Chemistry & Biology
Article

In Vivo Characterization of Nonribosomal Peptide Synthetases NocA and NocB in the Biosynthesis of Nocardicin A

Jeanne M. Davidsen[1] and Craig A. Townsend[1,*]
[1]Department of Chemistry, Johns Hopkins University, Baltimore, MD 21218, USA
*Correspondence: ctownsend@jhu.edu
DOI 10.1016/j.chembiol.2011.10.020

SUMMARY

Two nonribosomal peptide synthetases (NRPS), NocA and NocB, together comprising five modules, are essential for the biosynthesis of the D,L,D configured tripeptide backbone of the monocyclic β-lactam nocardicin A. We report a double replacement gene strategy in which point mutations were engineered in the two encoding NRPS genes without disruption of the *nocABC* operon by placing selective markers in adjacent genes. A series of mutants was constructed to inactivate the thiolation (T) domain of each module and to evaluate an HHxxxDR catalytic motif in NocA and an atypical extended histidine motif in NocB. The loss of nocardicin A production in each of the T domain mutants indicates that all five modules are essential for its biosynthesis. Conversely, production of nocardicin A was not affected by mutation of the NocB histidine motif or the R828G mutation in NocA.

INTRODUCTION

The biosynthesis of nocardicin A, isolated from the fermentation of *Nocardia uniformis* subsp. *tsuyamanensis*, has been of interest as the paradigm of a monocyclic β-lactam antibiotic and for its activity against gram-negative bacteria and relative stability to β-lactamases (Aoki et al., 1976; Hashimoto et al., 1976; Kojo et al., 1988). Before discovery of the nocardicin A gene cluster (Figure 1A), precursor incorporation studies have shown that the D,L,D tripeptide backbone of this antibiotic family originates from 2 units of L-*p*-hydroxyphenylglycine (L-pHPG) and one unit of L-Ser (Townsend and Brown, 1983). A double-label experiment demonstrated nocardicin G, a D,L,D tripeptide β-lactam, to be the earliest isolable biosynthetic intermediate of this pathway (Townsend and Brown, 1983; Townsend and Wilson, 1988). On the basis of these early experiments and the known structure of nocardicin G, the nocardicin A gene cluster had been expected to encode a three module nonribosomal peptide synthetase (NRPS), whose initiation and termination modules installed D-pHPG into the peptide core by activation and epimerization of L-pHPG and a central module that activated and incorporated L-serine to form the tripeptide core of the nocardicins. This expectation was founded on δ-(L-α-aminoadipyl-L-cysteinyl-D-valine (ACV) synthetase, the NRPS responsible for biosynthesis of the immediate tripeptide precursor of isopenicillin N, a fused bicyclic β-lactam, and one of the few comparatively well-characterized NRPSs at the time. The 400-425 kDa ACV synthetase had been isolated from both bacteria and fungi and exemplified the classical "linear" NRPS architecture depicted in Figure 2 (Martin, 2000). The order of adenylation (A), thiolation (T) or peptidyl carrier, and condensation (C) domains, along with a C-terminal epimerase (E) and thioesterase (TE), fully accounted for synthesis of the Arnstein L,L,D-tripeptide. Also prior to characterization of the nocardicin gene cluster, ~200 kDa and ~150 kDa proteins that demonstrated ATP/PPi exchange in the presence of L-pHPG were isolated from wild-type *N. uniformis*. Fortuitously, the higher molecular weight protein also exhibited ATP/PPi exchange in the presence of L-serine (Gunsior et al., 2004), but reconstitution experiments failed to produce nocardicin G. Against expectation, when the nocardicin A gene cluster was discovered, it revealed two overlapping genes encoding a pair of NRPSs, *nocA* (11.1 kbp) and *nocB* (5.8 kbp), which were predicted to translate a five-module NRPS system and contained only a single epimerization domain (Figure 1C) (Gunsior et al., 2004). The ~200 kDa and 150 kDa proteins isolated from wild-type *N. uniformis* were identified as NocB and a proteolytic fragment of NocB, respectively—not of NocA—in keeping with earlier failed reconstitution attempts.

A combination of in vitro, gene inactivation, and bioinformatic experiments has been successfully applied to define the role of each protein encoded in the nocardicin A gene cluster: *nocF*, *nocG*, and *nocN* encode proteins responsible for the biosynthesis of the nonproteinogenic amino acid precursor L-pHPG (Figure 1B) (Gunsior et al., 2004); *nat*, *nocJ*, and *nocL* encode proteins responsible for the late-stage biosynthetic steps—the addition and epimerization of the homoseryl side chain and oxime formation (Figure 1D) (Kelly and Townsend, 2002, 2004; Reeve et al., 1998). NocR has been demonstrated to be a transcriptional activator for the *nocABC* operon (Davidsen and Townsend, 2009), NocI belongs to the MbtH family of proteins, recently shown to be involved in activating A domains in some NRPSs (Felnagle et al., 2010; Heemstra et al., 2009; Zhang et al., 2010), and NocH is homologous to membrane transport proteins of the major facilitator family (MFS) (Gunsior et al., 2004). Proteins encoded by *nocK*, *nocD*, *nocE*, and *nocO* have been shown by in vivo gene knockout experiments to be nonessential for nocardicin A biosynthesis (Davidsen and Townsend, 2009; Kelly and Townsend, 2005).

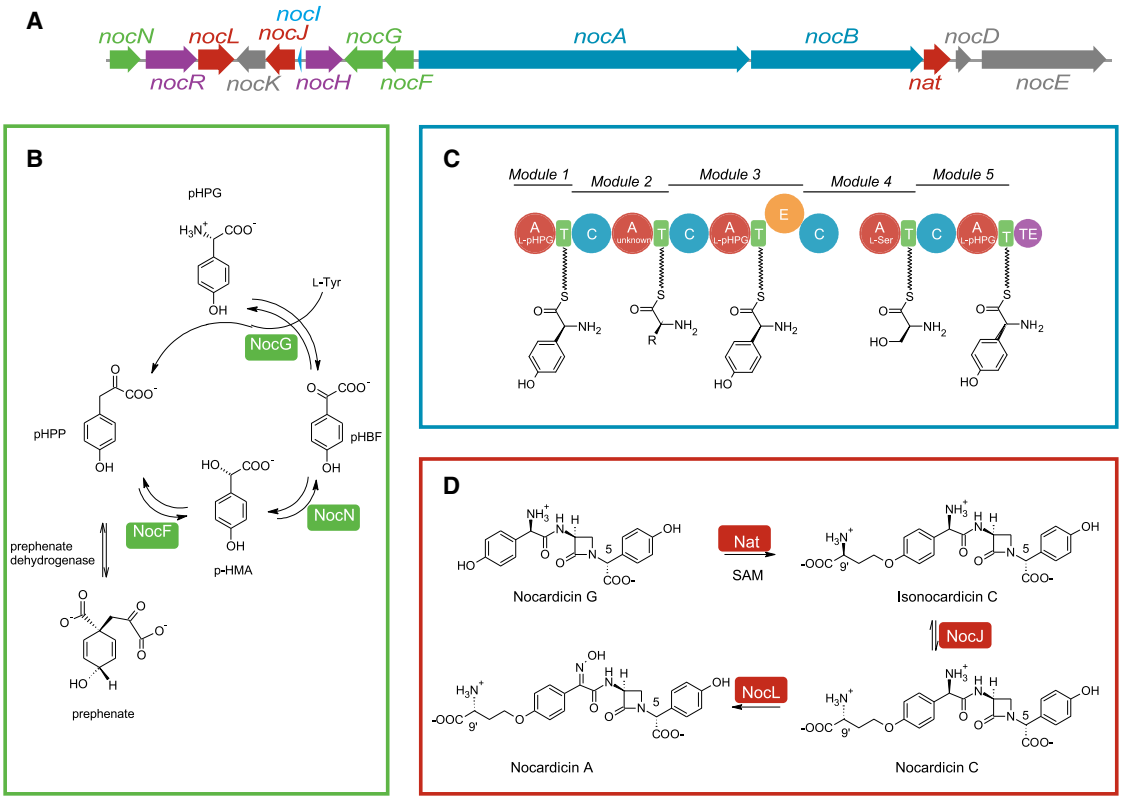

Figure 1. Biosynthesis of Nocardicin A in *N. uniformis*

(A) Gene cluster for the biosynthetic pathway, which includes genes encoding two NRPSs (blue), tailoring enzymes (red), proteins involved in regulation and transport (violet), proteins involved in biosynthesis of pHPG (green), and proteins shown to be nonessential for nocardicin A biosynthesis (gray).

(B) *p*-HPG biosynthetic pathway.

(C) Predicted domain and module organization of Noc A and NocB. Substrates predicted by bioinformatic analysis of the A domain active sites are shown.

(D) Late-stage biosynthetic steps; conversion of nocardicin G to nocardicin A.

The NRPSs of the gene cluster, NocA and NocB, are likely responsible for the synthesis of the nonribosomal D,L,D-tripeptide core of nocardicin G based on A domain substrate prediction algorithms consistent with the activation of L-pHPG by modules 1, 3, and 5 and L-Ser by module 4 (Challis et al., 2000; Gunsior et al., 2004). The substrate of the module 2 A domain is less defined but has a signature suggesting L-N^5-hydroxyornithine (Gunsior et al., 2004). Because of the gap in biosynthetic logic between the five-module NRPS encoded in the gene cluster and the requirement for a three-module NRPS suggested by the structures of the isolated nocardicins, several experiments were undertaken to further characterize the nocardicin NPRSs. Unfortunately, attempts to heterologously express NocA and NocB separately or partially as individual modules in *E. coli* and *Streptomyces* for further in vitro analysis were unsuccessful. Because of the high titer of nocardicin A produced from wild-type *N. uniformis* coupled with the successful isolation of NocB and the expectation that NocA and NocB would be translated in equal amounts from cotranscription of the *nocABC* operon, the isolation of NocA from the native bacterium was again pursued. However, despite assiduous attempts and the re-isolation of NocB and its 150 kDa fragment, the ~345 kDa NocA protein was never observed.

In the face of these setbacks, we returned to in vivo mutagenesis experiments to determine if NocA and/or NocB were essential for nocardicin A biosynthesis and, if so, to determine if they function in a nonlinear manner either by using module skipping or iterative logic. Because the genes encoding NocA, NocB, and NocC (Nat) appear in a single operon, however, insertional mutagenesis of *nocA* or *nocB* would likely affect transcription of the downstream genes, automatically altering the production of nocardicin A. Seeking precedents where the *nocABC* operon would not be disrupted, it was found that in *Streptomyces* and *Bacillus subtilis* double-gene replacement strategies to introduce markerless targeted mutations into PKS or NRPS systems have been successfully developed (Butz et al., 2008; Khosla et al., 1992; Mootz et al., 2002a; Stachelhaus et al., 1995; Uguru et al., 2004). Of particular relevance are module deletion, module substitution methods, and module exchange experiments in NRPS systems (Alexander et al., 2010; Baltz, 2009; Butz et al., 2008; Khosla et al., 1992; Mootz et al., 2002a; Nguyen et al., 2010; Powell et al., 2007; Stachelhaus et al., 1995; Uguru et al., 2004).

In an extension of the genetic system previously developed for insertional mutagenesis studies of *N. uniformis* (Kelly and Townsend, 2005), described here are double replacement

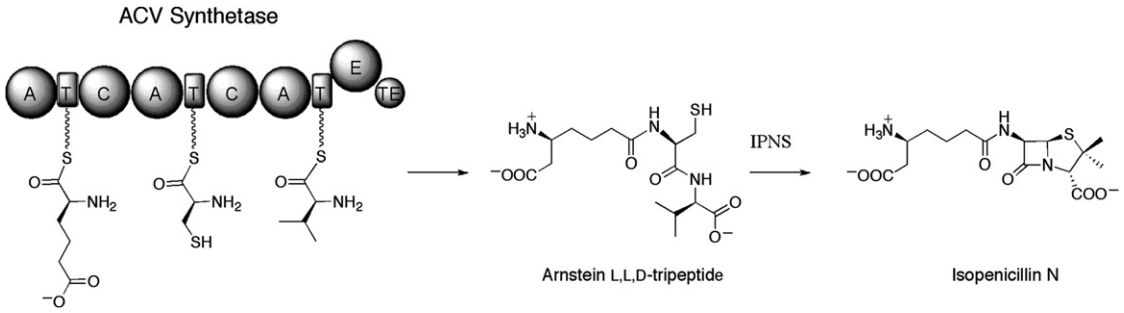

Figure 2. Biosynthesis of the Arnstein L,L,D-Tripeptide, the Precursor to Isopenicillin N by ACV Synthetase
IPNS, isopenicillin N synthase.

experiments in which a dual function selection marker was used in the second, "knock-in" step. A new strategy was devised that can be particularly amenable to probing natural product biosynthetic gene clusters and that takes advantage of the methods developed for insertional mutagenesis experiments successfully completed on genes *nocF* and *nocE* located adjacent to the *nocABC* operon. The preparation of 12 point mutants is presented, which can be divided into three key areas of interest. First, is the preparation of S to A point mutants for each T domain (total = 5) of NocA and NocB. These experiments primarily test the hypothesis that one or more modules of the NRPS is inactive. A second experiment addresses the unusual HHxxxDR catalytic motif of C2 of which the R residue is unexpected (Rausch et al., 2007). An R828G point mutant was prepared to determine if this unusual feature is critical for antibiotic production. Finally, an atypical "extended His-motif," reminiscent of that critical for catalysis in C and E domains in NRPS systems, was noted in the A5 domain of NocB. The third set of point mutants addressed whether the residues of this motif play a role in the biosynthesis of nocardicin A.

RESULTS AND DISCUSSION

Considerations for the Two-Step Gene Replacement Experiment

Our goal was to develop an in vivo mutagenesis system for *N. uniformis* for the preparation of markerless point mutants in NocA and NocB, as was done for the *Streptomyces* and *Bacillus* systems described earlier. Several blocked mutants of genes in the nocardicin A cluster have been prepared in previous studies in which an antibiotic cassette, *tsr* or *apr*, was inserted into the gene of interest (Kelly and Townsend, 2005). The preparation of an analogous *N. uniformis* insertional mutant is the first step of the double replacement experiment. Protoplasts of wild-type *N. uniformis* were prepared, transformed with a pULVK2 *Nocardia-E.coli* replicating shuttle vector, grown for several rounds without selection, and successfully screened for homologous recombination mutants. However, the second step of the double-gene replacement protocol proved more problematic given the unavailability of a suicide or temperature-sensitive plasmid compatible with *N. uniformis* of the type that was successfully employed for gene replacements in *Streptomyces* (Khosla et al., 1992). Initial studies in which a second pUVLK2 vector, engineered with a replacement sequence, transformed into the knockout or deletion mutant, failed to undergo homologous recombination and produce the desired engineered mutant. Further experimentation revealed the vector could not be maintained without antibiotic selection.

Given the scarcity of plasmids available for *Nocardia* systems, a gene replacement strategy was developed to incorporate antibiotic selection markers in genes adjacent to the *nocABC* operon. Previous studies had shown that the downstream genes encoding NocD and NocE are not essential in nocardicin A biosynthesis (Davidsen and Townsend, 2009). Upstream of the NRPS operon, the *nocA-nocF* intergenic region is essential for the binding of the pathway-specific transcriptional activator NocR. The immediate upstream target, *nocF*, encodes a *p*-hydroxymandelate synthetase involved in the biosynthesis of the pHPG precursor. Incorporation experiments in which isotopically labeled pHPG was added to fermentation cultures of wild-type *N. uniformis* showed efficient incorporation into nocardicin A (Townsend and Brown, 1983). Thus, it was anticipated that insertional inactivation of *nocF* should be chemically complemented by addition of pHPG to the fermentation medium.

A *nocF::apr* insertional inactivation mutant was prepared and evaluated for antibiotic production. The culture supernatants of *nocF::apr* mutants confirmed by Southern analysis were bioassayed against *E. coli* ESS and characterized by high-pressure liquid chromatography (HPLC) analysis. Figure 3 compares chromatograms of the *nocF::apr* mutant with wild-type *N. uniformis*. Nocardicin A (retention time = 14.6 min) was not detected in the *nocF::apr* mutant but was substantially restored by the addition of 0.5 mM L-pHPG to the fermentation culture, as anticipated. Another notable difference between the chromatograms of the wild-type and mutant is the minimal depletion of tyrosine (retention time = 5 min) and absence of *p*-hydroxybenzoyl formate (pHBF) accumulation (retention time = 8.6 min) observed in the *nocF::apr* mutant growths. This observation is consistent with previous studies of the pHPG biosynthetic cycle (Figure 1B) (Hubbard et al., 2000; Müller et al., 2006) However, the ability to restore nocardicin A production in the *nocF::apr N. uniformis* mutant confirmed that this insertional mutant could be efficiently chemically complemented by fortification of the culture medium with L-pHPG.

The first step, therefore, involved the preparation of deletion mutants T2KO and T3KO for targeted gene replacements in *nocA* (Figure 4A) and T45KO for targeted gene replacements in *nocB* (Figure 4B), in which the region between the location of

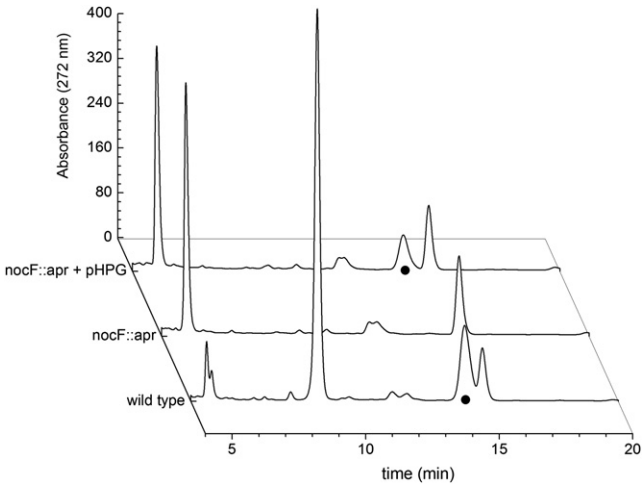

Figure 3. HPLC Chromatograms of the Culture Supernatants of *nocF::apr N. uniformis* with and without Chemical Complementation with L-pHPG Compared to Wild-Type *N. uniformis*

Key components are tyrosine (retention time = 5 min), *p*-hydroxybenzoyl formate (retention time = 8.6 min), and nocardicin A (retention time = 14.6 min), indicated by a dot.

the *apr* cassette to be inserted in the second replacement step and the NRPS domain of interest was deleted and replaced with the *tsr* gene. As expected based on previous studies (Gunsior et al., 2004), nocardicin A production was absent in each of these deletion mutants. In preparation for the second step, pULVK2 vectors were cloned containing *nocF::apr-nocA* (Figure 4A) for *nocA* gene replacement studies or *nocBC-apr-nocE* (Figure 4B) for *nocB* gene replacement studies. In both cases, the pULVK2 knock-in vector encoded the gene, which contained the *apr* resistance cassette, and the NRPS sequence to at least 1,000 bp past the desired mutation. The step 1 deletion mutants and the encoding of pULVK2 vectors for the step 2 transformations were designed so that homologous recombination would result in only one type of double crossover mutant. The resulting mutant has the Apr^r Ts^s phenotype and encodes the engineered *nocABC* operon. This protocol allows for the construction of NRPS mutants without disrupting the regulatory elements of the gene cluster (Davidsen and Townsend, 2009).

For each step 1 deletion mutant, a control knock-in mutant was prepared, encoding the native sequence. Thus, successful control experiments should result in the restoration of nocardicin A production. The restoration of antibiotic production in all three deletion mutants, T2KO, T3KO, and T45KO was observed by replacement of the *tsr* cassette with their respective native sequences of *nocA* or *nocB*, validating the strategy of the overall experiment and providing additional evidence for accuracy in the cloning of the pULVK2 vectors. In addition, because an *apr* resistance marker disrupts *nocF* in T2KO and T3KO knock-in experiments, antibiotic production should only be observable in native knock-in mutants with the addition of pHPG to the fermentation medium, a result that was observed in all the control (native sequence) knock-in experiments. Although this two-step gene replacement strategy requires a proximal nonessential or chemically complemented gene for placement of a selection marker, it was attractive for several reasons but particularly because this method proved robust in producing the desired engineered mutants and selecting for them using antibiotic sensitivity for the phenotype. After antibiotic selection, 1–4 putative mutants of each type were screened by Southern analysis, followed by sequence analysis. Mutants were identified and confirmed from only one round of screening. This strategy should be generally applicable to the investigation of other natural product gene clusters.

Characterization of Mutants

To test the hypothesis that one or more modules of the NocA+ NocB NRPS system may be nonessential or skipped, we engineered a serine-to-alanine mutation at the conserved phosphopantethienylation site of the T domain for each module and prepared mutants using the double gene replacement strategy outlined in Figure 4. Each of the resulting mutants was characterized for antibiotic production, as assessed by the *E. coli* ESS bioassay and HPLC analysis for nocardicin A production. A summary of results is shown in Table 1.

Although most NPRS systems are linear (i.e., the primary sequence of the peptide product formed is colinear with the ordered modules of its corresponding synthetase), there are now a number of known "nonlinear," Type C, NRPS systems in which module skipping, iteration, or a combination of these have been shown to occur in the formation of their peptide products (Haynes and Challis, 2007). Based on the hypothesis that the D,L,D- configured tripeptide backbone of nocardicin G originates from NocA + NocB, the nocardicin NRPS system was classified as a nonlinear Type C NRPS (Mootz et al., 2002b). Furthermore, within modules 1 and 2 were noted several atypical regions in which additional sequences of short amino acid repeats were found, leading to the hypothesis that perhaps modules 1 and 2 were skipped or inactive and, thus, the three remaining modules were responsible for the biosynthesis of D-pHPG-L-Ser-D-pHPG (Gunsior et al., 2004). Evidence for module skipping due to a defective T domain in which the conserved active site serine is not located in the expected position was reported in myxochromide biosynthesis in *Stigmatella aurantica.* As a consequence, proline is not incorporated into the peptide chain, despite an active A domain (Wenzel et al., 2005, 2006).

However, contrary to this hypothesis, antibiotic production was not detected in any of the T domain point mutants. Figures 5A–5C shows the chromatograms of the culture supernatants for each mutant, comparing them to the chromatograms of their respective first-step deletion mutant, positive control, and wild-type *N. unformis*. Figures 5A and 5B plot chromatograms for the *nocA* T1PM (S626A), T2PM (S1671A), and T3PM (S2782A) mutants and reveal few distinctions between the T1PM, T2PM, and T3PM point mutants and their parent T2KO and T3KO deletion mutants, each grown in culture medium supplemented with 0.5 mM L-pHPG. Chromatograms for *nocB* T4PM (S571A) and T5PM (S1648A) are plotted in Figure 5D. Again, the chromatograms of T4PM and T5PM lack a peak for nocardicin A and resemble the chromatograms of their parent T45KO deletion mutant. Because the *apr* resistance marker in the second step of the *nocB* gene replacement is located at

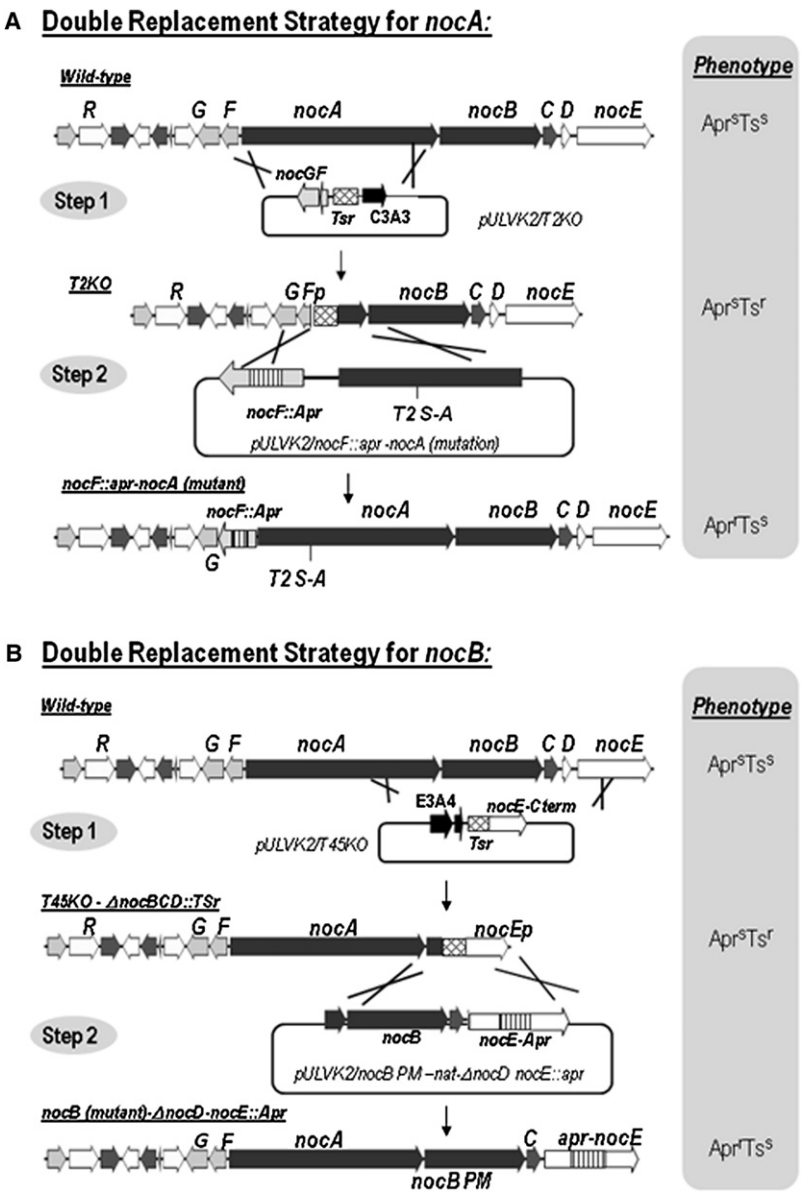

Figure 4. In Vivo Two-Step Gene Replacement Strategy

The first step required the preparation of a deletion mutant. Transformation of the deletion mutant with a vector containing the native or engineered sequence followed by homologous recombination gives the desired mutant.

(A) Strategy for mutagenesis of *nocA*, in which an *apr* gene cassette is placed in the adjacent gene *nocF*. The *nocF::apr* mutant is chemically complemented by the addition of L-pHPG to the culture medium.

(B) Strategy for mutagenesis of *nocB*, in which an *apr* gene cassette is placed in the nonessential gene *nocE*.

the end of the gene cluster and, thus, does not perturb pHPG biosynthesis, the depletion of tyrosine and the accumulation of HBF are observed in these mutants. In addition, no new peaks that might correspond to NRPS derailment products were seen in the HPLC chromatograms of the T-domain mutants. In all cases, control experiments in which native sequences were restored to the T2KO, T3KO, and T45KO deletion mutants demonstrated significant restoration of nocardicin A production, unlike the T-domain mutants, indicating that each of the five modules of this NRPS is essential for nocardicin A biosynthesis.

Mutagenesis of the *nocA* C2 domain was prepared to determine whether replacement of the arginine from the unusual catalytic motif HHxxxDR to the consensus HHxxxDG would affect nocardicin A production. The canonical catalytic motif of C domains is HHxxxDG, also called the "His motif" (Rausch et al., 2007). Mutagenic studies on the *N*-terminal C domain of TycB (tyrocidine) showed that the second histidine and aspartate residues of this signature are essential for C domain activity (Bergendahl et al., 2002; Stachelhaus et al., 1998), but there is conflicting evidence as to whether the terminal glycine of this motif is also essential. Although the HHxxxDR motif is also found in the C13 domain of daptomycin synthetase, catalyzing the addition of L-kynurenine to the nascent peptide in *S. roseosporus* (Miao et al., 2005), studies on the C domain of EntF (enterobactin) found the terminal G residue of the HHxxxDG motif to be essential. When a G-to-L mutation was introduced, it abolished activity, which was thought to result from steric occlusion of the substrate channel (Roche and Walsh, 2003). This observation posed the question whether the arginine residue of the HHxxxDR motif in C2 might render this domain inactive and block transfer of the growing peptide from the first two modules to the third. A precedent for the presence of an inactive C domain that is compensated for by the next downstream active C domain has been observed in bleomycin biosynthesis (Du et al., 2003). The *nocA* R828G mutant was found to retain antibiotic production (Table 1). Based on HPLC analyses of the culture supernatant of this mutant (Figure 5C), this mutation also did not result in a difference in nocardicin A production compared to the native control, and no new peaks were observed. This result is consistent with the structural analysis of the TycC6 C domain (Samel et al., 2007), in which the second H residue is catalytic and the D residue is essential for providing interactions that stabilize the active site. In the crystal structure, a sulfate ion sits in the active site and is "presumed" to be situated to mimic the thiol terminus of the pantethienyl moiety. This sulfate is hydrogen bonded to the peptide backbone at the G residue, suggesting that the side chain of the amino acid residue at this position is not critical and thus less conserved.

The observation that each of the five T domains is required for nocardicin A biosynthesis allows us to draw several conclusions. First, this study demonstrates that both NocA and NocB are essential for nocardicin biosynthesis, and there is no evidence that module skipping occurs in this NRPSs system. This was

Table 1. Summary of *N. uniformis* Mutants

N. uniformis Strain	Mutation/Description	Milligrams of Nocardicin A/Liter of Culture Supernatant	Bioassay
Wild-type	None	280	+
T2KO	Δ*nocF*-Δ*nocA* (M1-2)::*tsr*	0	–
Control/T2KI	*nocF::apr*	50	+
T1PM	*nocF::apr; nocA* S626A	0	–
T2PM	*nocF::apr; nocA* S1671A	0	–
C2PM	*nocF::apr; nocA* R828G	37	+
Control/T3KI	*nocF::apr*	33	+
T3KO	Δ*nocF*-Δ*nocA* (M1-3)::*tsr*	0	–
T3PM	*nocF::apr; nocA* S2782A	0	–
T45KO	Δ*nocBCDE*::*tsr*	0	–
Control/T45KI	*nocE, apr*	106	+
T4PM	*nocB* S571A	0	–
T5PM	*nocB* S1648A	0	–
M3-4	*nocB* H1411A	81	+
M4-8	*nocB* H1412A	65	+
M6-2	*nocB* E1417A	104	+
M7-2	*nocB* D1418A	54	+

See also Figure S1.

unexpected. The absence of wild-type NocA, but the comparatively ready isolation of NocB, led us to suspect that the former was swiftly proteolyzed, as was clearly observed for NocB, to smaller fragments that might act in trans. It was hypothesized that the unusual repeated sequences inserted after A1 and before module 3, for example, presented vulnerable sites where just such events could take place. Our observations make it less likely that a proteolytic product of NocA or NocB is acting in trans with NocB to produce a tripeptide. The other possibility previously entertained was that NocA was not essential and that an alternate starting module existed, as in anabaenopeptin biosynthesis in *Anabaena* (Rouhiainen et al., 2010), encoded in a yet unknown gene cluster but capable of crosstalking with the nocardicin gene cluster. Crosstalk between gene clusters has been observed in the biosynthesis of erythrochelin (Lazos et al., 2010; Robbel et al., 2011) and rhodochelin (Bosello et al., 2011).

There are several known examples of NRPSs with "too many modules." The exochelin gene cluster in *Mycobacterium smegmatis* encodes a six-module NRPS; however, exochelin is a pentapeptide. Whether all six modules are essential for exochelin biosynthesis is not known (Yu et al., 1998). Thiocoraline biosynthesis in *Micromonospora* is another system that appears to involve extra modules. The thiocoraline gene cluster encodes four NRPSs—TioR, TioS, TioY, and TioZ—of which, the roles of TioY and TioZ are unclear. Although it appears that only TioR and TioS should be required for the biosynthesis of the peptide precursor, in vivo mutagenesis experiments conclude that TioY and TioZ are also essential for thiocoraline biosynthesis (Lombó et al., 2006). A third example is the tandem TE domains that terminate the NRPSs involved in lysobactin, arthrofactin, massetolide, and syringopeptin biosyntheses. In vivo studies of the arthrofactin system showed both TE domains to be involved in efficient product formation (Roongsawang et al., 2007). Recent in vitro studies of the terminal module of the lysobactin NRPS demonstrated proteolytic cleavage of the terminal TE domain, leading the authors to propose that the terminal TE domain is also proteolyzed from the NRPS in the native *Lysobacter* to then function as an editing stand-alone type II TE domain (Hou et al., 2011).

The combination of bioinformatic analysis of the nocardicin A gene cluster, particularly the NRPS proteins NocA and NocB, as well as characterization of the in vivo NocA and NocB point mutants, indicate the likelihood that NocA+NocB is forming a pentapeptide or a pentapeptide β-lactam. If the NocA+NocB product is a pentapeptide, how and at what point in the pathway is it trimmed to a tripeptide? There are natural products with a closely related, inactive biosynthetic precursor that can be accumulated in the host until an appropriate time for release. In lantibiotic biosynthesis, the leader peptide has been proposed to act as an *in-cis* chaperone for posttranslational modification enzymes or a provider of stabilizing interactions that prevent degradation and assist in folding of the precursor peptide (Oman and van der Donk, 2010). In *Pseudomonas syringae,* the dipeptide precursor of tabtoxin, tabtoxinine-β-lactam, is trimmed by a periplasmic aminopeptidase encoded in the gene cluster to activate the β-lactam product (Kinscherf and Willis, 2005; Levi and Durbin, 1986). Because of the absence of a specifically encoded protease in the nocardicin cluster, the possibility that M2 might append a moiety such as N^5-hydroxyornithine or ornithine, and that cleavage of the first two residues might be autocatalytic, has been considered (Gunsior et al., 2004).

Another anomaly of nocardicin A biosynthesis considered in this study is the conversion of L-pHPG to its D antipode at two centers in nocardicin A when only one epimerase is found in its synthetase. D-amino acids are usually incorporated into nonribosomal products by the action of an epimerization domain, located immediately downstream of the T domain in a typical NRPS module, as seen in module 3 of NocA (Linne and Marahiel, 2000). Only rarely are D-amino acids substrates for direct activation by A domains in NRPSs, although cyclosporin, fusaricidin, and leinamycin synthetases have been shown to activate D-alanine for direct incorporation into the product peptide (Hoffmann et al., 1994; Li and Jensen, 2008; Tang et al., 2007; Zocher et al., 1986). An alanine racemase, located in the cyclosporin gene cluster, has been characterized, whereas the fusaricidin and leinamycin gene clusters do not appear to contain a similar racemase (Hoffmann et al., 1994; Li and Jensen, 2008). A third strategy for the addition of D-amino acids into nonribosomal products involves action of C domains that also catalyze epimerase activity. These dual C/E domains are observed in arthrofactin, syringomycin, syringopeptin, and ramoplanin synthetases (Balibar et al., 2005) and are characterized by an extended histidine sequence at the N terminus, HH(l/L) xxxxDG, in addition to the conventional His motif (HHxxxDG) known to be essential for condensation of the peptide bond. A fourth, and thus far unique, example for epimerization has been found in the PchE protein of pyochelin synthetase. Between the A8 and A9 motifs of the cysteine-activating A

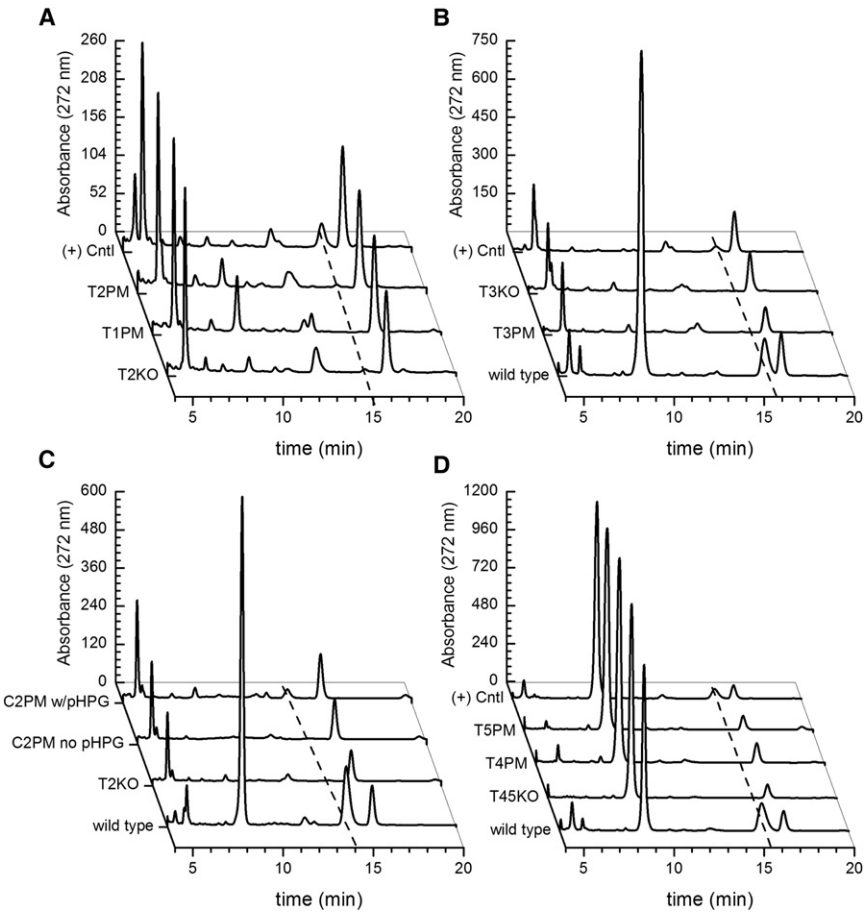

Figure 5. HPLC Chromatograms of the Culture Supernatants from Each Set of *N. uniformis* Mutants Prepared in This Study Compared to Wild-Type, Analyzed after Fermentation for 5 Days

In these studies, the culture medium was supplemented with 0.5 mM L-pHPG, except where noted otherwise. The peak for nocardicin A is indicated by a dashed line.

(A) Chromatograms for deletion mutant T2KO, T domain mutants *nocA* S626A (T1PM) and *nocA* S1671A (T2PM), and the native knock-in experiment (represented as (+) Cntl), in which the native *nocA* sequence was restored.

(B) Chromatograms for deletion mutant T3KO, T domain mutant *nocA* S2782A (T3PM), and a positive control (represented as (+) Cntl), in which the native *nocA* sequence was restored compared to wild-type *N. unformis*.

(C) Chromatograms for mutant *nocA* R828G, located in the C2 domain are compared to the deletion mutant T2KO and wild-type *N. unformis*. For comparison, the chromatogram of the C2PM mutant, grown without pHPG supplementation, is shown.

(D) Chromatograms for deletion mutant T45KO, T domain mutants *nocB* S571A (T4PM) and *nocB* S1648A (T5PM), and a positive control (represented as (+) Cntl), in which the native *nocB* sequence was restored, are plotted.

domain exists an approximately 300-amino-acid insert resembling a methyltransferase domain. However, mutagenesis of histidine 1204 to alanine was shown to eliminate racemization of the tethered benzoylcysteine intermediate, suggesting that this insert functions as an epimerization domain (Patel et al., 2003).

Because of the absence of a second E domain, a dual C/E domain or a racemase in the gene cluster and the pyochelin precedent, the discovery of an extended histidine motif, HHTCAPEDG, between Motifs A5 and A6 of the A5 domain posed the question of whether this could be an epimerization motif. In addition, the lack of epimerization domains in the initiation modules of several glycopeptides was of interest (Sosio and Donadio, 2006). Alignment of the A1 domains of chloroeremomycin, balhimycin, A47934, complestatin, and teicoplanin synthetases shows conservation at the second histidine and aspartate residues, which correspond to the catalytic residues of epimerization motifs. (Figure 6) All the mutants prepared in the A5 domain—H1411A, H1412A, E1417A, and D1418A—produced nocardicin A at levels virtually unchanged compared to that of the native control (Table 1; Figure S1 available online), indicating that this motif is not essential for formation of the peptide precursor and thus is unlikely to be involved in an epimerization reaction.

Several hypotheses can account for conversion from the L- to the D-antipode of the C-terminal pHPG in nocardicin A biosynthesis. The possibility that this activity is cryptically embedded in nocardicin synthetase has not been eliminated. Second, a racemase encoded outside the known gene cluster may have a role. Other than D-Ala and D-Glu, known components of bacterial peptidoglycan, other D-amino acids have been found in a variety of prokaryotes, along with the discovery of a broad-spectrum racemase (Lam et al., 2009). Finally, synthetic studies have shown the C5 position to be base labile, suggesting that epimerization at C5 might occur in tandem with amide deprotonation and β-lactam formation (Salituro and Townsend, 1990).

SIGNIFICANCE

Two NRPS enzymes, NocA and NocB, act centrally in the biosynthesis of the nocardicin monocyclic β-lactam antibiotics. Bioinformatic comparisons of these proteins reveal several anomalies as, for example, extended repeat sequences and atypical histidine motifs that call into question whether these multidomain enzymes are conventionally functional, particularly modules 1 and 2 of NocA, and what their roles are in the biosynthesis. While NocA and NocB comprise five modules, the last three would appear sufficient to generate the tripeptide core of this antibiotic family. In beginning to answer these questions, we found that expression of these peptide synthetases in *Escherichia coli* and *Streptomyces* hosts proved intractable. A two-step, in vivo gene replacement strategy reported here enabled the preparation of a series of point mutants to evaluate the roles of

Figure 6. Comparison of Extended His Motif Observed in the NocB A5 Domain to the Initiation A Domains of NRPSs for Chloroeremomycin, Balhimycin, Complestatin, A47934, and Teicoplanin

Identical residues are shaded. The conserved histidine and acidic residues of the extended His motif are noted with an asterisk. Numbers indicate amino acid residues from the N terminus of the protein. Gene Bank accession numbers are as follows: chloroeremomycin (CepA), AJ223999; balhimycin (BpsA), Y16952; complestatin (ComA), AF386507; A47934 (StaA), U82965; and teicoplanin (TcpA), AJ605139.

individual residues and modules in NocA+NocB without disruption of promoter or regulatory elements. The use of chemical complementation of an ancillary precursor biosynthetic gene in the critical second "knock-in" step proved to be robust and reliable and may be more widely useful in analogous studies of natural product biosynthetic pathways. Using this method the phosphopantethiene attachment site in each of the five T domains was specifically mutated to alanine and, in each instance, proved essential to antibiotic production. An uncommon HHxxxDR catalytic motif in C2 was mutated to the canonical HHxxxDG, but both were equivalently active in nocardicin A biosynthesis. A similar extended His motif in A5, potentially responsible for the cryptic C-terminal epimerization characteristic of all known nocardicins, was unaffected by mutation. These data suggest that all modules of NocA and NocB are required for nocardicin G synthesis that and these unusually modified proteins, in fact, appear to function normally. Their interplay is now more sharply defined, but more complex questions remain in the full orchestration of precursor peptide assembly, editing, C-terminal epimerization, and β-lactam formation.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmid Construction

Strains and plasmids and oligonucleotide primers used in PCR amplification and Quick Change reactions are included in the Supplemental Information. We performed PCR amplification reactions using previously prepared cosmid or plasmid templates with *Pfu* (Stratagene, LaJolla, CA) DNA polymerase and using primers purchased from IDT-DNA (Coralville, IA). Amplified products were typically subcloned into pT7B3 (EMD Biosciences, Gibbstown, NJ) or pUC19 using standard protocols. We performed further manipulation of DNA for plasmid preparation using standard procedures (Maniatis et al., 1982).

Point mutations were engineered into plasmids of interest using QuickChange (Stratagene) (Weiner and Costa, 1995). DNA polymerases *Pfu* Ultra or *Pfu* Turbo (Stratagene) were used combined with a manual "hot start" PCR amplification protocol. Following PCR, products were treated with *Dpn* I to remove template DNA. DNA was concentrated using Pellet Paint (EMD Biosciences) before transformation into *E. coli* XL1-Blue by electroporation. The construction of all plasmids was confirmed by DNA sequencing at the Biosynthesis and Sequencing Facility, Johns Hopkins Medical School, Baltimore MD.

Culture and Assay Conditions

Nocardia uniformis subsp. *tsuyamanesis* ATCC 21806 (wild-type strain), producer of the antibiotic nocardicin A, was maintained on ISP2 solid medium (Difco Laboratories, Detroit, MI) at 28°C. Seed cultures were prepared in trypic soy broth (TSB) medium (Difco Laboratories) and incubated at 28°C, with shaking, for inoculation of fermentation medium (containing, per liter, 10 g peptone, 4 g yeast extract, 10 g KH_2PO_4, 4 g NaH_2PO_4, 2.4 g $MgSO_4$, 2 g glycine, 2 ml trace minerals, 20 g soluble starch, 1 g tyrosine, and 75 mg L-methionine) as previously described (Reeve et al., 1998). During the 5- to 7-day growth period for *N. uniformis*, aliquots of the culture supernatants were sampled and assayed for the production of nocardicin A and related precursors by bioassay versus *E. coli* ESS and quantitative HPLC. 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 application of 200 μl of culture supernatant to paper discs placed on solid Luria broth medium inoculated with *E. coli* ESS. Plates were analyzed for developed zones of antibiosis after incubation at 37°C overnight. We performed quantitative analyses of nocardicin A production using an Agilent 1100 HPLC system equipped with a diode array detector. Filtered supernatants (nylon, 0.45 μm) were injected directly on to a Luna C18(2) column (250 mm × 46 mm) (Phenomenex, Torrance, CA), using an isocratic mobile phase: 90:10 water:acetonitrile with 0.08% trifluoroacetic acid at a flow rate of 1 ml/min. Analytes were detected by absorbance at 272 nm and were quantified by comparison to a standard curve.

Protoplast Transformation of *N. uniformis* and Preparation of Mutants

Before protoplast transformation into *N. uniformis,* the constructed pULVK2 (*Nocardia–E.coli*) shuttle vectors were transformed into *E. coli* JM110 cells to provide nonmethylated DNA. The protocol for PEG-mediated transformation of *N. uniformis* with pULVK2 vectors has been described previously (Kelly and Townsend, 2005). *N. uniformis* pULVK2 vector transformants were selected by a 200 μg/ml kanamycin and 100 μg/ml apramycin or 25 μg/ml thiostreptone overlay. The Apr^r Kan^r or TS^r Kan^r phenotype was confirmed by plating on ISP2 solid medium containing 200 μg/ml kanamycin and 100 μg/ml apramycin or 25 μg/ml thiostreptone. Transformants were usually subjected to three rounds of nonselective plating on ISP2 to obtain double crossover mutants. However, selective propagation on ISP2, using 100 μg/ml apramycin, was used after the transformation of the T2KO, T3KO, and T45KO mutant *N. uniformis* strains. Putative double crossover mutants were identified by their predicted phenotype and confirmed by Southern analysis and sequencing of genomic DNA.

Plasmid Construction for *nocF::apr* Disruption Mutant

The *nocF* gene (1,035 bp) was amplified using primers nocF_For and nocF_Rev and subcloned into pT7B3 (EMD Biosciences). The resulting pT7B3/*nocF* plasmid was linearized by digestion with *Bbs I* and blunted with Klenow. The *apr* insert was prepared by digestion from plasmid pT7B3/*apr* and ligated into the linearized *nocF* plasmid. The disrupted gene cassette *nocF*::*apr* (3.1 kbp) was ligated into the *EcoR I* site of pULVK2 to generate pULVK2/*nocF*::*apr*.

Plasmid Construction for Two-Step Gene Replacement Experiments

Detailed procedures for the cloning of plasmids for both the step 1 deletion mutants and the step 2 knock-in mutants are described in the Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chembiol.2011.10.020.

ACKNOWLEDGMENTS

This work was supported by NIH Grant AI014937. We thank Dr. D.M. Bartley for construction of plasmid pCRBlunt/*T1PM* and Ms. Anne Rigby for help constructing the figures in the manuscript. Drs. T. Bililign and R.F. Li are thanked for their technical expertise and the Greenberg group at Johns Hopkins

University for the use of their phosphoimager and providing space and support for radiochemical experiments.

Received: May 31, 2011
Revised: August 12, 2011
Accepted: October 24, 2011
Published: February 23, 2012

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