Identification and Characterization of NocR as a Positive Transcriptional Regulator of the β-Lactam Nocardicin A in Nocardia uniformis

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Nocardicin A is a monocyclic β-lactam isolated from the actinomycete Nocardia uniformis, which shows moderate activity against a broad spectrum of gram-negative bacteria. Within the biosynthetic gene cluster of nocardicin A, nocR encodes a 583-amino-acid protein with high similarity to a class of transcriptional regulators known as streptomycetes antibiotic regulatory proteins. Insertional inactivation of this gene resulted in a mutant showing morphology and growth characteristics similar to the wild type, but one that did not produce detectable levels of nocardicin A or the early precursor p-hydroxybenzoyl formate. Similar disruptions of nocD, nocE, and nocO yielded mutants that maintained production of nocardicin A at levels similar to the wild-type strain. In trans complementation of the nocR::apr mutant partially restored the wild-type phenotype. Transcriptional analysis of the nocR::apr mutant using reverse transcription-PCR found an absence of mRNA transcripts for the early-stage nocardicin A biosynthetic genes. In addition, transcription of the genes responsible for the biosynthesis of the nonproteinogenic p-hydroxyphenylglycine (pHPG) precursor was attenuated on the nocR disruption mutant. NocR was heterologously expressed and purified from Escherichia coli as an N-terminal maltose binding protein-tagged fusion protein. DNA binding assays demonstrated that NocR is a DNA binding protein, targeting the 126-bp intergenic region between nocE and nocA. Within this intergenic region is the likely binding motif, a direct hexameric repeat, TGATAA, with a 5-bp spacer. These experiments establish NocR as a positive transcriptional regulator of the nocardicin A biosynthetic pathway, coordinating the initial steps of nocardicin A biosynthesis to the production of its pHPG precursor.

Gram-positive soil bacteria of the Actinomycetales family produce structurally diverse secondary metabolites that include many antibiotics. Nocardicin A is a monocyclic β-lactam, isolated from Nocardia uniformis subsp. tsuyamanensis, which shows moderate activity against a broad spectrum of gram-negative bacteria (4). A large number of antibiotics and other secondary metabolites of interest have been isolated from members of the Streptomyces genus, a well-studied actinomycete. Nocardia, like Streptomyces, is part of the Actinomycetales family and when grown on solid medium forms branching filaments that form a mycelial mass, but it is distinguished from Streptomyces by the absence of spore formation (52).

Like nocardicin A, many antibiotics are produced by complex biosynthetic pathways in which genes encoding these products are typically clustered together with at least one regulatory gene. In many cases a biosynthetic cluster contains a cluster-situated regulator (CSR) which activates or represses the transcription of the biosynthetic and resistance genes within a cluster. The regulation of secondary metabolite gene clusters is of interest due to the economic benefit of being able to control and optimize the production of their biologically active products. As the debate continues on how new antibiotics will be discovered and developed into useful drugs (11), the ability to turn on the many newly discovered cryptic biosynthetic pathways by the induction of their associated pathway-specific transcriptional activators (7) ensures continued interest in the regulatory pathways of actinomycetes.

In Streptomyces, a hierarchy of regulation factors controls the life cycle of the bacterium and links the onset of secondary metabolite production to the ability of the bacterium to develop aerial mycelia (hyphae) and form spores (10). In Streptomyces coelicolor A3(2), the pleotropic regulator AfsR has been characterized and found to control the production of actinorhodin, undecylprodigiosin, and calcium-dependent antibiotic (CDA). There are many examples of CSRs that have been characterized and shown to regulate transcription of genes of a single secondary metabolite biosynthetic cluster (12). These late-stage regulators, usually transcriptional activators, are represented by several families, including the Streptomyces antibiotic regulatory protein (SARP) family characterized by an N-terminal OmpR-like domain, the StrR family characterized by a helix-turn-helix motif in its central region, and the LAL family characterized by a LuxR-like C-terminal DNA binding motif (8). Recent DNA microarray experiments on the CSR proteins of the antibiotic pathways of S. coelicolor A3(2) suggest that CSRs are not pathway specific but can influence the transcription of other antibiotic gene clusters (18).

The gene cluster of nocardicin A contains 14 open reading frames, including one gene, nocR, whose deduced product is homologous to transcriptional regulators (15). Three genes, nocF, nocG, and nocN, encode proteins responsible for the biosynthesis of the nonproteinogenic l-p-hydroxyphenylglycine (pHPG) precursor of nocardicin A. Biosynthesis of nocardicin A is initiated by the presumed formation of a tripeptide from pHPG and serine by nonribosomal peptide synthetases (NRPSs) encoded by nocA and nocB. Three genes, nat, nocI, and nocL, encode tailoring enzymes (23, 24, 38). The predicted product of nocH is homologous to membrane transport pro-
teins and thought to be involved in exporting nocardicin A from the cell. The functions of the four remaining genes in the cluster, nocK, nocI, nocD, and nocE, are unclear. The protein encoded by nocI is homologous to the Mbt-H family of small proteins that are found in gene clusters of many peptide antibiotics and siderophores, including the CDA and cobelichin biosynthetic clusters of *S. coelicolor*, and is essential for their biosynthesis (26). Previous experiments indicate that nocK is not essential for nocardicin A biosynthesis (24).

This study focused on the gene product of nocR, a 583-amino-acid protein. A previous BLAST analysis reported high similarity to CdaR (35% identity, 46% similarity) and the pleotropic regulator AfsR, both from *S. coelicolor* A3(2) (2). CdaR is a putative transcriptional regulator located within the gene cluster encoding the biosynthesis of CDA (16). Current BLAST analysis gives essentially the same result, except for the addition of several putative regulatory proteins annotated from full-genome sequencing projects, including a putative protein described from the sequenced genome of *Nocardia farcinica* (20).

In addition, to more clearly define the boundaries of the nocardicin A biosynthetic cluster, insertional mutants of nocD, nocE, and nocO were also prepared for this study. The boundaries of the nocardicin A biosynthetic cluster were predicted to be nocN and nocE, based on comparison to an almost identical cluster in *Actinosynnema* minum and the observation that no genes with apparent roles in nocardicin A production were found upstream of nocN or downstream of nocE (15). BLAST analysis of the gene product of nocD shows homology to the GNAT family of acetyl transferases, a subset of which is involved in antibiotic resistance. Analysis of NocE does not show any conserved domains, and the only homology is to hypothetical proteins identified in *Streptomyces clavuligerus, Salinispora arenicola*, and *Streptomyces castellanea*. The deduced product of the 1,339-bp nocO, located immediately upstream of nocN, is a member of the cytochrome P450 superfamily.

The effects of nutrients on growth and secondary metabolite formation in some species of *Nocardia* have been studied to optimize titers, but no characterized transcriptional activators of the *Nocardia* genus have been reported in the literature. This work presents the first characterization of a SARP in *Nocardia*. Insertional mutagenesis experiments show that NocR is essential for the biosynthesis of nocardicin A. High-performance liquid chromatography (HPLC) analysis of culture supernatants combined with transcriptional analysis, using reverse transcription-PCR (RT-PCR), and DNA binding studies show that NocR is a positive transcriptional regulator of the nocardicin A biosynthetic pathway, coordinating the biosynthesis of the hPGP precursor to the initial stage of nocardicin A biosynthesis.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and culture conditions.** *Nocardia uniformis* subsp. *tayuanensis* ATCC 21806 (wild-type strain), producer of nocardicin A, was grown on ISP2 solid medium (Difco Laboratories) at 28°C. Seed cultures were prepared in tryptic soy broth (TSB) medium (Difco Laboratories) grown at 28°C for inoculation of nocardicin fermentation medium (containing, per liter, 10 g peptone, 4 g yeast extract, 10 g KH2PO4, 4 g Na2HPO4, 2.4 g MgSO4, 2 g glycine, 2 ml trace minerals, 20 g soluble starch, 1 g t-tyrosine, 75 mg t-methionine) as previously described (38). Plasmids pBSIISK- (Stratagene) and pT7Blue3 (Novagen) were used for routine cloning and subcloning into *Escherichia coli* DH5α. Vector pULVK2 was provided by J. F. Martin (University of León), and the construction of vector pULVK2T has been described previously (15). *E. coli* JM110 (dcm dam) was used to propagate nonmethylated DNA. *E. coli* BL21(DE3) and *E. coli* (TB1) (New England Biosabs) were used for protein overexpression experiments. *E. coli* ESS was generously provided by A. Demain (Drew University, Madison, NJ).

**DNA isolation, manipulation, and sequencing.** Plasmid DNA isolation from *E. coli* was performed using the Qiaprep Spin Miniprep kit (Qiagen Inc.). Total DNA from *N. uniformis* was isolated using the Qiaprep Spin Miniprep kit (Qiagen Inc.). Total DNA was isolated from the pULVK2 vector transformants using the Qiaprep Spin Miniprep kit (Qiagen Inc.). Total DNA was isolated from the pULVK2 vector transformants using the Qiaprep Spin Miniprep kit (Qiagen Inc.). Total DNA was isolated from the pULVK2 vector transformants using the Qiaprep Spin Miniprep kit (Qiagen Inc.). Total DNA was isolated from the pULVK2 vector transformants using the Qiaprep Spin Miniprep kit (Qiagen Inc.). Total DNA was isolated from the pULVK2 vector transformants using the Qiaprep Spin Miniprep kit (Qiagen Inc.). Total DNA was isolated from the pULVK2 vector transformants using the Qiaprep Spin Miniprep kit (Qiagen Inc.). 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single band upon hybridization with a labeled apr probe and wild-type gene probe.

**Growth and characterization of N. uniformis strains.** N. uniformis was grown in nocardin fermentation medium for 5 to 7 days and assayed for the production of nocardin A and related precursors. Culture aliquots were centrifuged to separate the cell mass from the supernatant, and both were stored at −20°C. A paper disc bioassay analysis to detect antibiotic production was prepared by the addition of 200 µl of culture supernatant to paper discs placed upon solid LB medium inoculated with E. coli ESS. Plates were incubated at 37°C overnight and analyzed for developed zones of antibiotic. Culture supernatants were also analyzed by reverse-phase HPLC, employing an Agilent 1100 HPLC system equipped with a diode array detector. Filtered supernatants (nonyl, 0.45 µm) were injected directly onto a Luna C18(2) 250- by 46-mm column (Phenomenex), analyzed by reverse-phase HPLC, employing an Agilent 1100 HPLC system equipped with a diode array detector.

**In trans complementation of wild-type and nocR::apr N. uniformis.** For complementation studies, primers (forward, GAATTCCCTCGCGCTCGGCGCCTGGTCGGTCGCAAG CGTTGAGGTGCCTGCACACC 589 GCGACC; reverse, TATGAATTCTCACGGGCCGGTCGTGGCAAGTT) were designed to amplify a 500- to 800-bp region of each gene according to the instructions of the Qiagen OneStep RT-PCR kit (catalog no. 12603). Primers were designed to amplify a 500- to 800-bp region of each gene using the PE plate, 0.6

**DNA isolation.** A series of identical 100-ml-scale fermentations of wild-type and nocR::apr N. uniformis were prepared. Following 1 h of growth, cells were collected by centrifugation at 2,000 × g, flash frozen in liquid nitrogen, and stored at −80°C. A subsample of frozen cells was crushed to a fine powder with a diethyl pyrocarbonate- (HEPES) medium inoculated with E. coli ESS. Plates were incubated at 37°C overnight and analyzed for developed zones of antibiotic. Culture supernatants were also analyzed by reverse-phase HPLC, employing an Agilent 1100 HPLC system equipped with a diode array detector. Filtered supernatants (nonyl, 0.45 µm) were injected directly onto a Luna C18(2) 250- by 46-mm column (Phenomenex), analyzed by reverse-phase HPLC, employing an Agilent 1100 HPLC system equipped with a diode array detector.

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RESULTS

In silico analysis. The predicted product of nocR is a 61.8-kDa protein. A conserved domain search of NocR revealed an effector domain at its N terminus, followed by a bacterial transcriptional activator domain (15). The structure prediction for the N terminus of NocR (30) aligned with the three characteristic α-helices followed by two β-sheets of the N-terminal OmpR-like domains of SARP’s (Fig. 1A) (54). OmpR is part of the two-component global regulatory system controlling the expression of many genes in E. coli. Structural and mutational analyses of the winged helix-turn-helix motif of the C-terminal DNA binding domain of OmpR have elucidated the manner of interaction of this domain with DNA and RNA polymerase (31). Of note, the recognition helix, α3, interacts with the major groove of the targeted DNA sequence. The α3 helix of NocR, like members of the SARP family, has a hydrophobic residue at position 8, a conserved arginine at residue 15, and a positively charged amino acid (K/R) at residue 16. In OmpR, the three Arg residues in this helix each provide a positive charge for interaction with the DNA backbone. The C-terminal β-strands pair, and the “wing” connecting them also directly interacts with the DNA backbone. The second β-sheet begins at a conserved Gly-Tyr, which is essential for activity in DnrI, a member of the SARP family (45).

Further primary sequence comparisons of the recognition (α3) helices reveal high similarities among complementary proteins. Mutants deficient in ActII-Orf4 can be complemented by MtmR (29) DnrI (46), and AlpV (1), whose recognition helices show high identity, but not RedD (48) or CcaR (33), where only two to three residues are identical in this region. Thus, the lack of identity between NocR and ActII-Orf4 suggests that these proteins have different DNA recognition sequences, and the significant homology with CdaR (10 identical residues) implies a potential for complementarity between these proteins.

Analysis of the C-terminal half of NocR beyond the DNA binding domain (Fig. 1B) showed the presence of Walker A and Walker B nucleotide binding motifs, which are also present in AsfR and CdaR but not conserved in members of the SARP family. Although Walker A motifs are not exclusive to proteins that bind nucleotides, comparison of structural data between nucleotide binding proteins and nonnucleotide binding proteins found that the Walker A motif in nucleotide binding proteins is sandwiched between a β-strand and an α-helix, forming the P-loop (35). Domain prediction analysis of NocR suggested the presence of a P-loop between residues 280 and 293 and a Walker B motif located between residues 363 and 369. Mutations in the Walker A and Walker B motifs of AsfR destroyed ATPase and GTPase activity in vitro (27). Although NocR and CdaR do not appear to have a catalytic Asp/Glu in a position analogous to that identified for AsfR (27), NocR does exhibit the DE Walker B motif that is involved in ATP hydrolysis in bacterial enhancer binding proteins (22, 43). Disruption of the ATP/GTPase domain in AsfR resulted in a significant loss of production of actinorhodin, undecylenic acid, and CDA in S. coelicolor A3(2) (17) but was not required for DNA binding activity in vitro.

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(wt/vol) native polyacrylamide gel (11 cm by 8 cm) with 0.5× Tris-borate-EDTA running buffer. The gel was run for 65 min at 100 V, transferred to filter paper, dried, exposed to Kodak film for 12 to 14 h at −70°C, and developed.

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Construction and characterization of the nocR::apr inser- 
tional mutant. To determine whether NocR is essential for 
the biosynthesis of nocardicin A, insertional mutants were 
constructed by homologous recombination (Fig. 2). No dif-
ferences in growth or differentiation were observed between 
the wild-type and mutant N. uniformis grown on R2YE or 
ISP2 solid medium or in TSB liquid cultures. Three of the 
four randomly selected putative mutants were confirmed by 
Southern hybridization and grown for 10 days at 28°C in 
fermentation medium. Sample aliquots were analyzed after 
2, 3, 4, 5, 7, and 10 days of growth. Aliquots from each 
culture and each time point were used in a paper disc bio-
assay against /H9252-lactam-supersensitive E. coli ESS (Fig. 3).

The replicates of wild-type N. uniformis showed a zone of 
antibiosis unlike the N. uniformis nocR::apr mutants, which 
failed to inhibit the growth of E. coli ESS. The bioassay 
experiments for days 2, 3, 4, 5, 7, and 10 produced similar 
results. HPLC analyses of these fermentation cultures were 
also performed to detect differences between the wild-type 
and nocR::apr mutant cultures (Fig. 4). Nocardicin A, with a 
retention time of 14.2 min, was not detected in any of the 
nocR::apr mutant fermentation cultures, nor was there a 
peak that coeluted with nocardicin G (retention time, 7.8 
min), a known intermediate.

Further comparison of wild-type versus nocR::apr chro-
matograms of culture supernatants showed differences in-
dicative of alterations in the pHPG biosynthetic pathway.
Wild-type chromatograms indicated significant accumula-
tion of p-hydroxybenzoyl formate (pHBF; retention time, 
8.1 min), which was absent in the chromatograms for the 
mutants. In addition, tyrosine (retention time, 4.9 min), 
which was supplemented into the production medium at 5.5 
mM, appeared to be depleted over time in chromatograms 
with the wild type, but levels remained similar to the unin-

![FIG. 1. (A) Secondary structure prediction of the N-terminal region of NocR, aligned with the corresponding N-terminal OmpR-like domains 
of several SARPs. Origins of amino acid sequences and their database accession numbers are as follows: CdaR, Q9Z389; ActII-orf4, P46106; 
MtrR, Q19495; Dn1, P52047; AlpV, Q6VMH3; CcaR, P97066; RedD, P25941. Numbers indicate amino acid residues from the 
N terminus of the protein. Identical residues are shaded. The hydrophobic residue conserved in the α3 helix is marked by an open box. The 
positively charged amino acid residues generally conserved at the end of the α3 helix are marked with an asterisk. (B) Alignment of the C-terminal 
region of NocR with CdaR (entire protein) and AfsR (truncated). Conserved amino acids are highlighted. The positions of Walker A and Walker 
B motifs are noted by solid lines above the sequence.](http://jb.asm.org/ on September 22, 2017 by guest)
occluded fermentation medium in mutant cultures. These observations suggested that NocR influences pHPG biosynthesis.

**Complementation of the nocR:apr insertional mutant.** To determine if the lack of nocardicin A production in the nocR insertional mutants was due to the unavailability of the pHPG precursor, the mutants were grown in fermentation medium supplemented with 0.5 mM D,L-pHPG. HPLC analyses of the culture supernatants were similar to samples from unsupplemented medium. Neither nocardicin A nor pHBF production was restored with the addition of pHPG. Supplementation of the fermentation culture with 5 mM pHMA oxidase activity remained active in the nocardicin A biosynthetic pathway, restored the production of pHBF, indicating (pHMA), a direct precursor of pHBF in the pHPG biosynthesis.

To verify that the loss of nocardicin A production was the result of nocR disruption and not the result of a polar mutation, an in trans complementation of the nocR:apr N. uniformis mutant was performed. The pULVK2T/-300nocR replicating plasmid contained the nocR gene fragment, as well as a 300-bp region immediately upstream of the GTG start codon, thus containing the native promoter sequence for nocR. Transformants of pULVK2T/-300nocR into nocR::apr N. uniformis were grown in nocardicin production medium unsupplemented with pHPG and were analyzed by HPLC. Nocardicin A was detected in samples of the pULVK2T/-300nocR nocR::apr N. uniformis transformant taken at 6 days of growth. In comparison to wild-type N. uniformis samples grown under identical conditions, nocardicin A production was restored to ~20% of wild type (Fig. 4D). Supplementation of the production medium with 0.5 mM pHPG produced unchanged levels of nocardicin A in the complemented mutant. Nocardicin A was not detected in the pULVK2T/(no insert) nocR::apr N. uniformis transformant, a negative control. In contrast, restoration of pHBF biosynthesis by in trans complementation of the nocR gene was much more robust at 20 to 50% of wild type, and the concomitant depletion of tyrosine was also observed.

**Transformation of wild-type N. uniformis with a multicopy vector containing nocR.** In some streptomycetes, the introduction of multiple copies of transcriptional activators, either by incorporation into the genome or on a multiple-copy-number plasmid, results in the overproduction of the specific antibiotic (29, 40, 45), indicating the limiting role of the transcriptional activator secondary metabolite biosynthesis. Transformation of wild-type N. uniformis was successfully achieved with pULVK2T/-300nocR, the same plasmid used to complement the nocR::apr mutant. However, the production of nocardicin A did not change significantly in this transformant.

**Construction and characterization of nocD, nocE, and nocO disruption mutants.** To more clearly define the boundaries of the nocardicin A biosynthetic cluster, insertional mutants of nocD, nocE, and nocO were prepared by homologous recombination using the same strategy described above for nocR mutants. Mutants with the Kan’ Apr’ phenotype, confirmed by Southern hybridization, were grown in nocardicin fermentation medium. Sample aliquots taken at 3, 4, and 5 days were analyzed by paper disc bioassay against β-lactam-supersensitive E. coli ESS and HPLC. The bioassays indicated that the production of antibiotic in the nocD, nocE, and nocO disruption mutants was similar to wild-type N. uniformis. This result was confirmed by HPLC analyses of the culture supernatants. Chromatograms for all three mutants were similar to those of wild-type N. uniformis over the three time points analyzed.

**RT-PCR analysis of wild-type versus nocR:apr mutant N. uniformis.** The isolation of total RNA following the fermentation of wild-type and nocR:apr mutant N. uniformis culture supernatant is approximately half of that found in mature cultures. To detect mRNA transcripts of interest, primers were designed for three genes in the nocardicin A biosynthetic cluster, insertional mutants of nocR, nocD, and nocE, and nocO were primed by RT-PCR. After 2 days of fermentation, nocardicin A production is ongoing, and its accumulation in the culture supernatant is approximately half of that found in mature cultures. To detect mRNA transcripts of interest, primers were
designed to amplify a 500- to 800-bp region of each gene in the nocardicin cluster between noeN and nat (except for noeI, in which only a short 133-bp region was selected, due to its small size). The resulting cDNAs were subcloned and confirmed by sequencing. A 30-cycle RT-PCR analysis of wild-type and noeR::apr mutant N. uniformis was performed (Fig. 5). As would be expected during the linear phase of antibiotic production, cDNA transcripts were detected for all the genes in the cluster in the wild-type sample. Comparison of the wild-type data to the noeR::apr mutant showed an absence of transcripts for noeA, noeB, and nat as well as noeK, noeJ, and noeR. The RT-PCR was repeated using only 24 cycles of amplifica-

FIG. 4. HPLC results. (A) Nocardicin A authentic standard, 1.0-μg injection. Inset: structure of nocardicin A. (B) Wild-type N. uniformis culture supernatant at 4 days. (C) noeR::apr mutant N. uniformis culture supernatant, at 4 days. (D) Results from the complementation experiment with noeR::apr mutant N. uniformis transformed with pULVK2T-300necR culture supernatant at 6 days.

FIG. 5. Gene expression analysis of the nocardicin gene cluster by RT-PCR (30 cycles). A scheme representing the organization and sizes of intergenic regions of the nocardicin cluster is included. Analyses were carried out on wild-type (+) and noeR::apr mutant (-) N. uniformis.
tion (data not shown). In this experiment, cDNA transcripts for the pHPG biosynthetic genes, nocN, nocG, and nocF, as well as nocL, nocI, and nocH, were still visible in the wild-type sample but were not seen in the nocR:apr mutant. These data indicate that NocR plays a role in the transcription of all the genes in the nocardicin A biosynthetic cluster, but to varying degrees. The lack of any observable transcripts of nocA, nocB, nat, and nocJ is consistent with the analysis of the mutant cultures in which neither nocardicin A nor nocardicin G was detected. Interestingly, transcription of genes responsible for pHPG biosynthesis (nocF, nocG, and nocN), the gene likely responsible for self-resistance (nocH), as well as nocL and nocI were detectable in the nocR:apr mutant in the more-sensitive 30-cycle experiment, indicating that transcription of these genes can occur in the absence of NocR, but their transcription is upregulated, either directly or indirectly, in the presence of NocR.

Overexpression and purification of NocR. Heterologous expression of NocR in E. coli as an N-terminal His6 fusion protein was attempted to facilitate further analysis of its function. The coding sequence of nocR was cloned into the pET28b(+) expression vector and transformed into E. coli BL21(DE3) for E. coli expression vector and transformation into BL21(DE3). A significant proportion of MBP-NocR was found in the soluble fraction and was further isolated and purified by amylose affinity chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) analysis showed a band consonant with the expected 105-kDa fusion protein. The yield was 1.7 mg MBP-NocR per liter of culture as determined by Bradford assay and using bovine serum albumin as a standard.

EMSA studies. The RT-PCR and the insertional mutagenesis studies were consistent with the hypothesis that NocR acts as a transcriptional activator of the nocardicin A cluster (Fig. 6A) revealed a 126-bp intergenic region between nocF and nocA. A significant proportion of MBP-NocR was found in the soluble fraction and was further isolated and purified by amylose affinity chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) analysis showed a band consonant with the expected 105-kDa fusion protein. The yield was 1.7 mg MBP-NocR per liter of culture as determined by Bradford assay and using bovine serum albumin as a standard.

FIG. 6. DNA binding assay results with DNA fragments from the nocardicin gene cluster. (A) Nocardicin A biosynthetic cluster with the sizes of the intergenic regions noted. (B) Autoradiogram of PAGE gels following incubation of MBP-NocR with DNA amplified from four intergenic regions. Lanes: 1, control, no MBP-NocR; 2, control, no DNA; 3, control, 1.7 μg MBP; 4, 0.3 μg MBP-NocR; 5, 0.6 μg MBP-NocR; 6, 1.1 μg MBP-NocR; 7, 2.3 μg MBP-NocR; 8, 1.1 μg MBP-NocR and excess unlabeled DNA. (C) Competition experiments. The autoradiogram shows PAGE gels after incubation of 1 μg MBP-NocR with 32P-labeled DNA from the nocF-nocA intergenic region with and without two levels of competing (unlabeled) DNA fragments. Lanes: 1, control, no competing DNA; 2, control, no DNA; 3, 3-fold excess of DNA from the nocH-nocI intergenic region; 4, 10-fold excess of DNA from the nocH-nocI intergenic region; 5, 3-fold excess of DNA from the nocN-nocR intergenic region; 6, 10-fold excess of DNA from the nocN-nocR intergenic region; 7, 3-fold excess of DNA from the nocN-nocR intergenic region; 8, 10-fold excess of DNA from the nocN-nocR intergenic region.
nocR intergenic region and the region 100 bp upstream from the start codon of nocN show G+C content representative of the entire cluster. These four regions were chosen for DNA binding studies to determine if NocR directly influences the transcription of nocH, encoding a probable efflux pump for self-resistance, its own transcription, and/or the transcription of the biosynthetic genes nocN and nocF and the nocABnat operon.

Incubation of MBP-NocR with each 32P-labeled DNA fragment from the four intergenic areas was assessed (Fig. 6B) using a standard gel binding assay. Attempts to cleave MBP from NocR by digestion with factor Xa resulted in degradation of NocR, likely due to the precedent promiscuity of factor Xa (21). However, MBP-tagged proteins have been successfully used in EMSAs (9), notably in related experiments with MBP-DnrI (45). For each experiment three negative control reactions were performed: absence of protein, MBP (isolated separately), and absence of labeled DNA. The appearance of a retarded band was only observed upon incubation of MBP-NocR with the 126-bp nocF-nocA intergenic region. The intensity of the retarded band increased with increasing protein concentration and, as expected, was diminished by the addition of the same but unlabeled DNA. The specificity of binding of MBP-NocR to the 126-bp nocF-nocA intergenic region was tested by competition with the other three intergenic regions.

Addition of 3- to 10-fold-higher concentrations of competitor DNA failed to diminish the intensity of the 126-bp nocA-nocF retardation band (Fig. 6C).

To narrow the MBP-NocR DNA binding region further, a series 32P-labeled subsections of the 126-bp nocF-nocA intergenic region were prepared by PCR amplification, in which the length of the DNA fragment was reduced by 10 bp from the nocF side for each iteration (Fig. 7A). The region required to maintain MBP-NocR binding activity includes 75 bp upstream from the beginning of the ATG start codon of nocA (Fig. 7C). A 60-bp oligonucleotide overlapping this region without the putative RBS (GAGCGG) resulted in smeared bands under the same assay conditions (data not shown).

DISCUSSION

The experimental evidence presented supports the conclusion that nocR encodes a positive transcriptional activator that targets the nocardicin A biosynthetic cluster. Insertional mutants of nocR demonstrated the absence of nocardicin A production and a large reduction in pHBF, a precursor of pHPG, one of the amino acid precursors of nocardicin A. Chemical complementation by the addition of pHPG into the fermentation medium of the nocR::apr mutant failed to induce nocardicin A or pHBF production, indicating that the absence of
nocardicin A biosynthesis is not due to the lack of availability of pHPG. Previous incorporation studies have shown that pHPG is efficiently incorporated into nocardicin A (49). In trans complementation of nocR with its native promoter partially restored nocardicin A and pHBF production, indicating that nocR is essential for the biosynthesis of nocardicin A and affects the pHPG biosynthetic pathway.

The results of this study are consistent with experiments on other CSR proteins encoded in the biosynthetic gene clusters of various types of secondary metabolites produced by the Streptomyces genus. A number of CSR SARPs have been characterized as late-stage regulators, including CcaR from the β-lactam-producing Streptomyces clavuligerus. The disruption of ccaR results in mutants lacking both clavulanic acid and cephamycin production (33). In addition, disruption of actII-ORF4 (5), redD (32), dhrI (46), mmtR (29), tylS (6), and pimR (3) results in mutants deficient in the production of actinorhodin, undecylprodigiosin, daunorubicin, mithramycin, tylosin, and pimaricin, respectively. Similarly, disruption of the “angucyclic” SARPs, distinguished by an OmpR DNA binding domain at the C terminus, also resulted in mutants deficient in secondary metabolite production as exemplified by LндI (landomycin E) and AurIP (auricin) (36). In all cases, as with nocR, disruption had no apparent effect on the growth or differentiation of the bacterium, and genetic complementation restored secondary metabolite production. These observations are not unique to the SARP family. NovG, a member of the StrR family, has been characterized as a transcriptional activator for the novobiocin gene cluster (12). PikD, a member of the LAL family, has been characterized as a transcriptional activator for the pikromycin gene cluster (55), and most recently ThnU, a member of the Lys-R family, was found to be essential for the transcription of genes essential for thienamycin biosynthesis in S. ataycaya (39).

Wild-type N. uniformis grown in nocardicin fermentation medium typically yields 300 to 350 mg of nocardicin A per liter. Wild-type complementation studies with nocR cloned into pULVK2T did not significantly increase this already-high yield. It was noted, however, that in trans complementation of the nocR::apr mutant with nocR encoded in the pULVK2T plasmid did not fully restore nocardicin A production. Other studies that have shown that different plasmid vectors can produce varying product yields, as noted with studies on daunorubicin-producing Streptomyces peucetius (46) and tylosin-producing Streptomyces fradiae (44). However, when wild-type N. uniformis was transformed with pULVK2T containing no insert, no change in nocardicin A production was observed, consistent with previous in trans complementation experiments (23, 24). The current evidence indicates that low expression from the pULVK2T vector may have a limiting effect in N. uniformis.

Transcriptional analysis of the nocardicin A gene cluster showed a lack of detectable mRNA transcripts for nocA, nocB, and nat in the nocR::apr mutant, consistent with the HPLC results. Evidence suggests that nocardicin A biosynthesis is initiated by the putative formation of the tripeptide backbone by the NRPSs encoded by nocA and nocB followed by the addition of the homoseryl side chain, by the 3-adenomethionine-dependent transferase encoded by nat. Thus, transcription of the genes required to initiate nocardicin A biosynthesis is absent in the nocR disruption mutant. HPLC analyses also indicated a lack of accumulation of pHBF and depletion of tyrosine in the culture supernatant of the nocR disruption mutant compared to the wild type. RT-PCR analyses detected transcripts for all three genes known to be involved in pHPG biosynthesis, nocF (pHMA synthase), nocG (pHPG transaminase), and nocN (pHPG oxidase), in both the wild type and the nocR::apr mutant, although at lower levels in the mutant. In the pHPG biosynthetic pathway (Fig. 8), the conversion of p-hydroxyphenylpyruvate (pHPF), the transaminated product of tyrosine, to pHMA is catalyzed by NocF. pHBF is formed by the oxidation of pHMA by NocN and then converted to pHPG by NocG. In vitro studies implicate tyrosine as the nitrogen source for the transamination reaction catalyzed by NocG; thus, an equivalent of pHPP, the substrate for NocF, is produced for each equivalent of pHBF converted to pHPG. It has been proposed that the cycle is initiated by the conversion of prephenate to HPP by prephenate dehydrogenase (19). A gene encoding a prephenate dehydrogenase is not located in the nocardicin cluster but is present in the gene clusters of several pHPG-containing antibiotics, including CDA (16), chloroeremomycin (51), and A47934 (Streptomyces toyoacensis) (34), and is presumed present in the Nocardia genome. In this catalytic cycle, inactivation of one enzyme would disrupt the cycle, preventing the depletion of tyrosine and the accumulation of pHBF. Supplementation with pHPMA showed restoration of pHBF formation in the nocR mutant, suggesting that nocN is transcribed and active, despite the lowered levels of transcription observed in the mutant. Thus, the impairment in the pHPG biosynthetic cycle is either due to the lowered activity or transcription of a prephenate dehydrogenase required to initiate the cycle or a similar effect on nocF and/or nocG, which are required to perpetuate the cycle.

DNA binding analyses on MBP-NocR heterologously expressed in E. coli demonstrate DNA binding activity to the 126-bp nocF-nocA intergenic region. The region bounded by −75 to +1 positions upstream of the nocA start codon was found to be the minimal fragment required for observable activity by EMSA. The size of this region is compatible with DNA footprinting analysis of DnrI, which was reported to

![FIG. 8. Proposed biosynthetic pathway for pHPG. pHPP is transformed to pHMA by NocF, which is oxidized to pHBF by NocN. The transamination of pHBF to pHPG and L-tyrosine to pHPP is catalyzed by NocG.](http://jb.asm.org/content/191/5/1075.full.html#sec-8-8)
regulatory genes in the act sequence of a number of Interestingly, the TTA codon is also present in the coding

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many of which are late-stage regulatory proteins. However, in the regulation of growth and differentiation, particularly spore

nocO (50). The absence of activity in the nocN-nocR intergenic region indicates that NocR does not influence its own transcription, unlike Lndl (landomycin E cluster of Strepomyces globisporus) and CcaR (S. clavuligerus), which show DNA binding activity to their own promoter regions (37, 41). In some Strepomyces strains, transcriptional activation of secondary metabolite bio-
synthetic genes is triggered by the relief of repression of the pathway-specific transcriptional activator by quorum sensing.

The presence of a TTA leucine codon in the coding sequence of transcriptional activators links their upregulation to another gene, bldA, which encodes the rare tRNA_{UUA}. This mechanism has been shown to play a role in the regulation of growth and differentiation, particularly spore formation, in S. coelicolor and is linked to the transcription of regulatory genes in the act cluster, including actII-orf4 (13). Interestingly, the TTA codon is also present in the coding sequence of a number of Strepomyces transcriptional activators, including CcaR (33), ActII-Orf4 (13), NovG (12), MtmR (29), SanG (28), and Lndl (36), but it is not present in the coding sequence of NocR of the nonsporulating N. uniformis.

It is possible that the transcription of nocR is regulated by another transcription factor, as in the actinorhodin system in which the transcriptional activator AtrA, which is not associated with any antibiotic gene cluster, regulates the transcription of actII-orf4 (50).

NocR and CdaR are more homologous to the well-studied AsfR, a SARP with pleiotropic regulatory properties in S. coelicolor A(3)2. Conserved domain predictions for both proteins indicate a nucleotide binding motif following the bacterial transcriptional activator domain as seen in AsfR. The activity of AsfR has been shown to be regulated by phosphorylation by AsfK, a protein serine/threonine kinase (42). Phosphorylation of AsfR increases both its DNA binding and ATP/GTPase activities in vitro, while the DNA binding activity remained independent of ATPase activity (27). The ATPase domain, however, is essential for the production of actinorhodin in vivo. Following recruitment of RNA polymerase to the promoter region of the AsfR target, the dsaS promoter, it has been postulated that the energy from ATP hydrolysis contributes to the conversion from the closed complex to a transcriptionally active open complex (47). Functional comparisons of NocR to CdaR are tenuous given the demonstrated role of other proteins in the regulation of CDA biosynthesis. The transcription of a number of operons in this cluster have been shown to be directly regulated by AbsA1 and AbsA2, a two-component regulator (40). More recently, DNA microarray analyses of the regulation of CDA and other antibiotic pathways in S. coelicolor indicated that constitutive expression of cdaR increased the abundance of transcripts of genes in the CDA biosynthetic cluster (18).

In conclusion, NocR is a transcriptional activator of the nocardicin A biosynthetic cluster affecting the biosynthesis of nocardicin A and its pHPG precursor in N. uniformis. NocR contains an OmpR-like DNA binding domain at the N terminus, like other members of the SARP family, but is the first to be characterized in Nocardia. RT-PCR and DNA binding studies provide evidence that NocR binds to the nocF-nocA intergenic region, directly affecting the transcription of the nocABnat operon, thus controlling nocardicin A production from its initial biosynthetic steps. A perfect hexameric repeat, TGATAA, within the nocF-nocA intergenic region is the probable binding motif for NocR.

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