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 (DHK). 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
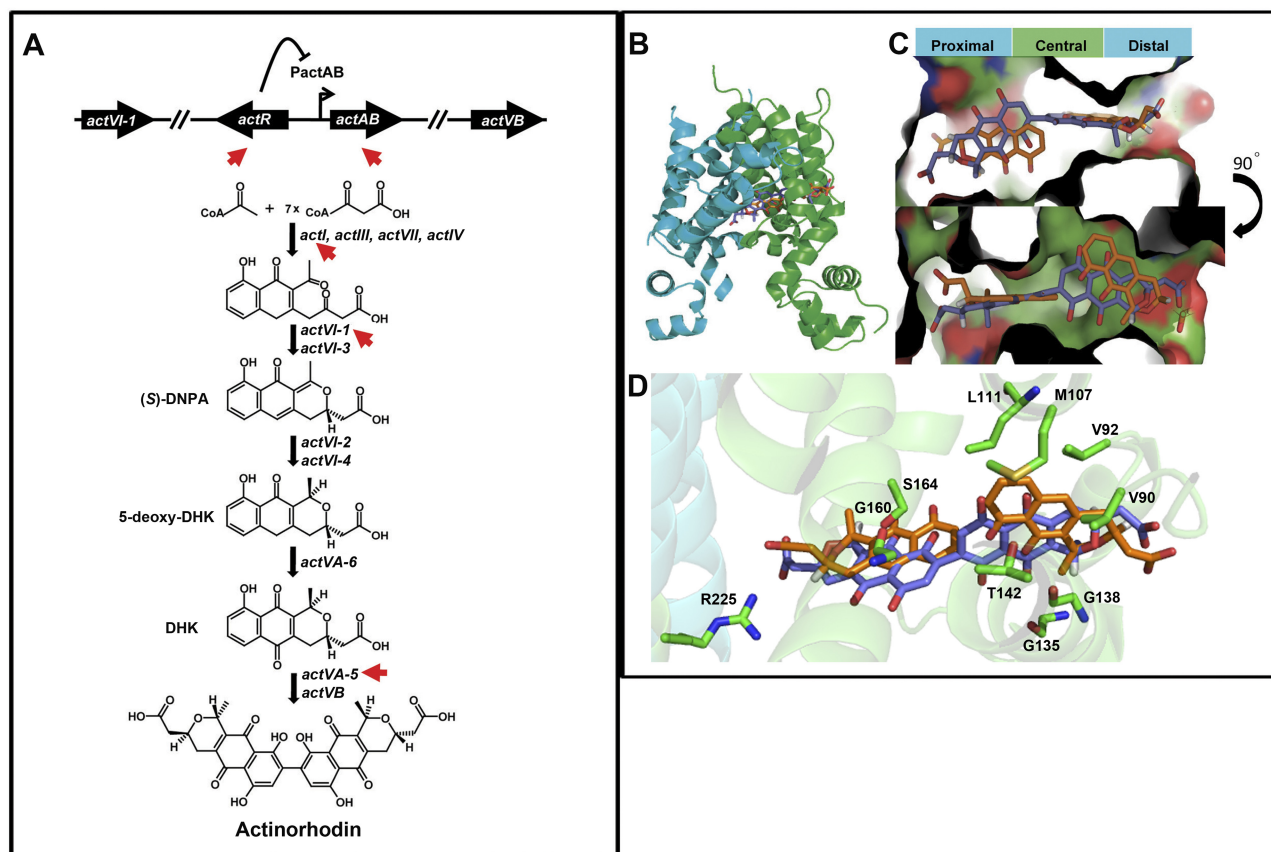
FIG 1 Actinorhodin biosynthesis and ligand binding by ActR. (A) The actinorhodin backbone is assembled by the polyketide synthase encoded by the *actI* operon. Tailoring enzymes convert this to a 2-ring molecule, and the ActVI-1 and ActVI-3 enzymes convert this to a 3-ring molecule. Following additional tailoring steps, two DHK molecules are ligated together by ActVA-5 to create the 6-ring molecule actinorhodin, which is then exported from the cell. Genes indicated with red arrows were inactivated in this work. CoA, coenzyme A. (B) ActR is composed of two monomers (green and cyan), which consist of a ligand-binding domain (top) and a DNA-binding domain (bottom). (C) The ligand-binding cavity of ActR consists of a central hydrophobic ring with two hydrophilic regions that are proximal and distal to the axis of symmetry of the dimeric protein. Structural work showed that each cavity binds one actinorhodin molecule (blue) or two molecules of the intermediate (*S*)-DNPA (orange). (D) Residues in the ActR ligand-binding cavity that were mutated in this work.

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 *actI* 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 P*actAB* 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 P*actAB* *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 P*actAB*-dependent luminescence. This confirms that a strain that can produce the 3-ring intermediates but not actinorhodin can still activate P*actAB* *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 P*actAB*. 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
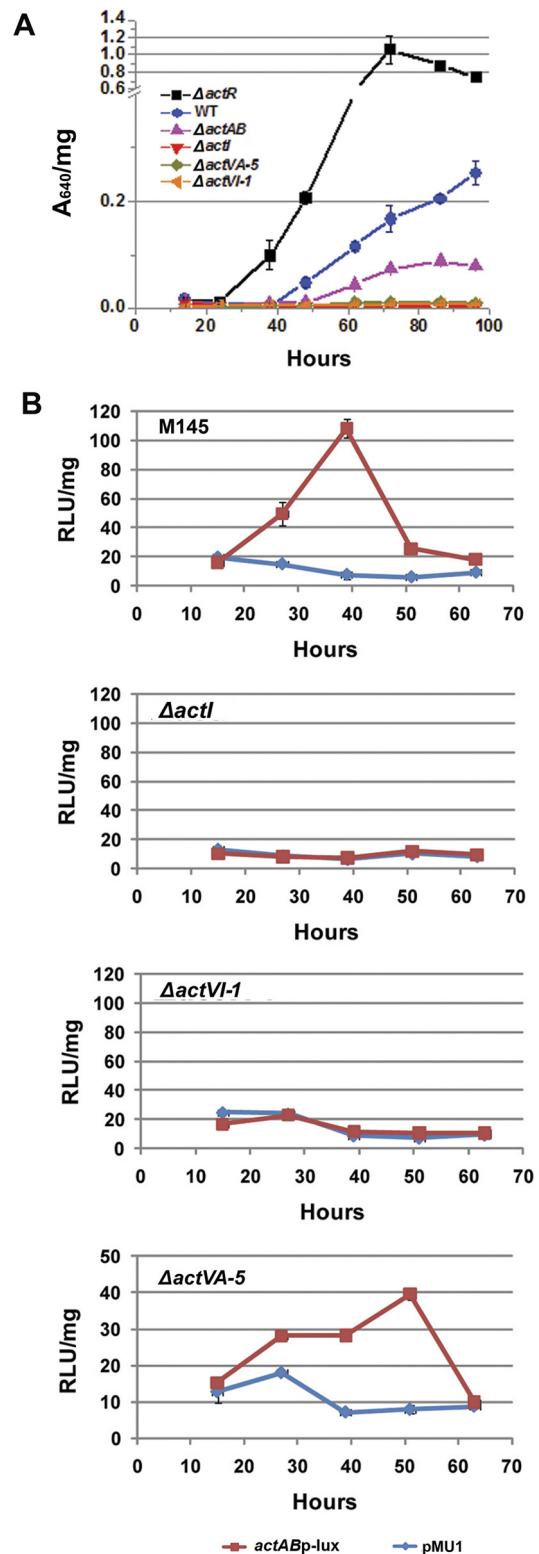
FIG 2 Effects of biosynthesis and export gene mutations on actinorhodin production and P*actAB* activation. (A) The strain M145 produces actinorhodin after ~40 h of culture; strains bearing mutations in *actI*, *actVA ORF1*, or *actVA ORF5* do not. Strains bearing a deletion of *actAB* or *actR* produced reduced (pink line) or enhanced (black line) levels of actinorhodin, respectively. (B) Luminescence readings from strains containing a fusion of P*actAB* to *luxCDABE* (red curves) or a control vector (blue), pMU1.

TABLE 1 ActR mutants

Mutation	Helix	Ligand-binding pocket	Protein purification	DNA binding	Actinorhodin production	(S)-DNPA
V90W	$\alpha4/\alpha5$	Distal	Yes	No	NA[a]	NA
V92W	$\alpha4/\alpha5$	Distal	Yes	No	NA	NA
V92Y	$\alpha4/\alpha5$	Distal	Yes	Yes	Induction	Induction
M107W	$\alpha5$	Distal	Yes	No	NA	NA
L111W	$\alpha5$	Distal	Yes	Yes	Induction	NA
G135A	$\alpha7$	Distal	Yes	Yes	Induction	NA
G138A	$\alpha7$	Distal	Insoluble	NA	NA	NA
G138F	$\alpha7$	Distal	Unstable	NA	NA	NA
G138Y	$\alpha7$	Distal	Unstable	NA	NA	NA
G138W	$\alpha7$	Distal	Yes	Yes	Induction	NA
G138L	$\alpha7$	Distal	Yes	Yes	Induction	NA
T142Y	$\alpha7$	Distal	Yes	Yes	Induction	Induction
T142W	$\alpha7$	Distal	Yes	Yes	No induction	Induction
GTWY	$\alpha7$	Distal	Yes	No	NA	NA
GTYW	$\alpha7$	Distal	Yes	Yes	No induction	Induction
GTWW	$\alpha7$	Distal	Yes	Yes	Induction	NA
G160W	$\alpha8$	Proximal	Yes	No	NA	NA
S164W	$\alpha8$	Proximal	Yes	No	NA	NA
S164Y	$\alpha8$	Proximal	Yes	No	NA	NA
R225A	$\alpha11$	Proximal	Insoluble	NA	NA	NA

[a] NA, not announced.

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-only" 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 *actO* 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 *luxCD-ABE* 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 *actR*-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), peaking at 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-
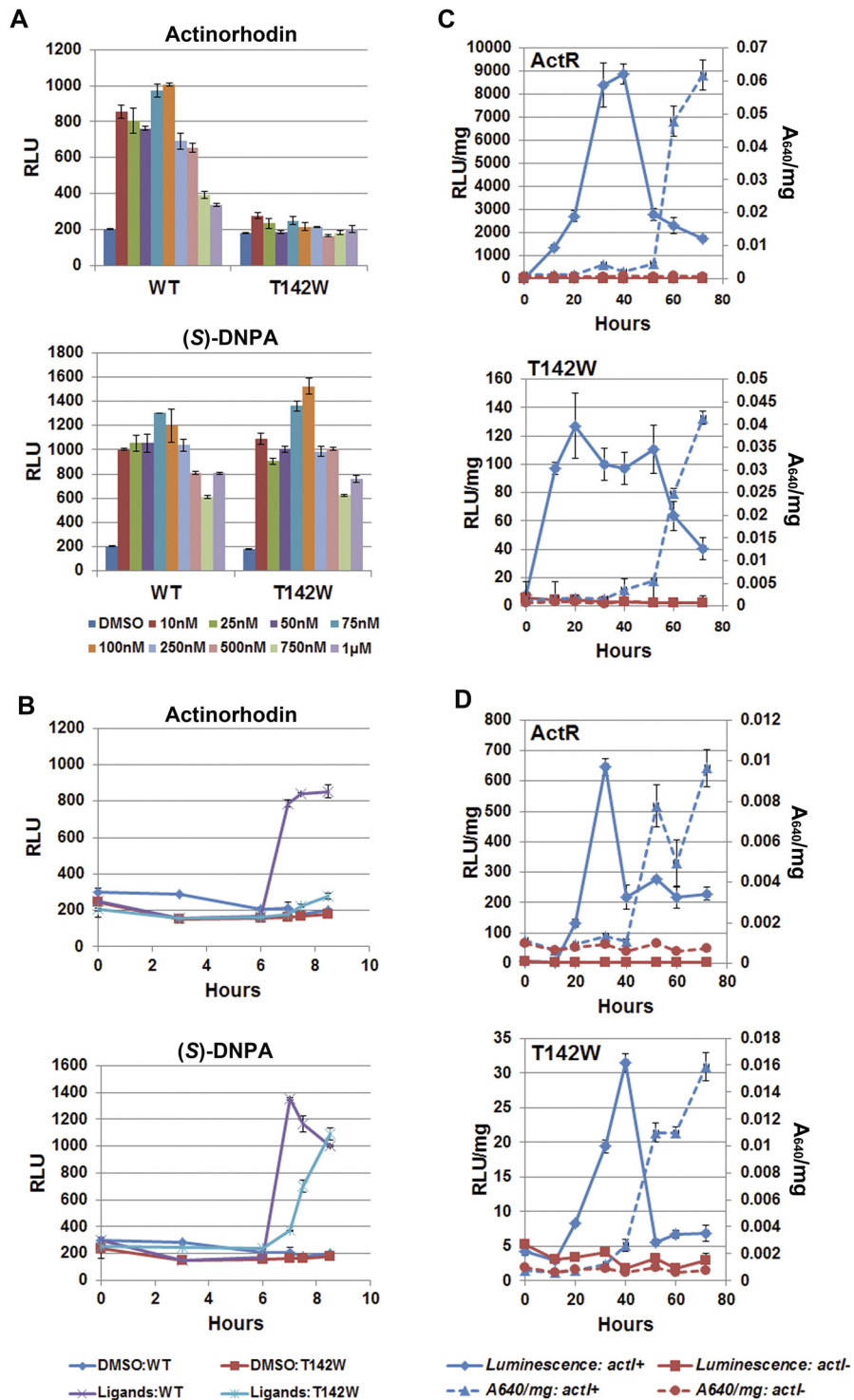
FIG 3 ActR responds to intermediates from the actinorhodin biosynthetic pathway *in vivo*. (A) An *actI ORF1-3* mutant bearing a fusion of P_{actAB} to *luxCDABE* and expressing either wild-type (WT) *actR* or the T142W mutant was incubated with various concentrations of actinorhodin or (*S*)-DNPA as 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_{actAB} 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_{actAB}-*lux* reporter activity was markedly lower in the mutant: ~9,000 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 action. 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_{actAB} to *lux-CDABE* (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_{actAB} 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_{actAB} 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_{actAB}, 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_{actAB}-*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_{actAB}. When M145 was used as a donor, it was able to activate P_{actAB} in recipient strains where the promoter was under the control of wild-type ActR (Fig. 4A, top), as expected. However, when P_{actAB} was 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_{actAB} 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_{actAB} in nonproducing cells is actinorhodin. Consistent with this, we note that the activation of the P_{actAB} 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_{actAB} activity that is amplified by actinorhodin signaling. To explore P_{actAB} 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 microcopy (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 (A and B) or 24 h (C and D) after resuspension in actinorhodin production medium. At the early time point, there is low-level fluorescence being driven by P_{actAB}-*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_{actAB}-*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_{actAB}-*egfp* was under the control of the T142W mutant, a majority of cell clusters (28/44) 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-
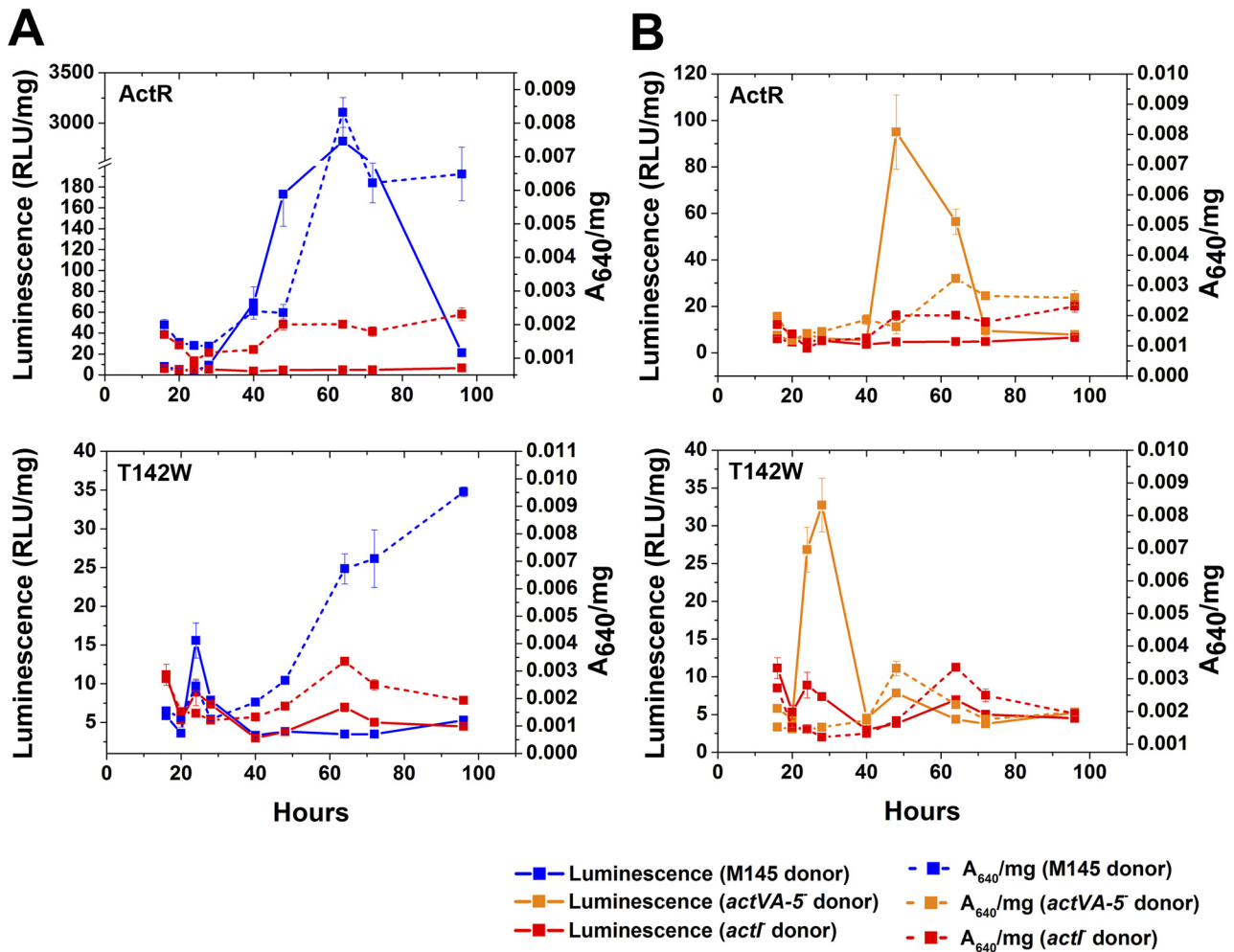
FIG 4 Actinorhodin, but not its biosynthetic intermediates, can act as an intercellular signal in wild-type cells. An *actI ORF1-3* mutant recipient strain bearing a fusion of P_{actAB} to *luxCDABE* was grown with wild-type strain M145, which can induce P_{actAB}-*lux* when it is under the control of wild-type ActR (top) but not the T142W mutant (bottom) (A), or the intermediate producer *actVA-5* mutant, which can induce P_{actAB} under the control of both wild-type ActR (top) and the T142W mutant (bottom) (B). A_{640}, indicative of actinorhodin production, is shown with dashed lines, and luminescence in the recipient strains, indicative of P_{actAB} activity, is shown with solid lines. The blue and orange lines show the results of mixed cultures where M145 or an *actVA-5* mutant acts as donor (A and B, respectively). The red lines show the effects when an *actI* deletion strain serves as donor.

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 congenic 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
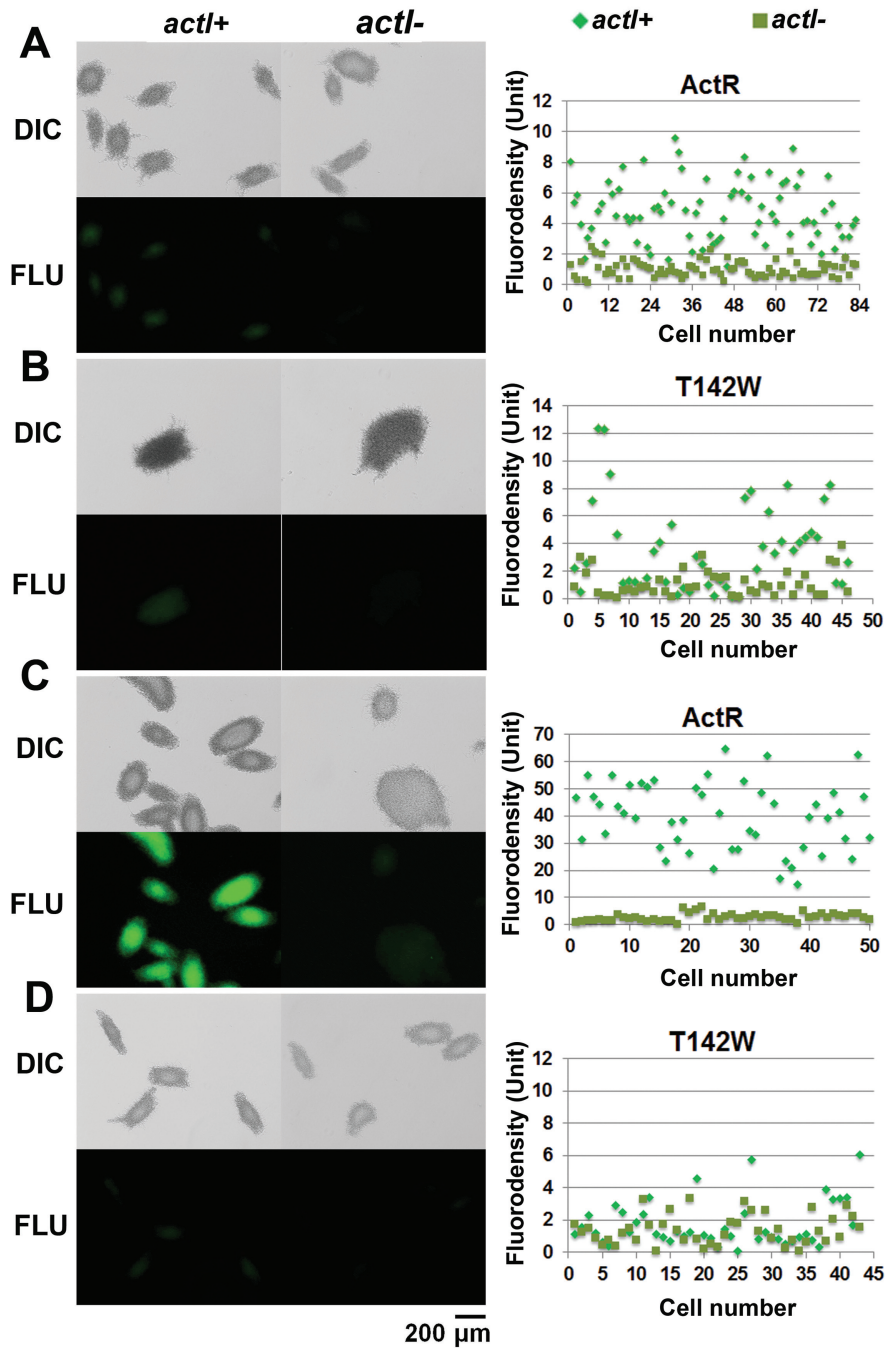
FIG 5 Visualization of P_{actAB} activity in *S. coelicolor* microcolonies. P_{actAB}-*egfp* fusions expressing wild-type ActR (A and C) or the T142W mutant (B and D) were introduced into an *actR* null mutant (*actI+*) or into an *actR actI* double null mutant (*actI-*) as indicated, such that the reporter 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 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
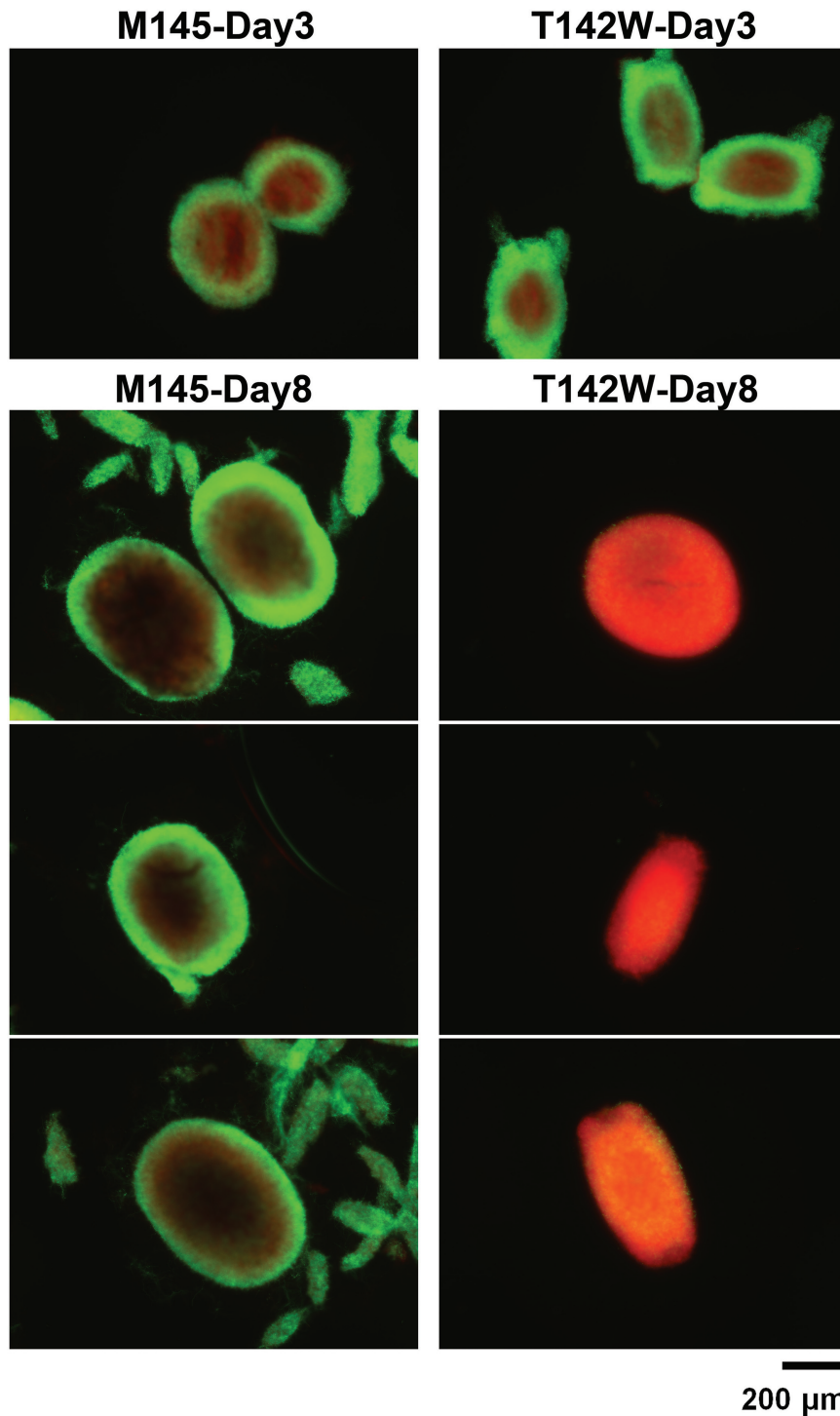
FIG 6 Live/dead staining assay for wild-type M145 and T142W mutant. Comparison of cell growth phenotypes was made between M145 (left) and a congenic T142W mutant (right) on day 3 and day 8 following inoculation. Cells that stain green are impermeable to PI, while those that stain red are permeable and therefore have damaged cell walls. The day 3 M145 and T142W microcolonies are indistinguishable by this assay, consisting of a red fluorescent core of dead cells and an outer ring of green viable cells. On day 8, all of the M145 microcolonies again consisted of a core of dead cells surrounded by viable cells, while ~50% of microcolonies of the T142W mutant were smaller and stained red throughout.

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 (ActC?) 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 landomycin 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 RG-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$_R$ 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 actI* double mutant, the *actI* mutagenic cosmid was used to inactivate the *actI* ORF1, -2, and -3 genes in an *actR* 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_{640} 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 Spc^r 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^r gene in the backbone and does not replicate in *S. coelicolor*. This construct was introduced into the *actR*::Spc^r mutant, and apramycin-resistant exconjugants were selected. These were grown nonselectively 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* reporters for monitoring P$_{actAB}$ 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 0.5×10^6 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-actAB* 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 pIJ8660 to give pWT+IGR+eGFP/pT142W+IGR+eGFP. The generated plasmids were introduced into the ΔactR mutant and the ΔactR ΔactI mutant by conjugation, to give four *Streptomyces* reporter strains: ΔactR/pWT+IGR+eGFP, ΔactR/pT142W+IGR+eGFP, ΔactRΔactI/pWT+IGR+eGFP, and ΔactRΔactI/pT142W+IGR+eGFP.

All four *Streptomyces* strains consisting of the enhanced green fluorescent protein (eGFP) reporter were inoculated in R2YE liquid medium at 0.5×10^6 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, 10× 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 ΔactR ΔactI 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 ΔactR 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 ΔactR ΔactI control and the ΔactR 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 nucleic acid affinity. The cell suspension and the stain mixture were mixed by pipetting or inverting the tubes and left to stand at room temperature for 20 min in the dark. Ten microliters of the suspension was deposited on a clean slide and observed under a Zeiss Axio Imager M2 microscope (AxioVision release 4.8, 10× objective). Images from green and red channels were merged and processed by Photoshop CS5.1.

SUPPLEMENTAL MATERIAL

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

Text S1, DOCX file, 0 MB.
Figure S1, TIF file, 0.4 MB.
Figure S2, TIF file, 8 MB.
Figure S3, TIF file, 0.6 MB.
Figure S4, TIF file, 0.4 MB.
Figure S5, TIF file, 0.2 MB.
Figure S6, TIF file, 3.2 MB.
Figure S7, TIF file, 1.5 MB.
Table S1, DOCX file, 0 MB.
Table S2, DOCX file, 0 MB.

REFERENCES

1. **Wang J, et al.** 2006. Platensimycin is a selective FabF inhibitor with potent antibiotic properties. Nature **441**:358–361.
2. **Goh EB, et al.** 2002. Transcriptional modulation of bacterial gene expression by subinhibitory concentrations of antibiotics. Proc. Natl. Acad. Sci. U. S. A. **99**:17025–17030.
3. **Tsui WH, et al.** 2004. Dual effects of MLS antibiotics: transcriptional modulation and interactions on the ribosome. Chem. Biol. **11**:1307–1316.
4. **Vetsigian K, Jajoo R, Kishony R.** 2011. Structure and evolution of *Streptomyces* interaction networks in soil and in silico. PLoS Biol. **9**:. http://dx.doi.org/10.1371/journal.pbio.1001184e1001184.
5. **Shinkawa H, Sugiyama M, Nimi O, Nomi R.** 1985. Molecular cloning and expression in *Streptomyces lividans* of a streptomycin 6-phosphotransferase gene from a streptomycin-producing microorganism. FEBS Lett. **181**:385–389.
6. **Pootoolal J, et al.** 2002. Assembling the glycopeptide antibiotic scaffold: the biosynthesis of A47934 from *Streptomyces toyocaensis* NRRL15009. Proc. Natl. Acad. Sci. U. S. A. **99**:8962–8967.
7. **Fernandez-Moreno MA, et al.** 1994. DNA sequence and functions of the actVI region of the actinorhodin biosynthetic gene cluster of *Streptomyces coelicolor* A3(2). J. Biol. Chem. **269**:24854–24863.
8. **Caballero JL, Martinez E, Malpartida F, Hopwood DA.** 1991. Organisation and functions of the actVA region of the actinorhodin biosynthetic gene cluster of *Streptomyces coelicolor*. Mol. Gen. Genet. **230**:401–412.
9. **Sciara G, et al.** 2003. The structure of ActVA-Orf6, a novel type of monooxygenase involved in actinorhodin biosynthesis. EMBO J. **22**:205–215.
10. **Valton J, Mathevon C, Fontecave M, Nivière V, Ballou DP.** 2008. Mechanism and regulation of the two-component FMN-dependent monooxygenase ActVA-ActVB from *Streptomyces coelicolor*. J. Biol. Chem. **283**:10287–10296.
11. **Wright LF, Hopwood DA.** 1976. Actinorhodin is a chromosomally-determined antibiotic in *Streptomyces coelicolar* A3(2). J. Gen. Microbiol. **96**:289–297.
12. **Caballero JL, Malpartida F, Hopwood DA.** 1991. Transcriptional organization and regulation of an antibiotic export complex in the producing *Streptomyces* culture. Mol. Gen. Genet. **228**:372–380.
13. **Fernández-Moreno MA, Caballero JL, Hopwood DA, Malpartida F.** 1991. The act cluster contains regulatory and antibiotic export genes, direct targets for translational control by the bldA tRNA gene of *Streptomyces*. Cell **66**:769–780.
14. **Bystrykh LV, et al.** 1996. Production of actinorhodin-related "blue pigments" by *Streptomyces coelicolor*, A3(2). J. Bacteriol. **178**:2238–2244.
15. **Tahlan K, et al.** 2007. Initiation of actinorhodin export in *Streptomyces coelicolor*. Mol. Microbiol. **63**:951–961.
16. **Jiang H, Hutchinson CR.** 2006. Feedback regulation of doxorubicin biosynthesis in *Streptomyces peucetius*. Res. Microbiol. **157**:666–674.
17. **Ostash I, et al.** 2008. Coordination of export and glycosylation of landomycins in *Streptomyces cyanogenus* S136. FEMS Microbiol. Lett. **285**:195–202.
18. **Ramos JL, et al.** 2005. The TetR family of transcriptional repressors. Microbiol. Mol. Biol. Rev. **69**:326–356.
19. **Reichheld SE, Yu Z, Davidson AR.** 2009. The induction of folding cooperativity by ligand binding drives the allosteric response of tetracycline repressor. Proc. Natl. Acad. Sci. U. S. A. **106**:22263–22268.
20. **Hillen W, Berens C.** 1994. Mechanisms underlying expression of Tn10 encoded tetracycline resistance. Annu. Rev. Microbiol. **48**:345–369.
21. **Ahn SK, Tahlan K, Yu Z, Nodwell J.** 2007. Investigation of transcription repression and small-molecule responsiveness by TetR-like transcription factors using a heterologous *Escherichia coli*-based assay. J. Bacteriol. **189**:6655–6664.
22. **Tahlan K, Yu Z, Xu Y, Davidson AR, Nodwell JR.** 2008. Ligand recognition by ActR, a TetR-like regulator of actinorhodin export. J. Mol. Biol. **383**:753–761.
23. **Taguchi T, et al.** 2000. Chemical characterisation of disruptants of the *Streptomyces coelicolor* A3(2) actVI genes involved in actinorhodin biosynthesis. J. Antibiot. (Tokyo) **53**:144–152.
24. **Valton J, Filisetti L, Fontecave M, Nivière V.** 2004. A two-component flavin-dependent monooxygenase involved in actinorhodin biosynthesis in *Streptomyces coelicolor*. J. Biol. Chem. **279**:44326–44329.
25. **Cole SP, Rudd BA, Hopwood DA, Chang CJ, Floss HG.** 1987. Biosynthesis of the antibiotic actinorhodin. Analysis of blocked mutants of *Streptomyces coelicolor*. J. Antibiot. (Tokyo) **40**:340–347.
26. **Craney A, et al.** 2007. A synthetic luxCDABE gene cluster optimized for expression in high-GC bacteria. Nucleic Acids Res. **35**: e46. http://dx.doi.org/10.1093/nar/gkm086.
27. **Willems AR, et al.** 2008. Crystal structures of the *Streptomyces coelicolor* TetR-like protein ActR alone and in complex with actinorhodin or the actinorhodin biosynthetic precursor (S)-DNPA. J. Mol. Biol. **376**:1377–1387.
28. **Fernandez M, Sanchez J.** 2001. Viability staining and terminal deoxyribonucleotide transferase-mediated dUTP nick end labelling of the mycelium in submerged cultures of *Streptomyces antibioticus* ETH7451. J. Microbiol. Methods **47**:293–298.
29. **Foulston L, Bibb M.** 2011. Feed-forward regulation of microbisporicin biosynthesis in *Microbispora corallina*. J. Bacteriol. **193**:3064–3071.
30. **Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA.** 2000. Practical *Streptomyces* genetics. The John Innes Foundation, Norwich, UK.
31. **Sambrook J, Fritsch EF, Maniatis T.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
32. **Gust B, Challis GL, Fowler K, Kieser T, Chater KF.** 2003. PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc. Natl. Acad. Sci. U. S. A. **100**:1541–1546.
33. **Redenbach M, et al.** 1996. A set of ordered cosmids and a detailed genetic and physical map for the 8 Mb *Streptomyces coelicolor* A3(2) chromosome. Mol. Microbiol. **21**:77–96.
34. **Straight PD, Fischbach MA, Walsh CT, Rudner DZ, Kolter R.** 2007. A singular enzymatic megacomplex from Bacillus subtilis. Proc. Natl. Acad. Sci. U. S. A. **104**:305–310.