dependent on the DnrN protein (Furuya & Hutchinson, 1996). On the other hand, the apparent lack of *dnrI* expression in all three of the regulatory gene null mutants studied here (Fig. 3c) is a puzzle that cannot be explained on the basis of the current data. It is possible that the *dnrI::melC* fusion is not a valid indicator of the relative level of *dnrI* expression in different genetic backgrounds (this must be validated by studies of the *dnrI* mRNA directly), or that the lack of anthracycline metabolite production in the *dnrI* null mutant has reduced *dnrI* expression in some way. The latter caveat must also be considered for *dnrO* and *dnrN* since expression of these two genes also was unexpectedly low in certain genetic backgrounds [e.g. *dnrO* and *dnrN* in the *dnrI* null mutant and *dnrO* in the *dnrN* null mutant (Fig. 3a, b)].

Jadomycin biosynthesis in *Streptomyces venezuelae* also involves two divergently transcribed regulatory genes, *jadR1* (L. C. Vining, personal communication) and *jadR2* (Yang *et al.*, 1995). The former gene encodes a positively acting transcription factor similar to the OmpR-PhoB superfamily of response regulators (Gross *et al.*, 1989) and thus the SARP proteins (Wietzorrek & Bibb, 1997), whilst the *jadR2* gene encodes a negatively acting repressor protein similar to known repressors like EnvR (Klein *et al.*, 1991), TetC (Scholtmeier & Hillen, 1984) and TcmR (Guilfoile & Hutchinson, 1992). Disruption of *jadR2* relieves the stress response needed to induce jadomycin production in the wild-type strain, and when a *jadR2* mutant is cultured under stressful conditions (heat shock or toxic concentrations of ethanol), jadomycin is overproduced (Yang *et al.*, 1995). Consequently, *dnrO* is not a functional homologue of *jadR2* since the *dnrO::aphII* mutant does not produce DNR or DXR precociously or in greater amount than the wild-type strain.

With the exception of the region surrounding the HTH DNA-binding domain, DnrO shares little similarity with other characterized proteins, suggesting that DnrO may represent a new type of regulatory protein. Its closest relatives in the database are the hypothetical proteins YobV from *Bacillus subtilis* (GenBank accession no. 2619048) and ORF11 from *Rhodococcus erythropolis* (Nagy *et al.*, 1997), both of which show 25% identity to DnrO. Interestingly, a TBLASTN search of the unfinished *S. coelicolor* genome database (available at www.sanger.ac.uk/Projects/S-coelicolor/) revealed a hypothetical protein possessing high similarity to DnrO (39% identity). This putative protein also contains the consensus pattern for the DeoR family HTH DNA-binding motif as does DnrO.

As far as we are aware, the involvement of a third cluster-associated regulatory gene in antibiotic biosynthesis by a streptomycete is unusual. The closest example is the *redZ* and *redD* genes that are functional homologues of *dnrN* and *dnrI*, respectively, and control undecylprodigiosin biosynthesis in *S. coelicolor* (Guthrie *et al.*, 1998; White & Bibb, 1997). However, a *dnrO* homologue is not known to be involved in this system. DnrO appears to be expressed very early in *S. peucetius* (C. Olanó & C. R. Hutchinson, unpublished work), and it should prove interesting to examine how *dnrO* itself is controlled, whether in response to a physiological signal or by the anthracycline intermediates of the DNR and DXR pathway through some type of feedback regulation.

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REFERENCES

- Aceti, D. J. & Champness, W. C. (1998). Transcriptional regulation of *Streptomyces coelicolor* pathway-specific antibiotic regulators by the *absA* and *absB* loci. *J Bacteriol* **180**, 3100–3106.
- Bibb, M. J., White, J., Ward, J. M. & Janssen, G. R. (1994). The mRNA for the 23S rRNA methylase encoded by the *ermE* gene of *Saccharopolyspora erythraea* is translated in the absence of a conventional ribosome-binding site. *Mol Microbiol* **14**, 533–545.
- von Bodman, S., Hayman, G. T. & Farrand, S. K. (1992). Opine catabolism and conjugal transfer of the nopaline Ti plasmid pTiC58 are coordinately regulated by a single repressor. *Proc Natl Acad Sci USA* **89**, 643–647.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* **72**, 248–254.
- Buonocristiani, M. R., Howard, P. K. & Otsuda, A. J. (1986). DNA-binding and enzymatic domains of the bifunctional biotin operon repressor (BirA) of *Escherichia coli*. *Gene* **44**, 255–261.
- Burotowski, S. & Chodosh, L. A. (1996). Mobility shift DNA-binding assay using gel electrophoresis. In *Current Protocols in Molecular Biology*, pp. 12.2.1–12.2.11. Edited by F. M. Ausubel. New York: Wiley.

- Chater, K. F. (1993). Genetics of differentiation in *Streptomyces*. *Annu Rev Microbiol* 47, 685–713.
- Chater, K. F. & Bibb, M. J. (1997). Regulation of bacterial antibiotic production. In *Biotechnology*, 2nd edn, vol. 7, pp. 59–105. Edited by H. Kleinkauf & H. von Döhren. Weinheim: VCH.
- Dekleva, M. L., Titus, J. A. & Strohl, W. R. (1985). Nutrient effects on anthracycline production by *Streptomyces peucetius* in a defined medium. *Can J Microbiol* 31, 287–294.
- Della-Cioppa, G., Garger, S. J., Sverlow, G. G., Turpen, T. H. & Grill, L. K. (1990). Melanin production in *Escherichia coli* from a cloned tyrosinase gene. *Biotechnology* 8, 634–638.
- Fernandez-Moreno, M. A., Caballero, J. L., Hopwood, D. A. & Malpartida, F. (1991). The *act* cluster contains regulatory and antibiotic export genes, direct targets for translational control by the *bldA* tRNA gene of *Streptomyces coelicolor*. *Cell* 66, 769–780.
- Fisher, S. H. & Wray, L. V., Jr (1989). Regulation of glutamine synthetase in *Streptomyces coelicolor*. *J Bacteriol* 171, 2378–2383.
- Floriano, B. & Bibb, M. J. (1996). *afsR* is a pleiotropic but conditionally required regulatory gene for antibiotic production in *Streptomyces coelicolor* A3(2). *Mol Microbiol* 21, 385–396.
- Furuya, K. & Hutchinson, C. R. (1996). The DnrN protein of *Streptomyces peucetius*, a pseudo-response regulator, is a DNA-binding protein involved in the regulation of daunorubicin biosynthesis. *J Bacteriol* 178, 6310–6318.
- Furuya, K. & Hutchinson, C. R. (1998). The DrrC protein of *Streptomyces peucetius*, a UvrA-like protein, is a DNA-binding protein whose gene is induced by daunorubicin. *FEMS Microbiol Lett* 168, 243–249.
- Gross, R., Arico, B. & Rappuoli, R. (1989). Families of bacterial signal-transducing proteins. *Mol Microbiol* 3, 1661–1667.
- Guilfoile, P. G. & Hutchinson, C. R. (1991). A bacterial analog of the *mdr* gene of mammalian tumour cells is present in *Streptomyces peucetius*, the producer of daunorubicin and doxorubicin. *Proc Natl Acad Sci USA* 88, 8553–8557.
- Guilfoile, P. G. & Hutchinson, C. R. (1992). Sequence and transcriptional analysis of the *Streptomyces glaucescens* *tcmAR* tetracenomycin C resistance and repressor gene loci. *J Bacteriol* 174, 3651–3658.
- Guthrie, E. P., Flaxman, C. S., White, J., Hodgson, D. A., Bibb, M. J. & Chater, K. F. (1998). A response-regulator-like activator of antibiotic synthesis from *Streptomyces coelicolor* A3(2) with an amino-terminal domain that lacks a phosphorylation pocket. *Microbiology* 144, 727–738.
- Hintermann, G., Zatchej, M. & Hütter, R. (1985). Cloning and expression of the genetically unstable tyrosinase structural gene from *Streptomyces glaucescens*. *Mol Gen Genet* 200, 422–432.
- Hopwood, D. A., Bibb, M. J., Chater, K. F. & 7 other authors (1985). *Genetic Manipulation of Streptomyces: a Laboratory Manual*. Norwich: John Innes Foundation.
- Horinouchi, S., Kito, M., Nishiyama, M., Furuya, K., Hong, S. K., Miyake, K. & Beppu, T. (1990). Primary structure of AfsR, a global regulatory protein for secondary metabolism formation in *Streptomyces coelicolor* A3(2). *Gene* 95, 49–56.
- Huber, M., Hintermann, G. & Lerch, K. (1985). Primary structure of tyrosinase from *Streptomyces glaucescens*. *Biochemistry* 24, 6038–6044.
- Jendrisak, J. J. & Burgess, R. R. (1975). A new method for the large-scale purification of wheat germ DNA-dependent RNA polymerase II. *Biochemistry* 14, 4639–4644.
- Klein, J. R., Heinrich, B. & Plapp, R. (1991). Molecular analysis and nucleotide sequence of the *envCD* operon of *Escherichia coli*. *Mol Gen Genet* 230, 230–240.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Lawlor, E. J., Baylis, H. A. & Chater, K. F. (1987). Pleiotropic morphological and antibiotic deficiencies result from mutations in a gene encoding a tRNA-like product in *Streptomyces coelicolor* A3(2). *Genes Dev* 1, 1305–1310.
- Lomovskaya, N., Otten, S., Doi-Katayama, Y. & 8 other authors (1999). Doxorubicin overproduction in *Streptomyces peucetius*: cloning and characterization of the *dnrU* ketoreductase and *dnrV* genes and the *doxA* CYP450 hydroxylase gene. *J Bacteriol* 181, 305–318.
- Madduri, K. & Hutchinson, C. R. (1995). Functional and transcriptional analysis of the *dnrR1* locus, which controls daunorubicin biosynthesis in *Streptomyces peucetius*. *J Bacteriol* 177, 1208–1215.
- Nagy, I., Geert, S., Jos, V. & De Mot, R. (1997). Further sequence analysis of the DNA regions with the *Rhodococcus* 20S proteasome structural genes reveals extensive homology with *Mycobacterium leprae*. *DNA Seq* 7, 225–228.
- Narva, K. E. & Feitelson, J. S. (1990). Nucleotide sequence and transcriptional analysis of the *redD* locus of *Streptomyces coelicolor* A3(2). *J Bacteriol* 172, 326–333.
- Neal, R. J. & Chater, K. F. (1987). Nucleotide sequence analysis reveals similarities between proteins determining methylenomycin A resistance in *Streptomyces* and tetracycline resistance in eubacteria. *Gene* 58, 229–241.
- Otten, S. L., Stutzman-Engwall, K. J. & Hutchinson, C. R. (1990). Cloning and expression of daunorubicin biosynthesis genes from *Streptomyces peucetius* and *S. peucetius* subsp. *caesi*us. *J Bacteriol* 172, 3427–3434.
- Otten, S. L., Ferguson, J. & Hutchinson, C. R. (1995). Regulation of daunorubicin production in *Streptomyces peucetius* by the *dnrR2* locus. *J Bacteriol* 177, 1216–1224.
- Otten, S. L., Gallo, M. A., Madduri, K., Liu, X. & Hutchinson, C. R. (1997). Cloning and characterization of the *Streptomyces peucetius* *dnmZUV* genes encoding three enzymes required for biosynthesis of the daunorubicin precursor thymidine diphospho-L-daunosamine. *J Bacteriol* 179, 4446–4450.
- Paget, M. S. B., Hintermann, G. & Smith, C. P. (1994). Construction and application of streptomycete promoter probe vectors which employ the *Streptomyces glaucescens* tyrosinase-encoding gene as reporter. *Gene* 146, 105–110.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Scholtmeier, K. & Hillen, W. (1984). Transposon Tn10 contains structural genes with opposite polarity between *tetA* and IS10r. *J Bacteriol* 160, 499–503.
- Strohl, W. R. (1992). Compilation and analysis of DNA sequences associated with apparent streptomycete promoters. *Nucleic Acids Res* 20, 961–974.
- Stutzman-Engwall, K. J. & Hutchinson, C. R. (1989). Multigene families for anthracycline antibiotic production in *Streptomyces peucetius*. *Proc Natl Acad Sci USA* 86, 3135–3139.
- Stutzman-Engwall, K. J., Otten, S. L. & Hutchinson, C. R. (1992). Regulation of secondary metabolism in *Streptomyces* spp. and overproduction of daunorubicin in *Streptomyces peucetius*. *J Bacteriol* 174, 144–154.
- Tang, L., Grimm, A., Zhang, Y.-X. & 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.

Vara, J. A., Lewandowska-Skarbek, M., Wang, Y.-G., Donadio, S. & Hutchinson, C. R. (1989). Cloning of genes governing the deoxysugar portion of the erythromycin biosynthetic pathway in *Saccharopolyspora erythraea* (*Streptomyces erythreus*). *J Bacteriol* **171**, 5872–5881.

Ward, J. M., Janssen, G. R., Kieser, T., Bibb, M. J., Buttner, M. J. & Bibb, M. J. (1986). Construction and characterisation of a series of multi-copy promoter-probe plasmid vectors for *Streptomyces* using the aminoglycoside phosphotransferase gene from Tn5 as indicator. *Mol Gen Genet* **203**, 468–478.

White, J. & Bibb, M. J. (1997). The *bldA*-dependence of

undecylprodigiosin production in *Streptomyces coelicolor* A3(2) involves a pathway-specific regulatory cascade. *J Bacteriol* **179**, 627–633.

Wietzorrek, A. & Bibb, M. J. (1997). A novel family of proteins that regulate antibiotic productin in streptomycetes appears to contain a OmpR-like DNA-binding fold. *Mol Microbiol* **25**, 1181–1184.

Yang, K., Han, L. & Vining, L. C. (1995). Regulation of jadomycin B production in *Streptomyces venezuelae* ISP5230: involvement of a repressor gene, *jadR2*. *J Bacteriol* **177**, 6111–6117.

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