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Deciphering Deazapurine Biosynthesis: Pathway for Pyrrolopyrimidine Nucleosides Toyocamycin and Sangivamycin

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SUMMARY

Pyrrolopyrimidine nucleosides analogs, collectively referred to as deazapurines, are an important class of structurally diverse compounds found in a wide variety of biological niches. In this report, a cluster of genes from *Streptomyces rimosus* (ATCC 14673) involved in production of the deazapurine antibiotics sangivamycin and toyocamycin was identified. The cluster includes toyocamycin nitrile hydratase, an enzyme that catalyzes the conversion of toyocamycin to sangivamycin. In addition to this rare nitrile hydratase, the cluster encodes a GTP cyclohydrolase I, linking the biosynthesis of deazapurines to folate biosynthesis, and a set of purine salvage/biosynthesis genes, which presumably convert the guanine moiety from GTP to the adenine-like deazapurine base found in toyocamycin and sangivamycin. The gene cluster presented here could potentially serve as a model to allow identification of deazapurine biosynthetic pathways in other bacterial species.

INTRODUCTION

Pyrrolopyrimidine functional groups have been found in many secondary metabolites produced by various strains of *Streptomyces*. The molecules in which they are found range from cofactors involved in the biosynthesis of tetracycline antibiotics (McCormick and Morton, 1982; Miller et al., 1960) and DNA repair (Kuo et al., 1989) to compounds with herbicidal, antibacterial, antifungal, and antineoplastic activities (Isaac et al., 1991; Nishioka et al., 1991; Suhadolnik, 1970). The breadth of structural diversity in deazapurine-containing family of compounds is remarkable (Figure 1A). The strong resemblance between pyrrolopyrimidine nucleosides and purines suggest that these molecules could disrupt nucleic acid metabolism and kinase-related signaling cascades and be of potential use as therapeutic agents. The ability of deazapurines to enter nucleic acid pools of cells is well documented (Ritch and Glazer, 1982; Suhadolnik et al., 1967, 1968; Tavitian et al., 1969; Uematsu and Suhadolnik, 1973). Despite the ubiquity and potential usefulness of these molecules both as tools and therapies, their biosynthetic pathways have remained elusive.

The outlines of the biosynthetic steps leading to these molecules were established by elegant radiotracer experiments (summarized in Figure 1B) in which the deazapurine base was shown to be derived from a purine precursor (Smulson and Suhadolnik, 1967; Suhadolnik and Uematsu, 1970; Uematsu and Suhadolnik, 1970) by way of a process mirroring transformations involved in the formation of folate (Burg and Brown, 1966; Reynolds and Brown, 1964) and riboflavin (Bacher and Mailänder, 1973). When [2-^{14}C]- or [8-^{14}C]-labeled adenine and guanine were fed to *S. rimosus* cells in culture, the isolated toyocamycin was shown to have retained label at carbon-2 but lost the label at carbon-8. Intriguingly, the carbons 5 and 6 of the pyrrole moiety, and the cyano carbon appeared to derive from the C1′, C2′, and C3′ of the proffered ribose, respectively.

Though the exact nature of the enzymatic transformations that underlie the biosynthesis of deazapurines remains to be established, two studies have provided substantial hints. First, Suhadolnik and coworkers isolated a GTP cyclohydrolase protein from cells producing sangivamycin, whose activity paralleled the appearance of sangivamycin in the growth medium (Elstner and Suhadolnik, 1971, 1975); the protein was shown to produce a neopterin-like molecule, much like the product of GTP cyclohydrolase I (GCH I) of *E. coli,* which had been characterized by Brown and colleagues (Burg and Brown, 1968). The pyrimidine ring of sangivamycin or toyocamycin, however, resembles adenine and not guanine, suggesting that additional transformations must be involved, if the protein described in these early studies indeed catalyzes the first step in the biosynthesis of these deazapurines. The second intriguing observation was the description of a toyocamycin nitrile hydratase (TNHase) activity involving addition of a water molecule across the nitrile moiety of toyocamycin to produce sangivamycin (Uematsu and Suhadolnik, 1974, 1975; Figure 1B).

The hypermodified tRNA base queuosine (Kasai et al., 1975) is structurally homologous to the deazapurine base of pyrrolopyrimidine nucleosides that are produced by *Streptomyces*. Recently, four genes (*queC, queD, queE*, and *queF*) encoding proteins required for the biosynthesis of queuosine have been identified in *Bacillus subtillis* (Bai et al., 2000; Van Lanen et al., 2005). The importance of the four *B. subtillis* genes in the early steps was verified by gene knockout experiments. Though bioinformatics studies have revealed the potential biochemical

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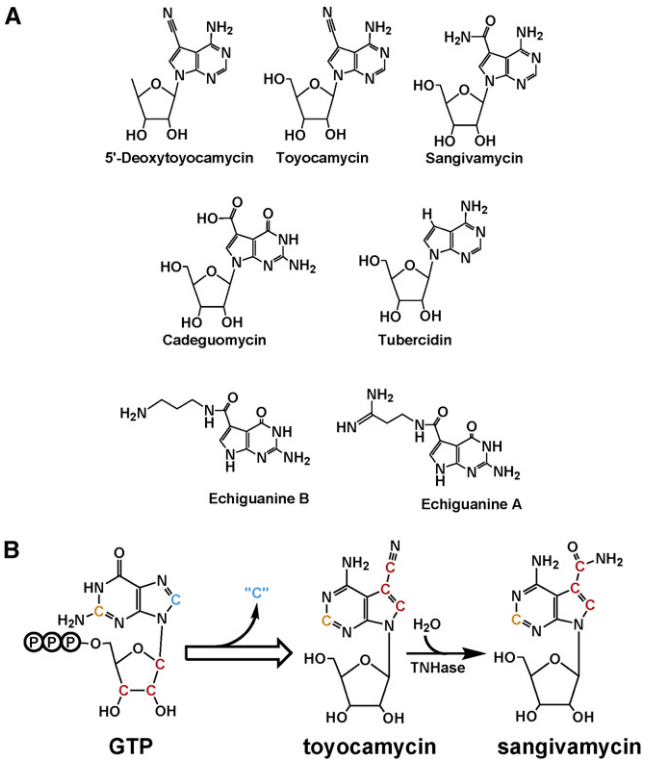

Figure 1. Deazapurines Are a Diverse Class of Purine-Based Secondary Metabolites

(A) Deazapurine secondary metabolites produced by *Streptomyces*.

(B) Deazapurine secondary metabolites are derived from purines in a pathway that retains C-2, as well as C-1′, C-2′, and C-3′ of the starting purine, but loses the C-8. Toyocamycin nitrile hydratase catalyzes the hydration of toyocamycin to produce sangivamycin. *S. rimosus* produces both sangivamycin and toyocamycin.

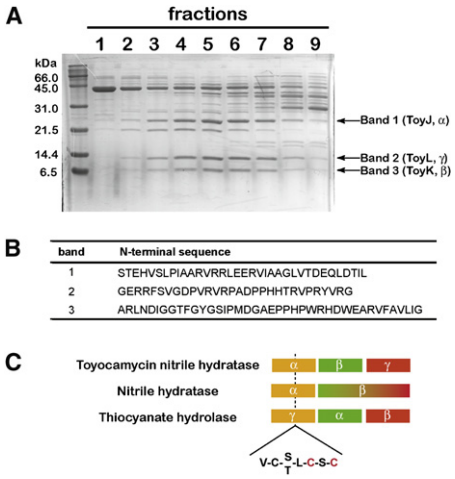

Figure 2. Identification of Toyocamycin Nitrile Hydratase of *S. rimosus*

SDS-PAGE analysis of proteins contained in consecutive fractions eluting from a HiPrep 16/60 Sephacryl S-200 analytical size exclusion column during purification of toyocamycin nitrile hydratase activity from *S. rimosus* (A). The intensities of protein bands 1, 2, and 3 correlate with TNHase activity over the range of fractions shown (Figure S1). N-terminal sequences of bands 1, 2, and 3 (B) were obtained by Edman degradation. TNHase is a three-subunit nitrile hydratase protein (C) with sequence similarity to nitrile and thiocyanate hydratase proteins. Each of these proteins has a metal ion in the active site (cobalt or iron), which interacts with two posttranslationally modified cysteine residues (shown in red).

functions for these proteins, in vitro characterization has only been carried out for QueF, which has been shown to catalyze the NADPH-dependent conversion of 7-cyano-7-deazaguanine (see preQ$_0$ in Figure 2B) to 7-aminomethyl-7-deazaguanine (preQ$_1$) (Lee et al., 2007; Van Lanen et al., 2005). However, the reaction(s) catalyzed for the remaining three have yet to be established. Recent X-ray crystallographic studies of QueC (Cicmil and Huang, 2008), QueD (Cicmil and Shi, 2008), and QueF (Swairjo et al., 2005) may provide additional insights into their biochemical function.

Streptomyces tend to organize genes that are responsible for the biosynthesis of secondary metabolites in distinct clusters (Hopwood, 1997); therefore, identification of a cluster of genes involved biosynthesis of any deazapurine secondary metabolite produced by *Streptomyces* could potentially inform on the biosynthetic pathway for these compounds. Because the genome sequence of *S. rimosus* is not available, identification of the genes responsible for biosynthesis of these secondary metabolites required a different approach. In this work we utilized forward genetics methods to identify the gene for TNHase in *S. rimosus*. Additional biochemical and bioinformatics analysis leave little doubt that this cluster is indeed the elusive deazapurine secondary metabolite biosynthesis cluster.

RESULTS

We undertook a forward genetic approach to isolate a cluster of genes that is responsible for the biosynthesis of deazapurines in *Streptomyces*, which are known to produce a large variety of deazapurine-containing secondary metabolites in the stationary phase of growth (Suhadolnik, 1970). *S. rimosus* (ATCC strain 14673) was chosen as the model organism in this study because it is known to produce two compounds: sangivamycin and toyocamycin (Elstner and Suhadolnik, 1971; Suhadolnik, 1970; Suhadolnik and Uematsu, 1970; Uematsu and Suhadolnik, 1970, 1974). Furthermore, Suhadolnik and colleagues had identified a TNHase in this organism (Uematsu and Suhadolnik, 1974, 1975); we reasoned that TNHase would be a good marker for the location of the biosynthetic cluster as *Streptomyces* tend to cluster the genes for the biosynthesis of secondary metabolites (Hopwood, 1997).

Isolation of the TNHase-Bearing Cluster from *S. rimosus* (ATCC 14673)

TNHase was partially purified from a 3-day-old culture growth by a combination of ammonium sulfate precipitation and seven chromatographic steps, which included anion exchange, hydrophobic interactions, and hydroxyapatite and size exclusion chromatography. At each step of the purification, the TNHase activity present in the fractions was monitored by an HPLC assay, which followed the conversion of toyocamycin to sangivamycin. Though SDS-PAGE of the resulting partially purified

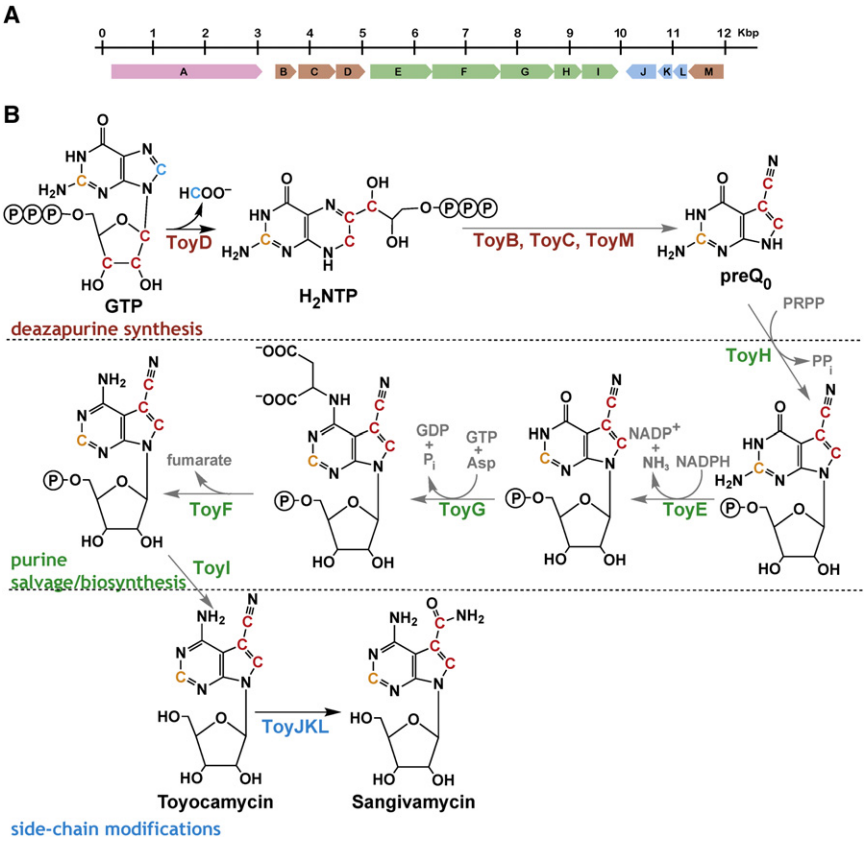

Figure 3. Toyocamycin and Sangivamycin Are Produced by a Cluster of Genes in *S. rimosus*

Organization of gene cluster (A) and putative biosynthetic pathway for production of sangivamycin and toyocamycin (B). Radiotracer experiments (Suhadolnik and Uematsu, 1970) have shown that during the conversion of purines to toyocamycin, carbon 2 (orange) is retained and carbon 8 (blue) is lost and that the pyrrole carbons in toyocamycin (red) are derived from the ribose on the nucleoside precursor (Uematsu and Suhadolnik, 1970.) Predicted functions of gene products are based on hits in PSI-BLAST analysis. The putative functions of the proteins are as follows: *toyA*, LuxR transcriptional regulator; *toyB*, 6-pyruvoyltetrahydropterin synthase; *toyC*, radical SAM family protein; *toyE*, GMP reductase; *toyF*, adenylosuccinate lyase; *toyG*, adenylosuccinate synthetase; *toyH*, phosphoribosylpyrophosphate transferase; *toyI*, phosphatase; *toyK*, QueC-related protein. Functions of ToyD (GTP cyclohydrolase I) and ToyJKL (toyocamycin nitrile hydratase) were demonstrated biochemically in this study.

protein eluting from the final column (Figure 2A) revealed a complex mixture, the intensities of three bands appeared to correlate with the magnitude of the toyocamycin→sangivamycin activity that was observed in each fraction (see Figure S1 available online). Therefore, we reasoned that these proteins were likely to comprise the TNHase protein that we had sought to purify.

The N-terminal amino acid sequences (≥30 amino acids) of each of the three bands were obtained by Edman degradation (Figure 2B). The 40-amino acid read obtained from the N terminus of band 3 permitted degenerate primers corresponding to N- and C-terminal portions of the sequence to be designed and used to amplify by PCR a 78 bp region from the *S. rimosus* genomic DNA. Cloning and sequencing of the PCR product revealed the genomic sequence encoding the amino acids 9–34 of band 3. A synthetic DNA oligomer containing this 78 bp sequence was used to probe a cosmid library of *S. rimosus* for the corresponding gene. Cosmids that hybridized to the probe were isolated, digested into smaller fragments by various restriction endonucleases, and subcloned. Sequencing of the subcloned DNA fragments revealed overlapping sequences, which were assembled into a 14.5 kbp stretch of DNA that contains the toyocamycin and sangivamycin biosynthesis cluster of *S. rimosus*, including three subunits that encode toyocamycin nitrile hydratase observed in the purification.

The open reading frames that encode the deazapurine biosynthesis cluster in this organism were identified and annotated manually (Figure 3A) . Potential ORFs were identified by the presence of potential start codons, ATG, GTG, and TTG, preceded by reasonable ribosome binding sites (containing a series of adenosine and guanosine bases), and in-frame stop codons. The putative functions of each *orf* were gleaned from analyzing the results of PSI-BLAST (Altschul et al., 1997) searches of the sequences against bacterial protein databases.

Pyrrolopyrimidine Nucleoside Antibiotic Biosynthesis Gene Cluster of *S. rimosus*

Sequence analysis revealed at least 13 putative ORFs clustered together, including the TNHase gene (Figure 3A). The ORFs that comprise the gene cluster are designated *toyABCDEFGHIJKLM*; the ORFs encoding protein bands 1, 2, and 3 of the TNHase protein isolated from *S. rimosus* are designated *toyJ*, *L*, and *K*, respectively. The ORFs in this cluster are listed in Table 1.

In addition to the TNHase genes, this group also includes a GCH I homolog, which has long been suspected to be one of the key enzymes required for the biosynthesis of deazapurines in *Streptomyces*. This result is completely in accord with the work of Suhadolnik and coworkers showing appearance of GCH I in cells during production of sangivamycin (Elstner and Suhadolnik, 1971, 1975). The cluster also includes genes encoding a putative 6-pyruvoyltetrahydropterin synthase (PTPS), a member of the radical SAM protein family (Sofia et al., 2001) and a member of the ExsB protein family; similar proteins (QueD, QueE, and QueC, respectively) have previously been shown to be involved in queuosine biosynthesis (Gaur and Varshney, 2005; Reader et al., 2004). Most importantly, however, our data show how the organism converts a guanine-based purine to an adenine-based deazapurine. This task appears to be accomplished by a group of purine salvage/biosynthesis proteins (phosphoribosylpyrophosphate transferase, GMP reductase, and adenylosuccinate synthetase and lyase homologs).

Table 1. ORFs Encoded by the Sangivamycin/Toyocamycin Biosynthesis Cluster of *S. rimosus*

ORF	Ribosome Binding Site	Start (stop)	% (G + C)	MW (residues)	Predicted/Known Function
toyA	AAAGA	ATG (TGA)	74.5	101,436 (961)	LuxR transcriptional regulator
toyB	GGGGAA	TTG (TGA)	65.4	15,114 (131)	Pyruvoyl-tetrahydropterin synthase
toyC	GGGAAGGGG	GTG (TGA)	71.1	26,743 (243)	Radical SAM
toyD	AGGAGAG	ATG (TGA)	71.2	22,140 (200)	GCH I (verified, this study)
toyE	AGAGAAAGAA	GTG (TGA)	74.0	39,492 (384)	GMP reductase
toyF	GGAGG	GTG (TGA)	72.6	49,005 (452)	Adenylosuccinate lyase
toyG	AAGGAGGA	ATG (TGA)	73.7	36,855 (351)	Adenylosuccinate synthetase
toyH	AAGGAG	ATG (TGA)	73.3	18,859 (176)	Phosphoribosyl-pyrophosphate transferase
toyI	GGAGG	GTG (TGA)	75.3	25,466 (241)	Haloacid dehalogenase superfamily
toyJ	GGAGGAGAAA	ATG (TGA)	71.8	21,215 (195)	Nitrile hydratase, α subunit (verified, this study)
toyK	AGAAGGGGA	GTG (TGA)	71.2	10,105 (91)	Nitrile hydratase, γ subunit (verified, this study)
toyL	GGGAAA	ATG (TGA)	66.3	11,543 (103)	Nitrile hydratase, β subunit (verified, this study)
toyM	AGGAGG	TTG (TGA)	69.2	25,583 (238)	ExsB family

Activity of Heterologously Expressed *S. rimosus* TNHase

To confirm that the *toyJKL* genes indeed encode the TNHase protein, which was the basis for the isolation of the gene cluster, these genes were cloned into vectors with compatible origins of replication and transformed into *E. coli* BL21(DE3) cells. Cell lysates of *E. coli* containing exogenously expressed TNHase subunits ToyJ, K, and L were incubated with toyocamycin and analyzed by HPLC. Control incubations of cell lysate from *E. coli* transformed with the corresponding expression vectors alone were performed as well. The HPLC analysis revealed conversion of toyocamycin to sangivamycin by cell lysate from cells containing expression constructs for ToyJ, K, and L (Figure 4). By contrast, TNHase activity was not observed in lysates of vector only controls. Moreover, in preliminary studies, purified TNHase (ToyJKL) exhibits the toyocamycin→sangivamycin activity (data not shown).

The three-subunit TNHase protein isolated in the course of these studies is unique in a number of respects. First, it catalyzes hydration of a cyanide moiety appended to a complex organic molecule, substantially expanding the repertoire of substrates for hydration, and may represent a mild alternative to chemical hydration of cyanide-containing compounds to nitriles. Second, TNHase contains three subunits, whereas other nitrile hydratase (NHase) enzymes studied to date contain only two (Kobayashi et al., 1992; see Figure 2C). BLAST sequence analysis reveals that ToyJ is similar to the α subunits found in other NHase enzymes. However, ToyL and ToyK show identity with the N- and C-terminal halves, respectively, of the β subunit of NHase enzymes. Curiously, thiocyanate hydrolase (SCNase) from *Thiobacillus thioparus* THI115, which catalyzes the conversion of thiocyante to carbonyl sulfide and ammonia, contains three subunits (Katayama et al., 1992), which are homologous to those that comprise *S. rimosus* TNHase. The α and β subunits of *T. thioparus* SCNase share similarities with the N- and C-terminal halves of the β subunit of typical NHase enzymes (Katayama et al., 1998); the γ subunit of thiocyanate hydrolase is homologous to the α subunit of NHase and TNHase (see Figure 2C).

The rarity of NHase enzymes is a consequence not only of the fact that they catalyze hydration of an uncommon biological moiety, an alkyl cyanide, but they do so using either a mononuclear nonheme iron or noncorrinoid cobalt (Banerjee et al., 2002). The metal ion is bound to the active site in an unusual mononuclear six-coordinate metal site, and sequence analysis reveals a signature amino acid sequence Val-Cys_1-Ser/Thr-Leu-Cys_2-Ser-Cys_3 in the α subunit; in all cases examined to date, two of the three Cys residues (Cys_2 and Cys_3) are modified to cysteine sulfinic acid (Cys_2-SO_2H) and cysteine sulfenic acid (Cys_3-SOH), respectively (Arakawa et al., 2007; Nagashima et al., 1998; Song et al., 2007). Iron or cobalt preference of the proteins is thought to be dictated as follows: proteins that have a Ser residue between Cys_1 and Cys_2 bind iron, where as the presence of Thr directs binding of cobalt (Banerjee et al., 2002; Payne et al., 1997). ToyJ encodes the amino acid sequence V-C-**T**-L-C-S-C; therefore, we presume that it is a cobalt-type nitrile hydratase. Additional studies on this fascinating protein are currently in progress.

Activity of Heterologously Expressed GCH I Homolog from *S. rimosus*

To determine if *toyD* encodes a GCH I homolog that catalyzes the canonical reaction, the gene was cloned, ToyD was overexpressed, purified, and assayed for its GCH I activity. GCH I is a zinc-containing protein (Auerbach et al., 2000; Kaiser et al., 2002) and catalyzes the conversion of GTP to 7,8-dihydroneopterin triphosphate (H_2NTP). Recombinant purified *S. rimosus* ToyD was shown to bind 0.76 M of zinc per mole of monomer.

The conversion of GTP to 7,8-dihydroneopterin triphosphate (H_2NTP) is readily monitored by UV-visible spectrophotometry. Changes in the spectral properties of GTP upon incubation with ToyD are shown in Figure 5A. Incubation with ToyD leads to a decrease in absorbance at 253 nm due to GTP, with a concomitant increase at 330 nm, which is consistent with conversion of GTP to H_2NTP (Pfleiderer, 1985). The turnover number for this reaction was determined to be ~0.4 min^{-1} (at pH 8.0). Though low, this value is consistent with the relatively low (0.2–2.5 min^{-1}) turnover numbers that have been observed with GCH I proteins studied to date (Bracher et al., 1999; El Yacoubi et al., 2006; Rebelo et al., 2003). The formation of H_2NTP was confirmed by LC/MS analysis of the reaction (Figure 5B). In these experiments, we compared the mass and retention time for the product produced by ToyD

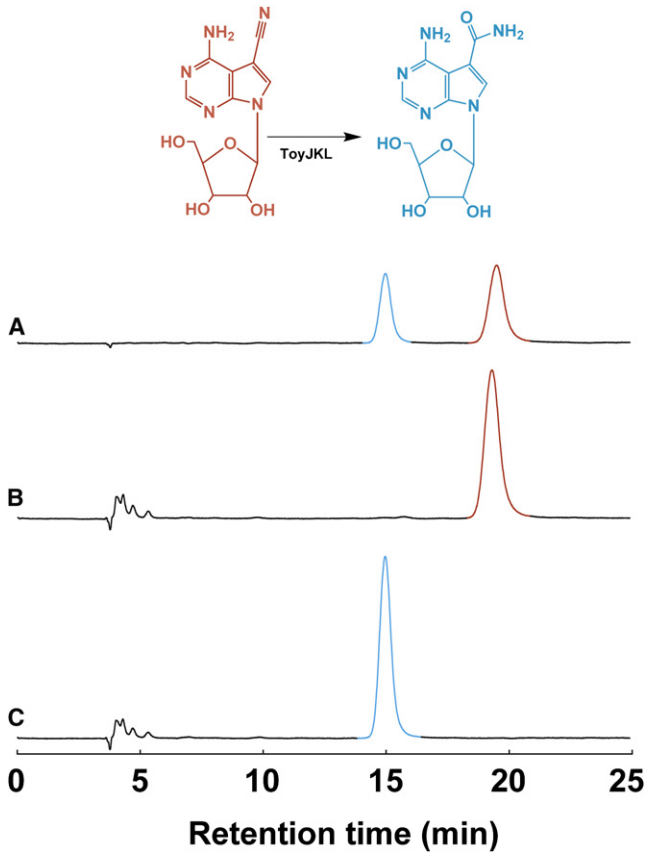

Figure 4. ToyJKL Encode Toyocamycin Nitrile Hydratase
Trace (A) is a standard mixture of sangivamycin and toyocamycin. The activity was assayed in crude extracts from *E. coli* strains that contained either the *toyJKL* genes (C) or the corresponding empty vectors (pACYCDuet-1 and pET29a) (B).

with that produced with *E. coli* GCH I (FolE, B2153). Under the conditions of the assays and analysis, H_2NTP is oxidized to neopterin triphosphate, as has been observed in the past (El Yacoubi et al., 2006). Extracted ion chromatograms of the GCH I reactions (in the negative ion mode) reveal a peak with product with *m/z* of 492 amu (Figure 5B), which elutes at an identical position as the product of ToyD. Furthermore, when equal quantities of the *E. coli* GCH I and *S. rimosus* ToyD reaction mixtures are combined and injected, a single peak is observed. Finally, when enzyme is excluded, the H_2NTP is not observed. Collectively, these results confirm that ToyD is indeed a GCH I homolog. Additional control experiments demonstrated that, as with GCH I, ToyD does not catalyze cyclohydrolase chemistry with GDP, GMP, ATP, ADP, or AMP (data not shown). To confirm that the GCH I activity observed in purified recombinant ToyD, exogenously expressed in *E. coli*, is not due to contaminating *E. coli* FolE, samples of the two purified proteins used in this study (ToyD and FolE) were analyzed by SDS-PAGE. Because FolE has a calculated molecular weight ~2.7 kDa greater than ToyD, the two are resolved by SDS-PAGE to reveal that the ToyD used in these experiments is not contaminated by *E. coli* FolE (Figure S2).

Whether ToyD is the same protein that Suhadolnik and coworkers purified from the organism in 1975 (Elstner and Suhadolnik, 1975) cannot be known for certain, as *S. rimosus* may encode multiple open reading frames that catalyze similar or identical reactions, as we have shown in the past for GTP cyclohydrolase II in *S. coelicolor* (Spoonamore and Bandarian, 2008; Spoonamore et al., 2006). Therefore, at present, we cannot rule out the presence of additional homologs of GCH I in *S. rimosus*.

DISCUSSION

We have identified the gene encoding the toyocamycin nitrile hydratase enzyme in *S. rimosus* and have sequenced a series of adjacent putative ORFs comprising what appears to be a cluster of genes for the biosynthesis of the pyrrolopyrimidine nucleosides sangivamycin and toyocamycin. A pathway for conversion of GTP to sangivamycin or toyocamycin is depicted in Figure 2B. The biosynthesis is proposed to occur in three phases: in the first phase, the starting purine nucleotide is tailored to the deazapurine base, the second phase involves conversion of the guanine-like base to an adenine-like base, and modifications in the 7-position occur in the third phase.

Our working model is that first phase in the biosynthesis of toyocamycin is achieved through four steps catalyzed by ToyD, ToyB, ToyC, and ToyM. According to this scheme, ToyD catalyzes the conversion of GTP to H_2NTP; in this article we show that ToyD has the expected enzymatic activity. Though the involvement of a GCH I protein in deazapurine biosynthesis has been hypothesized previously (Elstner and Suhadolnik, 1975; Reader et al., 2004), this report is the first demonstration that a GCH I protein with canonical activity is colocalized with the genes for the biosynthesis of a deazapurine. The additional steps involved in the transformation of H_2NTP to the deazapurine $preQ_0$ occur through the action of the ToyB, ToyC, and ToyM trio. The nature of the chemistry and order in which these enzymes act remain to be established. However, homologs of ToyB, ToyC, and ToyM are involved in the biosynthesis of $preQ_0$, which is a precursor the hypermodified base queuosine (Reader et al., 2004). The chemical transformations catalyzed by each of the three remain to be established; however, the presence of the same three genes in the toyocamycin biosynthesis pathway provides a universal paradigm for the biosynthesis of deazapurine-containing compounds in nature. The origin of the cyano nitrogen is not known. Though the most parsimonious path is one where it is derived from N-7 of the starting purine, we cannot exclude the possibility that it is eliminated and the cyano nitrogen is derived from another source.

We note in passing that the gene encoding the radical SAM homolog in the toyocamycin biosynthesis pathway encodes a TGA stop codon 30 bp from the GTG, which we have assigned as the start codon for the gene. Read-through of TGA stop codons, generally by insertion of a selenocysteine, is rare, but precedented. To our knowledge, *Streptomyces* species have not been shown to produce selenoproteins; moreover, the completed genome sequences of *S. avermitilis* and *S. coelicolor* lack genes encoding selenocysteyl tRNA, or other homologs of the bacterial selenocysteine incorporation systems. At this point, one cannot rule out that the selenocysteine incorporation pathways have diverged. Although PSI-BLAST searches reveal few examples of homologs that have similarity over the first ~11 amino acids of ToyC, significant similarity, including in the

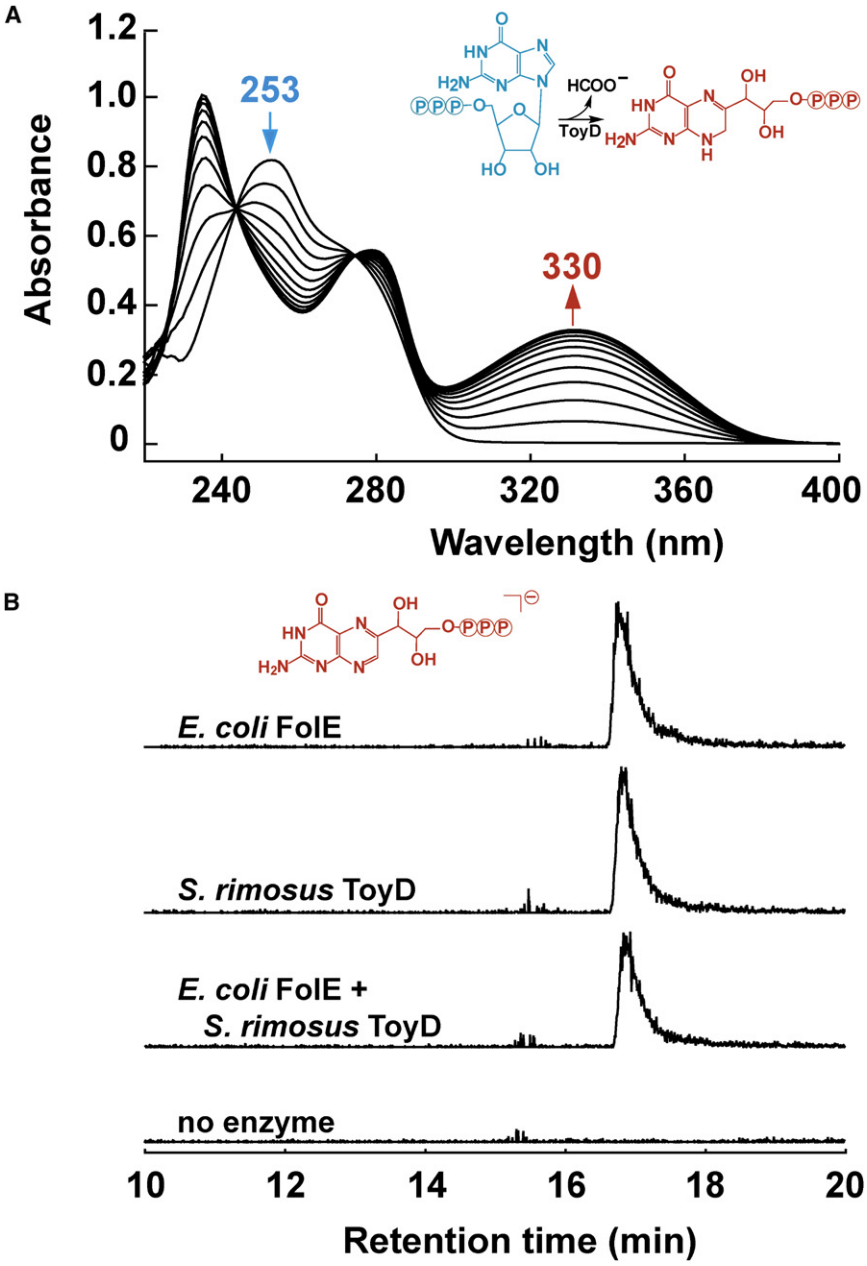

Figure 5. ToyD Is a GTP Cyclohydrolase I

UV spectrophotometric assays showing conversion of GTP to H_2NTP by *S. rimosus* ToyD (A) were carried out as described in Experimental Procedures. The identity of the product was confirmed by LC-MS analysis of the reaction and comparison to that observed with *E. coli* GCH I (FolE). (B) Extracted negative ion chromatograms of the GCH I product (at 492 amu for neopterin triphosphate) show peaks with identical retention times for the product of *E. coli* FolE and *S. rimosus* toyD. Identity of the product was confirmed by coinjection of a sample containing equal volumes of *E. coli* FolE and *S. rimosus* ToyD reaction mixtures. The product is not observed when the enzyme is omitted. 7,8-Dihydroneopterin triphosphate, which is the initial product of both reactions, is oxidized to neopterin triphosphate in the course of the analysis.

CXXXCXXC SAM radical motif (Sofia et al., 2001), is found between the in-frame TGA and the next potential start codon.

In the second phase of the biosynthetic pathway, we propose that the preQ$_0$ base is converted to the toyocamycin 5′-monophosphate by the successive action of ToyH, ToyE, ToyG, and ToyF as shown in Figure 3B. The functional assignments are based on sequence similarities. Intriguingly, these enzymes may have been ''borrowed'' from those involved in purine salvage/biosynthesis; it would be interesting to know if intragenomic homologs of these proteins, which carry out housekeeping functions, are present elsewhere in the chromosome. In this phase of the pathway, the only ORF whose functional assignment is questionable is ToyI. The *toyI* gene encodes a protein that is homologous to the HAD superfamily of enzymes, which includes a large family of phosphatases (Burroughs et al., 2006); we posit that ToyI could be involved in dephosphorylation of toyocamycin monophosphate, setting the stage for the third and final phase of the biosynthetic pathway.

The third phase of the pathway involves addition of water across the nitrile moiety of toyocamycin to yield sangivamycin. ToyJKL belongs to a rare metal-dependent nitrile hydratase family of proteins. Heterologous expression of the *S. rimosus* TNHase enzyme in *E. coli* and the observation of TNHase activity in the lysate from these cells establishes a solid connection between the *S. rimosus* protein bands for which N-terminal sequences were obtained and TNHase activity. Assays with the purified ToyJKL confirm this observation (data not shown); studies on the mechanisms of the reaction(s) catalyzed by this protein are currently underway.

The pathway shown in Figure 2B is not the only possible one that could have been proposed based on the available genes. For instance, one may imagine that the cyano group of preQ$_0$ could be hydrated first, prior to being appended to a ribose moiety. Access to the entire set of recombinant proteins encoded by the Toy cluster permits these questions to be addressed, and the chemical transformations that underlie conversion of purines to deazapurines to be unraveled.

SIGNIFICANCE

Deazapurine-containing compounds are widespread in nature. They are used in biological niches as diverse as the hypermodified tRNA base queuosine, which is found in nearly all organisms, to secondary metabolites that are

produced by various strains of *Streptomyces*. Deazapurine-containing secondary metabolites, such as sangivamycin, and their chemically modified derivatives are of importance clinically as they have long been known to partition into nucleic acid pools and have demonstrated antineoplastic, antiviral, and antimicrobial activity. Current research efforts aimed at examining the therapeutic potential of natural and derivatized deazapurines, as well as development of novel deazapurine synthetic routes, are active subjects of investigation. Though early radiotracer work has suggested that the biosynthetic routes toward diverse deazapurines proceed by way of similar steps, the absence of a complete set of deazapurine biosynthetic genes has made the exact chemical transformations difficult to decipher. The cluster presented here represents the first apparently complete set of genes required for the biosynthesis of a deazapurine secondary metabolite and is of significance for a number of reasons. First, the data clearly show a biosynthetic link between production of queuosine and formation of deazapurine-based secondary metabolites produced by *Streptomyces*. Second, access to the proteins involved in the biosynthesis of deazapurines could lead to development of semisynthetic paths toward known or novel deazapurines. Third, the gene cluster presented in this work will aid identification of deazapurines and deazapurine biosynthetic clusters that have yet to be discovered.

EXPERIMENTAL PROCEDURES

Isolation of the Sangivamycin/Toyocamycin Biosynthesis Cluster

Partial purification of TNHase, N-terminal sequencing, preparation and screening of the cosmid library, and annotation of the cluster are described in Supplemental Data.

Cloning, Expression, and Purification of Recombinant *S. rimosus* GCH I (ToyD)

The *toyD* ORF was amplified from *S. rimosus* genomic DNA by PCR, subcloned into pGEM-T easy vector and finally into the *Hind*III- and *Nde*I-digested pET29a for expression of native recombinant protein. Constructs were introduced by electroporation into *E. coli* BL21(DE3) for protein expression. One colony from transformants containing the expression plasmid, grown at 37°C on LB media containing 34 μg/ml kanamycin, was used to inoculate 0.05 liter LB starter culture containing 34 μg/ml kanamycin. The overnight starter culture was used to inoculate four single liters of LB media containing 34 μg/ml kanamycin. Cells were grown at 18°C to an OD_{600nm} ~1, at which point $ZnSO_4$ was added (0.1 mM final) and protein expression was induced by adding IPTG (0.1 mM). Cells were harvested after 16 hr by centrifugation (20,500 × g). All subsequent steps were carried out at 4°C. Cells were suspended in 20 mM Tris-SO_4 (pH 8.0) containing 5 mM dithiothreitol (buffer A) and 1 mM PMSF and lysed using a Branson 450D sonifier at 60% power. The cell lysate was centrifuged at 26,500 × g for 30 min. The soluble extract was loaded on a Q-Sepharose column (2.6 × 12.5 cm), which had been pre-equilibrated in buffer A. The column was developed with a linear gradient in buffer A first to 0.15 M NaCl over 0.1 liter, then washed with buffer A containing 0.15 M NaCl. Fractions containing ToyD (as judged by SDS-PAGE) were pooled, taken to 1 M ammonium sulfate by addition of an equal volume of a solution of buffer A containing 2 M ammonium sulfate, and loaded on a butyl-Sepharose column (2.6 × 12.5 cm) in buffer A containing 1 M ammonium sulfate. A 0.7 liter linear gradient in buffer A from 1 M ammonium sulfate to buffer A containing 30% ethylene glycol was applied. Fractions containing ToyD (as judged by SDS-PAGE) were pooled and dialyzed against 2 liters of buffer A with one buffer change. The protein was concentrated in an Amicon pressure cell (YM-10 membrane). Protein was quantified with a BCA assay, and metal content was determined by ICP-OES by Garratt-Callahan Company.

Activity Assays for *S. rimosus* ToyD and *E. coli* FolE

UV spectrophotometric assays used to determine k_{cat} for ToyD GCH I activity were carried out in the presence of 0.1 M Tris-HCl (pH 8.0), 0.1 M KCl, and 0.2 mM GTP. The UV spectra presented in Figure 5 were obtained from a reaction mixture containing 20 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, and 0.1 mM GTP; spectra shown were obtained at 5 min intervals. UV-visible spectra were recorded on an Agilent 8453 spectrophotometer after addition of 10 μM protein. The rate of conversion of GTP to H_2NTP was monitored by observing changes in absorbance at 330 nm ($\varepsilon = 6{,}300\ M^{-1}\ cm^{-1}$). GCH I assays for analysis by LC-MS were carried out in the presence of 0.02 M Tris-SO_4 (pH 8.0), 2 mM dithiothreitol, 0.1 M KCl, and 100 μM GTP. Reactions were allowed to proceed for 120 min at ambient temperature and quenched by centrifugation for 5 min at 14,000 × g through Nanosep (10 K MWCO) Omega centrifugal devices (Pall Life Sciences) to remove enzyme prior to being analyzed by reverse-phase HPLC. An aliquot (20 μl) of each reaction mix was injected onto a 100 × 4.6 mm 5μm Hypercarb column (Thermo Scientific) which had been pre-equilibrated in a buffer containing 95% 50 mM ammonium acetate, 0.1% diethylamine, and 5% acetonitrile at a flow rate of 0.5 ml/min. The column was developed with a linear gradient from 5% to 18% ACN between 2 and 14 min followed by an increase from 18% to 95% ACN between 17 and 27 min. UV-visible spectra were obtained from 220 to 500 nm using a ThermoFinnigan Surveyor photodiode array detector. Mass spectra were obtained in negative ion mode, scanning the mass-to-charge ratio range of 300–550 amu using ES ionization-equipped LCQ ThermoFinnigan Deca XP mass spectrometer. The instrument was set at 42 V ionization energy and 300°C ion source temperature.

Cloning and Assays of *S. rimosus* TNHase

Genes for the three subunits of TNHase (toyJ, toyK, and toyL) were amplified from *S. rimosus* genomic DNA by PCR. DNA encoding toyJ and toyK were cloned between *Nde*I/*Xho*I and *Nco*I/*Hind*III sites of pACYC DUET-1, respectively, for simultaneous expression of both proteins. DNA encoding toyL was cloned between NdeI and HindIII sites in pET29 vector. For the assays, pACYCDuet-1 containing toyJ and toyK and pET29 containing toyL were electroporated into *E. coli* BL21(DE3). Control strains containing the empty pACYCDuet-1 and pET29 vectors were also prepared. Transformants were plated on LB agarose plates. In this and all subsequent fermentations, 34 μg/ml each of kanamycin and chloramphenicol were included. A single colony was used to inoculate a 1 ml overnight culture containing the appropriate antibiotic. The overnight culture was added to a flask containing 0.05 liter of LB with the appropriate antibiotic(s) and grown to OD_{600nm} ~0.5; protein expression was induced by addition of IPTG to a final concentration of 0.1 mM. Because we suspected that TNHase was a cobalt-dependent protein (see text), the cultures were also supplemented with 0.1 mM $CoCl_2$. After 4 hr, 1 ml of the culture was removed, and cells were pelleted and suspended in 50 mM KPi (pH 7.4). Cells were lysed by sonication and clear lysates were obtained by centrifugation. Total protein concentration in the cleared lysate was determined by a BCA protein assay. A volume of cell lysate containing 5 μg total protein was combined with a 0.1 ml solution containing 0.05 M KPi (pH 7.4) and 0.1 mM toyocamycin. The samples were incubated 15 min at room temperature, and the enzyme was removed using a Microcon YM-10 centrifugal filtration device. An aliquot of the flowthrough (10 μL) was analyzed by HPLC over a Zorbax Eclipse C-18 column (4.6 × 250 mm). Baseline separation of sangivamycin and toyocamycin was achieved by isocratic elution with a mixture of 85% 0.1 M triethylammonium acetate (pH 6.8) and 15% methanol. Under these conditions, sangivamycin and toyocamycin elute at 15.2 and 19.7 min, respectively.

ACCESSION NUMBERS

The cluster of genes described in this article was deposited at the National Center for Biotechnology Information (NCBI) under accession number EU573979.

SUPPLEMENTAL DATA

Supplemental Data include two figures, one table, Supplemental Experimental Procedures, and Supplemental References, and can be found with this article online at http://www.chembiol.com/cgi/content/full/15/8/790/DC1/.

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