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The Hedamycin Locus Implicates a Novel Aromatic PKS Priming Mechanism

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Summary

The biosynthetic gene cluster for the pluramycin-type antitumor antibiotic hedamycin has been cloned from *Streptomyces griseoruber.* Sequence analysis of the 45.6 kb region revealed a variety of unique features such as a *fabH* homolog (KSIII), an acyltransferase (AT) gene, a set of type I polyketide synthase (PKS) genes, and two putative *C*-glycosyltransferase genes. As the first report of the cloning of the biosynthetic gene cluster for the pluramycin antibiotics, this work suggests that the biosynthesis of pluramycins utilize an iterative type I PKS system for the generation of a novel starter unit that subsequently primes the type II PKS system. It also implicates the involvement of a second catalytic ketosynthase (KSIII) to regulate this unusual priming step. Gene disruption is used to confirm the importance of both type I and II PKS genes for the biosynthesis of hedamycin.

Introduction

Aromatic polyketides are widely distributed in bacteria, fungi, and plants, and many are clinically valuable agents (e.g., tetracyclines and daunorubicin) or exhibit other fascinating biological activities. In bacteria these compounds are usually biosynthesized by type II polyketide synthases (PKSs), which minimally consist of the KS_α/KS_β heterodimer, also referred to as the ketosynthase-chain length factor (KS-CLF); an acyl carrier protein (ACP); and a malonyl-CoA:ACP transacylase (MCAT). This minimal PKS initiates polyketide biosynthesis by decarboxylation of malonyl-ACP, which is catalyzed by the KS_α/KS_β complex and leads to incorporation of an acetate starter unit. Despite the widespread occurrence of acetate-primed polyketides, some bacterial aromatic PKSs deviate from the decarboxylation mechanism and are primed by different starters [1]. Even though the mechanism of attachment of alternative primers on type II PKSs is not thoroughly understood, recent findings support two alternative pathways for unique starter unit discretion by aromatic PKS systems. In the case of benzoate-, salicylate-, or malonamate-derived polyketides, the corresponding biosynthetic gene cluster contains a monofunctional CoA ligase and an acyltransferase (AT) that are putatively responsible for activation and transfer of the parent starter units onto the PKS [1]. In the case of daunorubicin (**1**, dps), aclacinomycin (**2**, akn), frenolicin (**3**, fren), and R1128 (**4–7**, zhu) biosynthesis (Figure 1A), where the PKS is primed by short-chain fatty acids, a second catalytic ketosynthase module (KSIII), an AT, and, in the latter two, an additional ACP are postulated to be involved [1–6].

Hedamycin (Figure 1B, **14**) is an aromatic polyketide produced by *Streptomyces griseoruber* [7] and has gained considerable recognition as a highly selective DNA alkylating agent. Unlike simple DNA alkylating agents that react preferentially at guanines found in polyguanine sequences, **14** acts both via reversible DNA intercalation and irreversible alkylation of a guanine residue at specific 5′-PyG-3′ motifs, with 5′-CGT sites favored over 5′-TGT sites [8–12]. While this natural product demonstrates a significant range of biological responses [13, 14], it is not currently used clinically because of a low therapeutic index. Hedamycin is a member of the classical pluramycin antitumor antibiotics (Figure 1B) and consists of a planar 4-*H*-anthra (1,2-*b*)pyran chromophore, which carries two proximal amino sugars critical for activity, α-L-*N,N*-dimethylvancosamine (3-dimethylamino-2,3,6-trideoxy-3-*C*-methyl-α-L-*lyxo*-hexopyranose) and β-D-angolosamine (3-dimethylamino-2,3,6-trideoxy-β-D-*arabino*-hexopyranose), and a distal bisepoxide-containing side chain required for DNA alkylation [9, 12, 15]. The biosynthetic origin of this unique side chain is unknown, but its unusual structure suggests the possibility of an alternative priming of an aromatic PKS.

Hedamycin is also clearly distinguished by its unique di-*C*-glycosidic architecture. The chemistry of *C*-glycosides, defined as compounds in which an *exo*-cyclic oxygen atom of an *O*-glycoside is replaced by a carbon atom, has been of considerable interest over the last 20 years (Figure 1C) [16]. Unlike their *O*-glycoside counterparts, *C*-glycosides are unaffected by hydrolytic or enzymatic cleavage and possess a level of chemical stability comparable to that of cyclic ethers. Thus, *C*-glycosides are ideally suited for use as stable sugar mimics. Based upon synthetic chemistry precedent, some naturally occurring aryl *C*-glycosides are presumed to arise via an *ortho-O-C* rearrangement, giving rise to a preferred β-substitution [17, 18]. However, this biosynthetic postulation has not been tested. Furthermore, while a similar mechanism might be invoked to explain the novel *ortho-*, *para*-di-*C*-glycosyl substitution in **14**, chemical precedent has not been demonstrated. Thus, the **14** system not only presents the potential to

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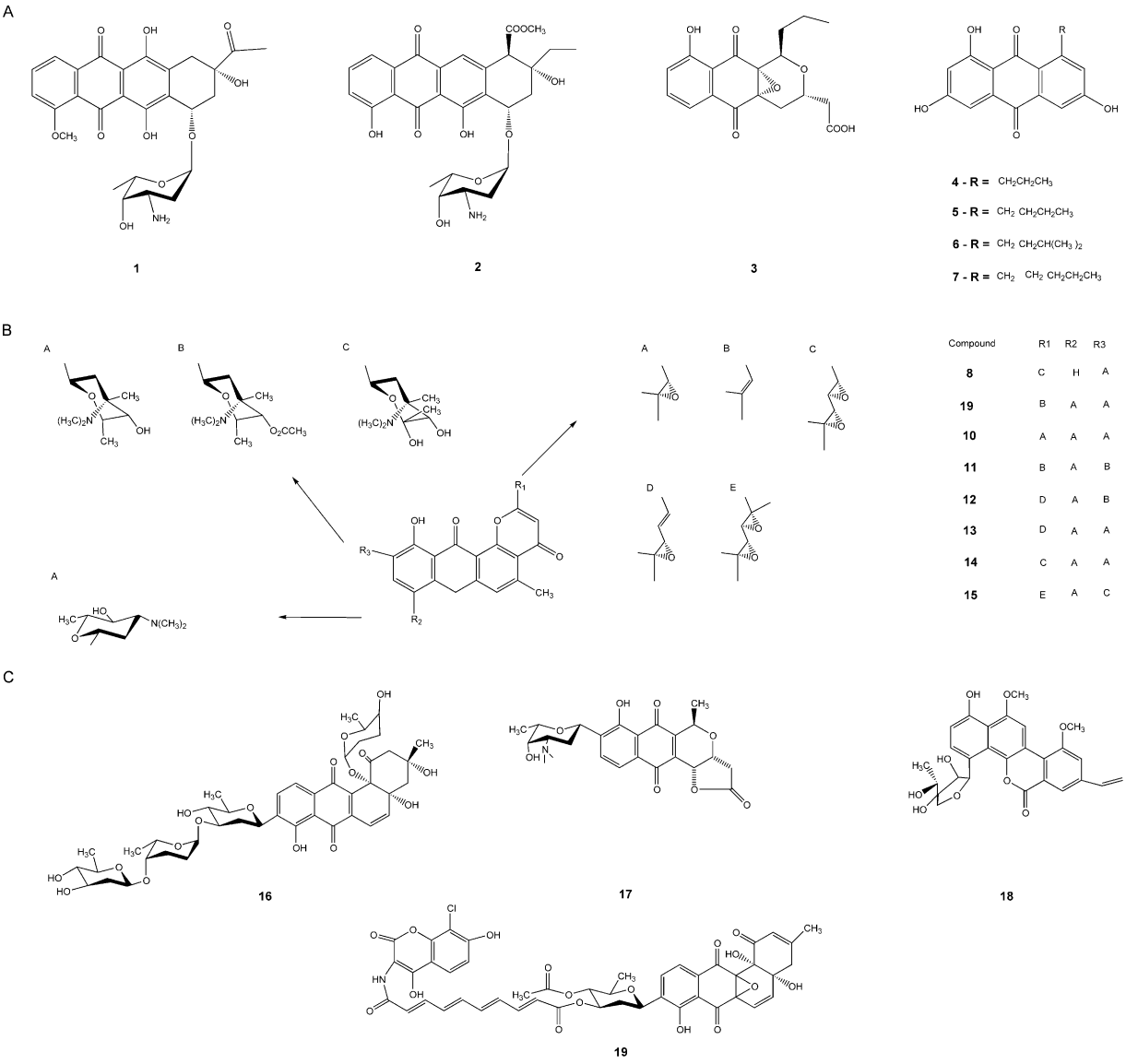

Figure 1. Representative Naturally Occurring Aromatic Polyketides

(A) Metabolites that are primed by nonacetate starter units: Daunorubicin. (**1**), Aclacinomycin (**2**), Frenolicin (**3**), and R1128 (**4–7**).

(B) Pluramycin antitumor antibiotics.

(C) Aryl-*C*-glycosides: Urdamycin (**16**), Medermycin (**17**), Gilvocarcin (**18**), and Simocyclinone (**19**).

explore unique type II PKS priming but also offers a superb opportunity to delve into the unknown mechanisms of both *ortho*- and *para*-*C*-glycosyltransfer.

Herein we report the cloning and characterization of the core of the biosynthetic gene cluster encoding for **14** biosynthesis (Figure 2), which reveals a starter unit-specific KSIII and an AT reminiscent of the dps, akn, zhu, and fren pathway. However, distinct from these previously elucidated loci, a unique feature of this cluster is the utilization of an iterative type I PKS system, HedT and HedU, which we propose to be critical for the generation of a hexenoate starter unit to serve as a special primer for subsequent aromatic polyketide biosynthesis. This work represents the first elucidation of a locus encoding for the biosynthesis of a pluramycin class antitumor antibiotic and should have direct relevance toward understanding the biosynthesis of other members within this important class of natural product. Moreover, the utilization of a novel mechanism of a type I and II PKS participation for the generation and transfer of a starter unit for aromatic polyketide biosynthesis is the first of its kind. These studies also provide the first available insights into di-*C*-glycosylation and cumulatively present a variety of potential new tools for combinatorial pathway engineering toward novel aromatic polyketides.

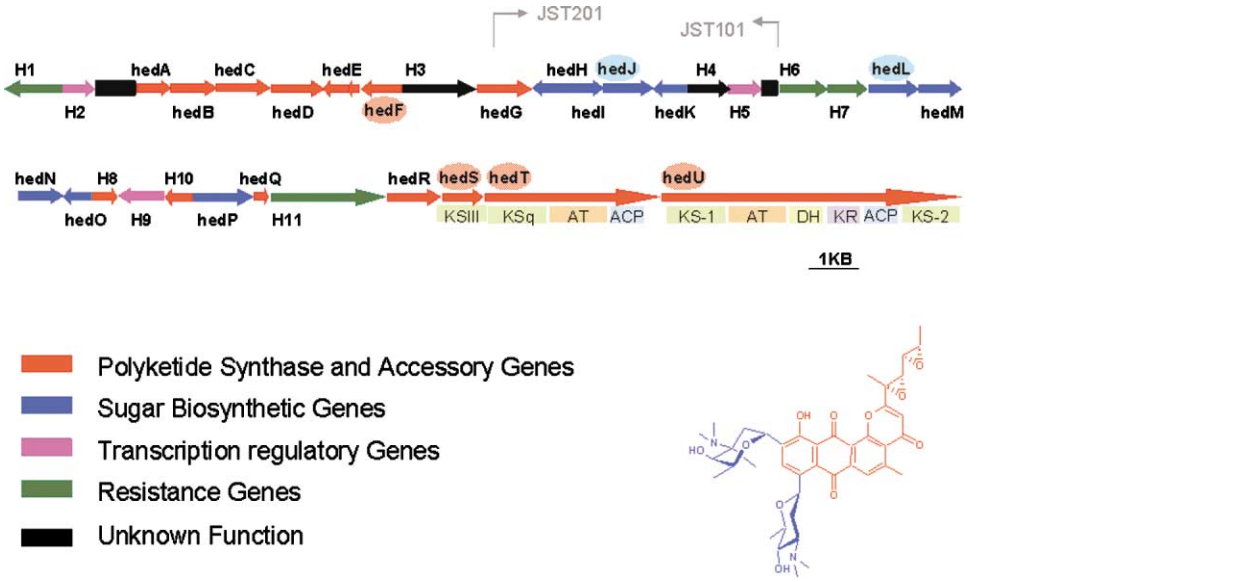

Figure 2. Organization of the **14** Biosynthetic Gene Cluster

The 32 ORFs indicated above derive from two overlapping cosmids JST101-201, the boundaries of which are highlighted. The genes unique to this cluster—namely the ones encoding the AT, two *C*-glycosyltransferases, KSIII, and the two modular PKS proteins—are highlighted by colored circles. Postulated functions associated with each of the genes and closest homologs are outlined in Table 1.

Results and Discussion

Cloning and Sequencing of the Hedamycin Biosynthetic Genes

In an effort to clone the **14** biosynthetic genes, a *S. griseoruber* genomic library in *E. coli* was generated. Degenerate primers were designed to amplify the highly conserved aromatic PKS gene, KSα, and similarly conserved sugar biosynthetic genes such as the NDP-hexose-4-6-dehydratase and glycosyltransferase genes [19–21]. The degenerate primer sets were directly used to screen the genomic library by PCR. Five hundred single colonies from the genomic library were picked and screened by pooling the colonies into groups of 50 for PCR. To deconvolute positive pools, the screen was repeated iteratively on a decreasing number of mixed colonies until a single clone was identified. A positive result was determined by amplification of the predicted size DNA fragment and confirmation by sequencing. Cosmid JST101 amplified all three target genes in the screen and was subsequently submitted to shotgun sequencing. Specific primers designed from the ends of cosmid JST101 were then used to rescreen the library and, in doing so, an overlapping cosmid, JST201, was identified and also shotgun sequenced. Analysis of the final contiguous 45.6 kb DNA segment revealed the presence of 32 open reading frames (ORFs) that encode proteins most homologous to enzymes involved in secondary metabolite production. Figure 2 shows a map of the completely sequenced cluster. The proposed function for the 32 ORFs and closest homologs are listed in Table 1. Similarly, a 25 kb DNA fragment from the pluramycin A (**12**) producer *S. pluricolorescens* was cloned and sequenced, and sequence analysis revealed most of the genes found in the **14** locus (T.B., C.-G.H., and J.S.T., unpublished data).

Confirmation of the Hedamycin Locus by PKS Disruption

As previously mentioned the KSα gene (*hedC* in the **14** locus) codes for an essential component of the KS$_\alpha$/KS$_\beta$ heterodimer, which catalyzes the Claisen-like condensation reaction iteratively in aromatic polyketide biosynthesis. To confirm that the identified locus is responsible for **14** biosynthesis, a KS$_\alpha$ disruption was performed. Specifically, protoplast transformation of wild-type *S. griseoruber* by an *E. coli* vector carrying the *hedC* internal fragment and the apramycin resistance gene (*aac(3)IV*) produced a resistant transformant named TBMC. Successful integration of the delivery vector via homologous recombination into the targeted gene on the chromosome was confirmed by Southern hybridization (Figures 3A and 3B). As expected disruption of this essential biosynthetic gene resulted in the complete loss of **14** production in this mutant strain, indicating that the correct locus has been cloned (Figure 4).

To determine the significance of the type I PKS genes in hedamycin production, a *hedT* disruption mutant was generated by using conjugational transfer of plasmids from *E. coli*. An apramycin resistant exconjugant named TBMT was analyzed as above (Figures 3C and 3D). As illustrated in Figure 4, disruption of *hedT* also completely abolished antibiotic production, which highlights the essential nature of the type I PKS genes for **14** biosynthesis.

Aglycon Assembly

In most cases the minimal PKS, consisting of a KS$_\alpha$/KS$_\beta$ heterodimer and the ACP, is primed via decarboxylation of a malonyl unit to yield an acetyl-S-KS intermediate that is subsequently elongated. However, some bacterial aromatic PKSs are primed by alternative primer

Table 1. Deduced Function of the Hedamycin ORFs Shown in Figure 2

Gene	Amino Acid	Proposed Function	Closest Protein Homolog, Strain, Identity, Similarity (%)	Accession Number
orfH1	505	Membrane transport protein	Mct, *S. lavendulae*, 23, 33	AAD29366
orfH2	222	TetR-family regulatory protein	*S. avermitilis* MA-4680, 24, 36	BAC68287
hedA	259	Keto reductase	AknA, *S. galilaeus*, 67, 76	BAB72043
hedB	321	Cyclase	Med-ORF3, *S. sp*. AM7161, 43, 53	AB103463
hedC	422	KS_{α}	SimA1, *S. antibioticus*, 68, 77	AF3244838
hedD	421	KS_{β}	TcsE, *S. aureofaciens*, 46, 56	BAB12567
hedE	319	Cyclase/dehyd. and ACP fusion	KinD, *S. murayamaensis*, 47, 60 NcnC, *S. arenae*, 43, 62	AAO65350 AAD20269
hedF	325	Acyltransferase	AknF, *S. galilaeus*, 51, 60	BAB72049
orfH3	541	Transaminopeptidase	*S. lividens*, 27, 36	AAA92338
hedG	411	Monooxygenase	*Mesorhizobium loti*, 35, 48	NP_105618
hedH	242	*N,N*-di-Methyltransferase	DesVI, *S. venezuelae*, 53, 69	AAC68678
hedI	325	4,6-Hexoseketoreductase	SnogG, *S. nogalater*, 45, 55	CAA12010
hedJ	379	Glycosyltransferase	CmmGII, *S. griseus*, 39, 46	CAE17548
hedK	226	3,5-Epimerase	StaE, *S. sp. TP-A0274*, 57, 68	BAC55217
orfH4	364	Hypothetical protein	*S. avermitilis MA-4680*, 41, 54	CAB36600
orfH5	272	Regulatory protein	Dnr I, *S. peucetius*, 53, 69	P25047
orfH6	334	ATP-binding protein	AviABC1, *S. viridochromogenes*, 51, 68	AAG32068
orfH7	260	ABC transporter	AviABC2, *S. viridochromogenes*, 35, 57	BAB69284
hedL	380	Glycosyltransferase	Gra-orf14, *S. violaceoruber*, 44, 59	CAA09635
hedM	372	Aminotransferase	Med-ORF20, *S. sp*. AM7161, 77, 82	BAC79028
hedN	321	Diphospho-4-keto-2,3,6-trideoxyhexulose reductase	Med-ORF14, *S. sp*. AM7161, 48, 57	BAC79033
hedO	239	*N,N*-di-Methyltransferase	SnogX, *S. nogalater*, 57, 68	T46679
orfH8	228	ACP-phosphodiesterase	*S. coelicolor*, 39, 51	Q9S1U6
orf H9	348	Transcription repressor	DnrO, *S. peucetius*, 40, 60	AAD15248
orfH10	273	Putative hydroxylase	SnoaW, *S. nogalater*, 42, 57	AAF01810
hedP	480	2,3-Hexose dehydratase	SnogH, *S. nogalater*, 62, 73	CAA12009
hedQ	115	Anthrone oxygenase	AknX, *S. galilaeus*, 48, 58	BAB72044
orfH11	760	UvrA-like drug resistance protein	DrrC, *S. peucetius*, 49, 66	AAB39274
hedR	409	Cytochrome P450	*S. carbophilus*, 39, 54	JC4287
hedS	348	Putative 3-oxoacyl-ACP synthase	DpsC, *S. peucetius*, 48, 61	AAA65208
hedT	985	Type I PKS; **KS_Q** (20-428), **AT_L** (542-859), **ACP_L** (923-969)	OleAI, *S. antibioticus*, 44, 54	AAF82408
hedU	2125	Type I PKS; **KS** (48-471), **AT** (574-888), **DH** (918-1035), **KR** (1378-1557), **ACP** (1652-1712), **KS** (1766-2124)	BorA3, *S. parvulus*, 41, 53	CAE45669

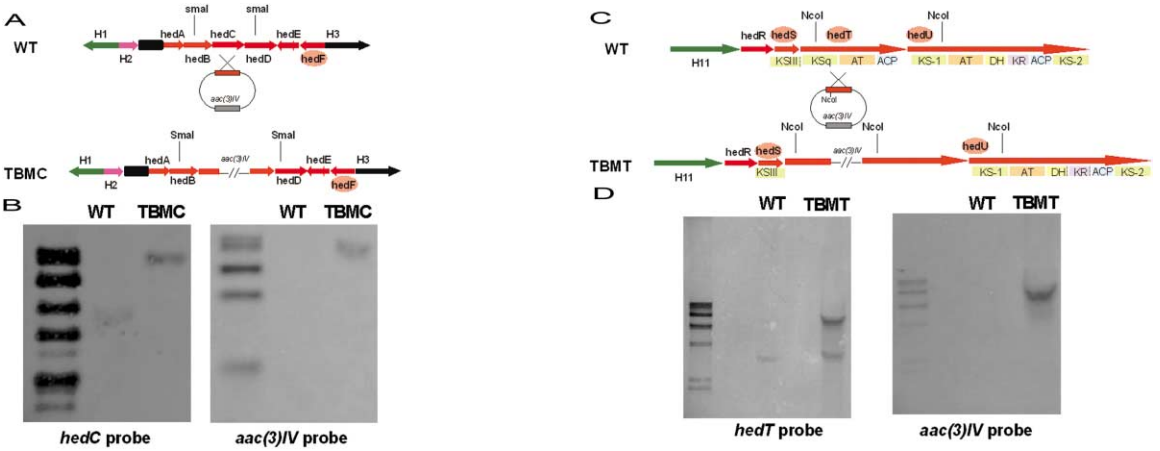

Figure 3. Generation of the PKS Mutants TBMC & TBMT

(A and C) Diagram showing insertion of the delivery vector containing *aac(3)IV* into the target genes *hedC* and *hedT* in the cluster via homologous recombination.

(B) Two replica blots containing DNA from the wild-type and the *hedC* mutant TBMC digested with SmaI were probed with a 1.1 kb *hedC* fragment and an *aac(3)IV* probe.

(D) Two replica blots containing DNA from the wild-type and the *hedT* mutant TBMT digested with NcoI were probed with a 2.75 kb *hedT* fragment and an *aac(3)IV* probe.

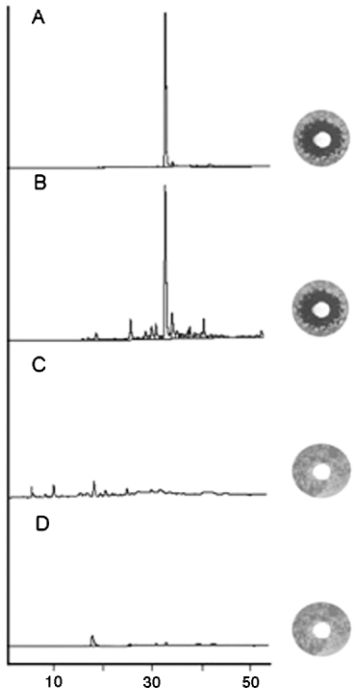

Figure 4. Phenotypic Characterization of the PKS Mutants TBMC and TBMT

HPLC profiles are presented on the left and *B. subtilis* bioactivity assays are illustrated on the right where zones of inhibition (e.g. [A] and [B]) represent positive activity. (A) A **14** standard. (B) An extract from a 100 ml culture of the **14**-producing *S. griseoruber*. (C) Extract from a 100 ml culture of TBMC. (D) Extract from a 100 ml culture of TBMT.

units. The presence of a second catalytic KSIII module, an AT, and in some cases an additional ACP, is believed to be indicative of nonacetate-primed aromatic PKSs [1]. In accordance with this model the **14** cluster contains *hedS* and *hedF*, which encode a KSIII and an AT, respectively. The closest homlogs of these genes, d*psC* and d*psD*, respectively, are found in the dps gene cluster, and recent studies have localized the observed specificity of the daunorubicin PKS toward a propionyl starter unit to the DpsC protein (*S. peucetius, S. sp*. Strain C5) [22, 23]. R1128 is the other well-studied aromatic PKS system that contains homologs to *dpsC* and *dpsD* and an additional ACP gene. In vitro characterization of the unique R1128 system has shown that the minimal PKS system and the KSIII in this cluster recognize orthogonal sets of ACPs, and the additional ACP is indispensable for the incorporation of nonacetate primer units [24]. More recently, Khosla et al. have successfully demonstrated the ability to introduce novel primer units to aromatic polyketides by using the R1128 initiation module [25].

The unprecedented physical link between a typical set of aromatic PKS genes and genes encoding two type I multimodular PKSs, a KSIII and an AT, as seen in both the **12** and **14** loci, suggests that the biosynthesis of pluramycins require a unique dependence of additional proteins beyond the normal aromatic polyketide assembly machinery. Furthermore, the type I and type II disruption experiments clearly support the critical nature of both sets of PKSs to **12** and **14** production. Based upon this information, we propose that a unique hexenoate starter unit is constructed by the type I PKS and is subsequently utilized to prime the type II PKS (Figure 5). One of the type I PKS proteins in this system, HedT, contains a KS, an AT, and an ACP domain. Consistent with other systems, the active site cysteine in the HedT KS domain is replaced by a glutamine, suggesting that it serves as the loading module [1]. In such systems the KS domain has been shown to be able to decarboxylate malonyl-CoA to produce an acetyl starter unit [26]. HedU carries KS, AT, ACP, ketoreductase (KR), and dehydratase (DH) domains and a second KS domain. Examination of the AT domains in these modular proteins show that both contain the conserved motifs required for malonyl-CoA transfer. Therefore we hypothesize that the second module is used iteratively to elongate the acetyl starter unit with two malonyl-CoA and subsequent 2-fold keto-reduction and dehydration gives **20** (Figure 5).

Although rare, iterative type I PKS systems have been reported to participate in forming the substituted orsellinc acid moieties of avilamycin and calicheamicin, and the naphthalinic acid moiety of neocarzinostatin [27–29]. There are also recent reports in which iteration, or the use of one module twice, occurs as a programmed event in the biosynthesis of modular PKS products such as stigmatellin, aureothin, and borrelidin [30–34]. In a similar manner, we postulate the **14** pathway utilizes a dedicated type I PKS iteratively to biosynthesize a specific hexenonate starter unit to prime aromatic polyketide biosynthesis. This remarkable participation of a type I and II system is not commonly seen in bacteria. However, the fungal aflatoxin-producers *A. nidulans* and *A. parasiticus* utilize a pair of specialized fatty acid synthases (Fas-1 A/Fas-2 A) to construct and present a hexanoate starter unit to a simple type I PKS (PksA), which then completes the synthesis of norsolorinic acid en route to aflatoxin [35, 36].

As in the case with daunorubicin biosynthesis, we postulate that the KSIII encoded by *hedS* is likely to play a role in the specificity of the type II PKS to utilize the hexenoate starter unit over the normal malonyl-decarboxylation mechanism [22, 23]. Knockout studies have shown that in the absence of *dpsC*, the daunorubicin type II PKS complex behaves promiscuously, utilizing both acetyl-CoA and propionyl-CoA as starter units. Comparison of HedS to the known KSIIIs from aromatic polyketide biosynthetic gene clusters (dps, akn, fren, zhu) reveals that it is more homologous to DpsC and AknE2 (61% and 54%, respectively) than to ZhuH and FrenI (20% and 43%, respectively). HedS, DpsC, and AknE2 also all lack the highly conserved active site cysteine, which is replaced by a serine, while ZhuH and FrenI maintain the conserved residue. The absence of an additional ACP in the three biosynthetic gene clusters is another common denominator, suggesting that these proteins perhaps function similarly to give the observed specificity of the type II PKS system for its respective nonacetate starter unit.

Once presented with the appropriate starter unit, the type II PKS in the **14** locus is proposed to catalyze the

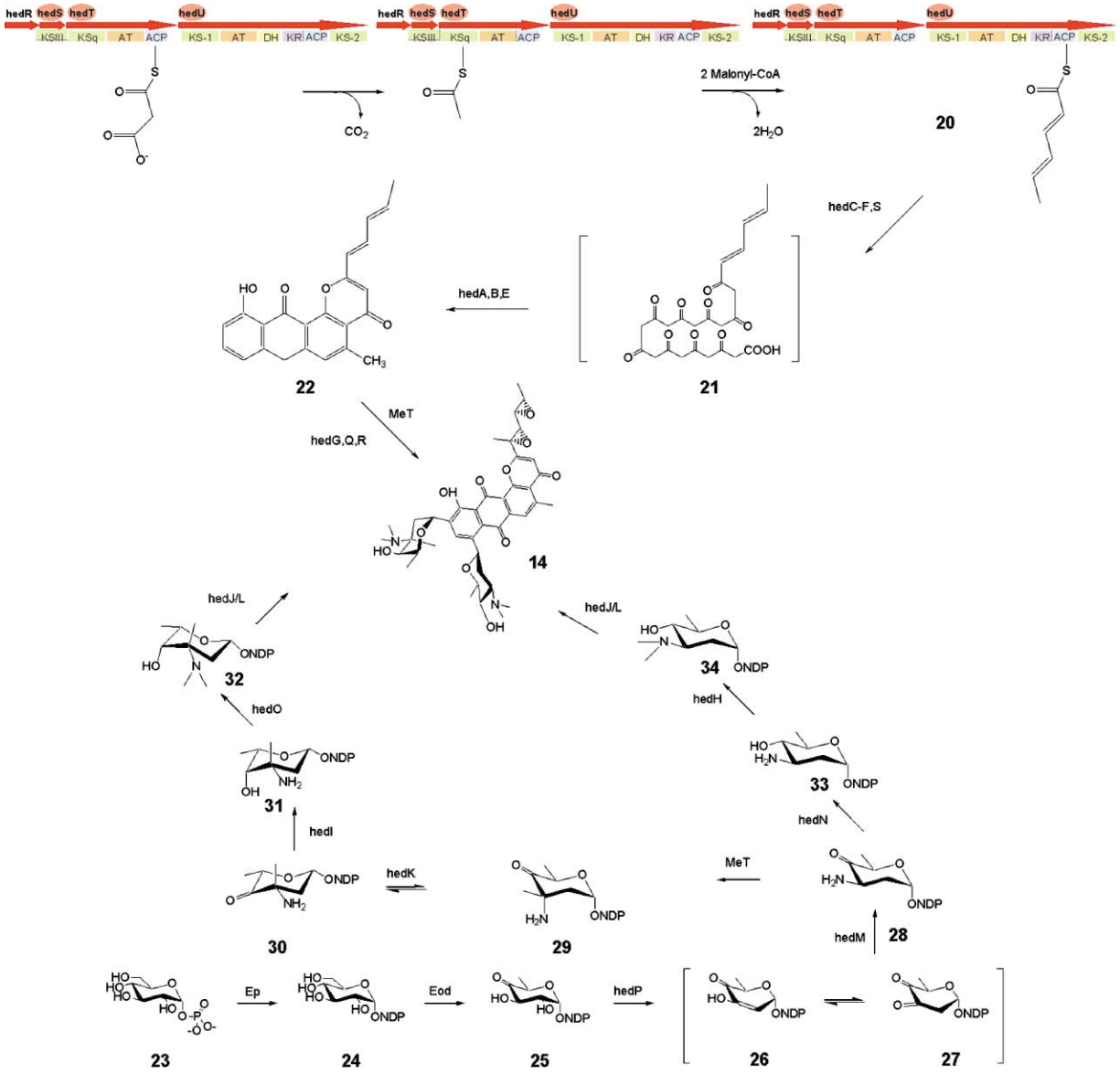

Figure 5. The Proposed Biosynthetic Pathway for 14 and Related Pluramycins

The collaborative action of the starter unit-specific HedS (KSIII) and HedF (AT), in conjunction with an adjacent iterative type I PKS system (HedT and HedU) for the generation and utilization of a de novo hexenoate primer for subsequent aromatic polyketide biosynthesis, is specifically noted.

elongation of intermediate **20** to make the β-polyketoacyl chain precursor **21** (Figure 5). The early biosynthetic enzymes closely related with the minimal PKS include a ketoreductase and aromatase/cyclase involved in modification of the nascent polyketide chain [19]. Homologs of these enzymes are found in this cluster and are presumed to be important for the regioselective aromatization of the open-chain precursor to form intermediate **22**. The final steps of aglycon biosynthesis are presumed to be catalyzed by the cluster encoded putative PKS oxygenases. The *hedG* gene encodes a protein with a low homology to FAD-dependent monooxygenase, and HedR is most similar to oxygenases of the cytochrome P450 family and bears 40% similarity to Grh03, which is presumed to catalyze the epoxide formation in griseorhodin A biosynthesis (*S. sp.* JP95) [37, 38]. We propose that these proteins likely play a role in epoxide formation, which constitutes the alkylating ability of the compound. *HedQ* encodes a protein with high similarity to oxygenases that have been proposed to be involved in quinone formation in the anthracycline polyketides such as aclacinomycins and nogalamycin. Despite the fact that we have not yet identified a methyltransferase gene in the current locus, we presume that one is involved in introducing the distal bis-epoxide methyl group.

Aglycon Modification

One of the novel features of **14** is the *C*-glycosidic attachment of two distinct sugars to the aglycon, a process anticipated to require two glycosyltransferases

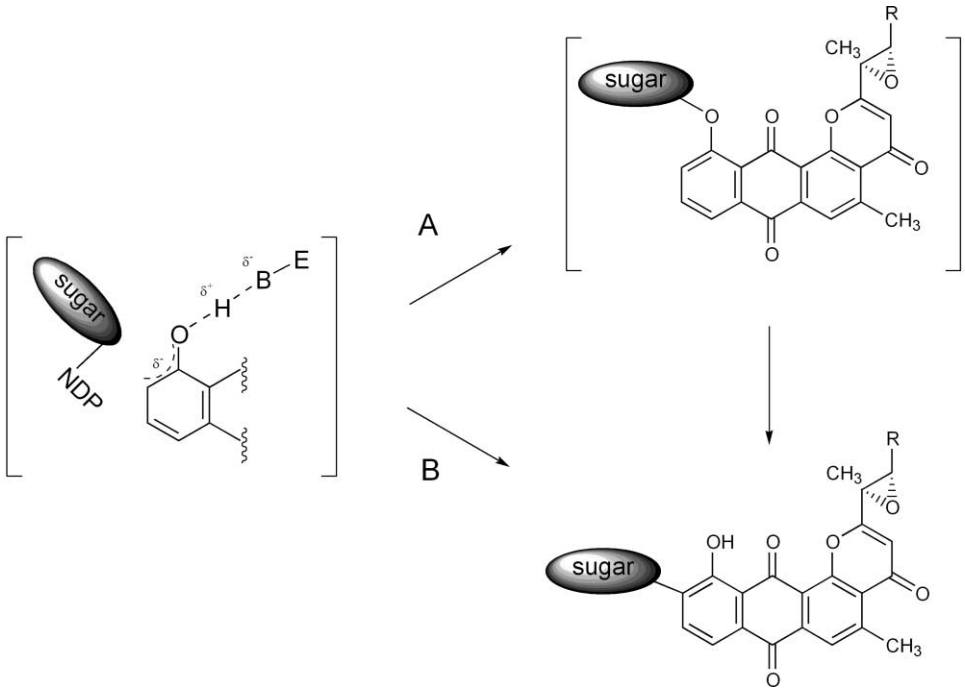

Figure 6. Two Proposed Mechanisms for *C*-Glycosylation

(A) Mechanism I illustrates an *O*-*C* rearrangement as proposed for the *C*-glycosylation of ortho-*C*-substituted *C*-glycosides.
(B) Mechanism II depicts direct aromatic substitution possibly promoted by the *ortho*/*para*-directing aromatic hydroxyl group.

with unique regio- and stereoselectivity. HedJ and HedL, homologous to various glycosyltransferases involved in secondary metabolite production, are the candidate *C*-glycosyltransferases (Figure 5). HedJ greatly resembles Med-Orf8 (45%), which is a *C*-glycosyltransferase responsible for angolosamine transfer in medermycin (**17**) biosynthesis (*S. sp*. AM7161) [39]. HedL shows significant homology to the single glycosyltransferase found in the granaticin biosynthetic cluster, which is postulated to be involved in the attachment of a 4-keto-2,6-dideoxy-D-glucose to the granaticin aglycon via two C-C bonds (*S. violaceoruber*) [40]. Amino acid alignment of the HedJ and HedL to UrdGT2 also shows that the proteins share significant identity (HedJ, 40% and HedL, 58%, respectively, *S. fradiae* Tü 2717). UrdGT2, a *C*-glycosyltransferase from the urdamycin (**16**) biosynthetic gene cluster, has thus far been established as the enzyme responsible for attaching the first D-olivose to the aglycon via a *C*-*C* linkage [41].

Most, if not all, UDP/TDP glycosyltransferases, which comprise by far the largest category of glycosyltransferases, fall into two different structural superfamilies [42]. These superfamilies have different folds, different active sites, and different mechanisms. The GT-A superfamily employs a DXD motif to bind a divalent metal ion (most commonly Mn^{2+}). The metal ion, which is essential for catalysis, helps anchor the pyrophosphoryl group of the UDP-sugar donor in the enzyme active site. The GT-B family carries a two-domain structure, and each domain adopts an α/β open-sheet motif similar to a classic Rossmann fold, which is thought to be involved in binding the glycosyl donors.

A more recent structural study of the UDP-glucosyltransferase involved in the biosynthesis of vancomycin-group antibiotics, GtfB, has revealed several additional conserved motifs and residues [43]. Particularly noteworthy are the highly conserved Gly-rich sequence HHGGAGT and the strictly conserved Pro and Asp (the potential catalytic base for proton abstraction of an acceptor hydroxyl) residues within these transferases. Interestingly, the Gly-rich motif and the Pro residue are also found in HedJ and HedL, while the Asp residue is replaced by the amino acids Lys and Ala, respectively, in these putative *C*-glycosyltransferases. Consistent with this observation, Ichinose et al. have postulated that this putative general base (Asp) is mainly restricted to *O*-glycosyltransferases [39]. Similar comparison of the two *C*-glycosyltransferases from the **14** cluster with all other *C*-glycosyltransferases so far identified and other close *O*-GT homologs illustrate that the most common replacement is with nonpolar residues such as Ala, Val, and Ile. In contrast, the *C*-glycosyltransferases HedJ and Gra-Orf-14 both contain a basic Lys substitution.

At least two mechanisms can be put forth for the *C*-glycosylation of the aromatic aglycon of **14**. The first mechanism (mechanism I) requires *O*-glycosylation of the phenolic hydroxyl followed by an *O*-*C* rearrangement and has been proposed for the *C*-glycosylation of *ortho*-*C*-substituted *C*-glycosides (Figure 6A). Chemical models promoted by Lewis acids exist, which demonstrate the facile rearrangement of *O*-aryl glycosides to the *ortho*-*C*-analogs and some minor *para*-*C*-substituted variants, with typically the β-glycoside as the predominate product [17, 18, 44]. The second mechanism (mechanism II, Figure 6B) is analogous, in a regiochemical sense, to a direct Friedel-Crafts aromatic substitution

reaction. Support for this mechanism derives predominantly from chemical models in which a distinctive Lewis acid, Cp_2ZrCl_2-$AgClO_4$, promoted the efficient coupling of methoxynapthalene derivatives with glycosyl fluorides to give both the *ortho*- and *para*-*C*-glycosides, the β-glycoside predominating [45, 46]. The regiochemistry of aromatic substitution patterns in these studies correlated well with the predicted reactivity of each methoxynaphthalene starting unit. While the products of mechanism II are most consistent with the formation of **14**, *C*-glycosyltransferase mechanistic studies are completely lacking and future efforts will be focused on in vivo and in vitro characterization of these proteins to determine this novel enzymatic mechanism.

NDP-Sugar Biosynthesis, Regulation, Resistance, and Unknown Function

Blast analysis indicates that the putative genes involved in the biosynthesis of the two deoxyamino sugars bear great protein similarity to the enzymes involved in the biosynthesis of dTDP-L-vancosamine. This well-studied pathway sets the foundation for the proposed biosynthetic scheme presented in Figure 5 [47]. The early biosynthetic steps of 2,6-deoxygenation and amino transfer are expected to follow precedent, providing the common precursor **28** from which the pathways diverge to **32** and **34**, respectively, via reduction or methylation/epimerization/reduction as illustrated. The equivalent genes encoding E_p and the methyltrasferase, not present in the current **14** locus, were identified in the **12** locus and are presumed to also be necessary for **14** biosynthesis (T.B., C.-G.H., and J.S.T., unpublished data).

Database searches revealed three genes with probable regulatory function in the cluster. The *orfH5* encoded protein shows similarity to transcriptional activators of the *Streptomyces* antibiotic regulatory protein (SARP) family. They are proposed to activate transcription of target structural genes by binding to direct heptameric repeats sometimes found overlapping the –35 regions of cognate promoters [48]. The protein product of *orfH2* exhibits slight homology to tetR-family of regulatory proteins. OrfH9 is a close homolog of DnrO, which is proposed to be a transcriptional repressor protein (*S. peucetius*) [49].

Self-resistance is an important requirement for antibiotic producing microorganisms, and the **14** cluster contains several genes that are self-resistance candidates. *OrfH1* encodes a protein with significant homology to the *mct* gene, which encodes a protein involved in a novel drug export system, from the mitomycin biosynthetic gene cluster (*S. lavendulae*) [50]. O*rfH6* and *orfH7* genes resemble those encoding ABC transporter systems from antibiotic-producing actinomycetes. These proteins show highest homology to the resistance factors, AviABC1-AviABC2 proteins, from the avilamycin biosynthetic gene cluster (*S. viridochromogenes)* [51]. O*rfH11* encodes a 760 amino-acid protein with strong sequence similarity to the *E. coli* and *Micrococcus luteus* UvrA proteins involved in excision repair of DNA. It also shares 66% homology to DrrC, an UvrA-like protein, which has been confirmed to be important for daunorubicin resistance in vitro (*S. peucetius*)[52].

Three genes were identified to have no obvious role in the biosynthesis of **14**. *OrfH3* encodes a protein with great homology to tripeptidyl aminopeptidase (TAP) from *S. lividans*. The *S. lividans* gene has been cloned, and cross-species hybridization experiments showed that homologs are present in most of the *Streptomyces* strains tested [53]. O*rfH4* and *orfH10* encode a highly conserved hypothetical protein and a hydrolase, respectively, found in most secondary metabolite producing *Streptomyces*.

Significance

Hedamycin is an aromatic polyketide distinguished for its di-*C*-glycoside architecture. It is a potent DNA alkylating agent and in-depth structural studies have shown that the sugar moieties play a crucial role for its specific interaction with DNA. The biosynthetic enzymes responsible for the sugar modification of this natural product provide a platform for elucidating the unknown mechanisms of enzymatic *C*-glycosylation and, moreover, offer an attractive tool for bioengineering novel *C*-glycosides. Toward this effort, the cloning and initial characterization of the core of the biosynthetic genes encoding for hedamycin biosynthesis revealed the candidate *C*-glycosyltransferase genes and additional unique enzymes that implicate a novel aromatic PKS priming mechanism. The proposed biosynthetic pathway involves the collaborative action of the starter unit-specific HedS (KSIII) and HedF (AT) in conjunction with an adjacent iterative type I PKS system, HedT and HedU, for the generation and utilization of a de novo hexenoate primer for subsequent aromatic polyketide biosynthesis. This remarkable assembly of a type I and type II PKS system for the biosynthesis of a natural product is an original finding and as the first elucidation of a locus encoding for the biosynthesis of a pluramycin class of antitumor antibiotics, this work should greatly impact upon understanding the biosynthesis of other members within this important class of natural product.

Experimental Procedures

Bacterial Strains, Culture Conditions, and Vectors

Streptomyces griseoruber ATCC 15422 cells were grown at 28°C and 225 rpm in baffled flasks containing beads (2.5 g). *Escherichia coli* DH5α, VCS257 (Stratagene), and GM2163 (New England Biolab) were cultured in LB at 37°C with the appropriate antibiotic selection at a final concentration of 100 μg mL^{-1} ampicillin, 50 μg mL^{-1} apramycin, 25 μg mL^{-1} chloramphenicol, and streptomycin. *Bacillus subtilis* ATCC 6633 was grown at 30°C in nutrient media according to the ATCC recommendations. Vectors pGEM-T easy vectors (Promega), pBS II SK^- (Stratagene) and pKC1138 were previously described. The shuttle vector pDW103, a pOJ446 derivative in which the apramycin resistance cassette is replaced by thiostrepton and ampicillin cassette, was used for cosmid library construction [54].

Genetic Procedures

Total DNA isolation, plasmid DNA preparations, restriction endonuclease digestions, ligations, and transformation were performed according to standard procedures for *E. coli*. Total cellular DNA isolation and methods for preparation of *S. griseoruber* spores and protoplasts were those described previously [55]. For cosmid library construction, genomic DNA from *S. griseoruber* was partially digested with Sau3A and calf intestine alkaline phosphatase treated

according to the manufacturing instructions. The fragments were ligated to BamHI-digested pDW103 and packaged into lambda phage particles by using Stratagene Gigapack III XL as per instruction and plated on *E. coli* VCS257. Single random bacterial clones were combined together in groups of fifty and the complete plasmid DNA isolated from the pools was screened by diagnostic PCR using three sets of degenerate primers that amplify deoxysugar biosynthetic genes such as the *d*TDP-glucose-4,6-dehydratase (5′-GTSACSGGSGGSGCSGGSTTCAT-SGG-3′ and 5′-CTGGTASGGS CCGTAGTTGTT-3′) and glycosyltransferase (5′-GCSTGGGCSCT SMR-SDSSGCSGGSCACGA-3′ and 5′-GTSCCSSHSCCSCCGTGG TGSA-3′), and the aromatic PKS gene KS_{α} (5′-GSMGSGTSGTSAT SACSGGSATSGG-3′ and 5′-CTGGAASCC- SCCGAASCCSSWSCC SAC-3′). Amplified PCR products were sublconed into pGEM-T easy vectors and sequenced.

DNA Sequencing and Analysis

Cosmid clones were shotgun sequenced by the Genome Center, University of Wisconsin, Madison, WI. Specifically, intact cosmids were mechanically sheered and fragments in the size range of 1–3 kb were purified by preparative agarose gel electrophoresis and subcloned into SmaI-digested plasmid Bluescript II SK^- (Stratagene). Plasmid subclones were isolated by using Qiagen columns. Automated sequencing was done on double stranded DNA templates. Sequence data obtained from single random subclones (700 for each cosmid) was assembled and edited by using the SeqMan software (DNAStar). ORF searches were done by using the frameplot software available at the website http://www.nih.go.jp/~jun/cgi-bin/frameplot-3.0b.pl. Database comparison was performed with the BLAST search tools on the server of the National Center for Biotechnology Information, Bethesda, MD. The DNA sequence has been deposited in the GenBank under the accession number AY196994.

Generation of Chromosomal Mutants of *S. griseoruber*

For the generation of *S. griseoruber* mutants, both protoplast transformation and conjugation with *E. coli* were used to introduce the disruption plasmids, with the latter method producing a higher transformant yield (>10-fold). The *hedC* knockout was constructed by using insertional inactivation via a single crossover homologous recombination event following standard protocol [55]. Specifically, a 1.1 kb *hedC* internal fragment was PCR amplified and cloned into pGEM-T easy vector for sequencing. The insert was isolated as an EcoRI fragment and subcloned into the conjugative *E. coli* vector pKC1138, which carries an apramycin selection marker. The new construct was passed through *E. coli* GM2163, and Qiagen preparation from this strain was first denatured by treatment with an alkaline solution before introduction into *S. griseoruber* protoplasts. For protoplast preparation, the producing *S. griseoruber* was cultured in 25 ml Yeast Extract-Malt Extract Medium (YEME) [55] overnight at 28°C in 500 ml baffled flasks. PEG-mediated protoplast transformations were plated on R2YE plates [54] and after 22 hr overlaid with soft R2 media [55] containing 50 μg mL^{-1} of apramycin.

Similarly, for construction of *hedT* disruption mutants, an internal fragment of *hedT* was isolated from a shotgun clone by using XbaI and HindIII and subcloned into pKC1138. A modified conjugation method was used to introduce the plasmid into *S. griseoruber* [55]. In preparation for conjugal transfer *S. griseoruber* spores (10^{10}) were heat shocked at 50°C for 10 min and then incubated in LB at 37°C for 4 hr. The methylation-deficient conjugative *E. coli* strain, ET12567, was transformed with the disruptive plasmid and fresh transformants were cultured at 37°C until an OD_{600} of 0.4–0.6 was reached. Equal volumes of the above treated strains were mixed together and plated on ISP II medium (Difco) and incubated at 37°C for 24 hr, at which point the plates were flooded with apramycin (50 μg mL^{-1}) and the *E. coli* selective eliminant naldixic acid (25 μg mL^{-1}).

Apramycin-resistant clones obtained from both of the transformation methods were analyzed by Southern hybridization. For isolation of total DNA from the wild-type strain and the mutants, each was grown in tryptic soy broth (TSB; Difco) overnight at 28°C, containing 25 μg mL^{-1} of apramycin for maintenance of the disruption mutants. Digoxigenin labeling of DNA probes, Southern hybridization, and detection were performed according to literature procedures and manufacturer recommendations (Roche).

Isolation and Detection of Hedamycin from *S. griseoruber* and Mutants

The strains were grown in production media consisting of 2.0% cerelose, 1.0% pharmamedia, 1.0% corn steep liquor, 0.40% calcium carbonate, 0.30% ammonium sulfate, and 0.003% zinc sulfate [7]. The fermentations were carried out in New Brunswick rotary shakers (260 rpm) in 500 ml baffled flasks containing 100 ml of the above medium for 4 days at 28°C. The organic layer from a whole-cell $CHCl_3$ extract was concentrated and analyzed on HPLC (C_{18} reverse phase chromatography, 250 × 4.6 mm, flow rate 1 ml min^{-1}, λ = 245 nm, 5% CH_3CN − 95% 0.1% aqueous H_3PO_4 to 58% CH_3CN − 42% 0.1% aqueous H_3PO_4, 45 min). The hedamycin production rate in these shake-flask fermentation conditions was comparable to a previously reported result of 60 mg L^{-1} from large scale fermentors [7]. A **14** standard under the above HPLC conditions had a retention time of 32 min.

Biological Tests for Hedamycin Antibacterial Activity

Growth inhibition tests on *B. subtilis* were used to visualize in vivo antibacterial activity [7]. Specifically, aliquots of the $CHCl_3$ whole-cell extracts were added to paper discs and placed on a lawn of *B. subtilis* overlaid onto nutrient agar plates. The plates were incubated at 30°C overnight. Standard **14**/$CHCl_3$ solutions were used as positive controls while $CHCl_3$ and $CHCl_3$ whole-cell extract from nonproducing strains (e.g., *S. lividans* K4-114) served as negative controls.

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Note Added in Proof

Evidence to support mechanism II (Figure 6B) for UrdGT2 has recently been reported. Durr, C., Hoffmeister, D., Wohlert, S.-E., Ichinose, K., Weber, M., von Mulert, U., Thorson, J.S., and Bechthold, A. (2004). The glycosyltransferase UrdGT2 catalyzes both C- and O-glycosidic sugar transfers. Angew. Chem. Int. Ed. *43*, 2962–2965.