Chemistry & Biology, Vol. 12, 191–200, February, 2005, ©2005 Elsevier Ltd All rights reserved. DOI 10.1016/j.chembiol.2004.11.006

Production of Branched-Chain Alkylprodiginines in *S. coelicolor* by Replacement of the 3-Ketoacyl ACP Synthase III Initiation Enzyme, RedP

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Summary

The enzyme RedP is thought to initiate the biosynthesis of the undecylpyrolle component of the antibiotic undecylprodiginine produced by *Streptomyces coelicolor*. RedP has homology to FabH, which initiates fatty acid biosynthesis by condensing the appropriate acyl-CoA starter unit with malonyl ACP. We have generated a *redP*-deletion mutant of *S. coelicolor* M511 (SJM1) and shown that it produces reduced levels of prodiginines and two new analogs, methylundecylprodiginine and methyldodecylprodiginine. Incorporation studies with perdeuterated valine were consistent with these being generated using methylbutyryl-CoA and isobutyryl-CoA as starter units, respectively. Plasmid-based expression of a streptomycete *fabH* in the SJM1 mutant led to restoration of overall prodiginine titers but the same overall ratio of undecylprodiginines and novel prodiginines. Thus, the *redP* FabH can be replaced by FabH enzymes with different substrate specificities and provides a method for generating novel prodiginines.

Introduction

Actinomycetes and other eubacteria produce alkylprodiginines (prodigiosins), a family of red-pigmented antibiotics [1]. The chemical composition of "prodigiosin" was established over 40 years ago, and it seems possible that by virtue of their color, these types of compounds may have been observed for centuries [2]. These antibiotics have a broad range of activity against bacteria, protozoa, and pathogenic fungi, but are not used clinically because of their toxicity [2, 3]. Interest in clinical development of prodiginine-type drugs as antitumor agents and immunosuppressants has been stimulated in recent years by a number of factors. It has been shown that they have immunosuppressive activity at noncytotoxic doses and operate by a different mechanism of action than either cyclosporine and FK506 (coadministration consequently gives a synergistic effect on the immune system) [2]. Numerous prodiginine and related compounds (including roseophilin) [4] have also been shown to have potent apoptotic effects on human cancer cells [5, 6]. More recently, synthetic efforts have made it possible to generate new prodiginine analogs with improved therapeutic indices [2, 5].

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In addition to ongoing synthetic efforts, a genetic and biochemical understanding of prodiginine biosynthesis has begun to emerge [3]. This work has provided the basis for rational approaches to engineer the biosynthesis of novel prodiginines, potentially with improved immunosuppressant or antitumor activities. Undecylprodiginine (Figure 1) is the most well studied of this structural class of compounds, and labeling studies have shown that it is generated from multiple acetate units as well as one unit each of proline, glycine, and serine [7–9]. This and its oxidatively cyclized form, butyl-*meta*-cycloheptylprodiginine, have been shown to be the principal components of the red-pigmented antibiotics produced by *Streptomyces coelicolor* A3(2) [10]. The recently completed *S. coelicolor* genome sequence has provided the entire *red* biosynthetic gene cluster [11]. A subsequent analysis of this cluster has resulted in proposed assignments for most of these genes in prodiginine biosynthesis [3].

Prodiginines are assembled by a convergent process involving a late-stage condensation of 2-undecylpyrrole (Figure 1) with 4-methoxy-2,2′-bipyrrole-5-carbaldehyde. It has been proposed that this unit is derived from the condensation of β-ketomyristoyl thioester with glycine, with concomitant loss of carbon dioxide from the latter [9]. Eight of the 23 genes in the cluster (*redX*, *redR*, *redQ*, *redP*, *redN*, *redH*, *redG*, and *redF*) have been proposed to be involved in formation of the 2-undecylpyrrole [3]. Of these, *redP*, *redR*, and *redQ* encode homologs of FabH (KASIII) and FabF (KASII) enzymes and an acyl carrier protein (ACP), respectively [3], components of the type II dissociated fatty acid synthase (FAS) in streptomycetes, *Escherichia coli*, and other microorganisms [12–15]. RedP is proposed to initiate the biosynthetic process by condensing an acetyl CoA starter unit with malonyl ACP (RedQ) [3] (Figure 1). The 3-keto group of the resulting acetoacyl thioester would be reduced to provide butyryl ACP, presumably by the appropriate type II FAS enzymes [3]. The *redR*-encoded FabF homolog might catalyze four subsequent elongation steps with malonyl ACP (RedQ), with appropriate 3-keto group processing after each step. The resulting dodecyl ACP (RedQ) is the proposed substrate for the unusual PKS encoded by *redX*, which generates the β-ketomyristoyl thioester using a malonyl extender unit bound to an ACP domain in the *redN* gene product [3] (Figure 1).

Evidence in the last few years has established that ketoacyl ACP synthases (KASs) involved in both FAS and PKS processes can exhibit significant ACP specificities, which enables these two processes to function independently [16]. In particular, it has been shown that the FabH, which initiates fatty acid biosynthesis in streptomycetes, reacts only with FAS ACPs and not ACPs from type II aromatic polyketide synthetic processes [16]. Thus, a FAS-type process which needs to interact with PKS must involve a specific set of KAS enzymes along with a dedicated ACP [17], as in the case of the proposed formation of the dodecyl ACP substrate for prodiginine biosynthesis. Nonetheless,

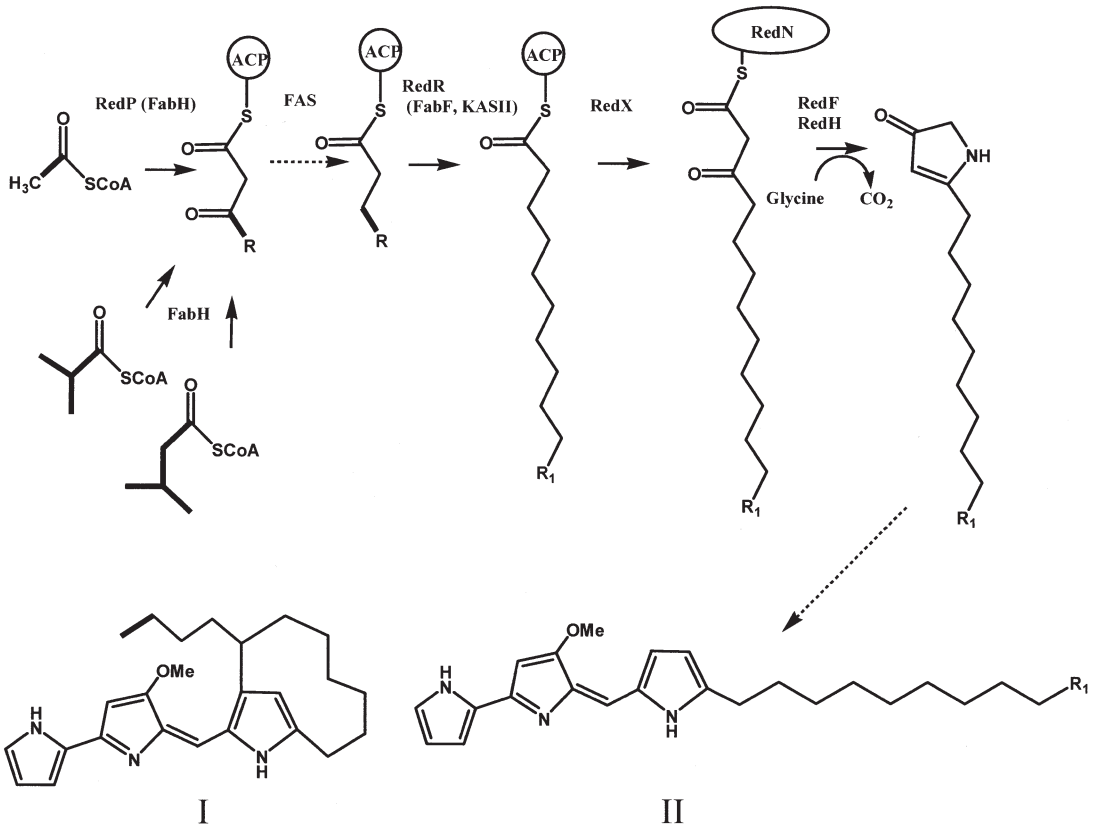

Figure 1. Proposed Roles of RedP and a FAS FabH in Initiating Alkylprodiginine Biosynthesis

Butyl-*meta*-cycloheptylprodiginine, undecylprodiginine [$R_1 = CH_2CH_3$], methylundecylprodiginine [$R_1 = CH(CH_3)_2$)], and methyldodecylprodiginine [$R_1 = CH_2CH(CH_3)_2$]. Biosynthesis of the known prodiginines from an acetyl CoA starter unit ($R = CH_3$) can be accomplished with either RedP or FabH and six elongation steps by RedR. Biosynthesis of the latter two novel branched-chain alkylprodiginines from 3-methylbutyryl CoA [$R = CH_2CH(CH_3)_2$] and isobutyryl CoA [$R = CH(CH_3)_2$] can only be observed when initiation is from FabH and involves five or six elongation steps by RedR, respectively. The ACP encoded by *redQ* is proposed to be involved in all the initial steps of the pathway.

the role of these enzymes and the absolute requirement for them has not been investigated. In this work, we have shown through genetic studies that the *redP* FabH product plays an important but not essential role in undecylprodiginine biosynthesis. Furthermore, we have provided, to the best of our knowledge, the first example of bioengineering of branched-chain alkylprodiginines by replacement of the RedP function with a streptomycete FAS FabH. Finally, we have shown that even when a streptomycetes FAS FabH initiates both fatty acid biosynthesis and prodiginine biosynthesis, the ratio of straight and branched-chain components of each is distinctly different. This last observation suggests that there is additional selectivity beyond the initiation step of these two processes.

Results

Deletion of *redP* in *S. coelicolor* Leads to a Decrease in Prodiginine Production in R2YE Media

The *redP* gene encodes a homolog of the *E. coli* FabH enzyme and was identified in the sc3F7 cosmid by the *S. coelicolor* genome sequencing project [11]. It has been proposed that this gene product could initiate prodiginine biosynthesis by catalyzing formation of an acetoacetyl ACP from an acetyl CoA starter unit (Figure 1) [3]. To test this hypothesis, we generated a new SJM1 mutant by replacing the *redP* gene in *S. coelicolor* M511 (a mutant which produces prodiginines but not actinorhodin) with the $Apra^R$-*oriT* cassette, using the recently described PCR-targeted gene replacement strategy developed for *Streptomyces* [18]. In R2YE solid medium, the *S. coelicolor* M511 and SJM1 grew and sporulated normally. The SJM1 also appeared to generate some level of a red-pigmented product, although at apparently lower levels than the M511 strain (Figure 2). To quantitate the effect of loss of *redP,* the two mutants were grown for 10 days in liquid culture and analyzed for both cell growth and overall prodiginine production. As shown in Figure 3, there was no observable difference in the growth of either strain. However, in the SJM1 strain, the maximal levels of prodiginines were at least 4-fold lower than that observed for the parent M511 strain. In both cases, the maximal levels of prodiginines were observed at approximately 96 hr. In neither case did continued incubation lead to higher levels.

Figure 2. Growth of *S. coelicolor* M511 and SJM1 in R2YE Agar Plate after 5 Days of Incubation at 30°C

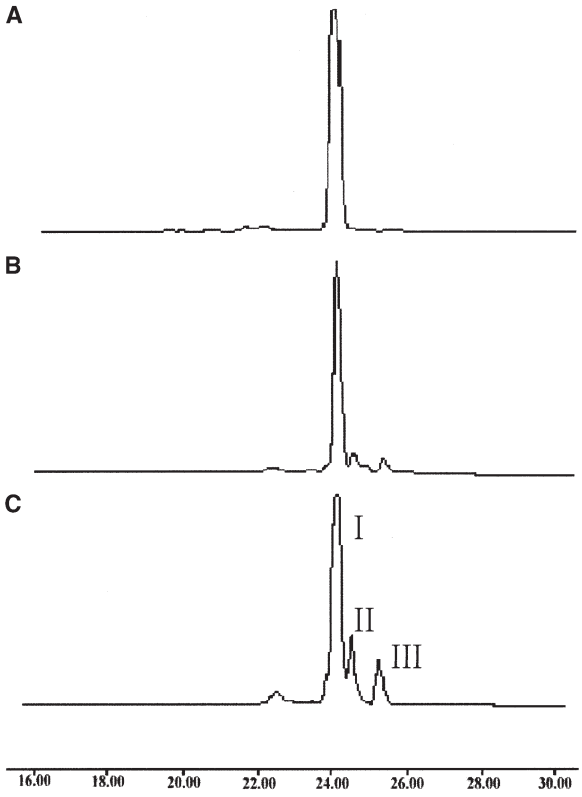

Figure 4. HPLC Analyses of Alkylprodiginine Production by *S. coelicolor*

(A), (B), and (C) are from *S. coelicolor* M511, SJM1, and SJM1/pSW7, respectively. Prodiginines were resolved by reverse-phase HPLC and detected by both absorbance at 530 nm (data shown) as well as mass spectral analysis. I, undecylprodiginine; II, methylundecylprodiginine; III, methyldodecylprodiginine.

LC-MS Analysis of Prodiginine Production in *S. coelicolor* SJM1 and M511

HPLC analyses of the M511 (Figure 4A) and SJM1 mutant (Figure 4B) and the corresponding pSE34 transformants (a *Streptomyces* expression plasmid) were carried out using both mass spectrometry and absorbance (530 nm) as detection methods. In all cases, the major prodiginine product eluted at 24 min (the levels of this were significantly higher in the M511 mutant). Mass spectrometry analysis demonstrated a parent ion $[M+H]^+$ with an *m/z* value of 394 (Figure 5A), consistent with the structure of undecylprodiginine. A smaller peak eluting slightly earlier was also consistently observed, and mass spectral analysis (*m/z* 392) indicated that this was likely the oxidatively cyclized product, butyl-*meta*-cycloheptylprodiginine. These observations are similar to those made previously made for *S. coelicolor*, where these two compounds have been shown to be the major prodiginines [10]. In the case of the *S. coelicolor* SJM1, lower levels of new prodiginines eluting with slightly longer retention times were observed by both absorbance and mass spectral analyses (see Figure 4B). These new prodiginines appeared to be composed of at least two separate compounds. Mass spectral

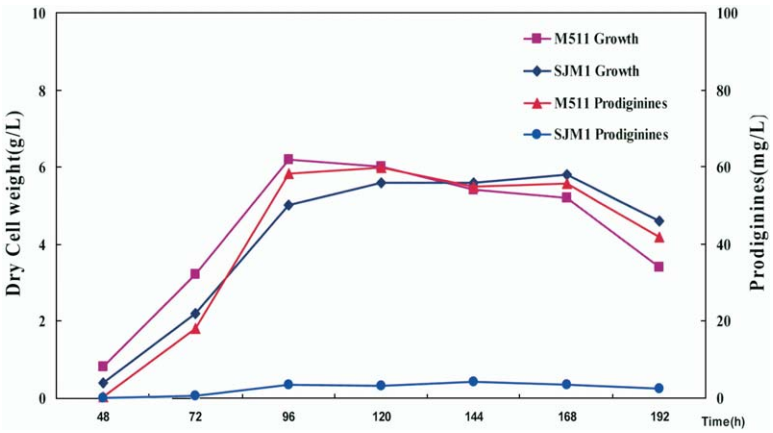

Figure 3. Growth and Prodiginine Production of M511 and SJM1 Strains in Liquid R2YE Medium

Production was measured in acidified methanol at 530 nm.

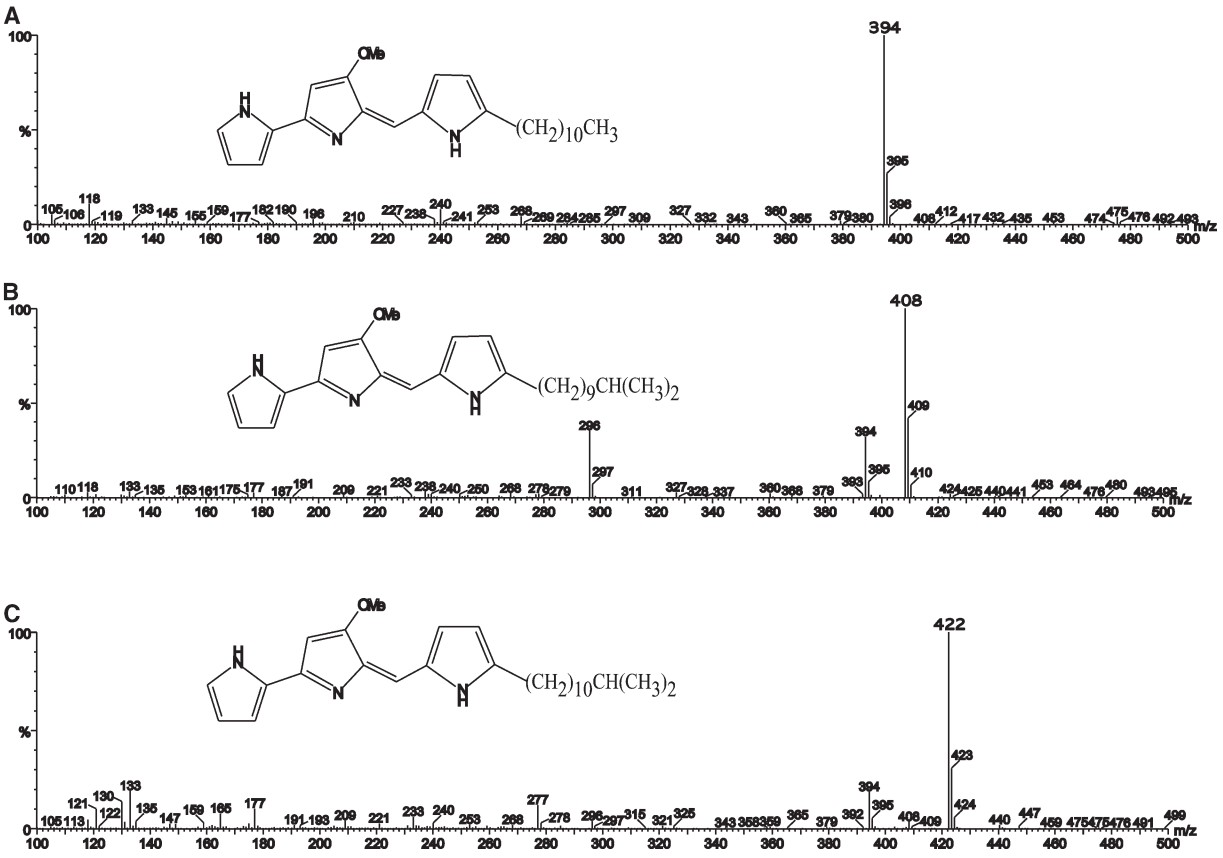

Figure 5. Mass Spectral Analysis of Prodiginines in the *S. coelicolor* SJM1 Mutant

(A) Undecylprodiginine with an *m/z* of 394.

(B) Methylundecylprodiginine with an *m/z* of 408.

(C) Methyldodecylprodiginine with an *m/z* of 422.

analysis revealed parent ions $[M+H]^+$ with *m/z* values of 408 and 422 (Figures 5B and 5C) for peaks eluting at 24.4 and 25.2 min, respectively. These masses are 14 and 28 AMU higher than the mass of the undecylprodiginine parent ion, indicating the presence of novel prodiginines containing longer (C_{12} and C_{13}) alkyl chains. HRFAB-MS was obtained for each of these peaks. The first peak had pseudomolecular ions observed at *m/z* 394.2840 and 394.2839 $[M+H]^+$ in agreement with the $C_{25}H_{35}N_3O$ molecular formula for undecylprodiginine (*m/z* 394.2859, $[M+H]^+$ calc.). The second peak had pseudomolecular ions observed at *m/z* 408.3001 and 408.3010 $[M+H]^+$ in agreement with the proposed $C_{26}H_{37}N_3O$ molecular formula for (*m/z* 408.3016, $[M+H]^+$ calc.). The final peak had pseudomolecular ions observed at *m/z* 422.3171 and 422.3174 $[M+H]^+$ in agreement with the proposed $C_{27}H_{39}N_3O$ molecular formula (*m/z* 422.3172, $[M+H]^+$ calc.). These compounds were consistently observed in the *S. coelicolor* SJM1 mutant and were not detected under the growth and analytical conditions used (including use of single-ion monitoring) with the *S. coelicolor* M511.

Low-level prodiginine production in the SJM1 mutant indicted that, while RedP is important in biosynthesis, its function can be replaced, albeit less efficiently, by an alternative enzyme or pathway. One candidate was the *S. coelicolor* fatty acid synthase (FabH), which is presumed to have different substrate specificities to RedP but catalyzes the same type of reaction (elongation of an acyl CoA primer with malonyl ACP). It has been shown previously that fatty acid biosynthetic enzymes, including FabH, are active during and after late log-phase growth, when antibitoics are produced [16]. The streptomycetes FabH has also been shown to have a relaxed acyl-group substrate specificity compared to the *E. coli* FabH enzyme and appears to be able to use isobutyryl CoA and even methylbutyryl CoA as substrates, thus allowing production of both branched-chain fatty acids and straight-chain fatty acids [19]. Priming of prodiginine biosynthesis by the *S. coelicolor* FabH might thus account for the production of branched-chain alkyl analogs. Use of methylbutyryl CoA with five elongation steps (carried out by FabH and RedR) would generate a ω-2 methyldodecoyl ACP product (Figure 1) and ultimately a methylundecylprodiginine. Use of an isobutyryl CoA starter and six elongation steps would provide a ω-2 methyltridecoyl ACP and ultimately a methyldodecylprodiginine. These two novel branched-chain prodiginines would have masses $[M+H]^+$ of 408.3016 and 422.3172, as observed in the analyses of SJM1 mutant.

Table 1. Prodiginine Production and FabH Activity of *S. coelicolor* M511 and SJM1

Strain	Overall Prodiginine Levels (%)	Ratio of Prodiginines (I:II:III)[a]	FabH Activity of Cell Extract[b] (cpm)
M511/pSE34	100 (±7)	100 (±2):0:0	1700 (±200)
SJM1/pSE34	20 (±1)	21 (±2):2 (±1):1 (±1)	150 (±50)
SJM1/pSW7	78 (±3)	63 (±1):5 (±2):4.5 (±2)	2700 (±200)
SJM1/pSW7+valine	—	75 (±2):3 (±3):6 (±2)	—
SJM1/pSE4	33 (±2)	—	630 (±100)
SJM1/pKR3	67 (±1)	—	1590 (±200)

[a] The ratios of undecylprodiginine (I), methylundecylprodiginine (II), and methyldodecylprodiginine (III) are expressed as a percentage of the overall prodiginine levels made in M511/pSE34. pSE34 is the control plasmid and was used to generate pSW7 (containing the *S. glaucescens* FAS *fabH*), pSE4 (containing the *E. coli* FAS *fabH*), and pKR3 (containing the *S. coelicolor redP*).
[b] Results from one set of FabH assays were conducted in duplicate on each cell extract containing equivalent protein content. Additional analyses provided different absolute values but the same ratio of activities.

A series of complementation experiments and incorporation studies with perdeutreated-labeled valine were used to confirm that a FAS FabH initiates prodiginine biosynthesis in the SJM1 mutant, leading to the generation of novel branched-alkyl-chain prodiginines.

Complementation of *S. coelicolor* SJM1

A complementation experiment in SJM1 using pKR3, a RedP expression plasmid, was carried out. This plasmid led to a 3-fold increase in the levels of prodiginines relative to SJM1 carrying a control plasmid (Table 1). A complementation experiment carried out using pSW7 for expression of *Streptomyces glaucenscens* FAS FabH (which has very high sequence identity to the *S. coelicolor* FabH) led to almost a 4-fold increase in overall prodiginine levels in the SJM1 mutant. These levels were close to that observed for the M511 strain. In contrast to these results, there were smaller increases in prodiginine production using the *E. coli* FabH expression plasmid, pSE4 (Table 1). LC analyses of prodiginine analogs produced by these complementation experiments in the SJM1 mutant were carried out. This analysis of the SJM1/pSW7 mutant revealed that, while there was an increase in the levels of all of the prodiginines produced by the SJM1 mutant, the ratio of the various analogs was unchanged (Figures 4C and 6B). In contrast, the levels of these new prodiginines, relative to undecylprodiginine, decreased in the SJM1 mutant with expression of either the RedP or *E. coli* FAS FabH. These observations clearly demonstrated that the *Streptomyces* FAS FabH can initiate prodiginine biosynthesis in the absence of RedP and apparently does so using at least three different starter units.

Relative FabH Activity Levels in Cell Extracts of SJM1 and M511

Support for the role of the FAS FabH initiating prodiginine biosynthesis in the SJM1 mutant was provided through FabH assays of cell extracts of both this strain and M511. The RedQ ACP encoded by the prodiginine biosynthetic gene cluster, the proposed substrate for RedP (Figure 1), was used for these assays and was expressed in *E. coli* as an N-terminal His-tagged protein in predominantly the *holo* form (>95%). Cell extracts of the SJM1 mutant strain exhibited approximately 10% of the corresponding activity of extracts of M511 strain, indicating that RedP present in the latter strain is responsible for the majority of FabH activity with RedQ ACP as substrate (Table 1). The drop in activity correlated closely with the decreased level of prodiginine production. Cell extracts prepared from SJM1 containing either the pSW7 (for expression of the *S. glaucescens* FAS FabH) or pKR3 (plasmid-based expression of RedP) exhibited at least the same levels of FabH activity as did extracts of M511. These observations, along with the partial restoration of prodiginine levels (Table 1 and Figure 6B), demonstrated expression of both of these proteins. Furthermore, the increase in FabH activity in SJM1/pSW7 relative to SJM1 is consistent with the proposal that a streptomycete FAS FabH can utilize the RedQ substrate and is likely responsible for the low-level FabH activity and prodiginine production associated with the SJM1 mutant. FabH activity levels in cell extracts of SJM1/pSE4 increased only slightly over that observed for the SJM1 (Table 1) and corresponded with the slight increase in prodiginine levels observed for complementation experiments using the *E. coli* FabH.

Incorporation Studies with Perdeutrated DL-Valine

Incorporation experiments with perdeutrated DL-valine demonstrated that the new prodiginines made in the SJM1 mutant likely contain branched alkyl chains. Efficient labeling of BCFAs by perdeuterated valine has previously been demonstrated [20]. In this case, valine is degraded to perdeutrated isobutyryl CoA, which is subsequently used to make even-numbered BCFAs, labeled intact with seven deuteriums. The first intermediate in this degradation pathway, α-ketoisovaleryl CoA, is an intermediate in leucine biosynthesis. Degradation of leucine provides 3-methylbutyryl CoA for the biosynthesis of odd-numbered BCFAs containing an ω-2 methyl group. These fatty acids are also labeled intact with seven deuteriums with perdeutrated valine incorporation studies, albeit to a lesser degree [20]. Similar observations to these were made with prodiginines made in the SJM1 mutant.

Mass spectral analyses of prodiginines isolated from an incorporation study on *S. coelicolor* SJM1/pSW7 showed an efficient (66%) labeling of the putative isobutyryl CoA-derived prodiginines, methyldodecylprodiginine, with seven deuteriums (masses 429 and 422 in Figure 6Ab). Less efficient labeling (33%) with seven deuteriums was observed for the putative methylbutyryl CoA-derived prodiginine, methylundecylprodigi-

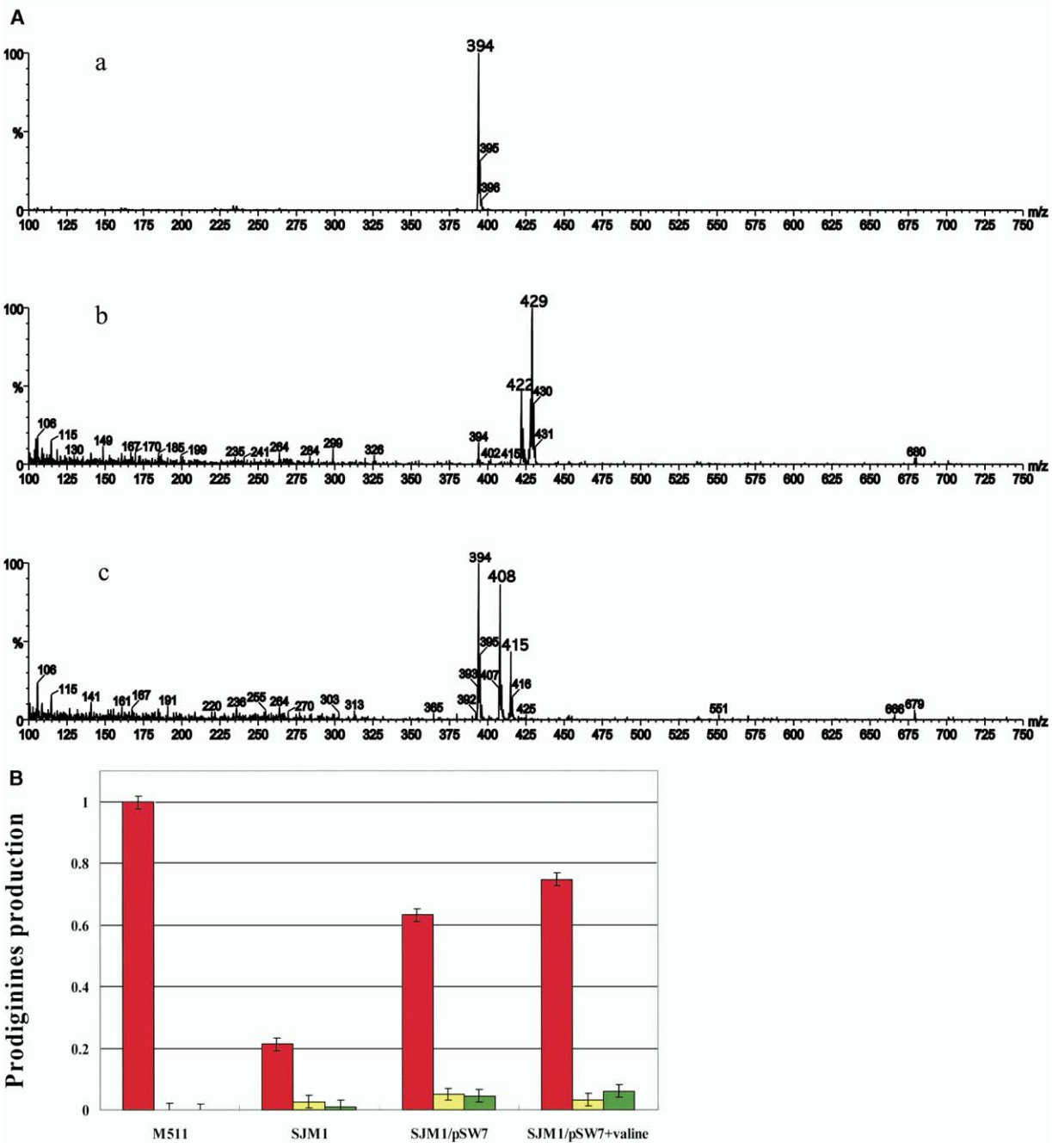

Figure 6. A Perdeuterated (D_8) Valine Feeding Study in the *S. coelicolor* SJM1 and SJM1/pSW7

(A) MS analysis of the SJM1/pSW7 reveals an efficient and predictable deuterium labeling (M+7) for the isobutyrate-derived (b) and methylbutyrate-derived branched-chain alkylprodiginines (c). No significant labeling was observed for the normal acetate-derived undecylprodiginine (a).

(B) Production of novel prodiginine analogs (yellow and green bars) was observed only in SJM1 mutant, and their ratio relative to undecylprodiginine did not alter with either expression of the *S. glaucescens* FabH (encoded by pSW7) or the addition of valine.

nine (masses of 415 and 408 in Figure 6Ac). This latter result provides compelling evidence that these prodiginines are produced by the leucine catabolite 3-methylbutyryl CoA. It is not possible to determine from these experiments if the isoleucine catabolite 2-methylbutyryl CoA is also used. In contrast to these observations, there was no significant deuterium labeling of the undecylprodiginine in the perdeuterated valine incorporation study (mass of 394 only in Figure 6Aa), consistent with the use of an acetyl CoA starter unit.

It has been demonstrated previously that in *Streptomyces* the addition of valine dramatically increases the relative levels of isobutyryl CoA-derived BCFA relative to other fatty acids [19]. Addition of valine might therefore also increase the levels of the methyldodecylprodiginine relative to the other prodiginines. This possibility

was investigated by determining the relative amounts of the various prodiginines in fermentations of the *S. coelicolor* SJM1/pSW7 in the presence and absence of valine. As shown in Figure 6B, branched-chain alkylprodiginines comprise approximately 16% of the overall prodiginine pool in SJM1 and SJM1/pSW7. In the presence of valine, this ratio does not increase. A small increase in the ratio of the methyldodecylprodiginine relative to the methylundecylprodiginine was observed. The low-level production of these prodiginines makes it difficult determine if this change is statistically significant. Analyses of the fatty acid pools generated under these conditions revealed that approximately 85% were BCFAs. Thus, the FAS FabH appears to use the same precursors to initiate fatty acid and prodiginine biosynthesis in the SJM1 mutant, yet the ratios of the product analogs in these two processes vary dramatically.

Discussion

It has been known for some time that initiation of fatty acid biosynthesis in a type II FAS is catalyzed by FabH [21] and that the acyl group specificity of this enzyme is likely a contributing factor in the type of fatty acids made by an organism [19, 22–26]. The catalytic steps involved in polyketide biosynthetic processes are analogous to those of fatty acid biosynthesis but use a more diverse range of initiation mechanisms [27, 28]. A limited but growing number of polyketide biosynthetic gene clusters contain genes encoding FabH homologs, indicating that, at least in some cases, the FAS and polyketide biosynthetic processes initiate via a similar mechanism [17, 29–32]. In vitro characterization of two of these enzymes, DpsC and ZhuG, has demonstrated that they exhibit the predicted catalytic activity and acyl group specificity for initiating their respective type II polyketide biosynthetic processes [32–34]. These observations raise important and related questions: (1) how are these specific initiation processes separate from that catalyzed by the FAS FabH, and (2) in other similar processes which initiate by a different mechanism, why does the FAS FabH not appear to play a role? The acyl carrier protein specificity of FabH and FabH homologs appears to be an important factor in this regard; the *S. glaucescens* FAS FabH has been shown to be able to utilize a variety of FAS ACPs but not ACPs encoded by polyketide biosynthetic processes [16]. The FabH homolog ZhuG can utilize either of the ACPs encoded by the R1128 polyketide biosynthetic gene cluster and even discriminates between them (the activity with a FAS ACP has not been reported) [17].

The role of FabH initiating these biosynthetic processes and determining the type of product made makes it an attractive target for engineering production of novel natural products. One approach to realize this goal may be protein engineering of the substrate specificity of a PKS FabH. To this end, the crystal structure of ZhuG has recently been determined [29]. We have taken an alternative approach and successfully replaced a FabH-type enzyme from a natural product biosynthetic process with a FabH with different acyl specificity. In doing so, we have achieved the goal of generating novel secondary metabolites. The streptomycete FAS FabH represents an attractive choice for the replacement enzyme, as an accumulation of evidence has suggested it can utilize a broad array of straight, branched, and cyclic acyl CoA substrates [19, 35]. The undecylprodiginine biosynthetic system was chosen because the proposed pathway [3] suggested that, with the exception of the elongation steps, FAS enzymes catalyze formation of the dodecanoyl ACP intermediate. The ACP (encoded by *redQ*) in this process was thus predicted to be a substrate for the *S. coelicolor* FAS enzymes as well as PKS enzymes (see Figure 1) and potentially more likely a substrate for the FAS FabH than an ACP from an aromatic PKS [16].

Low-level production of prodiginines in the *S. coelicolor* SJM1 mutant and the observation of novel branched-chain alkylprodiginines suggested an inefficient initiation of prodiginine biosynthesis by the endogenous FAS FabH. Supporting evidence is provided by the increased production levels of prodiginines in the same analog ratio with plasmid-based expression of this enzyme. FabH-catalyzed elongation of the various acyl CoA starter units using a malonyated RedQ is proposed to give rise to these prodiginines (Figure 1). (An alternative mechanism in which the FAS FabH utilizes the FAS ACP and there is a crossover from the fatty acid to the prodiginine biosynthetic process further down the pathway cannot be ruled out.) A lower catalytic efficiency with this RedQ substrate than corresponding FabC (the FAS ACP) or low levels of the FAS FabH at this stage of the fermentation [16] would contribute to the observed decreased level of the process. Indeed, FabH activity in SJM1 using malonylated RedQ is decreased dramatically in cell extracts of the SJM1 mutant relative to the M511 and restored by plasmid-based expression of the *S. glaucescens* FAS FabH. The inability of the *E. coli* FAS FabH to significantly enhance overall prodiginine levels in the SJM1 mutant may reflect low levels of expression of the enzyme and potentially poor catalysis with the malonylated RedQ. Indeed, our analyses of FabH activity levels in a *fabH* deletion strain of *S. coelicolor* using the FAS ACP (FabC) show much higher FabH levels from pSW7 than pSE4 (Y. Li, G. Florova, and K.A.R., unpublished data). Nonetheless, an apparent decrease in the ratio of the branched-chain to straight-chain alkylprodiginines in the SJM1/pSE4 versus SJM1/pSE34 may indicate that the *E. coli* and *S. coelicolor* FAS FabH compete to initiate the biosynthetic process.

It is important and intriguing that, despite the fact that the same enzyme initiates both processes, BCFAs and straight-chain alkylprodiginines predominate in *S. coelicolor* SJM1/pSW7. As these biosynthetic processes do not occur simultaneously, changes in the pools of the various acyl CoA precursors may account for this observation. However, there are many examples where significant levels of type I and type II polyketide products are made from branched-chain acyl CoA substrates [32, 36–39]. Alternatively, downstream enzymes specific to each process might exhibit substrate specificities and could account for these observations. For example, RedR (a FabF homolog) encoded by the prodiginine biosynthetic gene cluster may process straight-chain acyl ACP substrates most efficiently, while the FAS

FabF may exhibit greater efficiency with branched-chain acyl ACP substrates. In this case, more efficient production of branched-chain alkylprodiginines relative to the straight-chain analogs may require manipulation of the substrate specificity of RedR or other steps in the process. Similar issues of substrate specificity of downstream enzymes may also be valid for manipulation of other polyketide and natural product pathways initiated by FabH homologs.

Substrate specificity may account for the observation that methylbutyryl CoA and isobutyryl CoA appears to result only in two major new products, having two or one additional carbons, respectively. According to the proposed pathway (Figure 1), these observations would require the methylbutyryl CoA, isobutyryl CoA, and acetyl CoA primers to undergo a total of five, six, and six elongation steps (using RedP/FabH and RedR) to make C_{13}, C_{14}, and C_{12} acyl ACP substrates, respectively. There thus appears to be some selectivity based on the level of branching and chain length in the acyl ACP chain. For instance, a C_{12} acyl ACP derived from an isobutyryl CoA starter unit would be expected to give rise to a methyldecylprodiginine with the same mass as the undecylprodigine. LC-MS analyses of prodiginines generated in SJM1/pSW7 with deuterated valine suggest that this product may be present, but only at very low levels, and not resolved from the undecylprodiginine. These observations may reflect acyl substrate specificities in RedR, RedX (Figure 1), or enzymes catalyzing later steps in the process.

Significance

This work has provided, to the best of our knowledge, the first example of using a FAS FabH to initiate a complex natural product biosynthetic process, and a different approach for generating analogs of natural products. In principle, the approach demonstrated herein may also be applied to other natural product pathways that are initiated by FabH homologs and ultimately even those which initiate by different mechanisms. In this specific example, the use of a *Streptomycetes* FabH, able to use branched- and straight-chain acyl CoA substrates, has permitted the production of novel prodiginines. The ratio of the prodiginine analogs is different from the ratio of products made by fatty acid biosynthesis, despite the same enzyme initiating both processes. This observation demonstrates that factors beyond the substrate specificity of the initiation enzyme also contribute to the determination of the final product ratio. To date, attempts to make novel prodiginine derivatives with improved activities have depended upon chemical synthesis [2, 5]. The work described herein demonstrates that the cloning of the gene cluster and decoding of the pathway provide the basis for an additional and complementary rational bioengineering approach.

Experimental Procedures

Strains and Culture Conditions

All *E. coli* strains and *S. coelicolor* used in this study were grown following standard protocols [40, 41]. The template plasmids and strands used for PCR-targeted disruption (including *E. coli* BW25113, pIJ790, and pIJ773) were provided by Plant Biosciences Limited, Norwich, England and were developed Dr. Bertolt Gust at the John Innes Center. The *S. coelicolor* cosmid 3F7 was provided by Dr. Helen Kieser at the John Innes Center, Norwich, England. The prodiginine-producer *S. coelicolor* M511 [42] was provided by Dr. Greg Challis (University of Warwick, England). Determination of prodiginine production was carried out by inoculating a baffled 500 ml flask containing 100 ml of R2YE medium at pH 7.0 with 100 µl spore suspension and incubating on an orbital shaker (170 rpm) for 7 days at 30°C.

Allelic Replacement of *redP* in *S. coelicolor*

The *redP* gene of the prodiginine biosynthetic gene cluster was replaced with *aac(3)IV* resistance marker and *oriT* using the recently developed PCR-targeted *Streptomyces* gene replacement method [18]. The *aac(3)IV* resistance marker and *oriT* were amplified from the pIJ773 disruption cassette [18] using the following primer pairs: RedP-F1 5′-CACCGCACACCGCGGCCGCTCCGCGG GCGGTGCCCGGTGattccggggatccgtcgacc-3′ and RedP-R1 5′-TCT GCGCGCGCTTCGGCCGGGCCCGGCCGGCCGCCCTCAtgtaggctg gagctgcttc-3′ (pIJ773 homologous sequence is in lowercase). The PCR product was used to replace *redP*, first in *S. coelicolor* cosmid 3F7, and then in *S. coelicolor* M511 following standard methodologies [18]. The resulting mutants were shown to be Kan^S, $Apra^R$, indicating a double-crossover allelic exchange in *S. coelicolor*. Allelic replacement of the *redP* in the resulting mutant strain SJM1 (Δ*redP*::*oriT*, *aac(3)IV*) was confirmed by PCR amplification of chromosomal DNA using oligonucleotides which primed approximately 100 bp outside the region of recombination. The following primer pair was used: KOFabH-F 5′-ACTCCCCACACCTGCCGGGCC-3′ and KOFabH-R 5′-CGGCGTCGCCGCCCATCCAG-3′. The predicted 1.5 kb and 1.25 kb PCR products were obtained using chromosomal DNA from the SJM1 and M511 strains, respectively. The 1.5 kb product from the SJM1 mutant was subsequently cloned and sequenced, confirming the allelic replacement of *redP*.

Analytical Methods

To measure cell growth, duplicate samples (10 ml) of fermentation broth were collected every 24 hr, starting 48 hr after inoculation. Mycelia were collected on a preweighed filter (Whatman filter paper no. 1) by vacuum filtration. The samples were washed twice with distilled water, and the filters containing mycelia were dried at 80°C and weighed [43]. The overall prodiginine concentration was determined by absorbance at 530 nm (ϵ_{530} = 100, 500 M^{-1} cm^{-1}) of a methanolic mycelia extract [44].

Determination of the relative abundances of the various prodiginines was carried out by high-performance liquid chromatography (HPLC). The mycelia (1.0 g) were extracted with methanol, and the prodiginines in a 10 µl sample were resolved with a Supelco Discovery HS C_{18} column (4.6 × 250 mm) using 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 [45].

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 quadrapole mass spectrometer. High-resolution mass spectrometry of the prodiginines was also carried out at the Washington University Mass Spectrometry Resource.

Complementation of *S. coelicolor* SJM1

Complementation experiments with plasmids expressing three different 3-ketoacyl ACP synthase III enzymes were carried out. The plasmid pSW7 expressing the *S. glaucescens fabH* from P_{ermE*} has been described previously and was generated by inserting the corresponding *fabH* gene as a BglII fragment into the streptomyces expression plasmid pSE34 [22]. Similar approaches were used to generate the other two complementation plasmids. The *E. coli fabH* gene was obtained as a BamHI PCR fragment using *E. coli* chromosomal DNA and inserted into pSE34 to create pSE4. The primer set for this experiment was GAGAGAGGATCCACCCATGTATACGAA

GATTATTG (forward) and CTCTCTGGATCCTAGAAACGAACCAG (reverse). The *S. coelicolor redP* was obtained as a BglII and SphI PCR fragment from *S. coelicolor* cosmid 3F7 and cloned into the BamHI-SphI sites of pSE34 to create pKR3. The primer set for this experiment was CCGCACCAGATCTACCGCACACC (forward) and CGGTCGTCTGCATGCGCTTCGG (reverse). Protoplasts of *S. coelicolor* SJM1 were prepared following standard methods and transformed with pSW7, pSE4, and pKR3. The resulting thiostrepton-resistant transformants were obtained, and their respective prodiginine production levels were evaluated as described above.

DL-Valine Feeding Studies

Seed cultures of the *S. coelicolor* SJM1/pSE34 and SJM1/pSW7 were grown in R2YE media containing thiostrepton (12 μg/ml) for 3 days at 30°C and then used as a 5% inoculum for a 100 ml liquid culture of the same media. Perdeuterated DL-valine (D_8, 98%, Cambridge Isotope Laboratories) was added to a final concentration of 20 mM at 48 hr. The production cultures were grown for 7 days at 30°C as described above. Mycelia were collected on filter paper by vacuum filtration, washed twice with distilled water, and then extracted with methanol for LC-MS analysis.

Determination of Relative FabH Activity Levels in Cell Extracts

Seed cultures of the *S. coelicolor* M511/pSE34, SJM1/pSE34, SJM1/pSW7, SJM1/pSE4, and SJM1/pKR3 were grown in R2YE media containing thiostrepton (12 μg/ml) for 2 days at 30°C. Wet cells (1.0 g) from each fermentation were used to inoculate the same media, which was subsequently fermented for 4 days at 30°C. Cells were collected by centrifugation at 5000 × g for 20 min at 4°C, washed with 1× PBS buffer, resuspended in 5 ml buffer (50 mM Na^+-phosphate [pH 7.5], 1 mM DTT, 1 mM PMSF, 5% glycerol), and disrupted by sonication in an ice bath. Centrifugation at 20,000 × g for 20 min provided a cell extract, which was stored at –80°C.

FabH activity in these cell extracts was determined using standard methodologies to monitor the conversion of radioactive acyl-CoA and malonyl ACP substrates to a radiolabeled 3-ketoacyl-ACP [46]. A standard reaction mixture contained the following components in a final volume of 50 μl: 100 mM sodium phosphate buffer (pH 7.4), 10 μg (total protein) of the cell extract, 200 μM malonyl ACP (Sigma), and 10 μM [1-^{14}C]acetyl-CoA (Moravek Biochemicals, 50 μCi; specific activity, 50 mCi/mmol). The reaction was initiated by adding [1-^{14}C]acetyl-CoA and incubation at 30°C for 10 min. The reaction was terminated by addition of 10% trichloroacetic acid in the standard way [46].

Heterologous Expression and Purification of the *S. coelicolor redQ* Gene

The *redQ* gene was PCR amplified using cosmid 3F7 as a template. The forward primer (5′-GGCCCGCG*CATATG*AGCACCACCTACGA-3′) contained an NdeI site (italicized). The reverse primer (5′-ATG-CA*GGATCC*TCATGACGCGGTGGCCG-3′) contained a BamHI site (italicized) 3′ of the *redQ* stop codon. The PCR product was cloned as an NdeI-BamHI fragment in pET15b (Novagen) to create pSJ1 and used to transform *E. coli* BL21(DE3) (Novagen). Expression of *redQ* in the resulting ampicillin-resistant transformants was accomplished in LB fermentations (37°C) with IPTG (Sigma) induction (1 mM) when the optical density at 600 nm of the culture reached 0.4–0.7. Cultures were incubated for 3 hr after induction, and cells were collected by centrifugation at 20,000 × g for 10 min at 4°C. Cells were lysed and the RedQ was purified by metal chelate chromatography by following the recommended procedures provided by Novagen. Analysis of ACP purity was accomplished by electrophoresis with a 13% polyacrylamide gel containing 2.5 M urea in a manner similar to that described previously [22].

Acknowledgments

This work was supported by a Grant from the NIH (GM50541). We are very grateful to Dr. Greg Challis (University of Warwick, UK) for the M511 strain and for continued helpful discussions. The template plasmids and strains used for PCR targeting were developed at the John Innes Center by Dr. Bertolt Gust and kindly provided by Plant Biosciences Limited, Norwich, England. High-resolution mass spectrometry was provided by the Washington University Mass Spectrometry Resource, an NIH Research Resource (Grant No. P41RR0954).

Received: October 1, 2004
Revised: November 5, 2004
Accepted: November 5, 2004
Published: February 25, 2005

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