NOTES

Role of Phosphopantetheinylation Transferase Genes in Antibiotic Production by *Streptomyces coelicolor*^‡^ Ya-Wen Lu, Adrianna K. San Roman, and Amy M. Gehring* Department of Chemistry, Williams College, Williamstown, Massachusetts 01267

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The phosphopantetheinyl transferases (PPTases) catalyze the posttranslational modification of carrier protein domains by the transfer of a 4'-phosphopantetheine group from coenzyme A to a conserved serine residue (18, 31). This modification is essential for the function of the carrier protein; thus, PPTase activity is required for the biosynthesis of diverse cellular metabolites including fatty acids, polyketides, and nonribosomal peptides. *Streptomyces* and closely related actinomycetes are prolific producers of polyketide and nonribosomal peptide secondary metabolites, many of which have important pharmaceutical uses, particularly as antibiotics (5, 29). For those streptomycetes with sequenced genomes, the number of biosynthetic pathways requiring phosphopantetheinylation outnumbers the PPTase enzymes encoded, implying that a given PPTase is used in multiple pathways (1, 15, 22, 23, 32). Given the importance of PPTase specificity is important for the design of strategies for the given PPTase is used in multiple pathways (1, 15, 22, 23, 32). Given its likely role in fatty acid biosynthesis, it is expected that SCO4744 is essential, and indeed, our attempts to disrupt this gene have been unsuccessful to date. The redU gene is located in the undecylprodigiosin biosynthetic gene cluster and is the penultimate gene in a six-gene operon (4). A redU mutant that is defective for the production of 4-methoxy-2, 2'-bipyrrole-5-carboxaldehyde (MBC), an intermediate in undecylprodigiosin biosynthesis, has been described, and feeding experiments with implicaRedU in the phosphopantetheinylation of the RedO ACP (28). The SCO6673 gene product has not been studied, but it shows substantial similarity to the demonstrated PPTases Svp from the bleomycin producer *Streptomyces verticillus* (26) and SePptII from the erythromycin producer *Saccharopolyspora erythraea* (32). The SCO6673 gene is located downstream of and overlapping SCO6672, which encodes an unknown protein with a calcineurin-like phosphoesterase domain. An homologous pair of genes, sim18 and sim19, is found in the simocyclinone biosynthetic gene cluster of *Streptomyces antibioticus* Tü 6040 (8); however, SCO6673 is not located near any secondary metabolism genes (for example, the CDA gene cluster is SCO3210 to SCO3249) (1). We have constructed SCO6673 and redU single mutants as well as the corresponding double mutant to characterize the involvement of their encoded PPTases in antibiotic production and differentiation in *S. coelicolor*.

Construction and phenotypes of PPTase mutants. The SCO6673 and redU genes were disrupted in the chromosome of wild-type *S. coelicolor* by homologous recombination with a pUC19-based plasmid that contained the respective gene interrupted with a drug resistance cassette. For the SCO6673 knockout plasmid, SCO6673 and flanking DNA (3.8 kb total) were PCR amplified from genomic DNA with primers 1 and 2 (see Table 1 for all primer sequences) and cloned into the BamHI and HindIII restriction enzyme sites of pUC19 (34). The *aac(3)IV* (apr) gene (17), conferring

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apr amycin resistance, was amplified with PCR primers 3 and 4 and cloned into the unique BssHII site in SCO6673 (position 407 in the 681-bp gene). Finally, the omega fragment, conferring spectinomycin resistance and isolated by BamHI digestion of pH45 (position 407 in the 681-bp gene). To complete the redU knockout plasmid, the omega fragment was cloned into the unique XhoI site of SCO6673(24), was cloned into the BamHI site of pH45(24), was cloned into the BamHI site of pUC19. For the redU knockout plasmid, redU and flanking DNA (3.2 kb total) were PCR amplified from genomic DNA with primers 5 and 6 and cloned into the PstI and HindIII sites of pUC19. The knockout plasmids were alkali denatured prior to protoplast transformation using prototrophic S. coelicolor strain M145 (17, 21). Recombinants were selected by apramycin (50 μg/ml) or thiostrepton (10 μg/ml) flooding, respectively, for the SCO6673 and redU knockouts. Double-crossover recombinants were distinguished from single-crossover recombinants by spectinomycin sensitivity to give the SCO6673 mutant (SCO6673:apr) and the redU mutant (redU:tsr). For the double PPTase mutant (SCO6673:apr redU:tsr), protoplasts of the SCO6673 mutant were transformed with the redU knockout plasmid. For all three mutants, multiple double-crossover recombinants that exhibited identical phenotypes were isolated. One isolate of each mutant was chosen, and the genotype was confirmed by restriction analysis. The chromosomal DNA isolated from the single or double mutants was also transformed into wild-type S. coelicolor protoplasts, and apramycin or thiostrepton selection was applied (21), thereby demonstrating the complete linkage of the disrupted genes with the mutation causing the single-mutant phenotypes in the resulting transformants (data not shown).

The three PPTase mutants and the parent strain were plated onto rich R2YE medium (17) to visually assess the effects of the mutation(s) on morphological differentiation and antibiotic production (Fig. 1A and B). The redU mutant failed to produce RED and showed no apparent defects in aerial mycelium formation or sporulation. The SCO6673 mutant exhibited a delayed production of an aerial mycelium and, thus, delayed sporulation. However, for the double mutant, in which both SCO6673 and redU were disrupted, aerial mycelium formation and the production of the polyketides ACT and gray spore pigment were precocious and robust (Fig. 1A). Therefore, while SCO6673 appeared to retard morphological differentiation in the context of a single mutation, it is clearly not essential for these processes. An isolate of the double mutant constructed by the transformation of a redU mutant with the SCO6673 knockout plasmid showed the same robust sporulation and ACT production (data not shown). Deletion of the redD gene, encoding a transcriptional activator for the RED biosynthetic genes, has been shown to elicit elevated transcription of the whiE genes encoding the gray spore pigment biosynthetic machinery (13), perhaps helping to explain the strong production of gray spore pigment by the likewise RED-deficient double PPTase mutant.

The SCO6673 gene was PCR amplified (primers 9 and 10) and cloned into the BamHI site of the integrating vector pSET152S (9) to test for the complementation of the morphological defects in the SCO6673 mutant. Timely aerial mycelium formation and sporulation were restored to the SCO6673 mutant containing plasmid pSET152S-SCO6673 (introduced via conjugation from E. coli) (7) compared to the mutant with pSET152S alone (Fig. 1C). A plasmid was also constructed with redU under the control of a thiostrepton-inducible promoter (in pIJ6902) (14); however, this was unable to complement the RED biosynthesis defects in the redU mutant. It is likely that the redU mutation exerts polar effects on the downstream redV. The function of REDV has not been characterized in S. coelicolor (4); however, in Serratia, the homolog PigM is essential for MBC biosynthesis and is proposed to function as an oxidoreductase (33). Mutational analysis of the Serratia redU homolog pigL has also demonstrated its involvement in MBC biosynthesis in this organism (33).

The effects of the PPTase gene mutation on ACT and RED biosynthesis, each mutant was cultured in a liquid minimal medium [0.5% mannitol, 0.2% Casamino Acids, 1 mM KH2PO4, 25 mM-N-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES) (pH 7.2)] (27). This medium was chosen because it allowed the significant production of both ACT and RED by the wild-type strain. Cultures (400 ml) were inoculated with spores (107 spores) and incubated with shaking at 30°C. Every 12 h, triplicate samples of the culture were removed for determinations of dry cell weight.
(6 ml), ACT production (2 ml), and RED production (2 ml). To determine dry cell weight, cells were retrieved by vacuum filtration, washed once with water, and dried overnight at 75°C prior to weighing of the Whatman GF/A filter. Growths of all strains were equivalent, with the exception of perhaps an initial small delay in the growth of the double mutant (Fig. 2A).

Total blue pigments (ACT and congeners) were assayed, as previously described, by the addition of base (1 ml 5 M KOH) and measurements of the $A_{640}$ of the supernatant (3). The wild-type strain and both the SCO6673 and redU mutants produced substantial amounts of ACT over the time course (Fig. 2B). The double mutant showed delayed and reduced synthesis of ACT, which reached about 25% of wild-type levels after 5 days under these growth conditions.

RED was also assayed, as previously described, by overnight extraction of the cell pellet with acidified methanol and $A_{530}$ determinations (12). As expected, given the known role of RedU in MBC biosynthesis (28), RED production was abrogated in the redU and double mutants (Fig. 2C). RED synthesis by the SCO6673 mutant was reduced to ca. 30% of wild-type levels under these growth conditions.

**Actinorhodin and undecylprodigiosin production on solid rich medium.** Levels of ACT and RED production were also assessed for cells cultured on the glucose-based solid R2YE medium (Fig. 1). Spores ($10^8$ spores) were inoculated onto R2YE plates covered with cellophane. Cells were harvested from the cellophane after 3 or 7 days of growth at 30°C for assays of RED and ACT levels, respectively, as described.
above. All strains produced substantial levels of ACT on R2YE medium (Fig. 3A). ACT production defects for the double mutant were not apparent, but rather, this strain made more actinorhodin than either single mutant on this medium. Consistent with the liquid culture results, the redU and double mutants failed to make RED (<2% of wild-type levels). However, the SCO6673 mutant significantly overproduced RED compared to the production by the wild-type strain on this medium (Fig. 3B). The overproduction of RED was eliminated when the mutant was complemented with plasmid pSET152S-SCO6673 (Fig. 3B, right). An excess synthesis of RED by the SCO6673 mutant compared to that of the wild type on glucose-containing SMMS solid minimal medium was also observed (17), although total levels of RED were much lower (data not shown).

**CDA production.** CDA production by the PPTase mutants was examined using a previously described bioassay (17). Spores were spotted onto an Oxoid nutrient agar plate and incubated for 2 days at 30°C. Plates were then overlaid with soft nutrient agar containing *Bacillus mycoides* and calcium nitrate. CDA produced by *S. coelicolor* killed the *B. mycoides* cells, resulting in a zone of clearing on the plate. Both the wild-type strain and the redU mutant could clear the *B. mycoides* cells in a calcium-dependent manner, while the SCO6673 and double mutants could not; CDA production could be restored to the SCO6673 mutant by complementation plasmid pSET152S-SCO6673 (Fig. 3B, right). An excess synthesis of RED by the SCO6673 mutant compared to that of the wild type on glucose-containing SMMS solid minimal medium was also observed (17), although total levels of RED were much lower (data not shown).

**Concluding remarks.** Here, we demonstrate that SCO6673 is required for CDA biosynthesis. With an apparent role for the SCO6673 gene product in the phosphopantetheinylation of the CDA synthetase, it will be interesting to determine if this enzyme also participates in the synthesis of other *S. coelicolor* nonribosomal peptides such as the siderophore coelichelin (19). Our data are also consistent with a role for redU in undecylprodigiosin biosynthesis, which has previously been attributed to the necessary posttranslational modification of the RedO ACP (28). Given that the SCO6673 mutant can synthesize undecylprodigiosin in large quantities, it appears that SCO4744 (AcpS) is competent for the modification of the other ACPs in the RED biosynthetic machinery. AcpS is also sufficient for fatty acid, actinorhodin, and polyketide spore pigment biosynthesis as shown by the ability of the double mutant to produce these molecules, supporting the characterization of *S. coelicolor* AcpS as a “promiscuous” PPTase (6). We also observed that PPTase genes could influence antibiotic biosynthetic pathways for which they are not required, with different effects depending on growth conditions. For example, while the SCO6673 mutant showed diminished RED production when cultured in a particular liquid medium, the same strain highly overproduced RED on a glucose-based solid medium. The regulation of antibiotic production in the streptomycetes is very complex, presumably responding to many environmental and physiological cues and coordinated with morphological differentiation (2). Transcriptional profiling experiments revealed extensive cross-regulation between antibiotic biosynthetic pathways in *S. coelicolor* as well as the influence of some of the antibiotic regulators on genes involved in morphological differentiation (13, 14). We presume that the PPTase gene mutations, through the role of their products in an essential posttranslational modification, alter flux through secondary metabolism by reducing or eliminating the activity of certain biosynthetic enzymes (both those whose products were detected here and those that were not); the specific effect of...
these alterations in enzyme activity on the production of the three antibiotics assayed here might then vary depending on the overall metabolic state of the cell as influenced by growth conditions. Conditions for the overproduction of RED by the SCO6673 mutant are particularly noteworthy given the current interest in undecylprodigiosin as a breast cancer therapy (11). The inactivation of a PPTase gene has also been shown to elicit nystatin overproduction in Strep-
tomyces noursei (30). Mutation of PPTase genes may thus prove to be a useful approach to bias secondary metabolism toward a desired product in the actinomycetes.

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REFERENCES


