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Elucidation of the *Streptomyces coelicolor* Pathway to 2-Undecylpyrrole, a Key Intermediate in Undecylprodiginine and Streptorubin B Biosynthesis

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SUMMARY

The *red* gene cluster of *Streptomyces coelicolor* directs production of undecylprodiginine. Here we report that this gene cluster also directs production of streptorubin B and show that 2-undecylpyrrole (UP) is an intermediate in the biosynthesis of undecylprodiginine and streptorubin B. The *redPQRKL* genes are involved in UP biosynthesis. RedL and RedK are proposed to generate UP from dodecanoic acid or a derivative. A *redK*$^-$ mutant produces a hydroxylated undecylprodiginine derivative, whereas *redL*$^-$ and *redK*$^-$ mutants require addition of chemically synthesized UP for production of undecylprodiginine and streptorubin B. Fatty acid biosynthetic enzymes can provide dodecanoic acid, but efficient and selective prodiginine biosynthesis requires RedPQR. Deletion of *redP*, *redQ*, or *redR* leads to an 80%–95% decrease in production of undecylprodiginine and an array of prodiginine analogs with varying alkyl chains. In a *redR*$^-$ mutant, the ratio of these can be altered in a logical manner by feeding various fatty acids.

INTRODUCTION

Actinomycetes and other eubacteria produce prodiginines, a family of red-pigmented tripyrrole antibiotics (Figure 1; Gerber, 1975). These compounds have potent biological activity which has led to renewed interest in their synthesis (Fürstner, 2003; Fürstner et al., 2005), biosynthesis (Cerdeno et al., 2001; Mo et al., 2005; Stanley et al., 2006; Williamson et al., 2005), and mode of action (Azuma et al., 2000; Campas et al., 2003; Llagostera et al., 2005; Nakamura et al., 1989). It is notable that synthetic analogs of undecylprodiginine (**1**) have promising immunosuppressant activity (Mortellaro et al., 1999), whereas a synthetic analog of streptorubin B (**2**) and metacycloprodiginine (**3**), GX-15-070, is currently in phase I and II oncology clinical trials (Dairi et al., 2006).

Streptomyces coelicolor A3(2) has been reported to produce the prodiginine antibiotic undecylprodiginine and a cyclic derivative tentatively identified as butylcycloheptylprodigiosin (**4**) (Tsao et al., 1985). Recently, Fürstner and coworkers accomplished a total synthesis of **4** and, by comparison of their ^{1}H NMR data for synthetic **4** with the reported ^{1}H NMR data for the cyclic derivative of undecylprodiginine isolated from *S. coelicolor*, concluded that the original tentative structural assignment of this natural product was correct (Fürstner et al., 2005).

The biosynthesis of both **1** and prodigiosin (Figure 2) is proposed to be a bifurcated process involving the condensation of 4-methoxy-2,2′-bipyrrole-5-carboxaldehyde (MBC) with a monopyrrole (2-undecylpyrrole [UP] or 2-methyl-3-pentylpyrrole [MPP], respectively) (Figure 2; Cerdeno et al., 2001; Stanley et al., 2006; Williamson et al., 2005). Labeling studies have demonstrated that, for **1**, **2, and prodigiosin**, the MBC-derived portion is biosynthesized from proline, acetate/malonate, serine, and methionine (Gerber et al., 1978; Wasserman et al., 1973, 1974). The *red* and *pig* gene clusters directing biosynthesis of **1** and its cyclic derivative in *S. coelicolor* and prodigiosin in *Serratia*, respectively, have been cloned and sequenced, and putative biosynthetic roles for the various gene products have been proposed (Cerdeno et al., 2001; Harris et al., 2004). Deletion of genes required for MBC biosynthesis results in mutants in which prodiginine production can be restored through genetic and chemical complementation (either with synthesized MBC or through cross-feeding experiments with mutants which accumulate and excrete MBC) (Stanley et al., 2006; Williamson et al., 2005). The MBC pathway delineated from these studies appears to be highly conserved in these organisms.

In contrast, there is a stark difference in both the structure of the proposed monopyrrole intermediates and the proposed pathways that generate them (Williamson et al., 2005; Cerdeno et al., 2001). In the case of undecylprodiginine biosynthesis, UP has been observed in *S. coelicolor* mutants blocked in the biosynthesis of MBC, implicating it as either an intermediate or shunt product (Stanley et al., 2006). Precursor incorporation studies suggest that the UP is generated by a decarboxylative condensation of glycine with an acetate-derived 3-ketomyristic acid derivative (Figure 2). Initial analysis of the *red* gene cluster revealed *redQ* encoding an acyl carrier protein (ACP) and two genes *redP* and *redR* which encode homologs of the fatty acid biosynthetic enzymes FabH (3-ketoacyl-ACP synthase III) and FabF (3-ketoacyl-ACP synthase II), respectively (Cerdeno et al., 2001). It was proposed that RedP and RedR, in tandem with fatty

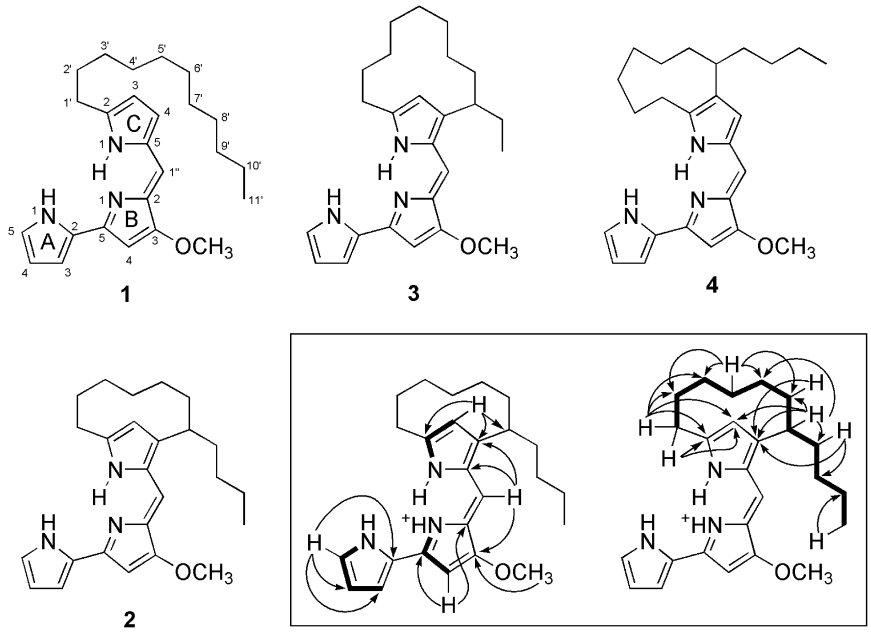

Figure 1. Structure of Undecylprodiginine, 1, Butylcycloheptylprodigiosin, 4, Metacycloprodiginine, 3, and Streptorubin B, 2
COSY (bold lines) and HBMC (arrows) correlations for streptorubin B. HCl isolated from *S. coelicolor* M511 are shown in the box.

acid biosynthetic enzymes, which catalyze the conversion of 3-ketoacyl-ACP to acyl-ACP, were responsible for formation of a RedQ-activated dodecanoic acid (Figure 2). The subsequent elaboration of dodecanoic acid to UP was originally proposed to be catalyzed by the RedX and RedN gene products, but these have since been shown to be involved in MBC biosynthesis, implicating RedL in UP biosynthesis instead (Stanley et al., 2006).

To date only the role of RedP, responsible for initiating the biosynthesis of the proposed UP intermediate, has been investigated (Mo et al., 2005). In a *redP* deletion mutant (*S. coelicolor* SJM1), there was an 80% decrease in prodiginine titers, which could be restored by plasmid-based expression of either *redP* or *Streptomyces glaucescens fabH*. FabH-catalyzed initiation of hydrocarbon-chain biosynthesis led to formation of two branched-chain alkyl prodiginines as well as the normal undecylprodiginine product. This observation was consistent with the demonstrated ability of this FabH to catalyze a condensation of malonyl-ACP with both straight- and branched-chain acyl-CoA substrates (Han et al., 1998; initiation of hydrocarbon-chain biosynthesis by RedP led only to formation of **1**, indicating that this enzyme has a substrate specificity for acetyl-CoA). In the SJM1 mutant, FabH-initiated hydrocarbon-chain biosynthesis leads to predominantly straight-chain prodiginine products and branched-chain fatty acid products (Mo et al., 2005). These observations suggested that another enzyme(s) in these two pathways is also responsible for exerting control over the nature of the hydrocarbon chain in the final products.

Herein we report a comprehensive genetic analysis of the entire UP pathway and provide direct evidence that UP is an intermediate in the biosynthesis of undecylprodiginine (**1**) in *Streptomyces coelicolor*. We also demonstrate that streptorubin B (**2**) is the cyclic derivative of undecylprodiginine produced by *S. coelicolor*, not butylcycloheptylprodigiosin (**4**) as previously proposed (Tsao et al., 1985; Fürstner et al., 2005), and that this too is generated from a UP intermediate. RedK and RedL are shown to be essential enzymes in this process and likely elongate a dodecanoyl thioester with malonyl-CoA and glycine. An analog of undecylprodiginine, with one of the hydrogen atoms on ring C replaced by a hydroxyl group, is produced as a shunt metabolite in an *S. coelicolor* mutant lacking the *redK* gene. RedR and RedQ as well as RedP play a role in biosynthesis of dodecanoic acid, the presumed precursor of the dodecanoyl thioester attached to RedL, and can also be replaced by their homologs from fatty acid biosynthesis. In each case, the process is less efficient and exerts less control over the structure of the hydrocarbon chain in the prodiginine products. In vivo evidence indicates (1) a major role for RedR and FabF in determining the product ratios of fatty acid and undecylprodiginine biosynthesis and (2) a complex interface between these two biosynthetic processes. Abrogated dodecanoic acid bioynthesis in a *redR*$^{-}$ *S. coelicolor* mutant permits efficient precursor-directed biosynthesis of prodiginine analogs with altered alkyl chains.

RESULTS

Structure Elucidation of the Cyclic Undecylprodiginine Derivative from *S. coelicolor*

Prodiginine antibiotics were extracted from harvested mycelia of *S. coelicolor* M511 (a prototrophic derivative of *S. coelicolor* A3[2] lacking plasmids and the ability to produce actinorhodin) grown on R5 agar medium (Kieser et al., 2000). LC-MS analyses of the extract identified two compounds absorbing significantly at 533 nm with m/z 394 ($[M+H]^+$) and m/z 392 ($[M+H]^+$; eluting with a slightly earlier retention time) as previously observed (Tsao et al., 1985). The hydrochloride salts of the antibiotics were purified from the extract by semipreparative HPLC. ^{1}H NMR and MS/MS analyses of the antibiotic of mass 393 daltons confirmed its structure as **1** (data not shown). The ^{1}H and ^{13}C NMR spectra of the antibiotic of mass 391 daltons were essentially identical to those reported for the hydrochloride salt of **2** isolated from marine actinomycete strain B 4358 (Laatsch et al., 1991; see the Supplemental Data available with this article online). On the other hand, they differed markedly from those reported for synthetic **4** (Fürstner et al., 2005). Structure **2** for this compound was confirmed by HSQC, COSY, and HMBC experiments (Figure 1). The HMBC experiments also showed that the signals for the 6′- and 8′-CH_2 groups of **2** in the ^{1}H and ^{13}C NMR spectra had been previously incorrectly assigned (Laatsch et al., 1991).

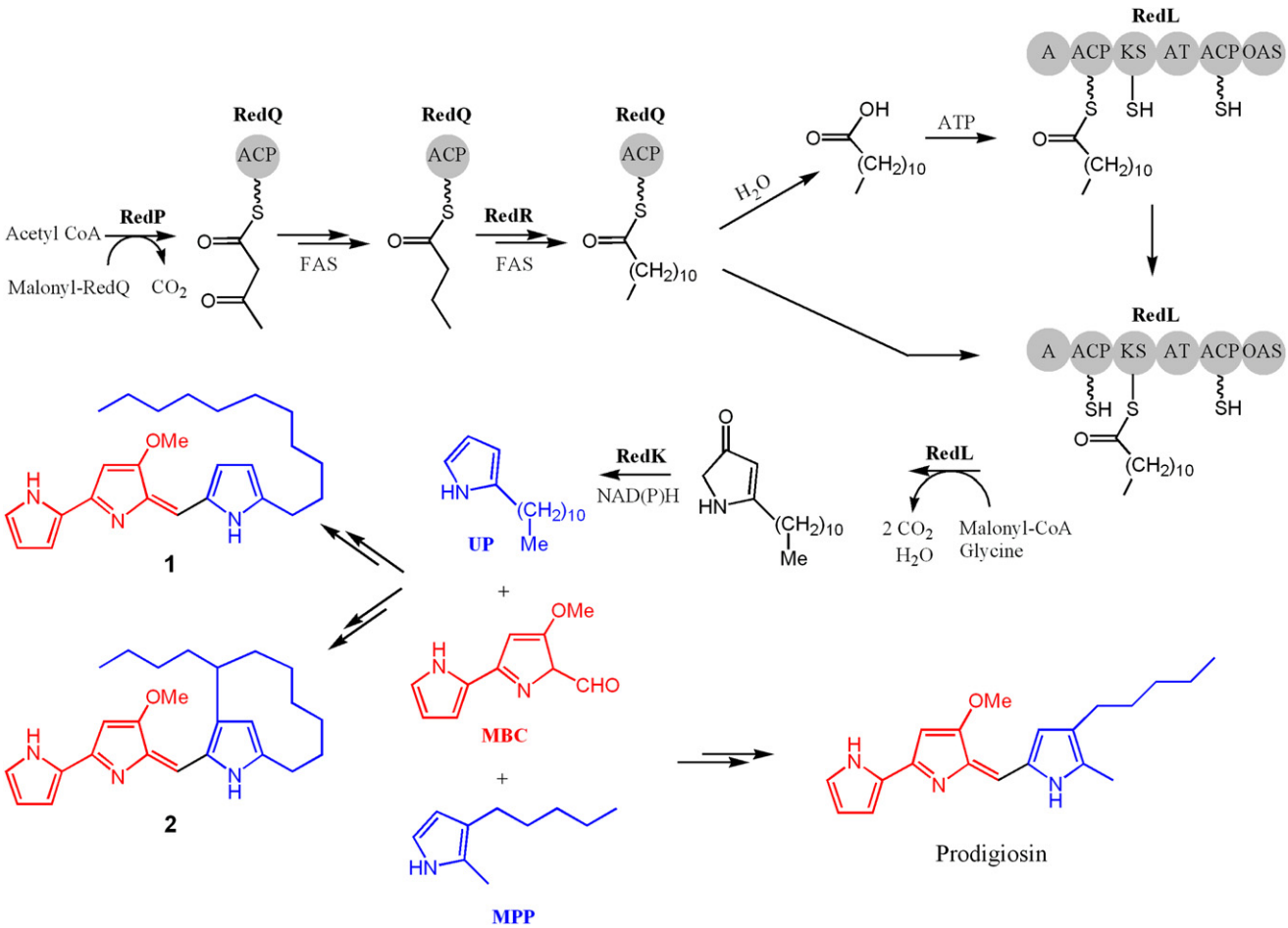

Figure 2. Proposed Role of RedP, RedQ, RedR, RedK, and RedL in Formation of the Undecylpyrrole-Derived Component of Undecylprodiginine and Streptorubin B

RedL is a multifunctional protein containing two acyl carrier protein (ACP) domains, an adenylation (A) domain, a ketosynthase (KS) domain, an acyltransferase (AT) domain, and an α-oxoamine synthase (OAS) domain.

Prodiginine Production in *redP*, *redQ*, and *redR* Deletion Mutants of *S. coelicolor*

S. coelicolor SJM1 was previously obtained by replacement of the *redP* gene in *S. coelicolor* M511 with an *oriT-aac(3)IV* cassette (Figure 3; Mo et al., 2005). In the current work, a similar approach was used to generate *S. coelicolor* SJM2, a *redQ* replacement mutant, and *S. coelicolor* SJM3, a *redR* replacement mutant (Figure 3). The *oriT-aac(3)IV* cassette in the SJM2 strain was deleted by FLP-mediated recombination (Gust et al., 2003) to generate *S. coelicolor* SJM5, a *redQ* deletion strain. A similar approach was used to generate *S. coelicolor* SJM4, a *redP* deletion mutant from the SJM1 strain (Figure 4). Replacement of *redR* in the SJM4 mutant by the *oriT-aac(3)IV* cassette generated *S. coelicolor* SJM6 (a *redR* replacement, *redP* deletion mutant).

On R2YE solid medium, *S. coelicolor* M511 and all the mutants derived from it grew and sporulated normally. Visual analysis indicated that all mutants generated some red-pigmented products, although at lower levels than the M511 strain. To quantitate prodiginine production, all of the mutants were grown for 10 days (240 hr) in liquid culture and analyzed throughout for cell growth and overall quantity of prodiginines (see the Supplemental Data). The highest prodiginine production levels were observed for the SJM4 strain (*redP* deletion mutant) at approximately 18% ± 4% of the levels seen for the M511 strain. The maximal prodiginine production in both *S. coelicolor* SJM4 and M511 was observed at approximately 96 hr. The SJM4 strain was indistinguishable in these ways from *S. coelicolor* SJM1 (a *redP* replacement mutant; Mo et al., 2005). Prodiginine levels in the *redR* replacement mutant (SJM3) were slightly lower (13% ± 3%) but not statistically different. In the SJM6 mutant, the levels were again lower at 8% ± 2% of wild-type levels. Thus, whereas all three mutants produced prodiginines, loss of both *redP* and *redR* impacted prodiginine yields greater than loss of just *redP* or *redR* alone. A much greater loss of prodiginine production was observed for deletion of *redQ* (SJM5) (3% ± 3% of wild-type levels). Replacement of *redQ* in the SJM2 strain led to a complete loss of detectable prodiginine production. The timings for maximal prodiginine production for the SJM3, SJM6, and SJM5 strains were 120, 168, and 216 hr, respectively. These mutants, particularly SJM5, also grew more slowly than the M511 strain.

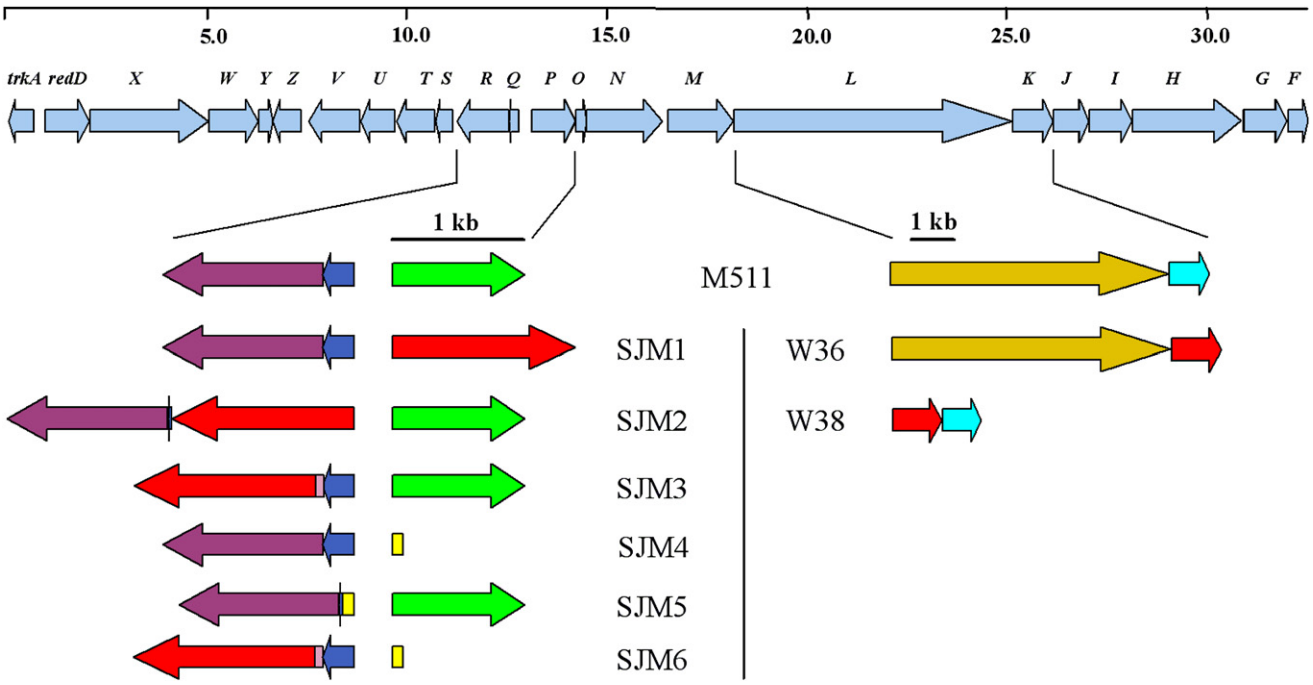

Figure 3. Organization of the *red* Cluster in Wild-Type *S. coelicolor* and the Mutants Constructed in This Study

The region containing the following genes is highlighted: *redR* (purple), *redQ* (blue), and *redP* (green), encoding homologs of fatty acid biosynthetic proteins, and *redL* (orange) and *redK* (light blue). SJM1–SJM5, W36, and W38 are mutants in which these genes have been replaced with *Apra*^{R} *oriT* (red) or deleted (yellow).

The prodiginine products made by the SJM mutants were analyzed by HPLC using mass spectrometry and absorbance (530 nm) as detection methods (Figure 4). As shown in Figure 5A, the M511 strain produced predominantly undecylprodiginine (**1**) and streptorubin B (**2**). Analogs with longer or shorter hydrocarbon chains were also present in very low quantities and could only be detected by LC-MS/MS analyses. The SJM4 mutant, like the SJM1 mutant (Mo et al., 2005), produced significant quantities of methylundecylprodiginine (**5**) (m/z 408 $[M+H]^+$) and methyldodecylprodiginine (**6**) (m/z 422 $[M+H]^+$) (Figure 4), the structures of which were confirmed as described previously (Mo et al., 2005). The SJM3 mutant produced the prodiginines (**1**, **2**, **5**, and **6**), and two further products, tetradecylprodiginine (**7**) (m/z 436 $[M+H]^+$, 42 AMU greater than **1**) and pentadecylprodiginine (**8**) (m/z 450 $[M+H]^+$, 56 AMU greater than **1**) (Figure 4C).

Growth of the SJM3 mutant in the presence of perdeuterated branched-chain amino acids led to the labeling of **5** and **6**, consistent with catabolism to perdeuterated isobutyryl-CoA or methylbutyryl-CoA which is then used by RedP to initiate UP biosynthesis. No labeling of **7** or **8** was observed in these experiments, indicating that these were not branched-chain alkylprodiginines but different linear alkylprodiginine products (this assignment was supported by feeding studies in the SJM3 mutant with fatty acids, as described below).

Using the model for undecylprodiginine biosynthesis involving elongation of an acetyl-CoA starter unit with a total of six malonyl-CoA extender units prior to condensation with glycine (Figure 2), **7** would be formed from a propionyl-CoA starter unit and seven extensions with malonyl-ACP and **8** from an acetyl-CoA starter unit and eight extensions with malonyl-ACP. A linear decylprodiginine (m/z 380 $[M+H]^+$, deriving from a propionyl-CoA starter unit and five malonyl-ACP units) and nonylprodiginine (m/z 366 $[M+H]^+$, deriving from an acetyl-CoA starter unit and five malonyl-ACP extender units) eluting at earlier retention times than undecylprodiginine were also produced at low levels in most fermentations of the SJM3 mutant. Variations in the relative levels of these shorter products and the presence of a longer linear heptadecylprodiginine were seen with multiple analyses of the SJM3 mutant. The presence of other linear- and branched-chain prodiginines within the complex mixture produced by the SJM3 strain could not be discounted. Cyclized forms of both the straight-chain and branched-chain prodiginines were also detectable in the fermentations of the SJM3 mutant. The ratio of each product to its cyclized derivative did not appear to differ in a clear way for branched-chain or straight-chain alkylprodiginines (suggesting the enzyme responsible for this step does not discriminate between them). In summary, deletion of *redR* relative to deletion of *redP* leads both to a slightly greater decrease in total prodiginine production and a greater variety of prodiginine products.

The same complex mixture of prodiginine products made in the *redR* deletion mutant was observed as a result of loss of both *redP* and *redR* (the SJM6 mutant) or deletion of *redQ* (the SJM5 mutant) (Figures 4D and 4E). No discernible difference in the relative ratios of the various prodiginine products was observed from multiple fermentations and analyses of these mutants.

Genetic Complementation of *S. coelicolor* SJM3

We previously reported that plasmid-based expression of *redP* (pKR3) in the SJM1 mutant led to restoration of both prodiginine yields and of undecylprodiginine and its cyclized derivative as the sole products. A similar experiment was used to complement the

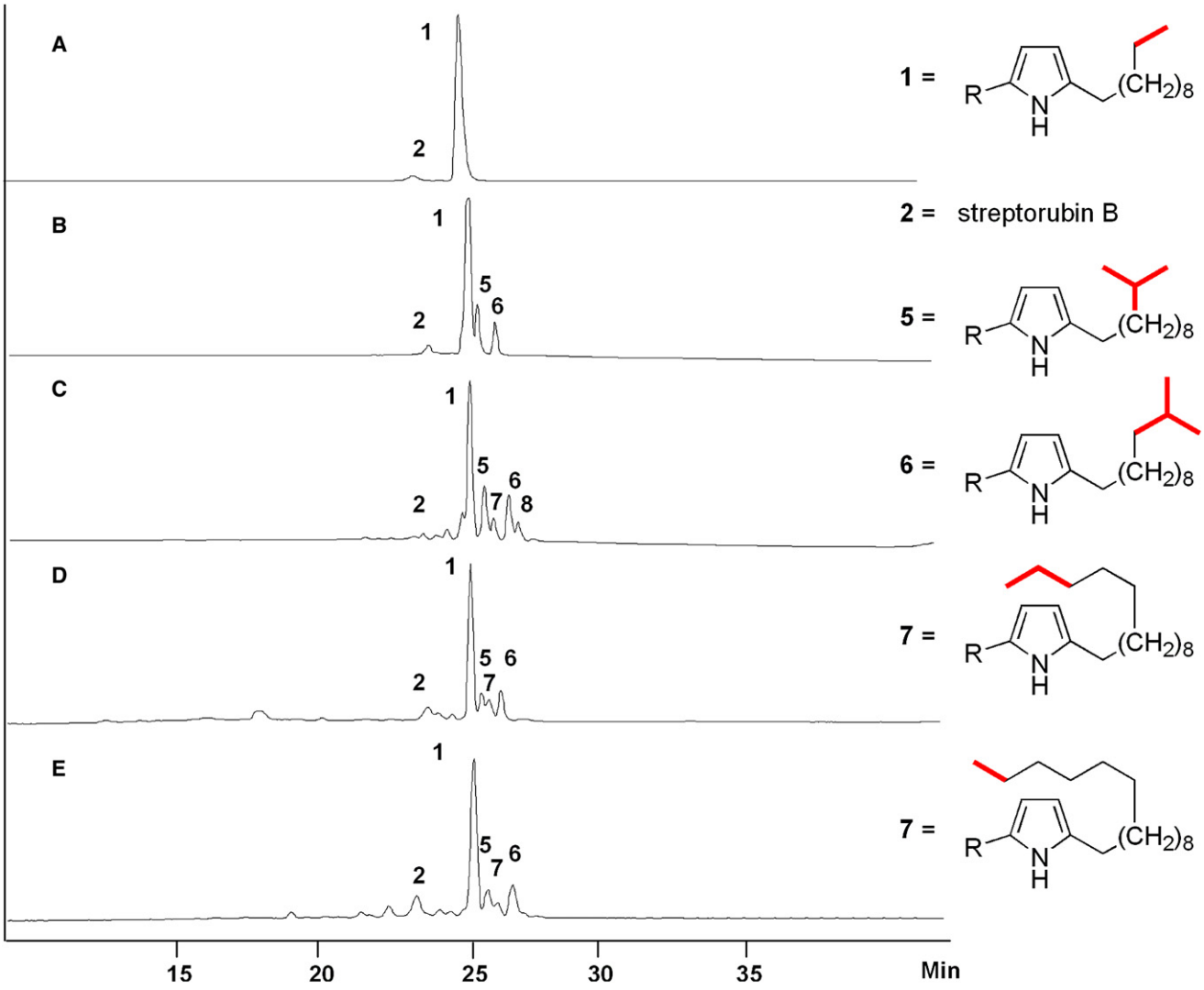

Figure 4. HPLC Analyses of Prodiginines Produced by *S. coelicolor* M511, SJM1, SJM3, SJM5, and SJM6

(A) M511.
(B) SJM1.
(C) SJM3.
(D) SJM5.
(E) SJM6.

1, undecylprodiginine; **2**, streptorubin B; **5**, methylundecylprodiginine; **6**, methyldodecylprodiginine; **7**, tetradecylprodiginine; **8**, pentadecylprodiginine. Left panel: prodiginines were separated by reverse-phase HPLC and detected by both absorbance at 530 nm (left panel) and mass spectral analysis. Right panel: alkylpyrrole component of prodiginines showing putative starter unit for alkyl chain biosynthesis in red.

SJM3 mutant with pSJM1 (a *redR* expression plasmid). Prodiginine production in the SJM3/pSJM1 strain was 78% ± 3% of that observed for the wild-type M511 strain, 4-fold higher than the 18% ± 4% observed with the SJM3 strain carrying the control pSE34 plasmid. Prodiginine levels of 28% ± 3% were observed in a complementation experiment carried out using pSJM2 for expression of *S. coelicolor* FAS *fabF*, less than a 2-fold increase from that of the SJM3/pSE34 strain. LC-MS analyses of prodiginine analogs produced by these complementation experiments in the SJM3 mutant were carried out. In the case of the SJM3/pSJM1 strain, the prodiginine profile was comparable to that of the M511 strain in which undecylprodiginine and its cyclized derivative were the only discernible products (data not shown). For the SJM3/pSJM2 and SJM3/pSE34 strains the product ratio was unchanged from that of the SJM3 strain, demonstrating that increased levels of FabF increased the rate of production of all prodiginines in this strain.

Directed Biosynthesis of Prodiginines in *S. coelicolor* SJM3

Reproducible and logical alterations in the ratio of prodiginine products made by the SJM3 mutant could be accomplished through growth in the presence of various fatty acids (Figure 5A). For instance, feeding pentadecanoic acid (PDA) to the SJM3

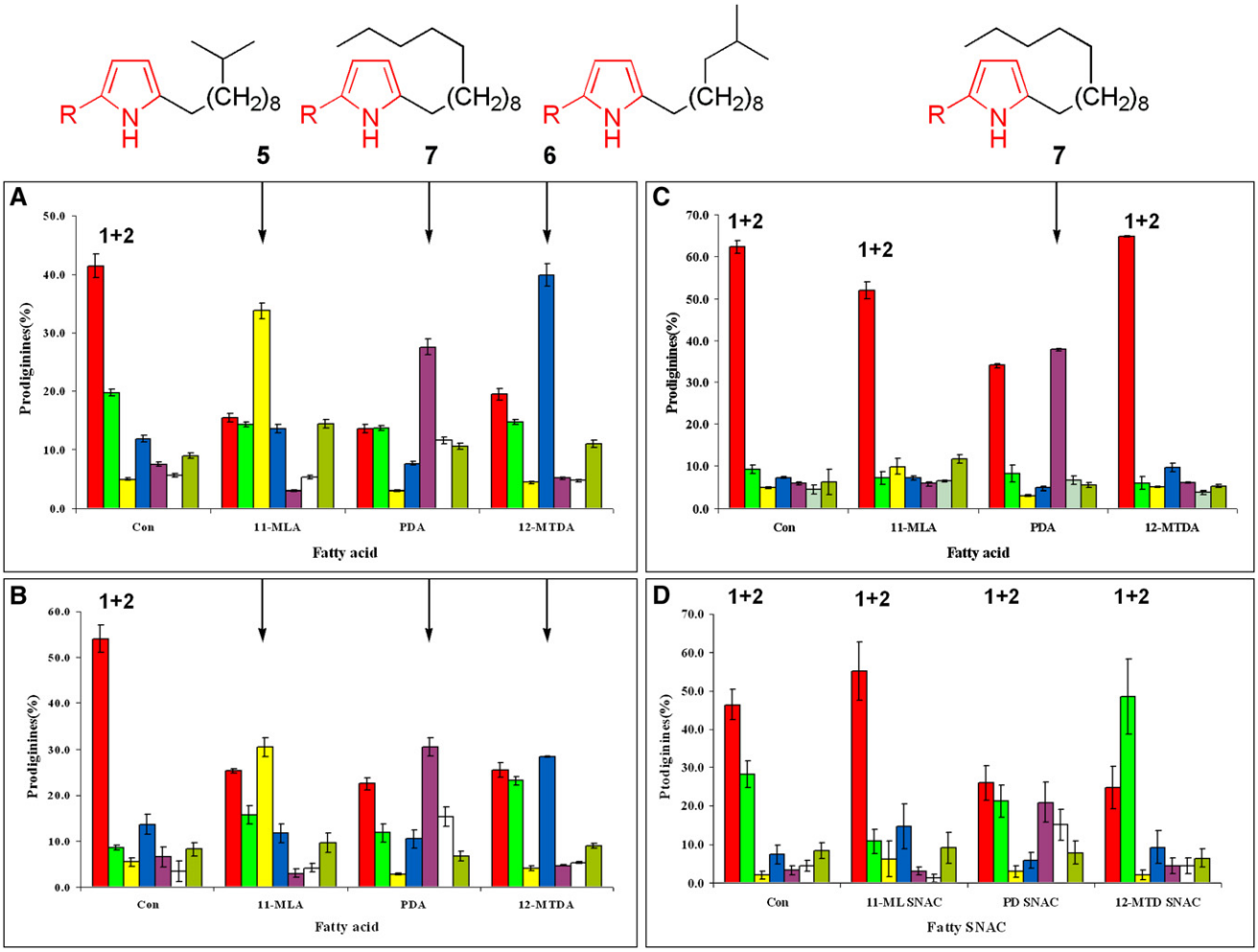

Figure 5. Prodiginine Composition of *S. coelicolor* SJM3 Grown in the Presence of Fatty Acids and Their N-Acetylcysteamine Thioesters

(A) SJM3 and free fatty acids.

(B) SJM3/pSE34 and free fatty acids.

(C) SJM3/pSJM2 and free fatty acids.

(D) SJM3 and fatty acid N-acetylcysteamine (SNAC) thioesters.

Fatty acids: 11-methyllauric acid (ll-MLA); pentadecanoic acid (PDA); and 12-methyltridecanoic acid (12-MTDA). Prodiginines: red, undecylprodiginine (**1**); green, streptorubin B (**2**); yellow, methylundecylprodiginine (**5**); blue, methyldodecylprodiginine (**6**); purple, tetradecylprodiginine (**7**); light sky blue, short-chain prodiginines; dark green, others. An arrow is used to depict cases where a different prodiginine product (not **1** and **2**) predominates. The structure of this major alkylpyrrole component is also given, with the carbon chain of the fatty acid chain depicted in black. Results are the mean (±SEM) of triplicate assays.

mutant gave rise to a clear increase in the corresponding tetradecylprodiginine product (**7**) and a relative decrease in the levels of the other prodiginines (Figure 5A). Feeding with 11-methyllauric acid (11-MLA) led to a clear increase in the levels of methylundecylprodiginine (**5**), whereas 12-methyltridecanoic acid (12-MTDA) led to an increase in methyldodecylprodiginine (**6**). In all cases, the prodiginine product whose levels increased was consistent with elaboration of the fatty acid to a monopyrrole via extension with one malonyl unit and glycine. Although these changes were clearly and reproducibly observed for the SJM3 mutant (each analysis was conducted on at least three separate fermentations), they were less clear for the other mutants (SJM1, SJM5, and SJM6), and no change was observed when the experiments were carried out on the M511 strain. The efficient directed biosynthesis with these fatty acids in the SJM3 mutant allowed selective production of different branched-chain and long-chain alkylprodiginines as the major products. Directed biosynthesis in the SJM3 mutant with these fatty acids activated as the corresponding N-acetylcysteamine (NAC) thioesters was much less effective. As shown (Figure 5D), no discernible change in the levels of branched-chain prodiginine products made by the SJM3 mutant was seen by growth in the presence of the NAC thioester of branched-chain fatty acids (there was variation in the ratio of undecylprodiginine to streptorubin B). An increase in tetradecylprodiginine was seen when the NAC thioester of PDA was fed, but this was less than that observed using the free acid (Figure 5A). Directed biosynthesis in the SJM3 mutant with fatty acids longer than 15 carbons, or bearing a halogen

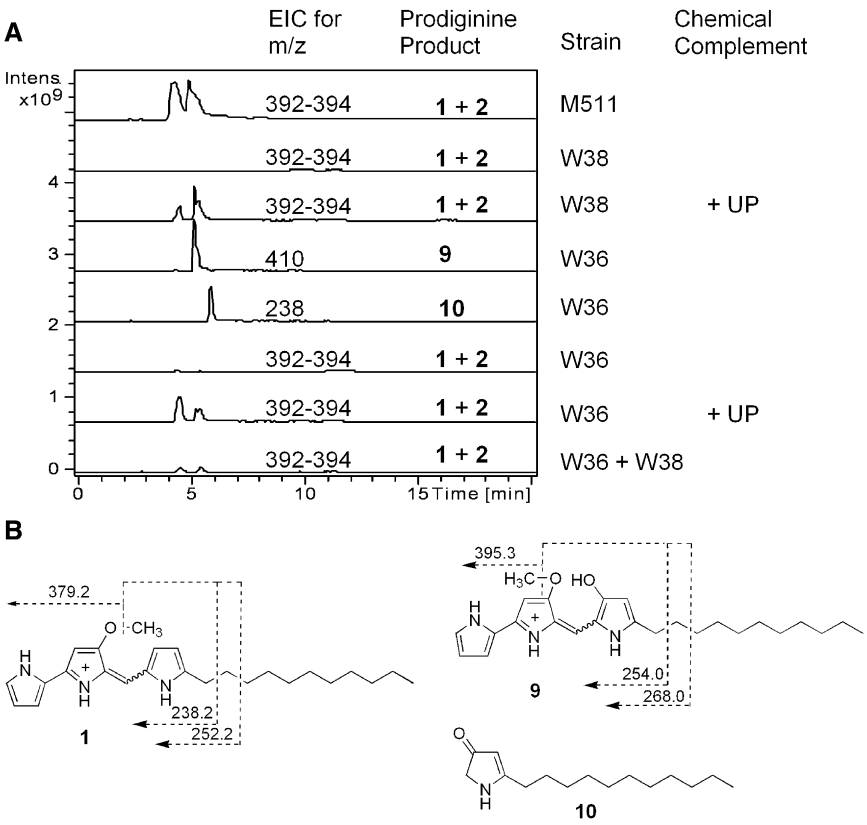

Figure 6. Analysis of Prodiginines Made by *S. coelicolor* M511, W36, and W38

LC-MS (A) and MS-MS (B) analyses of undecylprodiginine **1** and its hydroxylated derivative **9**. A tentative structure for **10** is provided in (B).

at the ω-2 position, did not result in production of the predicted prodiginine product, indicating substrate specificity in steps of the pathway subsequent to RedR.

Overall yields of prodiginines in directed-biosynthesis experiments with the SJM3 mutant were also determined (see the Supplemental Data). When straight-chain PDA was fed, there was a dramatic 10-fold increase in the levels of tetradecylprodiginine (**7**) product and a 2-fold increase in overall prodiginine production as a result (the levels of undecylprodiginine/streptorubin B and the other prodiginines remained essentially unchanged). When dodecanoic acid was added, the amount of undecylprodiginine/streptorubin B increased dramatically, and the overall prodiginine titers increased greater than 4-fold to almost that of the M511 strain. Thus, the SJM3 strain can be both genetically complemented (using a *redR* expression plasmid) and chemically complemented. Markedly different observations to these were made using the branched-chain fatty acids 11-methyllauric acid and 12-methyltridecanoic acid, where the overall prodiginine yield (including undecylprodiginine/streptorubin B) decreased.

Directed-biosynthesis experiments were also carried out with the SJM3 mutant carrying pSE34 (empty plasmid control) and pSJM2 (a *fabF* expression plasmid; Figures 5B and 5C, respectively). The same qualitative changes in the ratio of prodiginine products were seen for the SJM3/pSE34 strain as with the SJM3 mutant. Addition of each fatty acid led to an increase in the corresponding prodiginine product (the increase was approximately 2-fold higher in the absence of the control plasmid; Figure 5B). In stark contrast, only pentadecanoic acid caused an alteration in the prodiginine products in the SJM3/pSJM2 strain (the increase in the corresponding tetradecylprodiginine product was indistinguishable from that observed in the SJM3 mutant and the SJM3/pSJM2 strain). Feeding the branched-chain fatty acids 11-methyllauric acid and 12-methyltridecanoic acid led to no detectable changes in the prodiginine profiles of the SJM3/pSJM2 strain. The observation that only straight-chain fatty acids can alter the prodiginine profiles of the SJM3/pSJM2 strain, but that straight and branched-chain fatty acids alter the ratio in the SJM3 and SJM3/pSE34 strains, was shown to be highly reproducible.

Generation and Analysis of *redL* and *redK* Gene Replacement Mutants

The demonstration that RedX and RedN are not involved in UP biosynthesis, as originally proposed, but rather MBC biosynthesis (Stanley et al., 2006), suggests that RedL catalyzes the extension of an activated dodecanoic acid derivative with first a malonyl unit ($-CO_2$) and then a glycine-derived unit, followed by loss of a molecule of CO_2 and a molecule of water to yield 2-undecyl-4-pyrrolinone (Figure 2). Reduction of the keto group in this proposed intermediate, followed by elimination of water, would yield UP. RedK has been proposed to catalyze this reduction on the basis of comparative bioinformatic analyses (Williamson et al., 2005), but no experimental data to support this hypothesis have been available before now. To investigate the roles of RedL and RedK in prodiginine biosynthesis, we generated *redK::oriT-aac(3)IV* and *redL::oriT-aac(3)IV* mutants of *S. coelicolor* (designated W36 and W38, respectively) using PCR targeting (Gust et al., 2003). LC-MS analyses showed that neither mutant produced undecylprodiginine or streptorubin B when grown alone (Figure 6). These prodiginine products were observed in coculture experiments with the W36 and W38 mutants (Figure 6).

To further investigate the role of RedL and RedK in prodiginine biosynthesis, we fed chemically synthesized UP to the W36 and W38 mutants. In both cases, this restored production of undecylprodiginine and streptorubin B (Figure 6). In contrast, feeding of chemically synthesized MBC (Dairi et al., 2006) to the mutants did not restore prodiginine production (data not shown). To the best of our knowledge, these results provide the first direct evidence that UP is an intermediate in both undecylprodiginine and streptorubin B biosynthesis and show that RedL and RedK are required for the UP but not the MBC pathway.

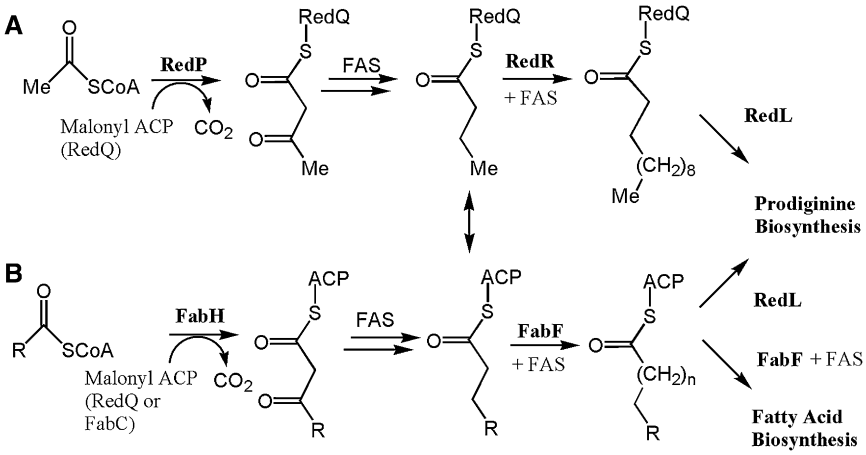

Figure 7. Interface of Fatty Acid and Undecylprodiginine Biosynthesis

(A) Initiation of UP biosynthesis by RedP, RedQ, and RedR.

(B) Initiation of the biosynthesis of UP and analogs by ketosynthase enzymes of fatty acid biosynthesis (FabH and FabF).

R = CH_3, CH_3CH_2, $(CH_3)_2CH$, and $(CH_3)_2CHCH_2$. n = 7–9.

LC-MS analyses of the W36 strain lacking *redK* also revealed that two compounds with m/z = 410.3 ($[M+H]^+$) and 238.0 ($[M+H]^+$) accumulate in it. The compound with m/z = 410.3 absorbs significantly at 533 nm, and high-resolution mass spectrometric analysis established its molecular formula as $C_{25}H_{35}N_3O_2$ (calculated for $C_{25}H_{36}N_3O_2^+$: 410.2802, found: 410.2806), suggesting that it is a hydroxylated derivative of undecylprodiginine. LC-MS/MS analyses provided further support for this interpretation and showed that the hydroxyl group is located on the tripyrrole nucleus, not the hydrocarbon side chain (Figure 6B). Feeding of UP to the W36 mutant restored undecylprodiginine production, indicating that the hydroxyl group is located on ring C of the undecylprodiginine analog accumulated by W36. Biosynthetic considerations lead us to propose structure **9** for this compound (Figure 6B). We tentatively propose that the product with m/z = 238.0 is 2-undecyl-4-pyrrolinone (**10**; Figure 6B), the product of RedL. The apparent instability of both compounds accumulated in the W36 mutant in the above experiments has thus far prevented us from isolating them in sufficiently pure form to allow confirmation of their structures by NMR analyses. The hydroxylated undecylprodiginine derivative has, to our knowledge, not previously been observed.

DISCUSSION

Streptorubin B Is the Cyclic Derivative of Undecylprodiginine Made by *S. coelicolor*

Undecylprodiginine was identified as one of the products of the *S. coelicolor red* gene cluster over 20 years ago, but the identity of the cyclic derivative of undecylprodiginine has remained unclear until now. It was originally proposed to be substituted at the *ortho* position (**4**), based on comparisons with data from early reports of the isolation of **4** from other actinomycetes. However, **2** was isolated from actinomycete strain B 4358 and its structure was unambiguously elucidated by 2D NMR experiments (Laatsch et al., 1991). These studies led Laatsch and coworkers to suggest that the structure of **4** was incorrect and that it should be reassigned as **2**. Recently, Fürstner and coworkers completed a total synthesis of **4** and, based on comparisons of the ^1H NMR data for synthetic **4** with the ^1H NMR data reported for the cyclic derivative of undecylprodiginine isolated from *S. coelicolor* (Tsao et al., 1985), suggested that this natural product was indeed **4** (Fürstner et al., 2005). However, in the present studies, we found no evidence for the production of **4** by *S. coelicolor*, and 2D NMR analyses of the cyclic undecylprodiginine derivative isolated from *S. coelicolor* unambiguously established its structure as **2**.

RedR Is Required for Efficient Formation of an Activated Dodecanoic Acid Intermediate in Prodiginine Biosynthesis

RedR has sequence similarity with FabF, an enzyme responsible for the elongation steps of fatty acid biosynthesis. FabF catalyzes a decarboxylative condensation of acyl-ACPs with malonyl-ACP. It has been proposed (Cerdeno et al., 2001) that RedR catalyzes an analogous reaction in undecylprodiginine biosynthesis, providing an activated dodecanoic acid precursor for formation of UP. The analysis of the *redR* replacement mutant (SJM3), where production of undecylprodiginine is dramatically reduced but restored by addition of dodecanoic acid, is consistent with this role.

FabF Likely Supports Prodiginine Biosynthesis in a *ΔredR* Strain

Continued production of undecylprodiginine in both the SJM3 and SJM6 strains demonstrates that an alternative pathway must provide some dodecanoic acid-derived substrate for prodiginine biosynthesis. We hypothesize that FabF serves as a replacement for RedR in these *S. coelicolor* strains (Figure 7B), accounting for the production of an array of different-length straight-chain alkylprodiginines as well as branched-chain alkylprodiginines. The *fabF* gene is located within a cluster of fatty acid biosynthetic genes (Revill et al., 1995) and is most likely responsible for catalyzing the elongation of both branched-chain and straight-chain acyl-ACP substrates to give the mixture of (C_{15}–C_{18}) branched-chain and straight-chain fatty acids made by streptomycetes (Figure 7B; this enzyme has yet to be characterized in vitro). The observation that plasmid-based expression of *fabF* in the SJM3 mutant increased overall prodiginine titers but did not alter the ratio of prodiginine products provides compelling additional support for the hypothesis that FabF can substitute for RedR. Clearly, FabF is less efficient and exerts less control than RedR in this role. RedR must therefore have substrate specificity which is preferential for elongation of straight-chain acyl-ACP substrates, and the RedR-catalyzed elongation process must also have a mechanism which ensures efficient elongation that terminates at an activated dodecanoic acid

(Figure 7A). Conversely, the FabF-catalyzed process would not be expected to terminate at this point, elongating fatty acids such that they are too long to prime the next step in prodiginine biosynthesis (Figure 7B). Given that branched-chain fatty acids predominate in streptomycetes, FabF might have a preference for elongating branched-chain acyl-ACP substrates (the initiation enzyme FabH in streptomycetes and bacilli has a preference for branched-chain acyl-CoA substrates; Choi et al., 2000; Han et al., 1998). These FabF properties might account for the observation that plasmid-based expression of *fabF* selectively blocks the ability of branched fatty acids (11-MLA and 12-MTDA) to alter the ratio of prodiginine products in the SJM3 mutant.

Prodiginine Biosynthesis in the Absence of RedP and RedR

Analysis of the SJM3 mutant suggests that FabF can substitute for the role of RedR, albeit inefficiently. Previous analyses of the SJM1 mutant have led to a similar conclusion regarding FabH and RedP. In the SJM6 mutant, neither RedP nor RedR are present, and an additional decrease in prodiginine yields is observed. In this mutant, all catalytic steps leading to an activated dodecanoic acid derivative are presumably catalyzed by the fatty acid biosynthetic process (Figure 7B). How much of this process proceeds using substrates activated as FabC and RedQ ACP thioesters must depend upon the abundances of these two ACPs, the ACP specificity of FabH and FabF, and the specificity of the enzyme or enzymes which catalyze the transfer of the ACP-activated species onto the next enzyme in the prodiginine biosynthetic process.

RedQ Is Not Essential for Prodiginine Biosynthesis

In the absence of RedQ (SJM5 strain), very low levels of prodiginine biosynthesis are observed. The observation that the range of prodiginine products is as broad as in the SJM3 and SJM6 strains indicates that the elongation process is likely proceeding at least in part via FabH and FabF using a FabC ACP (Figure 7B), as all evidence to date suggests that elongation using RedP and RedR would result in selective production of a straight-chain dodecanoic acid derivative. As RedP and RedR are present in this mutant, it follows that they are less efficient than FabH and FabF with FabC ACP thioesters. A RedQ-independent prodiginine pathway also suggests that an elongated acyl-FabC can function as an intermediate in undecylprodiginine biosynthesis. Nonetheless, the yields of prodiginines in the SJM5 mutant are the lowest of all of the mutants, demonstrating that the RedQ-independent process is very inefficient.

In summary, formation of an activated dodecanoic acid derivative for prodiginine biosynthesis requires a complex interface of RedP, RedQ, and RedR with the enzymes of fatty acid biosynthesis. In the presence of the three Red proteins, biosynthesis proceeds along a pathway which selectively produces dodecanoyl-RedQ (Figure 7A). RedP and RedR catalyze the elongation steps and direct the biosynthesis toward formation of a straight-chain substrate, but must depend upon the fatty acid biosynthetic enzymes for converting 3-ketoacyl-RedQ to the corresponding acyl-RedQ species. In the absence of RedP, RedR, or RedQ, the elongation steps of the process can be catalyzed by FabH and FabF, but with a reduction in both efficiency and selectivity (Figure 7B).

RedL and RedK Participate in UP Biosynthesis

Initial analysis of the undecylprodiginine biosynthetic process led to the proposal that a dodecanoyl chain was transferred from RedQ onto a ketosynthase domain of RedX and that the subsequent elongation and cyclization steps on the pathway to UP were catalyzed by the actions of RedX and RedN (Cerdeno et al., 2001). RedL was initially assigned a role in the MBC pathway (Cerdeno et al., 2001). More recent work has shown that RedX and RedN are required for formation of MBC (Stanley et al., 2006). A similar conclusion has been reached regarding the homologs PigH and PigJ from the prodigiosin system (Williamson et al., 2005; Garneau-Tsodikova et al., 2006). It has also been demonstrated that there is no RedL homolog in the prodigiosin biosynthetic gene cluster (Harris et al., 2004). By a process of elimination, these studies suggest that RedL is more likely responsible for steps in the UP pathway in *S. coelicolor*. The observations that a *redL::oriT-aac(3)IV* mutant does not produce detectable prodiginines, but can be chemically complemented with UP, provide clear experimental evidence for this role. It also provides unequivocal direct evidence that UP is an intermediate in the biosynthesis of undecylprodiginine and streptorubin B. The range of prodiginine products made in the *redP*$^{-}$, *redQ*$^{-}$, and *redR*$^{-}$ mutants and the observations that the ratio of these in the *redR*$^{-}$ mutant could be altered through addition of different fatty acids indicate that RedL and subsequent enzymes in the pathway can utilize a number of other fatty acids similar to dodecanoic acid. Nonetheless, a limit to the type of fatty acids that could be converted to prodiginines in the *redR*$^{-}$ mutant and the observation that only dodecanoic acid gave almost complete restoration of overall prodiginine yields indicate there is also substrate specificity in biosynthetic steps subsequent to those catalyzed by RedP and RedR. The reason why overall prodiginine yields decrease upon addition of branched-chain fatty acids to the *redR*$^{-}$ mutant, but increase with straight-chain fatty acids (dodecanoic acid or PDA), is unclear. Elongation of branched-chain fatty acids by the prodiginine biosynthetic pathway might be slow and inhibit biosynthesis of straight-chain alkylprodiginines. Such an explanation would not readily account for the observation that overall prodiginine yields also decreased when branched-chain fatty acids are added to the SJM3/pSJM2 strain, where the increased levels of FabF appear to stop the elongation of these fatty acids into prodiginines.

RedL contains an N-terminal adenylation (A) domain, followed by an ACP domain, a ketosynthase domain (KS), an acyltransferase domain (AT), and another ACP domain (Cerdeno et al., 2001; Figure 2). The C terminus of this protein contains an α-oxoamine synthase (OAS) domain showing similarity to known α-oxoamine synthases, such as 1-amino-2-oxononanoate synthase (AONS), with 34% identity to the C-terminal OAS domain of RedN (Cerdeno et al., 2001; Stanley et al., 2006). RedL has the catalytic domains needed for processing dodecanoyl-RedQ to 2-undecylpyrrolin-4-one (Figure 2). In this process, the AT domain presumably primes the adjacent ACP domain with malonyl-CoA, which undergoes a subsequent decarboxylative condensation with a dodecanoyl thioester in the active site of the KS domain. The resulting 3-ketomyristoyl-ACP could then undergo a condensation with glycine followed by decarboxylation and cyclization, catalyzed by the OAS domain. The KS domain might be primed

via a direct transfer from dodecanoyl-RedQ (Figure 2). Analysis of the putative RedL catalytic domains suggests an alternate mechanism in which dodecanoic acid, obtained from hydrolysis of dodecanoyl-RedQ, could be loaded by the N-terminal A domain onto the adjacent ACP domain and then transferred onto the KS domain (Figure 2). The observation that free acids (such as dodecanoic acid) were effective in directed biosynthesis in the SJM3 mutant, whereas the corresponding NAC thioesters were not, is consistent with the priming of RedL via the A domain. Analysis of prodiginine products of the *redQ*$^{-}$ mutant indicates that RedL priming through at least one of these pathways must also be possible for dodecanoyl FabC.

The observations that a *redK::oriT-aac(3)IV* mutant does not produce undecylprodiginine or streptorubin B but instead accumulates a ring C hydroxylated analog of undecylprodiginine and that feeding of UP to the mutant restores undecylprodiginine and streptorubin B production, together show that RedK participates in UP biosynthesis. RedK is similar to many known and putative NAD(P)H-dependent oxidoreductases and we therefore propose that it catalyzes reduction of the keto group in 2-undecyl-4-pyrrolinone, the putative product of RedL. Subsequent elimination of water from the product of this reaction would yield UP. It seems likely that the hydroxylated analog of undecylprodiginine that accumulates in the *redK::oriT-aac(3)IV* mutant is a shunt metabolite resulting from condensation of 2-undecyl-4-pyrrolinone with MBC. Small amounts of this hydroxylated analog can be detected in *S. coelicolor* M511 by LC-MS/MS analyses (C.C. and G.L.C., unpublished data).

The finding that UP is the precursor of streptorubin B in *S. coelicolor* shows that either UP or undecylprodiginine must undergo oxidative cyclization with loss of the hydrogen atom on carbon 4 of the pyrrole ring and a hydrogen atom from carbon 7′ of the hydrocarbon side chain to form streptorubin B, thus ruling out oxidation of the hydrocarbon chain at carbon 7′ during UP assembly. The *redG* gene encodes a protein with sequence similarity to known Rieske oxygenases and has been proposed to participate in the oxidative cyclization of undecylprodiginine to streptorubin B (Cerdeno et al., 2001). The stage is now set for elucidating the enzymes involved and mechanistic details of this remarkable oxidative cyclization reaction.

SIGNIFICANCE

Red-pigmented tripyrrole natural products are of interest for their unusual and diverse biosynthetic origins as well as their numerous biological activities and potential therapeutic applications. This study has established unequivocally that *S. coelicolor* produces streptorubin B, a cyclized derivative of undecylprodiginine. Both of these natural products are biosynthesized using undecylpyrrole (UP) as an intermediate. The RedK and multifunctional RedL proteins catalyze formation of UP from dodecanoic acid, malonyl-CoA, and glycine. The dodecanoic acid is generated through a redirection of the fatty acid biosynthetic process, accomplished by using RedP and RedR in place of FabH and FabF for the condensation steps. The products generated by fatty acid biosynthetic processes in *S. coelicolor* are shown to be controlled by condensing enzymes involved in elongation steps as well as the initiation step. Evidence indicates that the ACP specificities of these enzymes allows for efficient separation of two distinct biosynthetic processes, despite the apparent use of common enzymes for the other steps. Deletion of the *red* genes involved in UP biosynthesis and precursor-directed biosynthesis have been used to generate different prodiginine analogs.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Culture Conditions

All strains, plasmids, and oligonucleotides used are listed in the Supplemental Data. *S. coelicolor* manipulations were carried out as previously described (Gust et al., 2003; Mo et al., 2005). Prodiginine production was assessed on R2YE or R5 media (Mo et al., 2005; Stanley et al., 2006), and YEME liquid cultures or SFM solid cultures were used for genomic DNA isolation (Kieser et al., 2000). YEME liquid cultures were used for making protoplasts (Kieser et al., 2000). Luria-Bertani (LB) and SOB liquid media were used for growing *Escherichia coli*. Ampicillin (50 μg/ml), apramycin (50 μg/ml), chloramphenicol (25 μg/ml), kanamycin (50 μg/ml), nalidixic acid (25 μg/ml), spectinomycin (50 μg/ml), and streptomycin (50 μg/ml), all from Sigma, were added to growth media as required.

Replacements of *redQ* and *redR* by the *oriT-aac(3)IV* Cassette in *S. coelicolor*: Generation of SJM2 and SJM3

Disruptions of the *red* genes were carried out using PCR targeting as described previously (Gust et al., 2003; Mo et al., 2005). The primer set RedQ-F/RedQ-R and RedR-F/RedR-R were used to amplify the *oriT-aac(3)IV* cassette from pIJ773. The PCR products were used to replace *redQ* and *redR* in *S. coelicolor* cosmid 3F7 containing the *red* gene cluster. The modified cosmids SC3F7-2 and SC3F7-3 were used to make an allelic exchange in *S. coelicolor* M511 following standard protocols (Gust et al., 2003; Mo et al., 2005). The resulting SJM2 and SJM3 strains were Kan^{S} and Apra^{R}. Allelic replacement of *redQ* and *redR* was confirmed by PCR amplification of chromosomal DNA using oligonucleotides KORedQ-F/KORedQ-R and KORedR-F/KORedR-R, which primed approximately 100 bp outside the region of recombination. The predicted 1546 bp and 1619 bp PCR products were obtained using chromosomal DNA from SJM2 and SJM3, respectively. PCR amplification using chromosomal DNA of M511 gave products of 369 bp (*redQ*) and 1412 bp (*redR*). The PCR products from the SJM2 and SJM3 mutants were subsequently cloned and sequenced, confirming the allelic replacements of *redQ* and *redR*.

Deletion of *redP* and *redQ*: Generation of *S. coelicolor* SJM4 and SJM5

These gene deletions were carried out via FLP-mediated recombination (Gust et al., 2003). Briefly, electro-competent *E. coli* DH5α/BT340 cells were transformed with SC3F7-1 and SC3F7-2 cosmids. BT340 (pCP20; Amp^{R} and Cm^{R}; Table S2) has temperature-sensitive replication and heat-inducible expression of the FLP recombinase. Cm^{R} and Apra^{R} transformants were selected on LB at 30°C and propagated nonselectively at 42°C. FLP recombinase acts on FRT sites which flank the *oriT-aac(3)IV* cassette, leaving behind an 81 bp "scar" sequence (Datsenko and Wanner, 2000). Cm^{R}, Kan^{R}, and Apra^{S} colonies were selected, providing cosmid SC3F7-4 (loss of *oriT-aac(3)IV* from SC3F7-1) and SC3F7-5 (loss of *oriT-aac(3)IV* from SC3F7-2). PCR analysis with primers KoFabH-F/KoFabH-R and KoRedQ-F/KoRedQ-R provided bands of the predicted size (327 and 242 bp, respectively). The *neo* resistance gene in these cosmids was subsequently replaced by an *oriT-aadA* cassette through λ-Red-mediated recombination, permitting conjugation into the appropriate *S. coelicolor* host. Briefly, primers SuperNeo-F1/SuperNeo-R1 were used to amplify the *oriT-aadA* cassette from pIJ778. The purified PCR product was introduced into *E. coli* BW25113/pIJ790 containing the SC3F7-4 and SC3F7-5 cosmids. Selection for Spec^{R} and Kan^{S} transformants provided SC3F7-6 and SC3F7-7 (*neo* replacement was confirmed by PCR with primers Neo-UF1-Neo-UR1, which provided the predicted 1652 bp product). The SC3F7-6 and SC3F7-7 cosmids were introduced into the SJM1 and SJM2 mutants, respectively, by intergenic conjugation. An initial round of screening for Spec^{R} exconjugants, and then for Spec^{S} and Apra^{S}, gave rise to SJM4 and

SJM5. The genotypes of the resulting SJM4 and SJM5 mutants were confirmed by PCR analysis with the primers KoFabH-F/KoFabH-R and KoRedQ-F/KoRedQ-R.

Generation of *S. coelicolor* SJM6

Primers RedR-F/RedR-R were used to amplify the *oriT-aac(3)IV* cassette from pIJ773, which was used to replace *redR* with *oriT-aac(3)IV* in the SC3F7-4 cosmid (containing the *redP* deletion) by PCR targeting. The resulting SC3F7-8 cosmid DNA sample from transformants was shown to contain the desired gene replacement both by restriction analysis and PCR using KOFabH-F/KOFabH-R, which gave a predicted 327 bp PCR product confirming the *redP* in-frame deletion. PCR with KORedR-F/KORedR-R provided a 1619 bp PCR product consistent with replacement of *redR* with the *oriT-aac(3)IV* cassette. This cosmid was transferred by conjugation into the SJM4 *S. coelicolor* strain (containing a *redP* deletion), ultimately giving rise to $Apra^R$ and Kan^S SJM6 strains, the genotypes of which were confirmed by the same PCR analysis.

Generation of *S. coelicolor* W36 and W38

Disruption of the *redK* and *redL* genes was carried out using PCR targeting as described previously (Gust et al., 2003). The primer sets RedK-F/RedK-R and RedL-F/RedL-R were used to amplify the *oriT-aac(3)IV* cassette from pIJ773 and the PCR products were used to replace *redK* and *redL* in *S. coelicolor* cosmid 3F7, respectively. The modified cosmids SC3F7-9 and SC3F7-10 were used to make an allelic exchange in *S. coelicolor* M511 following standard protocols (Gust et al., 2003). The resulting W36 and W38 strains were Kan^S and $Apra^R$. Genomic DNA was isolated from these strains grown on SFM agar using the Fast DNA spin kit for soil (MB Biolabs). Allelic replacement of *redK* and *redL* in W36 and W38, respectively, was confirmed by PCR amplification from genomic DNA using the oligonucleotides KORedK-F/KORedK-R and KORedL-F/KORedL-R, which primed approximately 250 bp outside the region of recombination. The predicted 1971 bp and 1985 bp PCR products were obtained from the W36 and W38 strains, respectively. PCR amplification from genomic DNA of M511 with KORedK-F/KORedK-R gave a product of 1644 bp, but with KORedL-F/KORedL-R there was no product, probably because of inefficient amplification of the amplimer of the expected size (>7 kb) under the reaction conditions used.

Analytical Methods

The overall prodiginine concentrations were determined by absorbance at 530 nm (ε_{530} = 100,500 M^{-1} cm^{-1}) of a methanolic mycelial extract (Mo et al., 2005). Determination of the relative abundances of the various prodiginines was carried out by HPLC. The mycelia were extracted with methanol, and the prodiginines in a 10 μl sample were resolved using a Supelco Discovery HS C_{18} column (4.6 × 250 mm) with a linear elution gradient ranging from CH_3OH:CH_3CN:H_2O (40%:10%:50%) to CH_3OH (100%) in 0.15% trifluoroacetic acid at a flow rate of 0.5 ml/min with detection at 530 nm (Mo et al., 2005). For the quantitative analysis of each prodiginine, cyanidin chloride (1 mg/ml; Fluka) was used as an internal standard. This has a λ_{max} = 530 in methanolic HCl and a retention time of 10 min under the same HPLC conditions. The prodiginines were also resolved and identified by LC-MS analyses. LC-MS spectra (positive turbo-ion spray ionization mode; HPLC: Hewlett-Packard Series 1100; column: Luna C_{18} [4.6 by 250 mm] from Phenomenex; mobile phase: as described above) were obtained on a Perkin-Elmer SCIEX API 2000 pneumatically assisted electrospray triple-quadrupole mass spectrometer. Labeling of prodiginines with perdeuterated valine, leucine, and isoleucine was carried out as previously described.

Analysis of prodiginine production in *S. coelicolor* W36 and W38, with and without addition of chemically synthesized 2-undecylpyrrole and in coculture, was carried out using the method described previously (Stanley et al., 2006). LC-MS/MS analyses were carried out on an Agilent 1100 HPLC instrument connected to a Bruker HCT^+ mass spectrometer in auto MS/MS mode. High-resolution mass spectra were recorded on a Bruker MicroTOF instrument. Streptorubin B was purified from *S. coelicolor* M511 according to the procedure described in the Supplemental Data. NMR analyses of streptorubin B were carried out on a Bruker Avance 700 spectrometer equipped with a TCI cryoprobe.

Genetic Complementation of *S. coelicolor* SJM3

Plasmid pSJM1, expressing *S. coelicolor redR*, was created by inserting the corresponding *redR* gene as an *Xba*I and *Hin*dIII fragment into pSE34 (Smirnova and Reynolds, 2001). The PCR amplification was carried out using SC3F7 as a template and the primer set ComRedR-F and ComRedR-R. Similarly, the *S. coelicolor fabF* gene was obtained as an *Xba*I and *Hin*dIII PCR fragment from *S. coelicolor* cosmid 4A7, and inserted into pSE34 to create pSJM2. The primer set for this experiment was ComFabF-F and ComFabF-R. Protoplasts of *S. coelicolor* SJM1 were prepared following standard methods and transformed with pSJM1 and pSJM2. The resulting thiostrepton-resistant transformants were obtained, and their respective prodiginine production levels were evaluated as described above.

Feeding and Cosynthesis Studies

Seed cultures of *S. coelicolor* SJM3 were grown in R2YE medium for 3 days at 30°C and then used as a 5% inoculum for a 50 ml liquid culture of the same medium. 11-methyllauric acid, pentadecanoic acid, dodecanoic acid, and 12-methyltridecanoic acid (Sigma) and the corresponding N-acetylcysteamine thioesters were added to a final concentration of 10 mM at 48 hr. The production cultures were grown for 7 days at 30°C as described above. Duplicate mycelia were collected on filter paper by vacuum filtration, washed twice with distilled water, and then extracted with methanol for HPLC analysis. Complemented mutants (*S. coelicolor* SJM3/pSE34, SJM3/pSJM1, and SJM3/pSJM2) were fed the same fatty acids in R2YE media and extracted as described above.

Cultures of *S. coelicolor* W36, W38, and M511 (positive control) were grown on R5 agar plates that had been overlaid with sterile semipermeable (12,000–14,000 Da molecular weight cutoff, size 20) membranes prior to inoculation. After 2 days' growth at 30°C, 0.5 mg of 2-undecylpyrrole (Wasserman et al., 1976), prepared according to the procedure described in the Supplemental Data, was added in methanol to one plate containing *S. coelicolor* W36 and *S. coelicolor* W38. Cultures of *S. coelicolor* W36 and W38 with or without 2-undecylpyrrole and *S. coelicolor* M511 were grown for a total of 5 days at 30°C, after which the mycelia were harvested and extracted as described previously (Stanley et al., 2006). Spores of W36 and W38 were also grown together for 5 days on R5 agar plates and treated as above.

SUPPLEMENTAL DATA

Supplemental Data include four tables, Supplemental Experimental Procedures, and Supplemental References, and can be found with this article online at http://www.chembiol.com/cgi/content/full/15/2/137/DC1/.

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