Mutational Analysis and Characterization of Nocardicin C-9′ Epimerase*

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The biosynthetic gene cluster for the nocardicin A producer Nocardia uniformis subsp. tsuyamanensis ATCC 21806 was recently identified. Nocardicin A is the most potent of a series of monocyclic β-lactam antibiotics produced by this organism. Its activity has been attributed to a syn-configured oxime moiety and a D-homoseryl side chain attached through an unusual ether linkage to the core nocardicin framework. Notably present in the nocardicin biosynthetic gene cluster is nocJ, encoding a protein with sequence similarity to the pyridoxal 5′-phosphate (PLP)-dependent 1-aminocyclopropane-1-carboxylic acid deaminases. Insertional mutagenesis of nocJ abolished nocardicin A production, while the L-homoseryl isomer, isonocardicin A, was still observed. Expression of the disrupted nocJ gene in trans was sufficient to restore production of nocardicin A in the disruption mutant. Heterologous expression, purification, and in vitro characterization of NocJ by UV spectroscopy, cofactor reduction, chiral HPLC analysis of the products and their exchange behavior in deuterium oxide led to confirmation of its role as the PLP-dependent nocardicin C-9′ epimerase responsible for interconversion of the nocardicin homoseryl side chain in both nocardicin A with isonocardicin A, and nocardicin C with isonocardicin C. NocJ is the first member of a new class of β-lactam aminoacyl side chain epimerases, the first two classes being the evolutionarily distinct prokaryotic PLP-dependent isopenicillin N epimerase and the fungal isopenicillin N epimerase two protein system.

Since their introduction in the late 1940s, the β-lactam antibiotics remain among the most widely used clinical agents in the treatment of bacterial infections. Principal among these are the penicillins and cephalosporins/cephamycins whose commercial products are semi-synthetic variants of the natural products penicillin N (penam nucleus) and cephalexin/cephamycin C (cephem nucleus, Fig. 1). Three other structurally distinct families of naturally occurring β-lactam antibiotics are known. Two of these arose from mixed biosynthetic pathways, the clavams (e.g. clavulanic acid) (1–3) and the carbanems (e.g. carbapenem-3-carboxylic acid), (4, 5), but the third, the monobactams/multicyclic β-lactams (e.g. nocardicin A), like the penams and cephems, are solely derived from amino acids (6–8).

The nocardicins were isolated from Nocardia uniformis subsp. tsuyamanensis (ATCC 21806, N. uniformis) and their structures determined (9–12). Nocardicin A (Fig. 1) is the most potent of the series and contains unusual structural features critical for its activity. First, the oxime moiety, an uncommon feature among natural products, must be present in the syn-configuration relative to the β-lactam ring. Second, it can be seen that a D-homoseryl side chain has been attached to a p-(hydroxyphenyl)glycine residue. Its presence is also essential for optimal antibiotic potency. This side chain, however, like isopenicillin N, is initially formed bearing the natural L-configured α-amino acid terminus. Thus, isopenicillin N is synthesized from a tripeptide containing an α-aminoacidic acid residue while the corresponding side chain site in isonocardicin A arises by the rare intermediate transfer of an L-3-amino-3-carboxypropyl unit from S-adenosyl-L-methionine (AdoMet). Nature resorts, therefore, to the highly unusual inverted peptide incorporation of a nonproteinogenic amino acid in the formation of isopenicillin N, and an equally unusual transfer from AdoMet to achieve a flexible, linear N-acyl side chain of comparable length in isonocardicin A.

For oxidative ring expansion and subsequent transformations to occur to the cephalosporins and cephamycins, this terminal α-amino acid center must first be epimerized to the D-configuration. This pivotal process has been studied extensively and is known in prokaryotes to be catalyzed by a pyridoxal 5′-phosphate-dependent epimerase (13, 14). It has been recently determined, however, that in fungal cephalosporin producers two proteins are required, an acyl-CoA synthetase and an acyl-CoA epimerase. This is the first example of such a two-protein epimerization system in secondary metabolism (15).

Reverse genetics techniques from the AdoMet-dependent 3-amino-3-carboxypropyl transferase (Nat) have allowed the nocardicin biosynthetic gene cluster to be identified and characterized from N. uniformis (16, 17). Notably present in the cluster are two non-ribosomal peptide synthetases (NRPSs), genes required for generation of the non-proteinogenic amino acid p-(hydroxyphenyl)glycine, (17) nocL, which encodes a cytochrome P450 responsible for oxime formation in the nocardicins, (18) and nat providing the side chain AdoMet-dependent transferase. Also present in the cluster is an open reading frame, nocJ, whose predicted amino acid sequence shows similarity to the pyridoxal 5′-phosphate (PLP)1-dependent 1-aminocyclopropane-1-carboxylate (ACC) deaminases (17).

In the present studies we focus on the role of nocJ in nocardicin biosynthesis. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PLP, pyridoxal 5′-phosphate; ACC, 1-aminocyclopropane-1-carboxylate; HPLC, high performance liquid chromatography; MS, mass spectrometry; ESI, electrospray ionization; Noc A, nocardicin A; Iso A, isonocardicin A; HPLC, high performance liquid chromatography; MS, mass spectrometry; ESI, electrospray ionization.
cin A biosynthesis by *N. uniformis*. Insertional disruption of this gene was carried out, and the effect of this mutation and its subsequent complementation was evaluated. In addition, NocJ was overproduced as the N-terminal hexahistidine-tagged protein and examined for its dependence on PLP and the ability to catalyze epimerization at C-9 of the nocardicin side chain. The results from both in vivo and in vitro analyses of NocJ support a common finding that establishes its role as the nocardicin C-9’ epimerase.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids—** *Escherichia coli* SC12155 was obtained from the Squibb Institute for Medical Research (Princeton, NJ). Vectors pT7Blue-3 (Novagen, Madison, WI), pBSISK- (Stratagene, La Jolla, CA), and pET24b(+) (New England Biolabs, Beverly, MA) were obtained from commercial sources. Vectors pIJ4070 and pULVK2 were generous gifts of Professors Mervyn Bibb (John Innes Center, Norwich, England) and Juan F. Martín (Area of Microbiology, University of Léon, Spain), respectively. Vector pULVK2T was described elsewhere (17). Wild-type *N. uniformis* subsp. *tsuyamanesis* (ATCC 21806) was purchased from ATCC.

**Biochemicals, Chemicals, and Media—** Authentic nocardicin A was a gift of Fujisawa Pharmaceutical Co., Ltd., (Osaka, Japan) and sample of izonocardicin A was the gift of Drs. W. Hofheinz and H.-P. Isenring of Hoffman-La Roche (Basel, Switzerland). Nocardicin C was synthesized as previously described (19). Unless otherwise specified, biochemicals and reagents were from common commercial sources. *E. coli* strains were grown in Luria-Bertani (LB) medium and selected for their plasmid with the appropriate antibiotic (20). The nocardicin production medium was previously described (12). ISP-2 and TSB were purchased from Difco Laboratories (Detroit, MI).

**DNA Isolation, Manipulations, and Sequencing—** Plasmid DNA isolation from *E. coli* was carried out using the Qiaprep Spin Miniprep kit (Valencia, CA) and total DNA isolation from *N. uniformis* strains followed the Gram-positive bacteria protocol for the Qiagen DNAeasy Tissue kit. For Southern analysis, 32P-labeled probes were generated by random priming using the RadPrime DNA Labeling System (Invitrogen) and hybridizations were conducted according to protocol (20). DNA sequencing to confirm constructs was carried out at the Biosynthesis & Sequencing Facility, Johns Hopkins Medical School, Baltimore, MD.

**Construction of nocJ::apra *N. uniformis—** The gene *nocJ* was amplified from cosmid DNA by PCR (17). The forward primer (5’-AAGGATCC-GTGGGGCGGCGTG-3’) possessed a BamHI restriction site (underlined) and contained the wild-type start codon. The reverse primer (5’-AAGGATCTCAACTGACACGACGTCC-3’) also possessed a BamHI restriction site and the native stop codon. The amplified fragment was cloned into the EcoRV site of pT7Blue-3, and the resulting vector linearized by digestion with BamHI and shrimp alkaline phosphatase. The EcoRI insert containing *apra* was treated with Klenow fragment and ligated into the linearized vector. The resulting *nocJ::apra* fragment was excised with BamHI and cloned into pULVK2 to provide pULVK2::Apr. The vector was isolated from *E. coli* JM110 and used to transform *N. uniformis* protoplasts, selecting for transformants with 200 μg/ml kanamycin and 100 μg/ml apramycin. Individual colonies (8) were streaked onto ISP-2 solid medium containing 100 μg/ml apramycin. This operation was repeated, and spores of a single colony from each of the 8 plates were used to generate a mycelial stock. Potential mutants were identified according to renewed sensitivity to kanamycin and resistance to apramycin. Total DNA from wild-type and potential mutant strains was isolated and digested with DraIII and evaluated by Southern hybridizations to *apra* and *nocJ*.

**Evaluation of Nocardicin A Production in *N. uniformis* Strains—** Mycelial stock of the *N. uniformis* strain of interest was used to inoculate a seed culture of 10 ml of TSB medium (containing 50 μg/ml apramycin or both apramycin and 25 μg/ml thiostrepton, if appropriate) and grown at 30 °C for 48 h. At this time, a 500-ml Erlenmeyer flask with 160 glass beads containing 100 ml of nocardicin production medium supplemented with 0.5 mM L-methionine and L-4-hydroxyphenylglycine was inoculated with 2 ml of seed culture, and grown at 30 °C for 120 h. Samples of 1 ml were collected at 72, 96, and 120 h, and mycelia removed by centrifugation at 4000 × g for 10 min. An aliquot of 250 μl was applied to paper discs placed upon LB solid medium infused with *E. coli* SC12155. The plates were incubated overnight at 37 °C and examined the following morning for the appearance of antibiotic surrounding the discs. Additionally, 100-μl culture supernatants were diluted to 2 ml with 20 μM ammonium acetate (pH 3 with acetic acid)

[Fig. 1. Role of epimerases in penam/cephem and nocardicin biosynthesis.]
Characterization of Nocardicin C-9′ Epimerase

then applied to ~1.5 ml AG50W-X8 (Bio-Rad) resin pre-equilibrated with an 8 ml loading buffer. The resin was washed, and the bound material was released from the resin with 3 ml of 1% NH₄OH and collected in a tube containing 150 μl of 10% acetic acid to immediately neutralize the solution. The solvent was removed by lyophilization, and samples were analyzed by reverse-phase HPLC on a PerkinElmer 235C diode array detector and LC410 pump using the Phenomenex Luna C18 (2) 250 × 4.6 mm column (Torrance, CA) in 10% acetonitrile, 0.1% trifluoroacetic acid (aqueous) at 1 ml/min. Chiral HPLC analysis was performed with an Astec Chirobiotic T 250 × 4.6 mm column (Whippany, NJ) in a mobile phase of 20% methanol and 0.1% triethylamine (pH to 4.1 with acetic acid) at 1 ml/min. Absorbance was monitored at 272 nm.

Purification of Isonocardicin A from nocl::apra N. uniformis—isonocardicin A was purified from 3.2 liters of mutant culture. The cell paste was thawed in 40 ml of lysis buffer (10 mM phosphate, pH 7.4, 10% glycerol, 20 μM PLP, and 1 mM dithiothreitol) at 4 °C. The protein solution was finally applied (10 ml/min) to a Poros HS/M 100 × 4.6 mm column using the BioCAD system in 25 mM sodium phosphate, pH 7.4, 10% glycerol, and 20 μM PLP. A gradient was then applied from 0 to 1 M NaCl over 10 CV, and the fractions checked for protein content by their absorbance at 280 nm and 12% SDS-PAGE. Fractions containing NocJ were pooled together and concentrated with an Amicon PM-10 membrane. Protein concentration was determined by the method of Bradford using bovine serum albumin as a standard (22).

The pyridoxal 5′-phosphate content of NocJ was determined by measuring the optical spectrum of the protein in 0.1 M NaOH using ε_{280} of 6600 M⁻¹ cm⁻¹ (23).

Sodium Borohydride Inactivation of NocJ—To 100 μl of 2.53 μM NocJ in enzyme buffer, 5 μl freshly prepared 100 mM sodium borohydride in water was added. The buffer was exchanged with enzyme buffer. The activity of the protein was examined in a 100-μl reaction containing 0.2 μM of either NocJ obtained from PLP-free buffer or sodium borohydride-treated NocJ, 0.5 mM nocardicin A in 50 mM sodium phosphate pH 7.4, with and without the addition of 40 μM PLP. The reactions were incubated at 30 °C for 2 h and were quenched by heating at 95 °C for 5 min, as above. A 4-μl aliquot was analyzed by chiral HPLC.

Chiral HPLC Assay for NocJ-catalyzed Epimerization—In a final volume of 500 μl, 2 μM N-His₅-NocJ, 0.5 mM of nocardicin substrate, 40 μM PLP were mixed together in 50 mM sodium phosphate, pH 7.4. The reactions were set up in 8 ml as follows: 1 μM N-His₅-NocJ, 40 μM PLP, and 0.5 mM nocardicin A in 50 mM sodium phosphate, pH 7.4. Reaction was incubated at 30 °C for 20 h. At 18 h, the entire reaction was quenched by immersion of the reaction vessel in a boiling water bath for 5 min and filtered over a 0.45-μm filter. The diastereomeric mixture was purified by semi-preparative reverse phase HPLC, providing a 60% yield of nocardicin A (in dH₂O) provided a λmax of 272 nm; ESI-MS generated [M + H] ion peak at m/z 501.3 and an MS/MS analysis of that peak generated fragments at m/z 351.0 and 322.0; mp 210–215 °C dec. (lit. for nocardicin A (11) 214–216 °C dec.); 400 MHz 1H NMR (D₂O and K₂CO₃) 8.748 (2 H, d, J = 8.8 Hz, Ar), 7.23 (2 H, d, J = 8.4 Hz, Ar), 7.02 (2 H, d, J = 8.8 Hz, Ar), 6.89 (2 H, J = 8.4 Hz, Ar), 5.32 (1 H, s, H-5), 4.99 (1 H, dd, J = 4.8, 2.2 Hz, H-3), 4.23 (2 H, m, H-7), 3.90 (1 H, m, H-9), 3.84 (1 H, t, J = 5.4 Hz, Ar-α), 3.23 (1 H, dd, J = 2.2, 5.6 Hz, H-4β), 2.31 (2 H, m, H-8); [α]D = −138.6° (0.1 M potassium carbonate, 4°C).

Complementation of nocl::apra N. uniformis—nocl was amplified by PCR from cosmids DNA (17). The forward primer (5′-AACCATAT-GGGCGCGCTTGGCGCGCGTTG-3′) contained an NdeI restriction site (underlined) and the native Gateway start codon was replaced with ATG. The reverse primer (5′-GGGATCCTCACTACTGAAGCGAGCGCACTGC-3′) contained a BamHI restriction site and the native stop codon. The PCR product was ligated into the EcoRV site of pPT7Blue-5 to provide pPT7Blue-3/noclJ, then excised as the NdeI-BamHI fragment and ligated into appropriately digested pET24b (+). The XbaI-HindIII fragment containing noclJ behind the ribosomal binding site was excised and cloned into appropriately digested pLJ4070 to provide pLJ4070/noclJ. The open reading frame of this vector containing noclJ was excised with BglII and ligated into BamHI-digested pULVK2T to generate pULVK2T/noclJ. The plasmid was isolated from E. coli JM110 and used to transform nocl::apra N. uniformis protoplasts. Transformants were confirmed by isolation of total DNA from N. uniformis and re-isolation of the plasmid from E. coli DH5α.

Expression and Purification of NocJ—The NdeI-BamHI fragment from pPT7Blue-3/noclJ was cloned into appropriately digested pET28b (+) to provide pET28b (+)/noclJ. The vector was introduced into E. coli BL21(DE3), and then used to inoculate 50 ml of LB medium (50 μg/ml kanamycin), and the culture incubated at 37 °C and 300 rpm. The following day, 4 × 10 ml aliquots of the overnight culture were used to inoculate 2 × 1-liter baffled flasks of LB medium (50 μg/ml kanamycin). The cultures were incubated at 37 °C and 300 rpm until OD₆₀₀ = 0.6, and temperature reduced to 19 °C. Protein expression was induced with 10 μM isopropyl-1-thio-β-D-galactopyranoside, and cultures grown at 19 °C and 300 rpm for 8 h. Cells were harvested, frozen in liquid nitrogen, and stored at −80 °C overnight. The cell paste was thawed in 40 ml of lysis buffer (10 mM imidazole, 300 mM NaCl, 50 mM Na₂HPO₄, 1 mM phenylmethylsulfonyl fluoride, 12.9 mM benzamidine, and 200 μM PLP, pH 8.0). Lysosome was added to a final concentration of 1 mg/ml, and the cell suspension incubated on ice for 20 min, at which time 40 μl each of 10 mg/ml deoxyribonuclease I and ribonuclease A were added and incubated for 20 min on ice. Chromosomal lysates were sonicated with 10 s rests, followed by centrifugation at 17,000 × g for 40 min at 4 °C. To the CFE, 3.2 ml of Ni-NTA resin (Qiagen) was added, and the suspension incubated for 1 h at 4 °C. The protein was then eluted from the resin following the manufacturer’s guidelines. To the elution fraction, 0.125 mM EDTA was added. The elution fraction was dialyzed for 2 h against 0.1 M Tris buffer A (50 mM sodium phosphate, pH 7.4, 10% glycerol, 20 μM PLP, 0.2 mM phenylmethylsulfonyl fluoride, 1.29 mM benzamidine, 1 mM dithiothreitol, 0.13 mM EDTA). The protein solution was then applied (10 ml/min) to a Poros H/QH 100 × 4.46 mm column using the BioCAD 700E Perfusion Chromatography Work station (Perceptive Biosystems, Framingham, MA) in 50 mM bis-Tris and Tris, 10% glycerol, 0.1% Triton X-100, pH 7.6. Protein was eluted using a gradient from 0 to 1 M NaCl over 10 column volumes (CV). NocJ-containing fractions were pooled and dialyzed for 1 h against enzyme buffer (50 mM sodium phosphate pH 7.4, 10% glycerol, 20 μM PLP, and 1 mM dithiothreitol) at 4 °C. The protein solution was finally applied (10 ml/min) to a Poros H/QH 100 × 4.46 mm column using the BioCAD system in 25 mM sodium phosphate, pH 7.4, 10% glycerol, and 20 μM PLP. A gradient was then applied from 0 to 1 M NaCl over 10 CV, and the fractions checked for protein content by their absorbance at 280 nm and 12% SDS-PAGE. Fractions containing NocJ were pooled together and concentrated with an Amicon PM-10 membrane. Protein concentration was determined by the method of Bradford using bovine serum albumin as a standard (22).

The pyridoxal 5′-phosphate content of NocJ was determined by measuring the optical spectrum of the protein in 0.1 M NaOH using ε_{280} of 6600 M⁻¹ cm⁻¹ (23).
Characterization of Nocardicin C-9′ Epimerase

Evaluation of Nocardicin A Production in nocJ::apra N. uniformis—Wild-type and mutant strains of N. uniformis were incubated in nocardicin production medium for 120 h, and samples of the culture supernatant were withdrawn after 72, 96, and 120 h of growth and used in a paper disc bioassay against the β-lactam supersensitive E. coli SC12155 (11). Only the culture supernatant obtained from wild-type N. uniformis demonstrated antibiotic. All four nocJ::apra N. uniformis strains failed to generate even a diminished zone of growth inhibition surrounding the paper disc (Fig. 3).

The isolated culture supernatants were partially purified using strong cation exchange chromatography and analyzed by reverse phase HPLC. A peak co-eluting with nocardicin A was still present in all samples and possessed a UV absorption spectrum identical to authentic nocardicin A (data not shown). Isolation of this peak by reverse phase HPLC and analysis by electrospray ionization mass spectrometry (ESI-MS) revealed a [M+H] ion peak at m/z 501.3 and MS/MS of this peak generated fragment ions at m/z 351.0 and 322.0, identical that of nocardicin A. Analysis of the isolated material by chiral HPLC, however, demonstrated that it co-eluted at 5.7 min with a synthetic standard of isonocardicin A, and eluted 1.2 min prior to nocardicin A (tR = 6.9 min) (Fig. 4). The 400 MHz 1H NMR spectrum of the product isolated from 3.2 liters of culture was fully consistent with that reported for the synthetic sample of isonocardicin A (33). The specific rotation of the isolated isonocardicin A was [α]D = −138.2°, and can be compared with that of its diastereomer, nocardicin A, [α]D = −169.3°. Isonocardicin A is roughly two orders of magnitude less potent an antibiotic in bioassays, hence the absence of observed antibiosis (see “Discussion”).

Complementation of nocJ::apra N. uniformis—To confirm the loss of nocardicin A production was due directly to disruption of nocJ and not the result of a polar mutation, nocJ was re-introduced into the disrupted N. uniformis on a replicating plasmid. The in trans expression of nocJ was placed under control of the constitutively active ermE2 promoter in the E. coli-Nocardia shuttle vector pULVK2T to provide pULVK2T/nocJ (34). The pULVK2T-based vector, in addition to containing a kanamycin resistance gene, also carries the resistance gene for thiostrepton, to enable dual selection for transformants of the insertion mutants using apramycin and thiostrepton. The complementation plasmid was introduced into N. uniformis CAT J1–J4 by PEG-mediated protoplast transformation. Nocardia transformants were confirmed by introduction of the total DNA obtained from these strains into E. coli DH5α followed by subsequent isolation from E. coli and restriction digest analysis of the recovered plasmid.

The nocJ::apra N. uniformis (pULVK2T) strains were exam-
Co-eluted with synthetic standard of isonocardicin C synthetic cin C (Fig. 6 A), a new peak in the chromatogram formed that 38224/H11032 NocJ to mediate epimerization at the C-9 A NocJ revealed maxima at 280 and 420 nm, with an isonocardicin A (Fig. 5). These were isolated from: wild-type Iso A shown) (38). By obtaining the UV-visible spectrum of N-His6- with reduction of the imine bond of bound PLP (data not 420 nm, and the formation of a new band at 325 nm, consistent 386 with sodium borohydride, (37) resulting in loss of the peak at 420 nm is indicative of an internal aldimine linkage to the pyri- 344 phase HPLC purification revealed a small peak with identical spectral properties and retention time to nocardicin A (Fig. 4). Purification of NocJ—NocJ was heterologously expressed in E. coli BL21(DE3). The gene was cloned into pET28b(+) under control of the T7 promoter so that the recombinant protein would possess an N-terminal hexahistidine fusion. Following nickel-chelate chromatography, N-His6-NocJ was subjected to strong anion exchange, then strong cation exchange chromatography to provide the protein in greater than 95% homogeneity by SDS-PAGE (Fig. 5) A UV-visible spectrum of N-His6- NocJ revealed maxima at 280 and 420 nm, with an A280/A420 ratio of 2.2 (Fig. 5). The absorption maximum present at 420 nm C-9 mNocJ was rendered incapable of catalyzing the epimerization of a nocardicin A diastereomers was purified by reverse phase HPLC, and 341 by chiral HPLC. The mixture of nocardicin A and nocardicin A had identical UV-visible spectra to authentic material. The sodium borohydride-treated NocJ was rendered incapable of catalyzing the epimerization of nocardicin A to form isonocardicin A (Fig. 6B). As with nocardicin C, these apparent products with isonocardicin A and nocardicin A had identical UV-visible spectra to authentic material. The sodium borohydride-treated NocJ was rendered incapable of catalyzing the epimerization of nocardicin A to form isonocardicin A (Fig. 6B), adding further support for the requirement of the PLP cofactor and the role of NocJ.

Nocardicin A and N-His6-NocJ were incubated for 18 h in deuteronated buffer, and completion of the reaction was verified by chiral HPLC. The mixture of nocardicin A and isonocardicin A diastereomers was purified by reverse phase HPLC, and checked for integrity again by chiral HPLC. The UV-visible spectrum of the purified sample was found to be identical to authentic nocardicin A. When the recovered sample was analyzed by 400 MHz 1H NMR, the signal corresponding to H-9 was absent from the spectrum, confirming epimerization at this site by NocJ and specific incorporation of deuterium at this position (Fig. 7). The sample was also analyzed by ESI-MS, and
generated a [M+H] ion peak at m/z 502.5, while nocardicin A gives an [M+H] ion peak at m/z 501.3, consistent with incorporation of a single deuterium atom. Fragmentation of this ion peak provided fragments at m/z 352.0, 335.0, and 323.0, at the same relative intensity as for the nocardicin A standard, which affords the corresponding fragments at m/z 351.1, 333.9, and 322.1, as noted earlier. All these fragments contained the incorporated deuterium.

To determine the kinetic constants of NocJ, the reaction mixtures with isonocardicin A and nocardicin A were derivatized using 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) (39–41). Isonocardicin A had a $K_m$ of 70 ± 30 μM and a $k_{cat}$ of 0.59 ± 0.05 s$^{-1}$, while nocardicin A had a $K_m$ of 36 ± 15 μM and a $k_{cat}$ of 0.35 ± 0.02 s$^{-1}$. When the NocJ reaction with isonocardicin A and nocardicin A was permitted to reach equilibrium, the $d$-configuration of the final product at C-9’ is slightly favored, providing a ratio of 1:1.1 of isonocardicin A to nocardicin A. To determine whether a transamination side reaction could also be a property of the NocJ reaction, the enzyme was incubated with nocardicin A, and UV-visible spectra in the region of 300–500 nm were obtained over the course of 1 h. No loss of absorption at 420 nm was observed, nor any gain in absorption at 330 nm. In addition to chiral HPLC, the reaction was also monitored by reverse-phase HPLC to detect any side products of the reaction, potentially stemming from the transamination type (33). Therefore, for a comparable or even a 10-fold increase in the production level of isonocardicin A, a diminished or altogether absent antibiotic effect against E. coli strain would be expected. The importance of a stereocenter removed from the β-lactam warhead to antibiotic activity is not without precedent. Penicillin N is more active than its isopenicillin N isomer against the Gram-negative strains Salmonella typhimurium ATCC 13311 and Pseudomonas aeruginosa Pss, while the opposite is true for the Gram-positive species Staphylococcus aureus ATCC 25923 and Sarcina lutea ATCC 9341 (47).

The results obtained from the present bioassay suggest at least a significant reduction in nocardicin A production, an inability to epimerize the isonicardicin precursor, or possibly a transformation earlier in formation of the nocardicin framework has been affected.

HPLC analysis of the culture supernatants revealed the presence of a peak that co-eluted with an internal standard of nocardicin A. However, if a peak of this magnitude did corre-

**DISCUSSION**

The initial bioinformatic analysis of NocJ suggested that, akin to the ACC deaminases, it could also utilize the cofactor PLP for its catalytic function. The amino acid ACC was first identified from fruit juices in the late 1950s, and was later established as the key intermediate in the biosynthesis of the plant hormone ethylene (42–44). The enzyme ACC deaminase was first identified in Pseudomonas sp. ACP during a screen for prokaryotic and eukaryotic species capable of utilizing this unusual amino acid as the sole source of nitrogen (45). During this study, it was observed that a tightly bound PLP cofactor was required to direct cleavage of the cyclopropane ring to liberate ammonia and α-ketoacrylate.

The similarity of NocJ to ACC deaminases might, at an initial glance, suggest the same sort of reaction is mediated by NocJ. It is difficult, however, to reconcile this reaction type with the transformations needed to assemble the nocardicin scaffold. When considering the chemistry likely to be mediated by a PLP-dependent enzyme, it was tempting to propose NocJ to be an epimerase to introduce the $l$-configuration at C-9’ of the homoseryl moiety present in nocardicins A and C (Fig. 1). The side chain is attached by Nat from AdoMet, to initially present the $l$-configuration (16). Wilson et al. (46) reported a cell-free system from N. uniformis containing both transferase, and epimerase activities. The dialyzed cell-free extract did not require the addition of exogenous cofactors, such as PLP, for the epimerization to occur, but this does not necessarily rule out a PLP-dependent system. Many PLP-dependent enzymes remain tightly bound to their cofactor; for example the Salmo-

nella typhimurium alanine racemase and rat liver cystathion-
ase have $K_m$ values for PLP of 33 and 430 nM, respectively (35, 37). The original purification of ACC deaminase from Pseudo-
monas ACP did not require the addition of PLP to any purifi-
cation buffers or reaction mixtures to observe activity (45).

As one approach to evaluate the function of the gene product of nocJ in nocardicin A biosynthesis, the insertional disruption mutant was prepared. The effect of this mutation was initially examined by bioassay against a β-lactam supersensitive E. coli strain. An inherent limitation of the bioassay is the potential for false positives produced by β-lactam-containing biosyn-
thetic intermediates present in the culture supernatant. While nocardicin A is the most potent of the series, it is conceivable accumulation of isonocardicin A due to loss of the epimerase activity in a mutant could also produce antibiosis. It has been reported, however, that for E. coli 1024, Proteus vulgaris 1028 and Serratia marcescens 80315 the minimum inhibitory concentration of isonocardicin A is 200 μg/ml, while it is only 6.3 μg/ml for nocardicin A, differing by nearly two orders of magni-
tude (33). Therefore, for a comparable or even a 10-fold in-
crease in the production level of isonocardicin A, a diminished or altogether absent antibiotic effect against E. coli SC12155 would be expected. The importance of a stereocenter removed from the β-lactam warhead to antibiotic activity is not without precedent. Penicillin N is more active than its isopenicillin N isomer against the Gram-negative strains Salmonella typhi-
murium ATCC 13311 and Pseudomonas aeruginosa Pss, while the opposite is true for the Gram-positive species Staphylococcus aureus ATCC 25923 and Sarcina lutea ATCC 9341 (47).

The results obtained from the present bioassay suggest at least a significant reduction in nocardicin A production, an inability to epimerize the isonicardicin precursor, or possibly a transformation earlier in formation of the nocardicin framework has been affected.
The low nocardicin A production level by the complemented N. uniformis strain, was unknown, and in the absence of a characterizer, could be attributed to an inefficiency of N. lactamdurans. Amycolata species have been utilized previously for expression of proteins in antibiotic production. The ability of ermE2 to function effectively as a promoter in N. uniformis was unknown, and in the absence of a characterized promoter specific to this strain, ermE2 was the best option. The low nocardicin A production level by the complemented mutant, also observed with the complementation of nocardicin G, could be attributed to an inefficiency of ermE2 in N. uniformis. Additionally, thistreptone, although absent in the production medium, was used in the seed culture. This antibiotic has demonstrated the ability to alter expression levels of cellular proteins in Nocardia and other actinomycetes.

Following heterologous expression of NocJ and purification of the protein, it was found to require the PLP cofactor, as expected. Initial in vitro assays by HPLC and mass spectrometric analysis of NocJ confirmed its role as the C-9’ epimerase active with both isonocardicin A and nocardicin C, as well as the reverse reaction from nocardicin A and nocardicin C. It is a limitation that isonocardicin A and nocardicin A are indistinguishable by reverse phase HPLC, UV-visible spectroscopy, mass spectrometry, and 400 MHz 1H NMR. To overcome this obstacle, and to show unambiguously the epimerization at C-9’ of nocardicin A, the reaction was carried out in deuterated solvent. As the reaction approaches equilibrium, all hydrogens at that position will be exchangeable with deuterium, and result in a loss of the H-9’ signal in the 1H NMR spectrum. Indeed, this was seen to be the case, unequivocally establishing NocJ as the nocardicin C-9’ epimerase. The signals corresponding to the homoseryl side chain protons are all broadened in the recovered material from both the enzymatic and control reactions, and this could be attributed to the pH of the solution contributing to population of more than one protonation state of that moiety.

Certain amino acid racemases, particularly those with a relaxed substrate specificity, also facilitate transamination as a side reaction. This transamination half reaction eventually results in accumulation of PMP in vitro, as opposed to the epimerization reaction, which freely regenerates the PLP cofactor. Without an α-keto acid present to replenish the PLP cofactor, such as pyruvate, the absorption band at 420 nm diminishes over time, concurrent with an increase in absorption at 330 nm because of the increasing presence of PMP. The reaction of NocJ and nocardicin A gave no evidence that this derailing of the epimerization reaction occurs.

Another potential side reaction of NocJ with isonocardicin A or nocardicin A could lead to the generation of nocardicin E, the in vivo formation of which is poorly understood at this time.

The oxime-forming enzyme, NocL, is able to convert nocardicin C to nocardicin A; however no formation of nocardicin E from nocardicin G has been detected, leaving the origin of this nocardicin uncertain. NocJ could also effect a γ-elimination from either isonocardicin A or nocardicin A to afford nocardicin E (Fig. 8). No new peaks in the chromatogram were generated, however, when monitored by either chiral or reverse-phase HPLC, suggesting NocJ specifically catalyzes epimerization at C-9’ of the nocardicin homoseryl side chain, but not γ-elimination of the side chain or transamination. Furthermore, NocJ appears to be specific for its amino acid substrates exhibiting quite low KmA values, and it was unable to racemize any of the alternate amino acids with which it was presented.

NocJ is the third class of epimerases identified for the aminoacyl side chains of β-lactam antibiotics. The first of these, isopenicillin N epimerase, was initially isolated from the cephamycin C producer, Streptomyces clavuligerus, and then later from N. lactamdurans (13, 14, 55). These bacterial isopenicillin N epimerases are PLP-dependent enzymes, belonging to the aspartate aminotransferase structural family (56). Surprisingly, this same epimerization in the fungus Acremonium chrysoagenum, which produces cephalosporin C, was discovered to be due to the action of two separate enzymes: an acyl-CoA synthetase and an acyl-CoA racemase (15). Homologs for these genes have also been identified in the marine fungus Kallichroma tethys, suggesting this may be a universal solution to fungal epimerization of isopenicillin N (21). There is no significant primary sequence similarity between NocJ and any of the bacterial isopenicillin N epimerases. Instead, NocJ shows the greatest similarity to ACC deaminases of the tryptophan synthesis β structural family of PLP-dependent enzymes (56). This suggests NocJ, instead of following the evolutionary branch from prokaryotic isopenicillin N epimerase, diverged in function from ACC deaminase. In parallel with this observation, there is no cross-reactivity of NocJ with penicillin N despite the marked structural resemblance of its N-acyl side chain to that of nocardicin A (data not shown). Both bear an L-α-amino acid terminus on a nearby linear hydrocarbon chain, and both benefit greatly in their antibiotic activity by epimerization to the D-configuration during the biosynthesis. The absence of an aromatic ring, however, may thwart successful binding and reaction of penicillin N.

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