A Two-Step Mechanism for the Activation of Actinorhodin Export and Resistance in *Streptomyces coelicolor*

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**ABSTRACT** Many microorganisms produce secondary metabolites that have antibiotic activity. To avoid self-inhibition, the producing cells often encode cognate export and/or resistance mechanisms in the biosynthetic gene clusters for these molecules. Actinorhodin is a blue-pigmented antibiotic produced by *Streptomyces coelicolor*. The *actAB* operon, carried in the actinorhodin biosynthetic gene cluster, encodes two putative export pumps and is regulated by the transcriptional repressor protein ActR. In this work, we show that normal actinorhodin yields require *actAB* expression. Consistent with previous *in vitro* work, we show that both actinorhodin and its 3-ring biosynthetic intermediates [e.g., (S)-DNPA] can relieve repression of *actAB* by ActR *in vivo*. Importantly, an ActR mutant that interacts productively with (S)-DNPA but not with actinorhodin responds to the actinorhodin biosynthetic pathway with the induction of *actAB* and normal yields of actinorhodin. This suggests that the intermediates are sufficient to trigger the export genes in actinorhodin-producing cells. We further show that actinorhodin-producing cells can induce *actAB* expression in nonproducing cells; however, in this case actinorhodin is the most important signal. Finally, while the “intermediate-only” ActR mutant permits sufficient *actAB* expression for normal actinorhodin yields, this expression is short-lived. Sustained culture-wide expression requires a subsequent actinorhodin-mediated signaling step, and the defect in this response causes widespread cell death. These results are consistent with a two-step model for actinorhodin export and resistance where intermediates trigger initial expression for export from producing cells and actinorhodin then triggers sustained export gene expression that confers culture-wide resistance.

**IMPORTANCE** Understanding the links between antibiotic resistance and biosynthesis is important for our efforts to manipulate secondary metabolism. For example, many secondary metabolites are produced at low levels; our work suggests that manipulating export might be one way to enhance yields of these molecules. It also suggests that understanding resistance will be relevant to the generation of novel secondary metabolites through the creation of synthetic secondary metabolic gene clusters. Finally, these cognate resistance mechanisms are related to mechanisms that arise in pathogenic bacteria, and understanding them is relevant to our ability to control microbial infections clinically.

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A remarkable and seemingly universal feature of the streptomycetes is their capacity to produce “secondary” or nonessential metabolites that have potent biological activities. These molecules include inhibitors of transcription, translation, DNA replication, cell wall biosynthesis, and primary metabolism, and they can act on prokaryotic and/or eukaryotic cells. Many secondary metabolites have been developed for clinical use as antibiotics, anticancer agents, and other drugs, and these molecules remain an important source of new drug leads (1). While there is debate concerning the role played by these molecules in nature (2, 3), it is likely that they confer an evolutionary advantage on producers by allowing them to inhibit the growth and behavior of neighboring organisms (4).

Most secondary metabolites are produced by biochemical pathways encoded in discrete genomic islands. In addition to the biosynthetic enzymes, these islands often encode resistance mechanisms such as transmembrane efflux pumps that are related to mechanisms that confer clinical antibiotic resistance in pathogens (5, 6).

One model system for the investigation of secondary metabolism is the blue-pigmented polyketide antibiotic actinorhodin, produced by *Streptomyces coelicolor*. During biosynthesis, the polyketide synthase (ActI ORF1-3) generates a 16-carbon linear polyketide (Fig. 1A). Tailoring enzymes convert this to a two-ring intermediate molecule that is in turn converted to the 3-ring intermediate (S)-DNPA in a ring closure reaction that depends on ActVI-ORF1 and ActVI-ORF3 (7). (S)-DNPA is modified in subsequent steps to give rise to dihydrokalafungin (DHF). Two DHK molecules are then ligated by ActVA-ORF5 and ActVB to generate the 6-ring molecule actinorhodin (8–10).

Actinorhodin inhibits the growth of Gram-positive bacteria (11). Consistent with a requirement for a cognate export and resistance mechanism, the actinorhodin biosynthetic gene cluster encodes three putative export pumps (12–14). Two of these, ActII-ORF2 (ActA) and ActII-ORF3 (ActB), are encoded in the *actAB* operon, which is regulated by the transcriptional repressor...
ActR (Fig. 1A) (15). This is a common arrangement—export genes have been documented in the biosynthetic gene clusters of many known secondary metabolites, and they appear to be widespread in gene clusters for unknown molecules as well (16, 17).

ActR is a member of the TetR family of regulators (18). Like most TetR proteins, it is a homodimer of a polypeptide having an N-terminal helix-turn-helix DNA-binding domain and a larger, C-terminal ligand-binding domain (Fig. 1B). The role of the ligand-binding domain is to interact with small molecules that trap the protein in a conformation that does not bind DNA. This activates the target promoter (19, 20).

ActR binds actO (21), an operator in the actAB promoter (P_{actAB}). We showed previously that DNA binding by ActR can be prevented by actinorhodin, the probable substrate of the actAB-encoded pumps. Interestingly, we also found that some 3-ring intermediates from the actinorhodin biosynthetic pathway, including (S)-DNPA and a close structural relative of DHK, are also ActR ligands (15). Indeed, (S)-DNPA binds ActR more tightly than does actinorhodin and relieves DNA binding in vitro at lower concentrations (22). We suggested that the induction of export by intermediates could couple export/resistance to biosynthesis, preventing the accumulation of toxic levels of the mature antibiotic in the cytoplasm (15).

In subsequent work, we found that ActR binds a wider variety of molecules than we had originally anticipated (22), and this called the biological relevance of ActR’s interaction with 3-ring intermediates into question. Indeed, a critical and unanswered question is whether any of the 3-ring intermediates accumulate in the cytoplasm. It is possible that during normal biosynthesis they are converted to the mature, 6-ring molecule so rapidly that they have no opportunity to derepress the export genes. Alternatively, many biosynthetic intermediates are unstable—those intermediates that escape the biosynthetic apparatus could be subject to enzymatic or chemical degradation in the cytoplasm, again, preventing them from acting as export-inducing signals.

In this work, we show that normal actinorhodin yields depend on actAB expression, consistent with previous work (14). We show that the 3-ring intermediates can act as intracellular signals to activate actAB in vivo at levels sufficient for normal actinorhodin production. However, we also find that a second, intercellular signaling event mediated by actinorhodin is required for the bacterium to survive actinorhodin biosynthesis.

**RESULTS**

actR and actAB influence actinorhodin yields. We first constructed actR and actAB null mutants in the S. coelicolor strain.
M145 and tested them for actinorhodin production. As shown in Fig. 2A, actinorhodin yields were reduced ~5-fold by the actAB deletion. This was not simply due to a defect in actinorhodin export, because the level of intracellular actinorhodin was also compromised (see Fig. S1 in the supplemental material). In contrast, the actR mutant produced actinorhodin at levels 4- to 5-fold higher than did the wild type. Actinorhodin yields were restored by complementation of either mutation in trans (see Fig. S2). These experiments demonstrate that the activation of the actAB operon is an important step in actinorhodin production, consistent with previous work (14).

**Actinorhodin biosynthetic pathway intermediates can activate actAB.** We showed previously that 3-ring intermediates from the actinorhodin biosynthetic pathway can bind ActR in vitro and prevent its interaction with actO DNA (15). To determine whether this could be replicated in vivo, we created mutants that interrupted the pathway at steps that had been previously characterized biochemically and/or genetically (Fig. 1A). We first deleted the act operon, ablating the actinorhodin polyketide synthase and therefore the entire biosynthetic pathway. The second mutation, a deletion of actVI-1, eliminates the enzyme that closes the third ring in the first 3-ring intermediate. Such mutants block the pathway prior to the first 3-ring intermediate (S)-DNPA and accumulate inactive shunt products (23). The third mutation, actVA-5, eliminates an enzyme required for ligating two 3-ring intermediates to generate the 6-ring molecule actinorhodin (24, 25). Previous research showed that this mutant produces the 3-ring molecule DHK but no actinorhodin (25). As shown in Fig. 2A, while M145 produced blue-pigmented compounds having the characteristic absorption peak of actinorhodin, the actI, actVI-1, and actVA-5 mutants did not. We confirmed that the mutations in actVI-1 and actVA-5 could be complemented in trans (see Fig. S2 in the supplemental material).

To determine the effects of these mutations on actAB expression, we fused PaxAB to a luxCDABE reporter (26) (see Fig. S3 in the supplemental material) and introduced the fusion into M145 and the mutant strains. As shown in Fig. 2B, the PaxAB lux fusion was active in M145 but was inactive in the actI null mutant, consistent with the need for an actinorhodin biosynthetic pathway for the induction of the export genes. The actVI-1 mutant did not express the reporter either, consistent with a requirement for a 3-ring intermediate (including in particular the pyran ring characteristic of true pathway intermediates) and/or mature actinorhodin. In contrast, the actVA-5 mutant, which is blocked at the DHK step and produces no actinorhodin (Fig. 2A), supported PaxAB-dependent luminescence. This confirms that a strain that can produce the 3-ring intermediates but not actinorhodin can still activate PaxAB in vivo, and this in turn is consistent with a capacity for these molecules to act as export-inducing signals in vivo.

**ActR mutants that respond to (S)-DNPA but not actinorhodin.** The most important question concerning the signaling event that activates the expression of the actAB operon is what molecules contact ActR and release its transcriptional repression of PaxAB. To explore this question, we used ActR as a probe by creating mutants of the protein that interact with the 3-ring intermediate (S)-DNPA but not with the mature 6-ring antibiotic.

Our previous structural work (27) revealed that the ActR ligand-binding pockets each bind one 6-ring actinorhodin molecule or two 3-ring (S)-DNPA molecules positioned similarly to the two halves of...
actinorhodin (Fig. 1C). Our rationale was to introduce residues with larger side chains into the pocket and crowd sites occupied by the proximal or distal half of actinorhodin or by its central covalent bond, preventing a productive interaction. We reasoned that (S)-DNPA might be able to interact with some of these ActR mutants, as it is unconstrained by a central covalent bond. We generated 20 sequence changes at 10 positions (V90, V92, M107, L111, G135, G138, T142, G160, S16,4 and R225 [Fig. 1D]) to create candidate “intermediate” mutants (Table 1).

The G138A, G138F, G138Y, and R225A mutants were insoluble or unstable; however, the remaining 16 alleles generated protein of the correct size and at yields similar to those of wild-type ActR. We purified these proteins and compared their abilities to bind DNA by gel mobility shift assay. Seven of the mutants did not bind DNA; however, the remaining nine, V92Y, L111W, G135A, G138W, G138L, T142Y, T142W, GTYW (G138Y and T142W), and GTWW (G138W and T142W), bound actO with affinities similar to those of the wild-type protein (see Fig. S4 in the supplemental material).

We tested these proteins for response to actinorhodin and the 3-ring intermediate (S)-DNPA in vitro. Seven of the nine mutants responded to actinorhodin by releasing actO; however, the mutants T142W and GTYW (a G138Y and T142W double mutant) did not, even at concentrations as high as 200 μM. Consistent with our prediction, however, these mutants retained a productive response to (S)-DNPA (see Fig. S5 in the supplemental material). The double mutant GTYW includes the T142W mutation, so we focused on the single mutant for the rest of this work.

The T142W mutant responds to (S)-DNPA but not actinorhodin in living cells. To assess ligand responsiveness in living cells, we created transcriptional reporters in which the wild-type or T142W allele of actR controlled a fusion of P_{actAB} to a luxCDABE reporter (see Fig. S3, top, in the supplemental material). We introduced both reporters into a strain in which the actI operon and the actR gene had been deleted. The resulting reporter strains exhibited no luminescence, consistent with repression of P_{actAB} by the wild type or T142W mutant of ActR in the absence of actinorhodin biosynthesis (Fig. 3A and B).

We added actinorhodin to the reporter strain cultures at concentrations ranging from 10 nM to 1 μM and measured luminescence. Consistent with the behavior of these proteins in vitro, actinorhodin induced luminescence in the wild-type actAB-controlled reporter but not in the T142W mutant (Fig. 3A, top). In contrast, (S)-DNPA induced luminescence in both the wild-type and T142W reporters (Fig. 3A, bottom), demonstrating a productive interaction of both proteins with this 3-ring intermediate. Time course measurements of this reporter responding to 10 nM actinorhodin (Fig. 3B, top) and (S)-DNPA (Fig. 3B, bottom) indicated that ligand-induced luminescence took place ~6 h following the addition of the compounds in both cases. This demonstrates that the T142W mutation alters the ligand responsiveness of ActR such that it interacts productively with (S)-DNPA but not actinorhodin. These results also show that both (S)-DNPA and actinorhodin can cross the cell envelope.

**Activation of P_{actAB} by pathway intermediates.** To determine which molecules trigger actAB expression during normal actinorhodin biosynthesis, we introduced the wild-type actR and T142W mutant reporters into a strain in which actR was deleted from the actinorhodin biosynthetic gene cluster but the rest of the cluster was intact (see Fig. S3 in the supplemental material). As expected, the P_{actAB-lux} fusion was induced in cells where it was under the control of wild-type ActR (Fig. 3C, top), reaching 20 to 40 h. Actinorhodin accumulated to measurable levels within 50 h, following the activation of P_{actAB}. Both actinorhodin production and the induction of P_{actAB} depended on the actI genes as expected (Fig. 3C, top). When the same experiment was carried out in the strain where P_{actAB} was under the control of T142W, the result was very similar (Fig. 3C, bottom). P_{actAB} was active within 20 h of culture growth. Actinorhodin accumulated to measurable levels by 50 h and, importantly, reached a final yield that was the same as that of the strain bearing wild-type actR. Again, both actinorhodin production and the activation of T142W-regulated P_{actAB} re-

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**TABLE 1 ActR mutants**

| Mutation | Helix | Ligand-binding pocket | Protein purification | DNA binding | Actinorhodin production | (S)-DNPA |
|----------|-------|------------------------|----------------------|-------------|------------------------|---------|
| V90W     | α4/α5 | Distal                 | Yes                  | No          | NA*                    | NA      |
| V92W     | α4/α5 | Distal                 | Yes                  | No          | NA                     | NA      |
| V92Y     | α4/α5 | Distal                 | Yes                  | Yes         | Induction              | Induction |
| M107W    | α5    | Distal                 | Yes                  | No          | NA                     | NA      |
| L111W    | α5    | Distal                 | Yes                  | Yes         | Induction              | NA      |
| G135A    | a7    | Distal                 | Yes                  | Yes         | Induction              | NA      |
| G138A    | a7    | Distal                 | Yes                  | Yes         | Induction              | NA      |
| G138F    | a7    | Distal                 | Yes                  | Yes         | Induction              | NA      |
| G138Y    | a7    | Distal                 | Yes                  | Yes         | Induction              | NA      |
| G138W    | a7    | Distal                 | Yes                  | Yes         | Induction              | NA      |
| T142Y    | a7    | Distal                 | Yes                  | Yes         | Induction              | NA      |
| T142W    | a7    | Distal                 | Yes                  | Yes         | No induction           | Induction |
| GTYW     | a7    | Distal                 | Yes                  | Yes         | No induction           | Induction |
| GTWW     | a7    | Distal                 | Yes                  | Yes         | Induction              | NA      |
| G160W    | a8    | Proximal               | Yes                  | No          | NA                     | NA      |
| S164W    | a8    | Proximal               | Yes                  | No          | NA                     | NA      |
| S164Y    | a8    | Proximal               | Yes                  | No          | NA                     | NA      |
| R225A    | α11   | Proximal               | Insoluble            | NA          | NA                     | NA      |

*NA, not announced.*
FIG 3  ActR responds to intermediates from the actinorhodin biosynthetic pathway in vivo. (A) An actI ORF1-3 mutant bearing a fusion of P_{actAB} to luxCDABE and expressing either wild-type (WT) actR or the T142W mutant was incubated with various concentrations of actinorhodin or (S)-DNPA as indicated. (B) Time course response of an actI ORF1-3 mutant bearing a fusion of P_{actAB} to luxCDABE and expressing either wild-type ActR (purple) or the T142W mutant (cyan) to 10 nM actinorhodin or (S)-DNPA. Dimethyl sulfoxide (DMSO)-treated wild-type (blue) and T142W (red) reporter strains were shown as negative controls. (C) A_{640}, indicative of actinorhodin production (dashed lines), and P_{actAB} activity (solid lines) were monitored in strains expressing wild-type ActR or the T142W mutant. In these assays, the actR gene was adjacent to the P_{actAB} sequence in the lux reporter (see Fig. S3, top, in the supplemental material). (D) Actinorhodin production (dashed lines) and P_{actAB} activity (solid lines) were monitored in strains expressing wild-type ActR or the T142W mutant. In these assays, the actR gene was in the actinorhodin biosynthetic gene cluster P_{actAB} sequence in the lux reporter (see Fig. S3, bottom, in the supplemental material).
quired the actI operon. This suggests that the intermediate molecules accumulated to levels sufficient to bind ActR and derepress P_acrB during normal actinorhodin biosynthesis.

There was one striking difference between the behavior of the wild-type actR and T142W mutant reporters: while the levels of accumulation of actinorhodin were the same in the two, P_acrAB-lux reporter activity was markedly lower in the mutant: ~5,000 relative light units (RLU)/mg in the wild-type ActR strains, compared to only ~120 RLU/mg in the T142W strain. Given the importance of actAB in actinorhodin yields (Fig. 2A), this was a significant discrepancy, and it was reproducible in several different media (see Fig. S6 in the supplemental material).

We were concerned that the reporter arrangement, where the only source of ActR is outside the actinorhodin gene cluster, might have influenced repressor expression. We therefore constructed a strain in which actR was replaced with the T142W allele in the actinorhodin biosynthetic gene cluster in an otherwise normal M145 background. We then introduced a fusion of P_acrAB to luxCDABE (see Fig. S3 in the supplemental material) into M145 and the T142W strain and compared luminescence and actinorhodin production levels. For unknown reasons, lux signals were lower both under the control of wild-type ActR and under the control of the T142W protein (Fig. 3D). However, aside from this, the result was the same as those reported above (compare Fig. 3C and D): actinorhodin yields were identical in the two strains and yet the activities of the T142W and wild-type actR reporters differed by nearly 2 orders of magnitude. A reverse transcription-PCR (RT-PCR) experiment confirmed that the behavior of the lux reporter fusions was consistent with the expression status of actA itself in both M145 and the T142W mutant (see Fig. S7).

Since the T142W mutant does not interact productively with actinorhodin, these results demonstrate that P_acrAB was induced, at a much lower level, by biosynthetic intermediates. Since, however, the resulting yield of actinorhodin was normal, this intermediate-driven induction was clearly sufficient for a wild-type actinorhodin production process. Therefore, the mature antibiotic is not required for this signaling event—actAB induction by the 3-ring intermediates generates a sufficient level of ActA and ActB for normal actinorhodin production.

Actinorhodin can act as an intercellular signal. The discrepancy between the activation of P_acrAB regulated by the wild type and the T142W mutant of ActR could be explained by a scenario in which the intermediates induce actAB in actinorhodin-producing cells, perhaps a subset of the total culture, but where culture-wide induction depends on actinorhodin itself.

To determine whether actinorhodin or its biosynthetic intermediates can act as intercellular signals to activate P_acrAB, we carried out mixed culture experiments where one strain served as a donor of inducing molecules and an actR actI double null mutant harboring a P_acrAB-lux reporter (with the wild-type or T142W actR gene in cis to the lux genes—see Fig. S3, top, in the supplemental material) served as a recipient.

When an actI mutant was used as a donor in a mixed culture experiment, no luminescence was induced in either the wild-type ActR or the T142W recipient, confirming the requirement for actinorhodin biosynthetic pathway molecules for derepression of P_acrAB. When M145 was used as a donor, it was able to activate P_acrAB in recipient strains where the promoter was under the control of wild-type ActR (Fig. 4A, top), as expected. However, when P_acrAB was used under the control of the T142W mutant, activation was greatly reduced. We conclude that wild-type S. coelicolor does not export pathway intermediates at a level that is sufficient for high-level induction of(actAB (Fig. 4A, bottom).

We repeated the experiment using the actVA-5 deletion mutant as a donor. Recall that this mutant is unable to ligate two DHK molecules to form the 6-ring actinorhodin structure: the biosynthetic pathway can therefore progress only as the 3-ring intermediate DHK. In contrast to strain M145, this strain activated luminescence in both the actR- and the T142W-controlled P_acrAB recipients (Fig. 4B). We interpret the results in Fig. 4A and B as evidence that wild-type cells do not release significant levels of the intermediate molecules from the actinorhodin biosynthetic pathway. We conclude that the predominant intercellular signal for activation of P_acrAB in nonproducing cells is actinorhodin. Consistent with this, we note that the activation of the P_acrAB promoter in the recipient cells was coincident with the accumulation of actinorhodin produced by the donor during the mixed cell culture.

Intermediates trigger a burst of P_acrAB activity that is amplified by actinorhodin signaling. To explore P_acrAB activity at the level of whole colonies, we constructed a set of reporters in which luxCDABE was replaced with the enhanced green fluorescent protein (eGFP) vector pIJ8660 (5). These reporters were similar to those employed in Fig. 3A and B (see Fig. S3, top, in the supplemental material), except that the egfp gene replaced the luxCDABE operon. We introduced the fusions into cells in which the endogenous actR gene had been deleted and into an actR actI double mutant and examined the fluorescence of cells under microscopy (Fig. 5).

Filamentous S. coelicolor does not undergo cytokinesis or sporulation during growth in liquid culture; rather, cells grow as interconnected microcolonies (Fig. 5, differential interference contrast [DIC] images). The images in Fig. 5 show cultures 5 h after resuspension in actinorhodin production medium. At the early time point, there is low-level fluorescence being driven by P_acrAB-egfp in both the actR and T142W strains, and in both cases, this fluorescence depended on molecules from the actinorhodin biosynthetic pathway (compare fluorescence in actI+ cells with the lack thereof in actI-negative cells). Quantified fluorescence is shown in the scatter plots to the right. Microcolonies lacking actinorhodin biosynthesis generated a background fluorescence of 0 to 2 units, regardless of whether P_acrAB-egfp was under the control of the wild-type ActR protein or of the T142W mutant. In contrast, 79 of the 84 actI+ cells expressing the wild-type ActR exhibited fluorescence above 2 units with maximum signals of ~10. The fluorescence of 5 of these 84 microcolonies was at background level. In actI+ microcolonies where P_acrAB-egfp was under the control of the T142W mutant, a majority (32/44) also exhibited fluorescence of >2 units with maximum signals of ~9 units, and 16/44 were at background.

At the later time point, the phenotypes of the wild-type ActR and T142W mutants were markedly different. The majority (32/44) of the T142W mutant microcolonies were at background fluorescence, with only 8 exhibiting signals of 2 to 3 units, bordering on background levels. In contrast, the wild-type ActR microcolonies (50/50) exhibited greatly elevated fluorescence levels with maximum signals of 65 units.

These data further demonstrate that during the early stage of actinorhodin biosynthesis, intermediates can trigger the actAB promoter. Given that the intermediates do not act as intercellular signals (Fig. 4) in wild-type cells, we presume that this is an intracellular signaling step that is restricted to cells in which the acti-
The norhodin biosynthetic gene cluster has been activated. While this intermediate-triggered actAB expression is clearly sufficient for normal actinorhodin production (Fig. 2A), it does not drive sustained, culture-wide actAB expression: ActR must sense actinorhodin for this greater level of expression.

The phenotypic cost of the T142W mutation. The data presented above show that actinorhodin production is heavily influenced by actAB. Biosynthetic intermediates can trigger sufficient actAB expression of the operon for normal actinorhodin production, even though induction is at a low level and is short-lived. We wondered whether the inability of the T142W mutant to respond to actinorhodin-mediated signaling was detrimental to cell growth. To address this, we used a live/dead cell-staining assay, previously used in streptomycetes (28).

We visualized wild-type and T142W S. coelicolor microcolonies growing under the same conditions as those used for the reporter assays. We labeled M145 and the congeneric T142W microcolonies with SYTO 9 green and propidium iodide (PI) after 3 and 8 days in actinorhodin production medium. As observed previously (28), after 3 days of growth, during actinorhodin production we observed microcolonies having a core of red fluorescent dead cells surrounded by a ring of green fluorescent live cells in both the M145 and T142W microcolonies (Fig. 6). After 8 days of growth (Fig. 6), the appearance of the M145 strain was the same: a core of red fluorescent dead cells ringed by green fluorescent live cells. In striking contrast, after prolonged growth, the majority of the microcolonies in the T142W mutant exhibited red fluorescence throughout, indicative of widespread cell death. Given that this strain is identical to M145 except for the T142W mutation, we conclude that a second, actinorhodin-mediated signaling event is essential for S. coelicolor to survive its capacity to produce actinorhodin.

DISCUSSION
These results demonstrate that there is an intimate relationship between actinorhodin biosynthesis and export: actAB expression is a critical determinant of actinorhodin yields, and yet molecules

![Figure 4](mbio.asm.org)
from the biosynthetic pathway are clearly essential for actAB induction. We suggest that our results are most consistent with a two-step mechanism for the activation of these export genes.

The first step takes place in response to the activation of the actinorhodin biosynthetic genes. The fact that there is no difference in actinorhodin yields when actAB is controlled by wild-type ActR and when it is controlled by T142W mutant ActR shows that the 3-ring biosynthetic intermediates are sufficient for this activation. As wild-type cells do not export these intermediates, we suggest that this is an intracellular signaling event that serves as the primary or perhaps the exclusive determinant of initial actAB expression in actinorhodin-producing cells. By activating actAB in this way, the producer cells ensure that the pumps are available to drive efficient actinorhodin production and to protect themselves...

FIG 5 Visualization of P_{actAB} activity in S. coelicolor microcolonies. P_{actAB}egfp fusions expressing wild-type ActR (A and C) or the T142W mutant (B and D) were introduced into an actR null mutant (actI+) or into an actR actI double null mutant (actI-) as indicated, such that the reporter construct was the sole source of ActR protein. Fluorescence levels were quantified and expressed in arbitrary units. The green diamonds show the fluorescence levels in actI+ microcolonies, while the olive squares show the fluorescence levels in the actI microcolonies.
from self-killing before the mature antibiotic accumulates to significant concentrations. Apparently, however, this initial burst of actAB expression is not sustained and drops off with time.

We propose that the second actAB activation step leads to sustained, high-level actAB expression throughout the culture and that it is triggered primarily by actinorhodin itself. This second
step is clearly not required for efficient actinorhodin production but rather serves to protect *S. coelicolor* from the antimicrobial effects of the actinorhodin as it accumulates later on during cell culture.

At present, the manner in which *actAB* facilitates efficient actinorhodin production is unknown. It is clear that the lower total yield illustrated in Fig. 2A was not due strictly to a failure of export because the defect is even more severe when measured using an assay for intracellular actinorhodin. Indeed, it is a paradox that so much actinorhodin makes it out of the cells in this mutant. This could be due to the action of another export protein (*ActCT*) or to passive diffusion across the cell wall. Indeed, it could also be a result of the lysing of cells that die off during the course of normal growth or, perhaps in this case, that were killed as a result of the actinorhodin biosynthetic pathway. It is not possible to distinguish between these possibilities at present. We hasten to add that we have no concrete data showing that any of the proteins ActA, -B, and -C are actually actinorhodin exporters—although, of course, we assume that at least one of them is. Assuming that their role includes export, ActA and/or -B could facilitate actinorhodin production through several possible mechanisms. They could simply prevent product inhibition of one or more biosynthetic enzymes: the removal of actinorhodin from the cytoplasm could thereby accelerate flux through the entire pathway. Alternatively, one or more of these membrane proteins could coordinate the assembly of a membrane-associated multiprotein complex that is required for high-level actinorhodin production (34). Addressing the precise roles of these proteins is one of our current goals.

There is evidence consistent with an intermediate-driven resistance mechanism for at least two other polyketides: the antibiotic lanostadiene and the anticancer drug doxorubicin (16, 17). Both of these molecules are exported from producers by cognate efflux pumps, and in both cases, the pump genes are regulated by TetR family repressors. It has also been suggested that a conceptually similar mechanism acts during the biosynthesis of the lantibiotic microbisporicin (29). Our work demonstrates that this topic is clearly a rich source of important questions concerning these and other metabolites.

**MATERIALS AND METHODS**

**Bacterial growth.** Strains and plasmids are described in Table S1 in the supplemental material. *S. coelicolor* was grown in liquid YEME medium, R2 medium, actinorhodin production medium (23), and R2-2 medium (14) and on solid MS or R2 yeast extract (R2YE) agar (30). *Escherichia coli* was grown in liquid Luria-Bertani (LB) medium or LB agar (31). Apramycin, kanamycin, and spectinomycin were added at 50 μM, 30 μM, and 50 to 200 μM, respectively.

**DNA manipulation.** Oligonucleotides used in this study are listed in Table S2 in the supplemental material. Standard procedures were used for DNA purification, PCR, gene subcloning, and molecular analysis (30, 31). PCR was performed using Vent DNA polymerase (New England BioLabs), and all PCR products and site-directed mutants were sequenced.

**Gene disruption.** Chromosomal deletions were prepared by PCR-targeted mutagenesis of *S. coelicolor* strain M145 (32). BAC28G1 (33), which carries the actinorhodin biosynthetic gene cluster, was used for the mutagenesis. Each deletion was confirmed using PCR primers listed in Table S2 in the supplemental material. To construct the *actR actl* double mutant, the *actl* mutagenic cosmid was used to inactivate the *actl ORF1*, -2, and -3 genes in an *actl null* mutant.

**Measurement of actinorhodin.** Actinorhodin production was measured by treating 200 μl of cell culture with 1 N KOH, removing cells by centrifugation, and reading the *A*405 of the supernatant using the Bio-Tek Epoch microplate spectrophotometer (30).

**Construction of in situ actR point mutation in *S. coelicolor*.** The chromosomal *actR* gene was replaced with an SpcR disruption cassette using PCR targeting (32). A T142W mutant of *actR* with 1.1 kb of flanking sequence on either side was generated by PCR (31) and ligated to pOJ260, which has an ApR gene in the backbone and does not replicate in *S. coelicolor*. This construct was introduced into the *actR::SpcR* mutant, and apramycin-resistant exconjugants were selected. These were grown non-selectively through 4 generations, and spores that were sensitive to both spectinomycin and apramycin were identified—indicating removal of the plasmid and replacement of *actR* with the T142W allele. Finally, to confirm that *actR* had been reintroduced into the actinorhodin biosynthetic gene cluster, we amplified the gene and sequenced it, finding that it did indeed include the T142W mutation.

**Luminescence assays.** We constructed two sets of luxCDABE reporter monitoring *PactAB* activity (see Fig. S3 in the supplemental material) using a derivative of pMU1 (26). Reporter strains (including the promoterless pMU1 negative control) were inoculated in R2 liquid medium at 0.5 × 10^8 spores/ml and grown at 30°C with shaking at 200 rpm until the optical density at 450 nm (OD_{450}) reached approximately 0.1. Cells were recovered by centrifugation at 3,000 rpm, resuspended in fresh RG-2 (or other medium), and incubated at 30°C with continued shaking. Two-hundred-microliter aliquots were taken, and luminescence was measured using a Lumat 9507 luminometer (Berthold Technologies). Cells were then recovered by centrifugation and weighed, and luminescence values were normalized to the pellet (wet weight). All assays were done in triplicate, and standard deviations were determined using Microsoft Excel.

**Fluorescence microscopy.** To generate a transcriptional green fluorescent reporter that is directed by the *actAB* promoter and mediated by either wild-type ActR or mutated ActR, a gene fragment of *actR::actR* T142W along with an *actR::actR* intergenic region (IGR) was cut from the respective *lux* reporters (see Fig. S3, top, in the supplemental material) by BamHI and KpnI and ligated into the same restriction sites of pJL8660 to give pWT::IGR+eGFP/pT142W+::IGR+eGFP. The generated plasmids were introduced into the *DeltaactR* and *Deltaactl* mutant by conjugation, to give four *Streptomyces* reporter strains: *DeltaactR::pWT::IGR+eGFP, Deltaactl::pT142W+::IGR+eGFP, DeltaactRactl::pWT::IGR+eGFP, and DeltaactRactl::pT142W+::IGR+eGFP.

All four *Streptomyces* strains consisting of the enhanced green fluorescent protein (eGFP) reporter were inoculated in R2YE liquid medium at 0.5 × 10^8 spores/ml and grown at 30°C with shaking at 200 rpm until the OD_{450} reached approximately 0.1. Cells were recovered by centrifugation at 3,000 rpm, washed once with 0.85% saline, resuspended in RG-2 medium, and incubated at 30°C with shaking at 200 rpm for another 2 days. Fluorescence microscopy was performed on a Zeiss Axio Imager M2 microscope (AxioVision release 4.8, 10X objective) to detect mycelium grown at 0 h, 5 h, and 24 h after subculture in RG-2 liquid medium. To compensate for the autofluorescence of *S. coelicolor*, the two *DeltaactR actl* mutant hosts that were deficient in making ActR-responsive signaling molecules were used as negative controls to lower the visualization of fluorescence background, and the same parameters were applied to *DeltaactR* strains that host the same type of eGFP reporter. All the images were processed by Photoshop uniformly. The fluorescence intensity of each cell cluster was normalized and quantified by ImageJ and plotted by comparing density distributions between the *DeltaactR* and *Deltaactl* control and the *DeltaactR* reporter.

**Viability assay for M145 and T142W using fluorescence microscopy.** M145 and T142W were inoculated into R2YE medium, grown to exponential phase, and subcultured into RG-2 medium as described before. One milliliter of culture was taken on day 1, day 3, and day 8 after subculture. Samples were centrifuged for 5 min at 12,000 rpm, washed twice with 0.85% saline, and resuspended in 500 μl water. Each resuspension was mixed with 3 μl of SYTO 9 green fluorescent nucleic acid stain
plus propidium iodide (PI) red fluorescent nucleic acid stain (light bacterial viability kit; Invitrogen; L13152, prepared 1:1 [vol/vol], as recommended by the manufacturer). SYTO 9 labels all the cells, both those with intact and those with damaged membranes, while PI enters only the damaged cells, causing a reduction in the SYTO 9 stain because of its higher intact and those with damaged membranes, while PI enters only the damaged cells, causing a reduction in the SYTO 9 stain because of its higher

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00191-12/-/DCSupplemental.

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SUPPLEMENTAL MATERIAL

(AxioVision release 4.8, 10% objective). Images from green and red channels were merged and processed by Photoshop CS5.1.

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