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The DnrN Protein of *Streptomyces peucetius*, a Pseudo-Response Regulator, Is a DNA-Binding Protein Involved in the Regulation of Daunorubicin Biosynthesis

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DnrN, a protein essential for the transcription of the *dnrI* gene, which in turn activates transcription of the daunorubicin biosynthesis genes in *Streptomyces peucetius*, was overproduced in *Escherichia coli* and *S. peucetius*. The cell-free extract from *E. coli* was used to conduct DNA-binding assays. The results of gel mobility shift analysis showed that DnrN binds specifically to the *dnrI* promoter region with a high affinity (K_d = 50 nM). Neither acetyl phosphate nor ATP affected the binding ability, and there was no difference in binding between wild-type DnrN and a mutant form (D-55→N) lacking the putative phosphorylation site (aspartate 55) of a response regulator protein. Therefore, phosphorylation of DnrN apparently is not necessary for DNA binding. DNase I footprinting analysis indicated binding regions at 37 to 55 bp and 62 to 100 bp upstream of the transcriptional start point of *dnrI*. Interestingly, the sequence of these regions includes consecutive overlapping triplets [5′-(A/T)GC, 5′-(A/T)CG, 5′-(A/T)C(A/T)] that have been shown to be the preferential binding site of daunorubicin (J. B. Chaires and J. E. Herrera, Biochemistry 29:6145–6153, 1990). This may explain why daunorubicin appeared to inhibit the binding of DnrN to the *dnrI* promoter, which could result in feedback repression of daunorubicin production. The results of Western blotting (immunoblotting) analysis with His-tagged DnrN antiserum showed that *dnrN* expression is coincident with daunorubicin production and that the maximum level of DnrN is 0.01% of total protein in the wild-type *S. peucetius* strain. Since the level of DnrN was lowered in mutant strains that do not produce daunorubicin, we speculate that *dnrN* and *dnrI* expression are regulated by daunorubicin.

Actinomycetes are gram-positive bacteria that possess an interesting life cycle involving morphological differentiation (aerial mycelia and spore formation) and specialized secondary metabolism (antibiotic production). To regulate these complex processes, the bacteria appear to have evolved an intricate regulatory hierarchy with several different mechanisms. Some of these processes are unique to the *Streptomyces* spp., such as the *bldA* gene (20), and others are well-known in eukaryotes, such as hormone-like molecules (31) and serine-threonine or tyrosine kinases (24). Other mechanisms resemble ones common in prokaryotes. An increasingly important example of the latter is the two-component system (4, 17, 46, 50), which consists of a histidine kinase that undergoes autophosphorylation in response to environmental or developmental changes and then transfers its phosphate group to an aspartyl residue of a response regulator protein (34, 35, 40).

In our study of the biosynthesis of daunorubicin (DNR) and doxorubicin (DXR), commercially important antitumor drugs (Fig. 1), sequence analysis of the *dnrN* gene (see Fig. 2) showed that its product is very similar to response regulator proteins of the FixJ subfamily (13, 32). The N-terminal region of DnrN has a putative phosphorylation site (D-55) and other conserved sites required for activation by phosphorylation and characteristic of response regulators. However, the D-55→E (D55E) and D-55→N (D55N) substitutions at the putative site of phosphorylation resulted in a reduction rather than a complete loss of DnrN activity (32). Furthermore, analysis of the 60-kb region sequenced around *dnrN* has not uncovered a putative sensor kinase gene, which often resides near the response regulator gene. Since DnrN lacks two key characteristics of canonical response regulators, we call it a pseudo-response regulator.

Our previous results from mutant complementation (32) and transcriptional analysis (23) experiments showed that *dnrN* is essential for transcription of the *dnrI* regulatory gene and functions epistatically with respect to *dnrI*. DnrI, whose sequence is similar to other *Streptomyces* regulatory proteins such as AfsR (16), RedD (30), and *actII*-Orf4 (9), in turn activates the DNR and DXR structural and resistance genes (23). Among streptomycetes, this is a clear case in which a putative response regulator gene is known to govern expression of antibiotic biosynthesis and resistance genes in such a direct and essential manner.

In spite of considerable information about response regulator proteins in other bacteria (1, 11, 37, 48), so far none of them have been characterized from streptomycetes. Here we report that the DnrN protein binds specifically to the *dnrI* promoter region and is not likely to require phosphorylation for its binding. We also show that DNR has a positive effect on the DnrN level in *Streptomyces peucetius* and suggest how DNR could feedback repress *dnrI* expression.

MATERIALS AND METHODS

Biochemicals and chemicals. DNR, DXR, and ε-rhodomycinone (RHO) were obtained from Pharmacia and Upjohn (Milan, Italy). Thiostrepton was obtained from Sal Lucania at E. R. Squibb & Sons (Princeton, N.J.). ^{32}P-labeled dilithium acetyl phosphate was synthesized as described by Stadtman (39) except that 0.45 instead of 4.5 ml of 4 N LiOH was used. Purified PhoB protein was a gift from William R. McCleary at West Virginia University. Other reagents were obtained from standard commercial sources.

Bacterial strains and plasmids. *S. peucetius* ATCC 29050 was obtained from the American Type Culture Collection (Rockville, Md.). The high-copy-number

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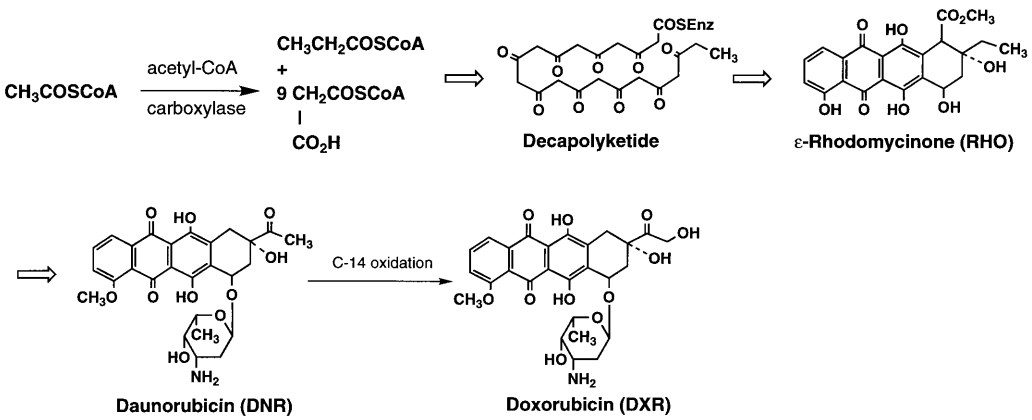

FIG. 1. DXR biosynthesis pathway in *S. peucetius*. Open arrows indicate multiple steps from the precursors and intermediates shown.

shuttle plasmid pWHM3 (43, 47) was used as the vector in *S. peucetius*. *Escherichia coli* NovaBlue and BL21(DE3) (Novagen, Madison, Wis.) were used for subcloning and expression hosts, respectively. The pET series plasmids (Novagen) were used as the expression vectors in *E. coli* BL21(DE3). Other plasmids and strains used in this study are listed in Table 1.

Protein analysis. Protein concentrations were determined by the Bradford method (2) with bovine serum albumin (BSA) as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (19), and the gels were Coomassie blue R stained. For the total protein and Western blot (immunoblot) analyses, the cells were suspended in SDS-PAGE sample buffer (50 mM Tris-HCl [pH 6.8], 100 mM dithiothreitol [DTT], 2% SDS [wt/vol], 0.1% bromophenol blue [wt/vol], and 10% glycerol [vol/vol]) and heated in boiling water for 3 min, and then the supernatant fractions were run on an SDS–15% polyacrylamide gel to observe the proteins produced in each sample. For Western immunoblotting, proteins were transferred to polyvinylidine difluoride (Immobilon-P; Millipore, Bedford, Mass.) membranes by a Bio-Rad electroblotting apparatus (Richmond, Calif.). The immunodetection assay was done with an ECL kit as instructed by the manufacturer (Amersham, Chicago, Ill.). A goat anti-rabbit immunoglobulin G horseradish peroxidase conjugate was used as the secondary antibody.

In vitro manipulation of DNA. Restriction endonuclease digestions, ligations, transformation of *E. coli*, and plasmid isolations were performed according to standard techniques (38). DNA fragments for labelling and subcloning were isolated with the Qiaex (Qiagen, Chatsworth, Calif.) gel extraction kit. Transformation of *S. peucetius* was done as previously described (33, 41) by using protoplasts prepared from 1-day-old mycelium growing in R2YE liquid medium (15) at 30°C and screening cell-wall regenerants for antibiotic resistance with thiostrepton (25 μg/ml) added to the R2YE medium.

Expression of *dnrN* in *E. coli* strains. To express *dnrN* in *E. coli*, an *Nde*I site was introduced at the ATG of the predicted translational start codon, and the first seven codons were changed to the preferred codons in *E. coli* by PCR methods with the following two primers: 5′-GCACGGATCCATATGACTATC CGTGTTGTTATCGCTGAAGAGCTGGAAATGGTC-3′ (the *Nde*I site is underlined and the altered third codon positions are in bold) and 5′-GCTGGAA TTCGTCGACATCGATGACCGTCAC-3′ (the *Sal*I site is underlined). Each primer (0.5 μg) was incubated with 30 ng of pWHM518 (32), which contains the 1.8-kb *dnrN* coding region, in a total volume of 99.5 μl of 10 mM Tris-HCl (pH 9), 1.25 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 100-mg/ml BSA, 60 μM (each) dCTP and dGTP, and 40 μM (each) dATP and dTTP. After being overlaid with mineral oil, the reaction mixture was incubated at 100°C for 5 min and then cooled to 70°C and 2.5 U of *Taq* polymerase (Promega) was added. The PCR was carried out in a model 480 DNA thermal cycler (Perkin-Elmer Cetus). Amplification was achieved with 25 cycles of denaturation at 97°C for 50 sec followed by annealing and extension at 70°C for 2.5 min. The resulting 170-bp fragment was recovered from a 1.5% agarose gel after electrophoresis and digested with *Nde*I and *Sal*I. The *dnrN* gene was reassembled by three-piece ligation of this 0.17-kb *Nde*I-*Sal*I fragment, the 0.48-kb *Sal*I-*Bam*HI fragment from pWHM518 containing the remainder of the *dnrN* coding region, and the 3.3-kb *Nde*I-*Bam*HI fragment of pET-17b to give pWHM401. The resulting *dnrN* gene was transferred as a 0.65-kb *Nde*I-*Bam*HI fragment into pET-26b for wild-type protein and into pET-16b for His-tagged-type protein between the *Nde*I and *Bam*HI sites to give pWHM402 and pWHM404, respectively. A 0.17-kb *Nde*I-*Sal*I fragment containing the entire region made by the PCR was subcloned from pWHM401 into pUC19. The DNA sequence of this region was verified by sequence analysis.

The *E. coli* BL21(DE3) strains transformed with these plasmids were grown in Luria-Bertani medium at 26°C, 29°C, or 37°C. At an optical density at 600 nm (OD_{600}) of 0.6, 1 mM isopropylthiogalactopyranoside (IPTG) was added, and the cells were collected after 3 h by centrifugation.

The pelleted cells were resuspended in one-tenth culture volume of a solution containing 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 0.1% Triton-X and 10% glycerol. After lysis by sonication, the cell lysate was centrifuged for 5 min at

TABLE 1. Plasmids and strains used in this study

Plasmid or strain	Description	Reference
Plasmids		
pWHM3	A high-copy-number *E. coli* and *Streptomyces* shuttle vector based on pIJ702 and pUC19	43, 47
pWHM401	The *dnrN* gene under control of the T7 RNA polymerase promoter of pET17-b	This study
pWHM402	The *dnrN* gene under control of the T7 *lac* promoter of pET26-b	This study
pWHM403	An N-terminal His-tagged *dnrN* gene under control of the T7 RNA polymerase promoter of pET14-b	This study
pWHM404	An N-terminal His-tagged *dnrN* gene under control of the T7 *lac* promoter of pET16-b	This study
pWHM405	The mutated *dnrN* (D55N) gene from pWHM542 (32) under control of the T7 *lac* promoter of pET26-b	This study
pWHM406	The mutated *dnrN* (D55E) gene from pWHM541 (32) under control of the T7 *lac* promoter on pET26-b	This study
pWHM408	The promoter region of *dnrI* on pUC19	This study
pWHM411	The *dnrN* gene under control of the *ermE** on pWHM860	This study
pWHM412	The promoter region of *dnrI* on pWHM860	This study
pWHM413	The presumed promoter region of *dnrN* and *dnrO* on pWHM860	This study
pWHM518	The *dnrN* gene and partial *dnrO* gene in pWHM601 (14)	32
pWHM534	The *dnrN* gene under control of its own promoter on pWHM3	32
S. peucetius		
WMH1535	*dpsB*-deleted mutant of *S. peucetius* ATCC 29050	12
WMH1445	*dnrI*::*aph*II-disrupted mutant of *S. peucetius* ATCC 29050	42

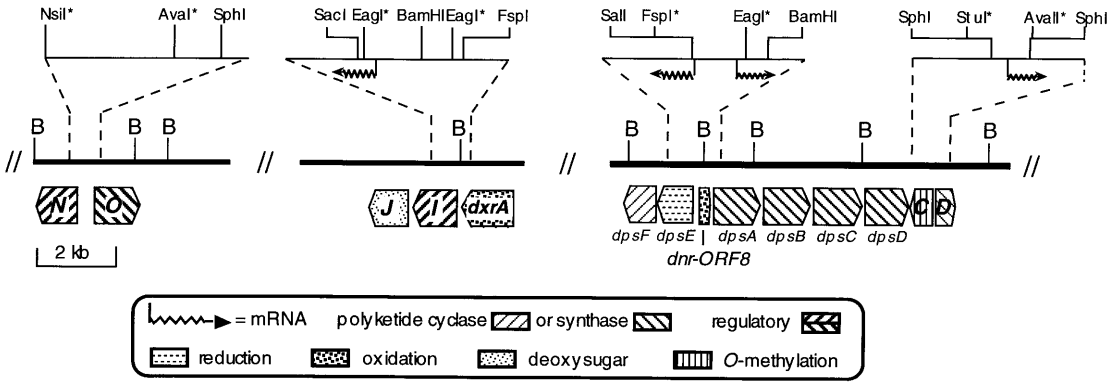

FIG. 2. A partial map of the cluster of *dnr* genes in *S. peucetius* ATCC 29050. The thick line shows the *Bam*HI (B) sites and the thin lines above it indicate the DNA template used for the gel mobility shift experiments described in the text. The genes are indicated by wedges oriented in the direction of transcription and are shaded to indicate the functions designated below the map, which were deduced from the results of sequence analysis, gene disruption, and/or expression experiments. The wavy arrows indicate transcripts that have been mapped by S1 nuclease protection and primer extension experiments. The four promoter regions discussed in the text are expanded above the map, and the restriction sites used for labeling the DNA with ^{32}P are marked with an asterisk.

14,000 rpm in an Eppendorf model 5414 microcentrifuge, and the supernatant was stored at −80°C.

Purification of His-tagged DnrN protein. *E. coli* BL21(DE3) harboring pWHM404 was grown at 37°C and lysed as described above. The insoluble pellet from the cell lysate was resuspended in the binding buffer with 6 M urea, the remaining insoluble material was removed by centrifugation as described above, and then DnrN was purified by using a Ni^{2+} affinity column (the buffers contained 6 M urea) as directed by the manufacturer (Novagen). Purified DnrN was precipitated by adding 2 volumes of H_2O for later use.

Refolding of the His-tagged DnrN protein. Refolding was carried out in two buffer systems. In the first system, purified protein (1 mg) was resuspended in 10 ml of phosphate-buffered saline (PBS) buffer (pH 7.5) containing 6 M guanidine and dialyzed against PBS buffer containing 10 mM $MgCl_2$, 10 mM DTT, and 50% glycerol. In the second system, purified protein (1 mg) was resuspended in 10 ml of HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 7.5) containing 6 M urea and dialyzed against HEPES buffer (pH 7.5) containing 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, and 50% glycerol. Each sample was dialyzed three times against 100 volumes of buffer for 10 h at 4°C.

Refolding experiments were carried out at 4°C and at room temperature with both buffers, with and without DTT, and the resulting DnrN was tested for its ability to be phosphorylated as described below.

Preparation of anti-DnrN antibody. Purified, insoluble His-tagged DnrN protein (130 μg) was mixed with Freund's complete adjuvant and injected into rabbits (6 pounds each; Hazelton, Madison, Wis.) by the intradermal route. The rabbits were booster injected every 4 weeks with a further 130 μg of protein and were bled 2 weeks after each booster injection. The antiserum was stored at −80°C.

Construction of *dnrN* expression plasmids for *S. peucetius*. To prepare pWHM411, a 0.69-kb *Nru*I-*Bam*HI fragment that contains 40 bp of upstream sequence and the entire coding region of the *dnrN* gene was cloned into pWHM860 (28) between the *ermE** promoter and a terminator.

Media and growth conditions for *S. peucetius*. *S. peucetius* strains were grown at 30°C for 7 to 10 days on ISP 4 medium (Difco Laboratories, Detroit, Mich.) for spore preparation and for 1 day in R2YE medium for liquid culture. For anthracycline production, the strains were cultured in the GPS particulate production medium as previously described (33) or in the soluble APM medium as follows. The APM seed and APM production media (14) both contained 15 g of MOPS (morpholinepropanesulfonic acid) sodium salt per ml and 4 g of glass beads (3-mm-diameter; Fisher, Pittsburgh, Pa.) in a 200-ml flask. Seed cultures (12.5 ml) were inoculated with 50 μl of glycerol stock mycelium (OD_{600} = 2.0), kept at −80°C, and incubated for 12 h. Then 100 μl of seed culture was transferred to 25 ml of the APM production medium and incubated at 30°C for 72 h. To determine anthracycline production, after the cultures were acidified with oxalic acid, heated at 60°C for 45 min, and adjusted to pH 8.5, they were extracted with chloroform and analyzed by high-performance liquid chromatography (33, 41).

Preparation of radiolabeled DNA. A 214-bp *Eag*I fragment containing the *dnrI* promoter region, a 212-bp *Ava*I-*Nsi*I fragment containing the putative divergent promoter regions for the *dnrN* and *dnrO* genes, a 205-bp *Eag*I-*Fsp*I fragment containing the divergent promoter regions for the *dnr-ORF8* and *dpsE* genes, and a 244-bp *Ava*I-*Stu*I fragment containing the *dnrD* promoter region (Fig. 2) were end-labeled with [α-^{32}P]dCTP (Amersham) and Klenow polymerase. For DNase I footprinting assays, the labeled *dnrI* promoter region was digested with *Ava*I to remove one labeled end containing 17 bp.

Gel retardation assays. Gel retardation assays were performed essentially as described by Chodosh (8). ^{32}P-labeled DNA fragments (1 ng; 2,000 to 4,000 cpm) were incubated with cell-free extract or purified DnrN protein for 10 min at 30°C in 20 μl total volume of buffer containing 8 mM HEPES (pH 7.8), 10 mM Tris-HCl (pH 7.8), 4 mM $MgCl_2$, 100 mM KCl, 0.2 mM EDTA, 1 mM DTT, 10 mM acetyl phosphate, 1.5 μg of poly(dI-dC) · (dI-dC), 1 μg of BSA, and 20% glycerol. Protein-bound and free DNA were resolved on 5% nondenaturing polyacrylamide gels run in a high-ionic-strength buffer containing 50 mM Tris, 380 mM glycine, and 2 mM EDTA (pH 8.5) at 4°C. The gels were analyzed by the ImageQuaNT program with a PhosphorImager SI (Molecular Dynamics, Sunnyvale, Calif.).

DNase I footprinting. Reactions were carried out in 60 μl of the DNA-binding reaction mixture described above with 3 ng of end-labeled *dnrI* promoter region (12,000 cpm). After incubation for 10 min at 30°C, 3 μl of DNase I solution (3 U/μl of DNase I [Boehringer Mannheim, Indianapolis, Ind.] in 200 mM $MgCl_2$–100 mM DTT) was added to each reaction mixture. The reaction was incubated at 30°C for 1 min and stopped by the addition of 60 μl of DNase I stop solution (1% SDS, 200 mM NaCl, 20 mM EDTA [pH 8.0] 40-μg/ml tRNA). The reaction mixture was extracted once with phenol:chloroform (1:1, vol/vol), and the DNA was ethanol precipitated with 2 μl of Pellet Paint (Novagen). The resulting pellet was resuspended in 5 μl of sequencing loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% Xylene Cynanol FF). After incubation at 87°C for 2 min, 3.5 μl was applied to a 6% polyacrylamide–10% formamide–6 M urea sequencing gel along with dideoxy DNA sequencing ladders. After electrophoresis, the gels were dried and analyzed by autoradiography.

Phosphorylation of proteins. Protein phosphorylation was carried out in 10-μl reaction mixtures containing 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 10 mM

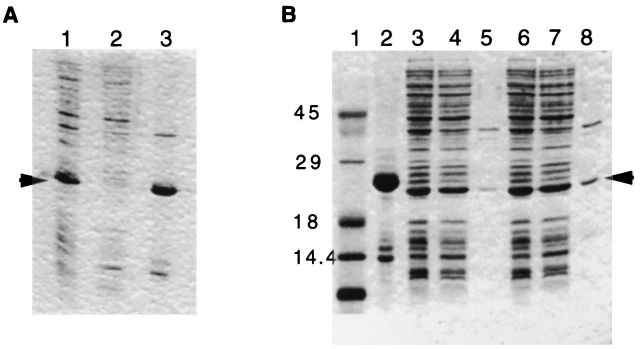

FIG. 3. Overexpression of the DnrN protein in *E. coli*. In each lane, the cell-free extract from 70 μl of culture was electrophoresed through an SDS–15% polyacrylamide gel. The arrows indicate the location of the DnrN protein. (A) *E. coli* BL21(DE3) harboring pWHM402 and grown at 37°C. Lane 1, total cell lysate; lane 2, soluble fraction; lane 3, insoluble fraction. (B) Lane 1, molecular mass markers (the numbers alongside indicate the molecular mass in kDa); lane 2, insoluble fraction from lysate of *E. coli* BL21(DE3) harboring pWHM404 and grown at 37°C. *E. coli* BL21(DE3) harboring pWHM402 was used in the following lanes: lane 3, total cell lysate from cells grown at 26°C; lanes 4 and 5, soluble and insoluble fractions, respectively; lane 6, total cell lysate from cells grown at 29°C; lanes 7 and 8, soluble and insoluble fractions, respectively.

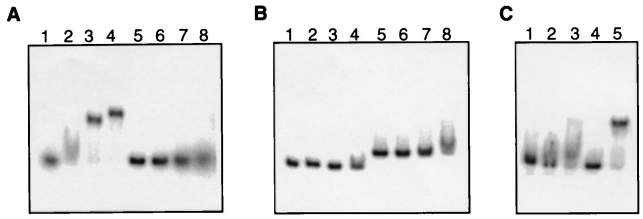

FIG. 4. Gel retardation analysis of four *dnr* gene promoter regions with cell-free extract of *E. coli* BL21(DE3) harboring pWHM402. (A) Lanes 1 to 4, *dnrI* promoter; lanes 5 to 8, putative *dnrNO* promoter. Lanes 1 and 5, no cell-free extract; lanes 2 and 6, 0.5 μg of cell-free extract; lanes 3 and 7, 1 μg of cell-free extract; lanes 4 and 8, 2 μg of cell-free extract. (B) Lanes 1 to 4, *dpsE* and *dnr-ORF8* promoter; lanes 5 to 8, *dnrD* promoter. Lanes 1 and 5, no cell-free extract; lanes 2 and 6, 0.5 μg of cell-free extract; lanes 3 and 7, 1 μg of cell-free extract; lanes 4 and 8, 2 μg of cell-free extract. (C) Inhibition by unlabeled competitor DNA. The *dnrI* promoter region and 1 μg of cell-free extract were used in each lane. Lane 1, 2 μg of pWHM408; lane 2, 10 μg of pUC19; lane 3, 2 μg of pUC19; lane 4, 10 μg of pWHM408; lane 5, no competitor DNA.

$MnCl_2$, 0.1 mM DTT, 0.1 mM EDTA, and 10% glycerol. The reaction was initiated by addition of 50 mM ^{32}P-labeled dilithium acetyl phosphate (3.6 mCi/mmol) or 33 nM [γ-^{32}P]ATP (30 Ci/mmol) and stopped by addition of twice-concentrated SDS-PAGE sample buffer after incubation at 30°C for 60 min. The reaction mixtures were promptly heated for 3 min at 55°C and separated by an SDS–15% polyacrylamide gel. The gels were analyzed by autoradiography.

RESULTS

Expression of the DnrN protein in *E. coli*. DnrN was overproduced in *E. coli* by using a T7 RNA polymerase expression system. An initial attempt using pWHM401, based on pET17-b, failed because we could not introduce pWHM401 into the BL21(DE3) strain by transformation, but expression of *dnrN* in pWHM402, which is based on pET26-b, which possesses a *lac* operator and *lacI* gene, in the BL21(DE3) strain was successful. After induction by IPTG at 37°C, almost all the DnrN was found in the insoluble fraction and was estimated to represent more than 20% of the total protein and 90% of the insoluble fraction by scanning of an SDS-polyacrylamide gel (Fig. 3A). When the culture temperature was decreased to 26°C, most of the DnrN was soluble (Fig. 3B) and was estimated to be 14% of total soluble protein. His-tagged DnrN behaved in a similar manner (Fig. 3B), and from the insoluble fraction, we purified and renatured the His-tagged DnrN protein. pWHM405 and pWHM406 were constructed for expression of the D55N and D55E mutant DnrN proteins, respectively. DnrN (D55N) was completely soluble, but DnrN (D55E) was insoluble even at 26°C (data not shown).

The DnrN protein binds specifically to the *dnrI* promoter region. Gel retardation assays with four promoter regions from the *dnr* genes (Fig. 2) were used to determine the specificity of DnrN binding. Although the *dnrI* promoter was the most likely candidate, two promoter regions for three biosynthesis genes, *dnrD* (23), *dnr-ORF8* (44), and *dpsE*, were also examined. To probe the possibility of the self-regulation of *dnrN* expression, the presumed *dnrN* and *dnrO* promoter region was also tested. Although 60 μg of cell-free extract of BL21(DE3) harboring pET26-b had no binding activity to all four promoter regions (data not shown), just 0.5 μg of cell-free extract of BL21(DE3) carrying pWHM402 showed a clear concentration-dependent retardation for the *dnrI* promoter region (Fig. 4A, lanes 1 to 4). DnrN showed a very weak binding affinity to the other three promoter regions (Fig. 4A and B). Although the results of inhibition of DnrN binding by unlabeled DNA indicated that the binding to the *dnrI* promoter region is specific (Fig. 4C, compare lane 1 with 5), the weak but clear inhibitory effect of pUC19 DNA at a high concentration suggested that DnrN has a weak nonspecific DNA binding activity.

The gel retardation assay was done at several concentrations of DnrN (Fig. 5A) to estimate the dissociation constant, K_d. Since the concentration of DNA was 0.2 nM in the reaction mixtures and the protein concentration far exceeded this value, $DnrN_{total} = DnrN_{free}$ and the protein concentration required for half-maximal binding is close to the K_d (5). From the graph of Fig. 5B, the K_d for DnrN is approximately 50 nM. This value is of the same order or smaller than that reported for other response regulator proteins of the FixJ subfamily (1, 11, 37, 48) to which DnrN belongs (32). For noncooperative binding, an increase of 1.81 log units in protein concentration is theoretically required to increase the bound fraction from 10 to 90% (5). Since the data in Fig. 5B show that an increase of only about 1 log unit in concentration of DnrN is required, its binding probably involves some type of positive cooperativity such as dimerization. A cell-free extract containing the D55N DnrN mutant protein did not show a significant difference of affinity compared with the wild-type DnrN (Fig. 5A).

If DnrN were tightly bound to the *dnrI* promoter in vivo, then multiple copies of this region might titrate out DnrN and result in a decrease in *dnrI* expression, which should lead to a decrease in RHO and DNR production. To test this idea, we constructed the high-copy-number plasmids pWHM412 and pWHM413 carrying the *dnrI* and *dnrN* promoter regions, respectively, and introduced them into *S. peucetius* 29050. Since neither plasmid had an effect on anthracycline production in either growth medium tested (data not shown), we assume that the binding between the plasmid-borne promoter regions is not tight enough or the plasmid copy number is not high enough to have such an effect.

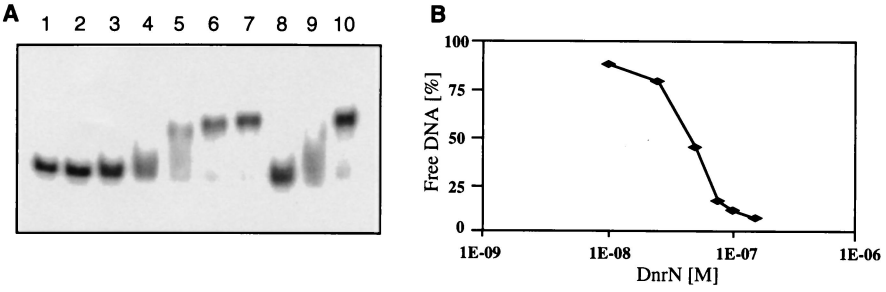

FIG. 5. Binding affinity of DnrN to the *dnrI* promoter region. (A) Gel retardation analysis with cell-free extract. Lanes 2 to 7, *E. coli* BL21(DE3) harboring pWHM402; lanes 8 to 10, *E. coli* BL21(DE3) harboring pWHM405. Lane 1, no cell-free extract; lane 2, 0.1 μg of cell-free extract; lane 3, 0.25 μg of cell-free extract; lanes 4 and 8, 0.5 μg of cell-free extract; lanes 5 and 9, 0.75 μg of cell-free extract; lanes 6 and 10, 1.0 μg of cell-free extract; lane 7, 1.5 μg of cell-free extract. (B) Binding curve calculated from the data shown in lanes 1 to 7 of panel A.

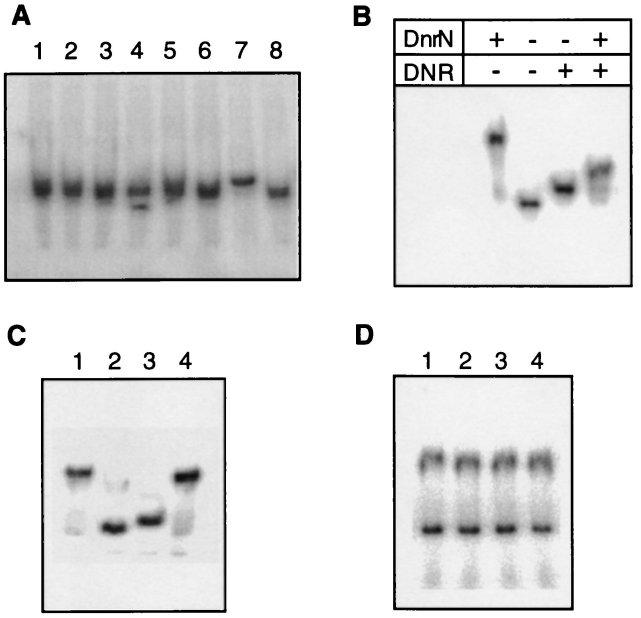

FIG. 6. Effects of several molecules on DnrN binding to the *dnrI* promoter region. (A) Here, 0.35 μg of cell-free extract from *E. coli* BL21(DE3) harboring pWHM402 was used for gel retardation analysis. Lane 1, 0.5 μM acetylCoA; lane 2, 50 μM acetylCoA; lane 3, 0.5 μM malonylCoA; lane 4, 50 μM malonylCoA; lane 5, 50-μg/ml A-factor; lane 6, 40 μM cAMP; lane 7, 25-μg/ml DNR; lane 8, no addition as control. (B) In the row labeled DnrN, "−" means no cell-free extract was added and "+" means 0.75 μg of the above cell-free extract was added. In the row labeled DNR, "−" means no DNR was added and "+" means 25 μg/ml of DNR was added. (C) In all lanes 0.75 μg of the above-described cell-free extract was used. Lane 1, no addition as control; lane 2, 25 μg of DNR per ml; lane 3, 25 μg of DXR per ml; lane 4, 25 μg of RHO per ml. (D) In all lanes, 0.6 μg of the above-described cell-free extract was used. Lane 1, no addition as control; lane 2, 10 mM acetyl phosphate; lane 3, 10 mM carbamyl phosphate; lane 4, 10 mM ATP.

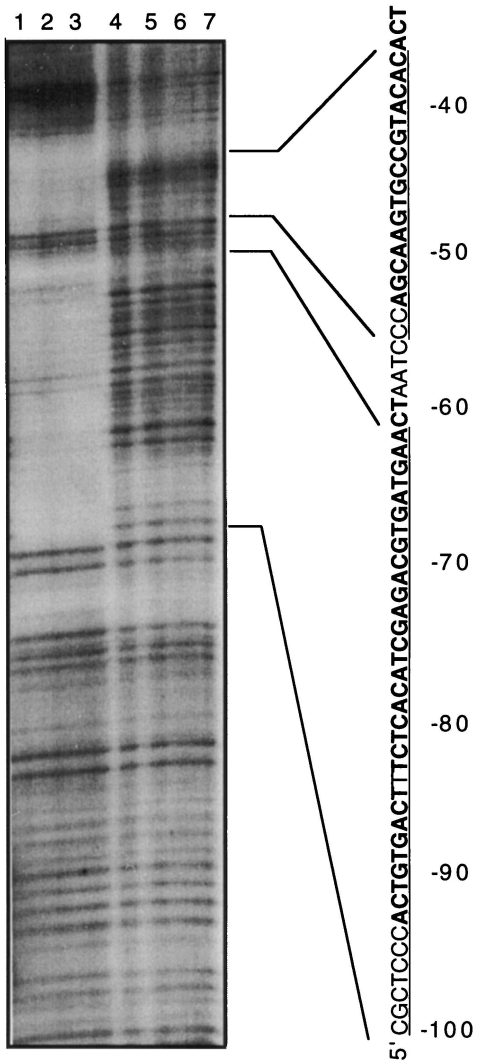

FIG. 7. DNase I footprinting analysis of DnrN binding to the *dnrI* promoter region. Protected bases are underlined. The nucleotides consisting of the preferred triplets for DNR binding are in boldface. The numbers along the right side indicate the distance from the transcriptional start point of the *dnrI* promoter. Lanes 1 to 3, cell-free extract from *E. coli* BL21(DE3) harboring pWHM402 was used; lane 4, no cell-free extract; lanes 5 to 7, cell-free extract from *E. coli* BL21(DE3) harboring pET26-b was used. Lanes 1 and 5, 0.33 μg of cell-free extract; lanes 2 and 6, 1 μg of cell-free extract; lanes 3 and 7, 2 μg of cell-free extract.

Since DnrN appears to be a key regulator of DNR biosynthesis (32), via its effect on *dnrI* expression (23), we examined the ability of different low-molecular-weight compounds to affect the binding of DnrN to the *dnrI* promoter. In the first set of experiments, we tested the DNR precursors acetyl-coenzyme A (CoA) and malonyl-CoA (Fig. 1), two signal transducing molecules (A-factor [31] and cyclic-AMP [45]), and DNR. Among these molecules, only DNR had an effect (Fig. 6A). To observe this inhibition more clearly, DNR was added in the presence or absence of a higher concentration of DnrN (Fig. 6B). The results showed that DNR at a concentration of 25 μg/ml apparently inhibits DnrN binding significantly (we cannot exclude the possibility DNR does not inhibit DnrN binding if the migration of a complex among DnrN, DNR, and the DNA is retarded less than the one formed between DnrN and the DNA) and by itself also retards the mobility of the *dnrI* segment, probably due to DNA intercalation. Among compounds related to DNR, DXR also inhibited the binding, but RHO, which lacks the deoxyamino sugar daunosamine, did not (Fig. 6C). In all these reaction mixtures, we had added 10 mM acetyl phosphate because many response regulator proteins have been shown to be activated by it (see reference 26 for a review). When we excluded it and added other phospho donor molecules to observe the effect on DnrN binding (Fig. 6D), the results showed that none of these phospho donors affected the binding of DnrN to the *dnrI* promoter region either.

DNase I protection of the *dnrI* promoter by DnrN. To show that DnrN recognizes specific nucleotides in the *dnrI* promoter and to identify its binding site, DNase I footprinting assays were carried out. Using the bottom strand from −180 to +15 bp with respect to the transcriptional initiation site of *dnrI*, we found that two regions, 37 to 55 bp and 62 to 100 bp upstream of the transcriptional start point, were clearly protected (Fig. 7). Interestingly, the sequence of these two regions includes consecutively overlapped triplets [5′-(A/T)GC, 5′-(A/T)CG, 5′-(A/T)C(A/T)] which have been shown to be the preferential binding site of DNR (7, 29, 49). These triplets are expected to occur at a frequency of 4.6/35 bp in a random sequence consisting of 60% GC content, but there are 15 such triplets in the 35-bp region from −61 to −95 of *dnrI* and all but one nucleotide in the 35 bp participate in such triplets. Furthermore, there are 8 such triplets in the 19 bp of the −35 to −53 region instead of the 2.5 expected for the random sequence.

TABLE 2. Effect of DnrN overproduction on the phenotype of *S. peucetius* strains grown on ISP 4 medium plates[a]

Plasmid	Host	Aerial mycelium	Red pigment
None	ATCC 29050	+	±
	WMH1535 (Δ*dpsB*)	+	−
	WMH1445 (*dnrI*::*aphII*)	+	−
pWHM860 (vector)	ATCC 29050	+	±
	WMH1535 (Δ*dpsB*)	+	−
	WMH1445 (*dnrI*::*aphII*)	±	−
pWHM411 (*ermE**::*dnrN*)	ATCC 29050	−	+++
	WMH1535 (Δ*dpsB*)	−	−
	WMH1445 (*dnrI*::*aphII*)	−	−

[a] +, effect was visible after 4 to 6 days of growth; −, not visible; +++, effect was markedly enhanced; ±, effect was slightly different from + or −.

Lack of phosphorylation of the DnrN protein by acetyl phosphate. Although the physiological meaning is not always clear, acetyl phosphate has been demonstrated to phosphorylate response regulator proteins in the absence of their cognate sensor kinases (26). As mentioned above, we have not identified a sensor kinase gene in the DNR gene cluster. Since acetyl-CoA is an important starting molecule in DNR biosynthesis (Fig. 1) and could be converted to acetyl phosphate by constitutive phosphotransacetylase, we considered the hypothesis that the DnrN activity is regulated by the level of acetyl-CoA via acetyl phosphate. Therefore, we explored the possibility that DnrN is phosphorylated by acetyl phosphate, even though this phospho donor did not affect the binding of DnrN to the *dnrI* promoter. No protein was phosphorylated after 3 to 60 min of incubation of [^{32}P]acetyl phosphate with 10 μg of cell-free extract from *E. coli* BL21(DE3) carrying pWHM402 (data not shown). Since the soluble His-tagged DnrN produced in *E. coli* did not bind to a nickel affinity chromatography column, the insoluble DnrN protein produced in *E. coli* was denatured in 6 M urea and purified by nickel affinity chromatography to give a single band in an SDS-polyacrylamide gel (data not shown). This denatured DnrN was refolded in eight different conditions by using two buffer systems, at 4°C and room temperature, with and without 5 mM DTT (see Materials and Methods). Although at least 5% of the renatured proteins were active in a gel retardation assay (data not shown), phosphorylation of renatured His-tagged DnrN by acetyl phosphate was not detected. As a positive control, the PhoB response regulator protein (25) was phosphorylated under the reaction conditions used (data not shown).

The DnrN level in *S. peucetius* is influenced by DNR metabolites. Since DnrN is one of the factors that regulates DNR production (32), we were interested in learning how the DnrN level changed with time as a function of metabolism and/or growth conditions. To facilitate these determinations, we introduced plasmids with the *dnrN* gene under control of its native promoter (pWHM534) or the *ermE** constitutive promoter (pWHM411) into *S. peucetius* 29050 (wild type) and two mutant strains that do not accumulate DNR metabolites, WMH1445 (*dnrI*::*aphII*) and WMH1535 (Δ*dpsB*), by transformation. The parent vector pWHM860 served as the control for the DnrN level due to expression of the chromosomal *dnrN* gene. Introduction of pWHM411 inhibited the aerial mycelium formation in all three strains grown on ISP4 solid medium and caused the copious formation of a red pigment presumed to be RHO or DNR by strain 29050 (Table 2). The time course of the change in DnrN level in cultures grown in the soluble APM medium was determined by Western immunoblotting by using anti-DnrN antibody raised in rabbits (Fig. 8A). The maximum amount of DnrN produced in the 29050 strain with pWHM860 was 0.01% of total protein between days 3 and 5 (Fig. 8C). In the 29050 strain bearing pWHM534, DnrN production began at the second day, reached its maximum amount at the third and fourth days, and then decreased. This pattern was coincident with the time of DNR accumulation in the broth (data not shown). Interestingly, the two DNR-nonproducing strains carrying pWHM534 produced much less DnrN than the 29050 (pWHM534) transformant (Fig. 8C). These results suggest that the maximum expression of *dnrN* is achieved in the presence of DNR. In contrast, there was only a small difference in the DnrN level among transformants harboring pWHM411 (Fig. 8D), in which *dnrN* was expressed constitutively, and the difference may not be subject to the effect of DNR. (The resulting level of DnrN produced from pWHM411 was approximately 10 times higher than that in the control strain carrying the pWHM860 vector [Fig. 8A].) Reprobing of the washed filter used for the experiment shown in Fig. 8A with anti-DnrI antiserum (44) revealed that the level of DnrI (lower band in Fig. 8B) did not change significantly in spite of the high level of DnrN in the cells. The presence of pWHM411 enhanced DNR production by the 29050 strain approximately twofold compared with the amount produced by the pWHM860 control in the GPS medium (data not shown; this is consistent with the behavior of *dnrN* described in reference 33), but there was no significant difference in DNR production between these two strains in the APM medium (data not shown). We could not detect DnrI reliably by Western blotting of gels made from cells grown in the GPS medium, but the level of DnrN was almost the same as that in the APM medium (data not shown). Thus, the increased level of DnrN may enhance *dnrI* expression (which would result in increased DNR production) in the GPS medium but not in the APM medium due to the lack of some modulator or the presence of a negative effector.

DISCUSSION

The data presented in this report, demonstrating that DnrN binds specifically to the *dnrI* promoter region, establish that a pseudo-response regulator protein of streptomycetes has DNA-binding activity. The results of the gel retardation and DNase I footprinting experiments show that this binding occurs in a sequence-specific manner. DnrN also has weak nonspecific binding activity, which we believe is due to its helix-turn-helix motif (3, 32). The nonspecific DNA-binding activity may explain why DnrN could only be produced in *E. coli* under tightly regulated conditions of *dnrN* expression. DnrN does not appear to be toxic in *S. peucetius* because there was no effect on growth of the wild-type strain in APM medium when the DnrN level was increased 10-fold. However, the increased level inhibited aerial mycelium formation even in the WMH1445 *dnrI*::*aphII* strain. Since DnrN and AmfR (46) have a very high amino-acid-sequence similarity in their C-terminal regions (data not shown), which determines the DNA sequence specificity, and an excess of AmfR exerts a negative effect on sporulation (46), we speculate that DnrN, independently of DnrI, can adventitiously regulate genes lying outside of the *dnr* gene cluster that are involved in aerial mycelium formation and sporulation.

Although the activity of many bacterial response regulators is modulated by phosphorylation and dephosphorylation, it is unlikely that DnrN must be phosphorylated to bind to the *dnrI* promoter. First, DnrN isolated from *E. coli* showed high DNA-

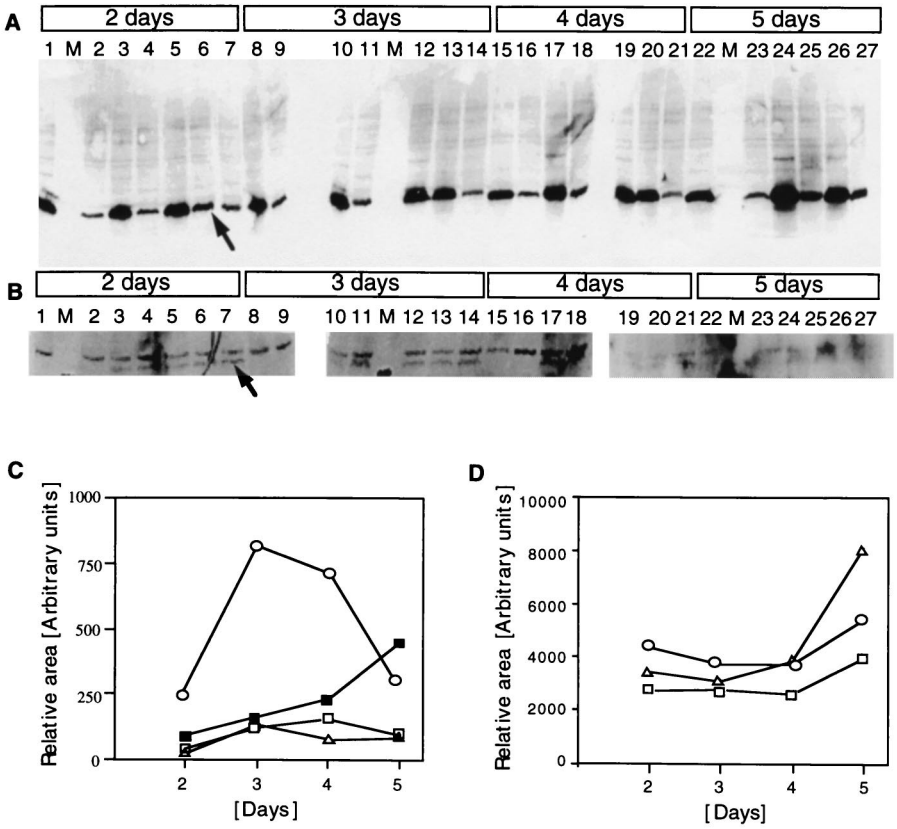

FIG. 8. Western immunoblot analysis of DnrN produced in *S. peucetius* transformants. Total proteins (15 μg) in cells grown in APM medium were subjected to SDS–15% PAGE and transferred to Immobilon-P membranes. Prestained molecular markers were used in lanes M to determine the molecular weight of each band. (A) Immunodetection by anti-DnrN. The arrow indicates the position of the DnrN protein. Lanes 1, 8, 15, and 22, pWHM411 in the WMH1445 strain; lanes 2, 9, 16, and 23, pWHM534 in the WMH1445 strain; lanes 3, 10, 17, and 24, pWHM411 in the WMH1535 strain; lanes 4, 11, 18, and 25, pWHM534 in the WMH1535 strain; lanes 5, 12, 19, and 26, pWHM411 in the 29050 strain; lanes 6, 13, 20, and 27, pWHM534 in the 29050 strain; lanes 7, 14, and 21, pWHM860 in the 29050 strain. (B) Reprobing of blot shown in panel A with anti-DnrI. The arrow indicates the position of the DnrI protein. Lanes are the same as in panel A. (C) and (D) Quantitative analyses of DnrN expression. The film shown in panel A was scanned and analyzed by the ImageQuaNT program (Molecular Dynamics). (C) Open square, pWHM534 in the WMH1445 strain; solid square, pWHM534 in the WMH1535 strain; circle, pWHM534 in the 29050 strain; triangle, pWHM860 in the 29050 strain (data for 5-days culture are not shown in panel A). (D) Square, pWHM411 in the WMH1445 strain; circle, pWHM411 in the 29050 strain; triangle, pWHM411 in the WMH1535 strain.

binding affinity and phospho donors such as acetyl phosphate, carbamyl phosphate, and ATP had no effect on the binding. Second, the DnrN D55N mutant protein, in which the putative phosphorylation site (D55) is replaced with a nonphosphorylatable residue, exhibited the same affinity for the *dnrI* promoter as the wild-type DnrN protein in the presence of the phospho donors. Third, purified and renatured His-tagged DnrN, which showed at least 5% of the DNA-binding ability of native DnrN, failed to undergo phosphorylation by ^{32}P-labeled acetyl phosphate in vitro. Response regulators that are phosphorylated in vivo normally can be phosphorylated in vitro by this phospho donor (36, 37), and many renatured response regulators have undergone phosphorylation in vitro (18, 27, 36). Phosphorylation of DnrN was also not observed when [^{32}P]ATP was added to a cell-free extract or a DnrN-supplemented cell-free extract of *S. peucetius*, even though the phosphorylation of several other proteins was seen (data not shown).

Although the activity of DnrN is thus not likely to be modulated by phosphorylation, some mechanism seems to regulate its activity independently of its cellular concentration, because overproduction of DnrN stimulated DNR production approximately twofold in the GPS medium compared with the level produced in the APM medium whereas the DnrN level was the same in both media. Since overexpression of *dnrN* resulted in a 10-fold increase in the DnrN level without an apparent increase in the DnrI level in the APM medium (Fig. 8A and B) or a significant increase in DNR production, a medium-dependent enhancement of DnrN binding to the *dnrI* promoter could explain why DNR production was stimulated twofold in the GPS medium, if we assume that the increased DNR production was due to an increased level of DnrI. This might involve oligomerization of DnrN since the binding curve (Fig. 5B) suggests that DnrN acts as a dimer or oligomer, and many activators and repressors of bacterial gene expression have been shown to be activated by oligomerization in the presence of an effector molecule (21, 51).

Although we did not find an activator of DnrN binding among the few molecules tested, DNR appears to inhibit the binding of DnrN to the *dnrI* promoter (Fig. 6B). We at first thought that this inhibition was the consequence of a change in the DNA structure imparted by the random intercalation of DNR (6). However, since the results of a DNase I footprinting assay showed that the two protected regions of the *dnrI* promoter include consecutively overlapped triplets that represent the preferred sites for DNR intercalation, DNR may compete for the same binding site as DnrN or specifically alter the topology of this site and thereby inhibit DnrN binding, which

could feedback repress *dnrI* expression and hence DNR biosynthesis. Furthermore, DNR or one of its biosynthetic precursors may also regulate *dnrN* expression since the DnrN level was much lower in the two DNR-nonproducing strains tested (Fig. 8C and D).

We speculate that DNR biosynthesis could be regulated by *dnrN* and *dnrI* as follows. In the early stage of cell growth before DNR is produced, we propose that *dnrN* is either not expressed or is expressed at a low level. This would prevent full activation of *dnrI* expression, whose product is required for expression of the DNR structural and resistance genes (23), and result in the production of only a small amount of DNR. This scenario is consistent with the low level of DnrN in the DNR-nonproducing strains (Fig. 8C). When the condition(s) for DNR production ensues, the rising level of DNR would trigger enhanced *dnrN* expression, and the increased DnrN level would fully activate *dnrI* expression, resulting in the increased expression of the DNR structural and resistance genes. Since DNR should be constantly excreted, most likely by the DrrA and DrrB proteins (14), when its rate of formation declines, the resulting lower level of DNR would not maintain *dnrN* expression and thence *dnrI* expression. In addition, once the DNR level is high enough to inhibit the binding of DnrN to the *dnrI* promoter, then DNR biosynthesis could be feedback repressed via a negative effect on the level of *dnrI* expression. This hypothesis implies that DNR binds to the *dnrI* promoter better than it binds to other regions of the *S. peucetius* chromosome. The recently described DrrC protein (22) may even promote this selectivity by interfering with DNR binding to these other regions.

Two important remaining questions are how does DNR affect the level of DnrN and what protein regulates the expression of the *dnrN* gene. The results of a gel retardation experiment (Fig. 4A) indicated that DnrN does not bind to the intergenic region between the *dnrN* and *dnrO* genes. Therefore, the putative DnrO repressor protein, encoded by the *dnrO* gene adjacent to *dnrN* in the divergently transcribed position (32), is the best candidate for a protein that binds to the *dnrN* promoter. We detected binding activity to the *dnrN* promoter region in a cell-free extract of *S. peucetius* (10) but do not know if this is due to DnrO. The mutational, expression, and DNA-binding studies in progress with *dnrO* and its product should answer this question.

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