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* 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 coordinate 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...
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

Fig. 1. (a) Physical map of the DNR and DXR production genes. The restriction sites BamHI (B), BglII (Bg) and EcoRI (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 restrictions sites mentioned in the text are indicated. The dashed arrows show the locations of direct or inverted repeats.
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 (Buoncristiani 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 e-rhodomycinone (RHO) were obtained from Pharmacia & Upjohn. Thioestreptom 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 DH5α 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 Vera et al. (1989), and the low-copy-number shuttle vector pWHM601 was from Guilfoyle & 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 pMT3001.1 (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. Wohleben (Universität Bielefeld, Germany).

**Media and growth conditions.** E. coli strains were grown in LB medium or 2 X YT medium (Sambrook et al., 1989) at 37 °C for preparation of ssDNA from strains containing M13. E. coli transformants were selected with 100 µg ampicillin ml⁻¹, 100 µg apramycin (Fluka Chemicals) ml⁻¹ or 50 µg 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 RYE medium (Hopwood et al., 1985) for protoplast preparation and the isolation of chromosomal DNA. Plasmid-containing Streptomyces strains were selected with 10 µg thiostrepton ml⁻¹, 25 µg apramycin ml⁻¹ or 25 µg 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 et al., 1999) were used to carry out melC expression experiments and quantification of anthracycline production in S. peucetius. Production of melacin was achieved by supplementing the growth medium with 0.0375% (w/v) t-lyotoreine, 5 mg CuSO₄, 5H₂O ml⁻¹ and 0.2 mM FeCl₃ (Paget et al., 1994). Spores from S. peucetius strains were incubated in 150 × 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 × 15 mm test tubes was inoculated with seed cultures to OD₅₄₀ 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 OD₅₄₀. 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 BglII fragment containing the melC operon (Hintermann et al., 1985) and the transcription terminators mnrI (Neal & Chater, 1987) and fd (Ward et al., 1986) flanking the coding region from pMT3003.1 (Paget et al., 1994) into pWHM601 digested with BamHI, resulting in pWHM2000.

To generate the dnrO::melC and dnrN::melC transcriptional fusions, a 450 bp SpbI–NsiI fragment from pWCM533 (Otten et al., 1985) containing the intergenic region between dnrN and dnrO (Fig. 1b) was cloned into SpbI/PstI-digested pMT3000 (Paget et al., 1994), resulting in pWHM2001. From pWHM2001, a BamHI–BglII fragment was cloned in both orientations in BamHI-digested pMT3003.1, leading to the constructions pWHM2002 (dnrO::melC) and pWHM2003 (dnrN::melC). From these constructions, BglII fragments containing the fusions were removed and ligated into the BamHI site of pWHM601 to obtain pWHM2004 and pWHM2005, respectively. The dnrI::melC transcriptional fusion was generated by cloning a 347 bp BamHI–SacI 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 BamHI–BglII fragment was cloned into BamHI-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::melC), pWHM2005 (dnrN::melC) and pWHM2007 (dnrI::melC) were introduced into the mutant strains WMH1777 (dnrI::aphII) WMH1530 (dnrO::apbII) (Otten et al., 1985) and WMH1445 (dnrI::apbII) (Stuzman-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 Sall, 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 guanidine 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 reisolated 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 dnrO gene (a) 5′-GGCCCCGACGCCGGTG-GCTACTCATCGACTG-3′ and (b) 5′-CGGGTGTCTACTATCGACTCTCCCGCTTCCT-3′; beginning 242 bp and 252 bp, respectively, upstream of the predicted dnrO ATG start codon; and for the dnrO gene (c) 5′-CGGCTTGGCC- TTACCTCCCGGAGATCGCC-3′ and (d) 5′-GCCCGTGTC- CACTCCCGGGAGAGTCCGCTGG-3′, beginning 206 bp and 211 bp, respectively, upstream of the predicted dnrO ATG start codon.

Low-resolution S1 mapping was conducted essentially as described by Neal & Chater (1987) using approximately 1 µM 32P 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 Sall–SacII DNA fragment containing the dnrO intergenic region and the N-terminal ends of the dnrO and dnrO genes, was labelled with [α-32P]dCTP using the Multiprime 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′-TATGACCGAAGCCCGTTCGACAG-3′ and 5′-ACTGGCTTTGGCCGAGGC-3′. The linker spanned the first eight codons of the dnrO gene from the predicted translational start codon (ATG) to the SphI site and introduced an Ndel 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 SphI–NotI fragment and the Ndel–NotI fragment of pET-26b to produce pWHM562. The dnrO gene was transferred from pWHM562 as a Ndel–XhoI 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) and 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 volume 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. TEPD buffer (TEPD 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% polyethyleneimine 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 supernatent 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 *SunI–Spbi* DNA fragment containing dnrN and the dnrO promoter region and a 212 bp *NsiI–SmaI* DNA fragment containing the dnrO promoter and dnrO promoter regions were end-labelled with \([\alpha^32P]dCTP\) (Amersham) and Klenow polymerase. The binding reaction contained the \([\alpha^32P]labeled 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 X 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–Stal* 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, thioesterron-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–Spbi* 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 carrying 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 *dnrl***

The response regulator protein DnrN positively regulates DNR biosynthesis by activating the transcription of Dnrl (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–Spbi* segment used in these experiments contains the *dnrN* promoter and DnrO-binding region identified below, we assume *dnrN* was
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 ± 1.19 μg ml⁻¹), and DNR levels (3.3 ± 1.23 μg 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. peucetius 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 A₅₉₀ 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 (OD₅₉₀) 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-
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 \( dnrI \) 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 32P-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
The DnrO protein binds specifically to the dnaN/dnrO\textsubscript{p1} promoter region

The phenotype of the dnaO::aphII mutant strain and the suppression of this mutation by the pathway-specific transcriptional activators dnaN and drrI, together with the large decrease in dnaN\textsubscript{p1}::melC expression in the dnaO null mutant, indicated that DnrO functions by activating the transcription of the dnaN gene. This led us to conduct mobility shift DNA-binding assays to determine if the DnrO protein binds specifically to the dnaNO 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 dnaN/dnrO\textsubscript{p1} promoter region as evidenced by the reduced mobility of the end-labelled 136 bp Sma–SphI promoter fragment on 5\% nondenaturing gels (Fig. 5a), whereas 10 ng 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 dnaN/dnrO\textsubscript{p1} promoter fragment resulted in the loss of DnrO binding, indicating that the DnrO protein recognizes the dnaN/dnrO\textsubscript{p1} promoter DNA in a sequence-specific manner (Fig. 5a). However, when the dnaO\textsubscript{p2} and dnaO\textsubscript{p3} promoter region as a 212 bp Nsi–SmaI DNA fragment was analysed by mobility shift assay, the protein concentration required for DNA binding increased 250-fold relative to the dnaN/dnrO\textsubscript{p1} promoter fragment, and only non-specific DNA binding was observed (Fig. 5b). Similar DNA-binding activity to the dnaN/dnrO\textsubscript{p3} 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 dnaO 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 point 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 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::aphBII 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::aphBII 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 (Scholteimer & Hillen, 1984) and TcmR (Guillfoile & 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::aphBII 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 undecylenodigosin 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. Olano & 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


Tang, L., Grimm, A., Zhang, Y.-X. & Hutchinson, C. R. (1996). Purification and characterization of the DNA-binding protein...


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