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β-Ketoacyl Acyl Carrier Protein Synthase III (FabH) Is Essential for Fatty Acid Biosynthesis in *Streptomyces coelicolor* A3(2)

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The *Streptomyces coelicolor fab* (fatty acid biosynthesis) gene cluster (*fabD-fabH-acpP-fabF*) is cotranscribed to produce a leaderless mRNA transcript. One of these genes, *fabH*, encodes a ketoacyl synthase III that is essential to and is proposed to be responsible for initiation of fatty acid biosynthesis in *S. coelicolor*.

Streptomyces spp. synthesize the majority of their fatty acids from branched starters such as isobutyryl, isovaleryl, and anteisovaleryl units to give odd- and even-numbered fatty acids with a methyl branch at the ω-terminus (80 to 90% of total fatty acid content); the remainder are synthesized from straight starters such as acetyl and butyryl units (11, 21). The fatty acid synthase (FAS) of *Streptomyces* spp. is, like that found in many other bacteria (including the best-studied example, that of *Escherichia coli*), a type II or dissociable system (13, 18). The type II FAS consists of several discrete proteins that form loose associations to synthesize the fatty acid. The assembly of fatty acids is initiated by the condensation of an acyl coenzyme A (acyl-CoA) starter unit and a malonyl-acyl carrier protein (malonyl-ACP) extender unit; this condensation is catalyzed by β-ketoacyl ACP synthase III (FabH), the product of the *fabH* gene. In vitro biochemical studies suggest that FabH determines the choice of starter unit to be used. *E. coli* FabH is specific for an acetyl-CoA starter unit, whereas *Bacillus subtilis* and *Streptomyces glaucescens* FabHs can accept a broader range of substrates, including branched- and straight-chain units (2, 8). In the case of the *S. glaucescens* FabH, the order of reactivity towards the different starters is isobutyryl-CoA > butyryl-CoA > acetyl-CoA. If FabH were solely responsible for the initiation of fatty acid biosynthesis in *Streptomyces* species, then one could hypothesize that its biochemical activity, together with the relative pool sizes of the different starter units in vivo, would account for the mix of branched and straight fatty acids (8). But when *S. glaucescens* was grown in the presence of high concentrations (480 μM) of the FAS inhibitor thiolactomycin, branched-chain fatty acid biosynthesis was inhibited and the proportion of straight-chain fatty acids increased (50% inhibitory concentration [IC_{50}] for purified *S. glaucescens* FabH 20 μM) (8). This result may be interpreted as evidence for a second; FabH-independent mechanism for fatty acid initiation in *Streptomyces* spp. Based on this second hypothesis, *fabH* should be dispensable to *Streptomyces* spp. In this study we provide further biochemical evidence for the role of a small cluster of presumed *fab* genes (which includes *fabH*) in *Streptomyces coelicolor* and a transcriptional analysis of the *fab* cluster, and we have attempted to disrupt *fabH* to determine if it is essential for the viability of the cells.

The *acpP* gene product stimulates long-chain fatty acid biosynthesis in vitro. The *S. coelicolor* FAS is still relatively poorly understood; a cluster of four *fab*-like genes has been identified on the *S. coelicolor* chromosome in the order *fabD-fabH-acpP-fabF* (cosmid SC4A7, *S. coelicolor* genome project [http://www.sanger.ac.uk/Projects/S_coelicolor/]; nucleotide sequence accession number AL133423). The deduced amino acid sequences of the *fab* genes are highly similar to components of the *E. coli* FAS, and at least some of the *S. coelicolor* genes are essential (13). We used a biochemical assay of fatty acid biosynthesis, dependent on the *acpP* gene product (ACP), to strengthen the evidence that these genes do encode the FAS of *S. coelicolor*. Cell extracts were prepared from *S. coelicolor* M145 grown for 20 h in YEME medium (12) and broken as previously described (3), with an additional clearing step by ultracentrifugation for 1 h at 100,000 × *g*. The supernatant was adjusted to 5 mg of protein/ml, a fresh ice-cold 10% (wt/vol) solution of streptomycin sulfate was added slowly while stirring on ice water to a final concentration of 1%; the mixture was then stirred for a further 20 min and centrifuged for 20 min at 14,000 × *g*. Endogenous ACP was removed from the cell extract by fractionation with a 60 to 80% ammonium sulfate cut as previously described (4), and this cut was dialyzed overnight against 1 liter of cell disruption buffer containing 2 mM dithiothreitol (DTT). Pure FAS holo-ACP was prepared as previously described (14) and reduced to the active monomeric form just prior to each assay by incubation at 30°C for 30 min in a solution containing 50 mM potassium phosphate, pH 7.2, and 10 mM DTT. Typical assay incubation conditions were as follows: 0.5 mg of *S. coelicolor* (60 to 80% ammonium sulfate cut) per ml, 100 mM potassium phosphate (pH 7.2), 2 mM DTT, 100 mM NADH, 100 mM NADPH, 20 μM ACP, and 100 μM [2-^{14}C]malonyl-CoA (0.02 Bq/pmol) in a total volume of 18 μl were preincubated for 10 min at 30°C; then 2 μl of isobutyryl-CoA was added (20 μM final concentration) to initiate the reaction (alternatively, buffer alone was added as a

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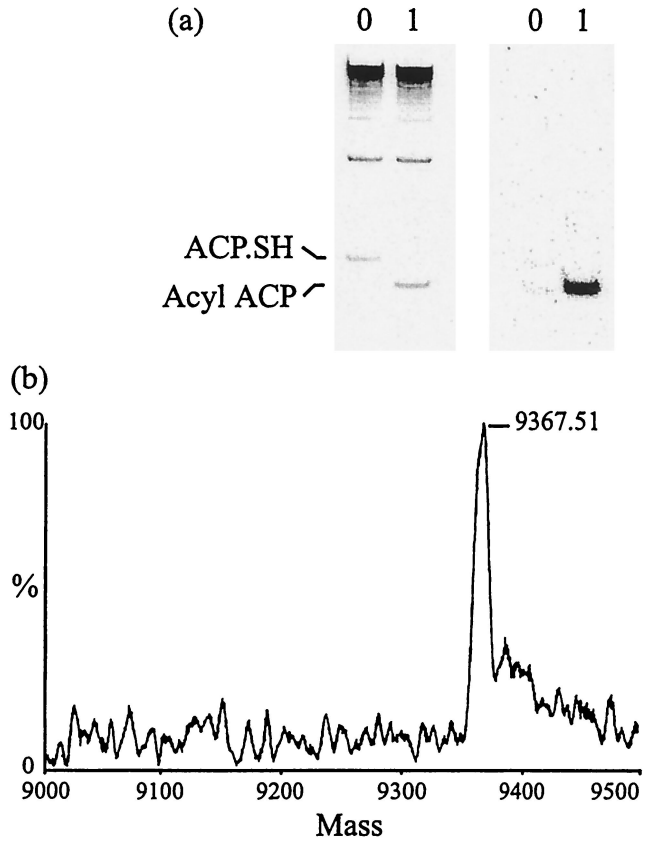

FIG. 1. Identification of the acyl-ACP products formed by in vitro FAS assay. (a) Left panel, CS-PAGE and Coomassie blue staining of material incubated in the assay. Lane 0, no acyl-CoA starter unit added; lane 1, isobutyryl-CoA added as a starter. Right panel, phosphorimage of the gel shown on the left. (b) Transformed electrospray mass spectrum of the repurified acyl-ACP after incubation with the isobutyryl-CoA starter. The major peak, with a molecular mass of 9,367.5 Da (±2.9Da) is in close agreement with the calculated mass of C_{16} acyl-ACP (9,366 Da).

negative control), and the incubation was continued for a total of 60 min. Assay products were analyzed in two different ways. First, conformationally sensitive polyacrylamide gel electrophoresis (CS-PAGE) was used to determine if any acyl-ACP product had been formed in the assay. The small highly acidic ACPs typically migrate faster than other proteins in CS-PAGE (9, 15), and this technique has been used previously to differentiate acyl adducts of the *S. coelicolor* ACP (14). After incubation in the presence of isobutyryl-CoA, ACP was depleted and a new, faster-migrating band appeared (Fig. 1a, left panel). The phosphorimage of this gel (Fig. 1a, right panel) showed that the new, faster-migrating ACP species was labeled by the extender unit, consistent with a role for this ACP in stimulating at least one round of condensation between the starter and extender units, catalyzed by the FAS components in the cell extract. Second, electrospray mass spectrometry (ESMS) was used to determine the exact mass of the acyl-ACP product identified by CS-PAGE. The assay incubation was run as described above, but it was scaled up 10-fold and cold malonyl-CoA was used. The acyl-ACP species were purified from the assay mixtures (with or without isobutyryl-CoA) using the Biocad Sprint purification system (Perkin-Elmer) with a PORO HQ/M column (4.6 by 100 mm) and eluted in a linear gradient of 0 to 800 mM NaCl in 20 mM Tris–bis-Tris propane, pH 7.2, over 15 column volumes (10 ml/min). Unmodified ACP (no isobutyryl-CoA, negative control) was eluted at 509 mM NaCl, and the acyl-ACP reaction product was eluted at 327 mM NaCl. After desalting (PD10; Pharmacia), the ACPs were analyzed by ESMS by John Crosby, School of Chemistry, University of Bristol, Bristol, England, as described previously (3). ACP purified from the assay mixture that lacked isobutyryl-CoA had a measured mass (mean ± standard deviation) of 9,126.6 ± 2 Da (expected mass, 9,128 Da), and the acyl-ACP (isobutyryl-CoA dependent) had a measured mass of 9,367.5 ± 2.9 Da, in close agreement with that expected for C_{16} acyl-ACP (9,366 Da) (Fig. 1b). This demonstrated that the *acpP* gene product is able to stimulate isobutyryl-CoA-dependent long-chain fatty acid biosynthesis in cell extracts of *S. coelicolor*. These data provide further evidence to substantiate the argument that *acpP*, and by implication its surrounding genes, does encode the FAS of *S. coelicolor*.

Transcriptional analysis of the *fab* genes. Reverse transcriptase PCR (RT-PCR) analysis was used to detect the presence or absence of continuous mRNA spanning the junctions between each of the *fab* genes and between *fabD* and SC4A7.14 (the gene on cosmid SC4A7 upstream of and colinear with *fabD*, named as such in the *S. coelicolor* genome project) (Fig. 2a). RNA was isolated from cultures of *S. coelicolor* M145 grown for 20 h in YEME medium as previously described (12) and incubated with DNase (free of RNase; Roche Diagnostics) to remove traces of contaminating DNA. Cotranscription of genes was analyzed by RT-PCR of intergenic regions using the Titan One Tube RT-PCR system (Roche Diagnostics) by following the protocol recommended by the manufacturer. The temperature profile was as follows: 1 cycle at 60°C for 30 min, 30 cycles of PCR (denaturation for 1 min at 96°C, annealing for 1 min at 65°C, and extension for 4 min at 72°C), and 1 cycle at 72°C for 10 min. The total reaction volume was 50 μl, and 10 μl was analyzed on an agarose gel. Oligonucleotides were as follows (Fig. 2b): SC4A7.14 forward, 5′-AAGTCGCTGATCG GGCCGTTCG-3′; *fabD* reverse, 5′-CGAGATCGAGTCCGA TGGCGTC-3′, *fabD* forward, 5′-GGCGAACGTGAACGGC GCCGGT-3′; *fabH* forward, 5′-GGAGCGGCTCCTGGCGA CCGGC-3′; *acpP* reverse, 5′-TGACGTCCTCGACCGGGAT GCC-3′; and *fabF* reverse, 5′-CGATCAGCGCGAACTGCG CCGA-3′. RT-PCR products were generated across the *fabD-fabH, fabH-acpP*, and *acpP-fabF* junctions but not across the *fabF*-SC4A7.19c interval (SC4A7.19c is downstream of and convergent with *fabH*, and so this served as a negative control) or the SC4A7.14-*fabD* junction. In all cases, the expected PCR product was generated when genomic DNA served as the template (initial RT incubation omitted), providing a positive control for each PCR (Fig. 2c). Because the *fabF*-SC4A7.19c-convergent genes gave a PCR product with genomic DNA as a template, but they did not give an RT-PCR product with RNA as a template (data not shown); there was no contaminating DNA in the RNA preparation. Additional controls included the following: no DNA or RNA template (no product seen), RNA template treated with RNase (no product), and DNA template treated with DNase (no product). These results strongly suggest that one long transcript originated from a

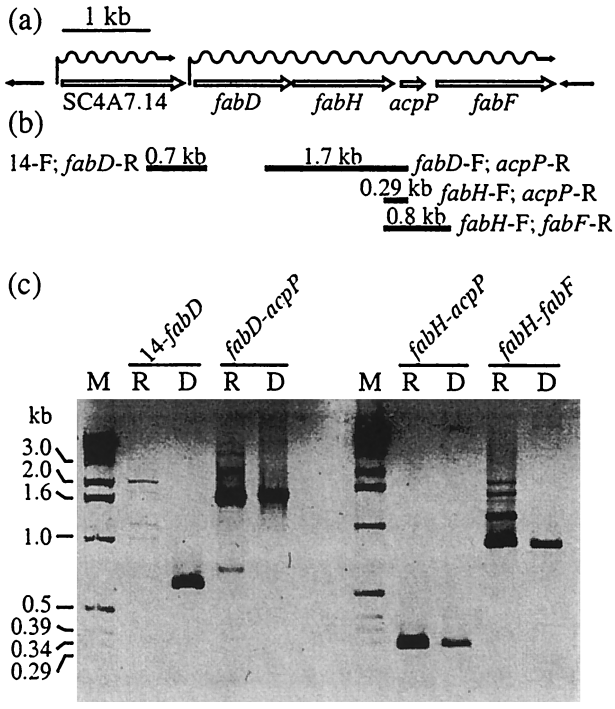

Fig. 2. RT-PCR analysis to detect transcriptional readthrough between *fab* genes. (a) Schematic representation of the *fab* cluster to show the organization of the genes on the chromosome (gene names are as in the *S. coelicolor* genome project). (b) Positions of oligonucleotides used in RT-PCR experiments and expected RT-PCR products. (c) Agarose gel of RT-PCR products showing no transcriptional readthrough between SC4A7.14 and *fabD*, whereas *fabD* and *fabH*, *fabH* and *acpP*, and *acpP* and *fabF* are cotranscribed. M, DNA molecular size markers; R, RNA template; D, DNA template.

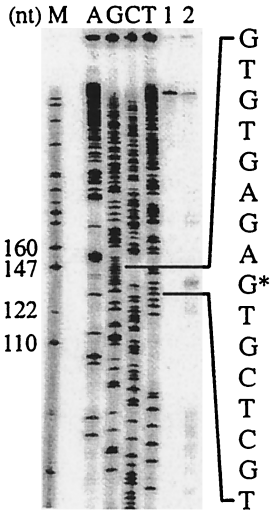

FIG. 3. High-resolution mapping of *fabD* promoter. A protected fragment (139 nt) of the *fabD* promoter probe (lane 2) comigrating with the GUG translational start point is indicated on the corresponding sequence ladder (lanes A to T). Lane 1, tRNA instead of mRNA mixed with the *fabD* promoter probe (negative control).

promoter upstream of *fabD* and continued through all four *fab* genes to terminate just 3′ of *fabF*. The gene upstream of and colinear with *fabD*, SC4A7.14, has end-to-end similarity with genes found in every prokaryote sequenced so far, including *Mycobacterium tuberculosis*, in which it is also located immediately upstream of *fabD*, though their functions are unknown. Even though a transcript was not detected between SC4A7.14 and *fabD*, this does not rule out a role for the SC4A7.14 gene product in fatty acid biosynthesis; it merely indicates that it is not cotranscribed with the *fab* genes, at least in cells grown to mid-log phase in a rich liquid medium.

High-resolution S1 nuclease protection analysis was used to locate the 5′ end of the long *fab* transcript. A *Sma*I-to-*Sty*I DNA fragment (883 nucleotides [nt], negative strand) encompassing the start of *fabD* was prepared with a γ-^{32}P label on the 5′ end of the minus strand (140 nt downstream of the *fabD* translational start site) and hybridized with RNA as previously described (12). A single 5′ end was identified which coincides with the GUG translational start point for *fabD* (Fig. 3) when run alongside a sequence ladder generated from the oligonucleotide 5′-CTTGGTGCCGAAGTGGGCGAGA-3′ (140 nt downstream of the *fabD* translational start site). This means that the *fab* operon is transcribed in the absence of an mRNA leader sequence, an unusual situation in bacteria but not uncommon in *Streptomyces* (10, 17). To confirm that this was the true transcriptional initiation point, we used an in vitro transcription assay comprising purified *S. coelicolor* holo-RNA polymerase, dinucleotide primers, and the same restriction fragment encompassing the promoter region of *fabD* as that used for S1 nuclease protection (12). A 140-nt runoff transcript was generated corresponding to that expected from initiation at the first nucleotide of the GUG translational start codon (data not shown). The translational start point had previously been determined from N-terminal sequence analysis of the purified protein (13). Interactions between the 3′ end of the bacterial 16S rRNA and sequences downstream of the start codon must initiate translation of mRNA sequences that lack a leader. A putative downstream box was identified within *fabD* (nt +13 to +24) that aligns well with consensus *Streptomyces* downstream box sequences (10, 17) and with a complementary sequence near the 3′ end of *S. coelicolor* 16S rRNA. Downstream-box-like sequences have also been found within *acpP* and *fabF* but not in *fabH*; the start codon of *fabH* overlaps the stop codon of *fabD* such that *fabD* and *fabH* could potentially be cotranslated, and so one may not necessarily expect to find a ribosome binding site. To our knowledge, this is the first example of this phenomenon for a primary metabolic gene in *Streptomyces*.

With these results we were able to design a strategy for the disruption of *fabH* such that there would be no unwanted polar effects on the transcription of the surrounding genes.

***S. coelicolor fabH* (encoding FabH) is essential for viability.** So far, four homologues of *fabH* have been found in the *S. coelicolor* genome (with 90% of the genome complete), and each has an amino acid sequence approximately 40% identical to the *fabH* product (*S. coelicolor* genome project). The roles of two of these open reading frames are unknown, but one possibility is that they might encode alternative FabHs for fatty acid initiation. pIJ8155 (Table 1) was introduced into *S. coelicolor* by conjugation from *E. coli* (as described in reference 6); apramycin-resistant colonies were picked, and putative single-crossover recombinants were confirmed by Southern hybrid-

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference
Strains		
S. coelicolor A3(2)		
M145	Prototrophic SCP1-SCP2 (wild type)	12
WP11	M145/pIJ8155	This work
WP21	WP11/pIJ84 (2nd copy of *fabH*)	This work
WP23	Δ*fabH* (*fab* cluster) derivative of WP21	This work
E. coli		
DH5α	General host for cloning	16
ET12567/pUZ8002	For conjugation with *Streptomyces*	6
Plasmids		
pUC118	General cloning vector	20
pOJ260	Suicide vector for *Streptomyces* containing *oriT* RK2 for conjugation from *E. coli* to *Streptomyces*	1
pIJ8600	Integrating vector for inducible expression of genes cloned under control of the *tipAp* promoter	19
pIJ8155	Derivative of pOJ260 containing 3.1 kb of *S. coelicolor fab* DNA (*Bgl*II to *Pst*I) spanning a 650-bp in-frame deletion in *fabH* (*Eag*I-*Eag*I); used for deletion of *fabH*	This work
pIJ84	Derivative of pIJ8600, with a *Sac*I-to-*Sal*I fragment encompassing *fabH* cloned (with *Bgl*II linkers) under control of the *tipAf* promoter and with *vph* (for viomycin resistance) cloned in place of *aacC*(IV) (for apramycin resistance); used to complement the deletion of *fabH*	This work

ization. Eleven out of 12 of the colonies showed integration of the plasmid by homologous recombination through the sequence to the left of the deletion in *fabH* (event 1) (Fig. 4a), and 1 (*S. coelicolor* WP11) out of 12 showed integration by homologous recombination through the right-hand sequence (event 2) (Fig. 4a and b, lane 2). Neither event was expected to disrupt transcription of the *fab* operon. WP11 was chosen as a parent from which to attempt to isolate a *fabH* disruptant because its low frequency of occurrence suggested that the recombination event leading to the deletion of *fabH* would be favored. Twenty-four apramycin-sensitive segregants were isolated among 21,553 colonies screened after three rounds of growth in the absence of apramycin. All had reverted to wild type via a reversal of the first crossover (event 2); as shown by Southern hybridization (Fig. 4b, lane 6); none had undergone the second crossover event to delete the *fabH* gene.

The likely interpretation of this result is that *fabH* is essential. To address this issue further, a second copy of *fabH* was

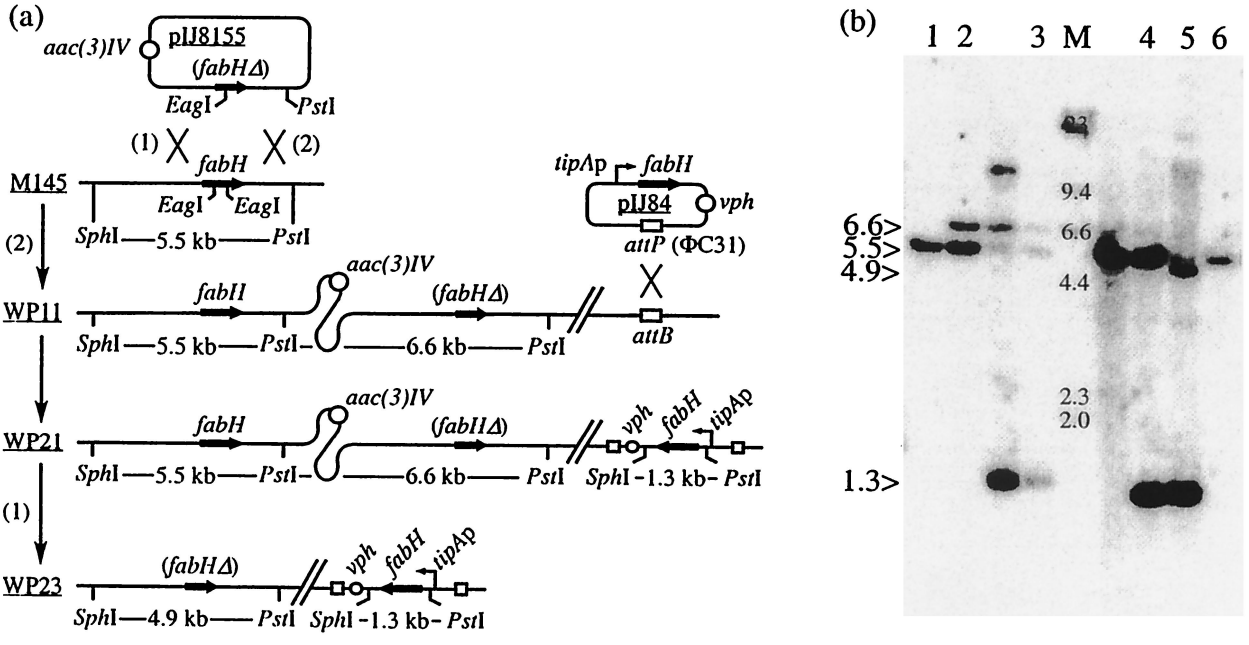

FIG. 4. Disruption of *fabH* by double crossover. (a) Schematic representation of the disruption events. (b) Southern hybridization analysis of the recombinant strains at each stage of the disruption. Genomic DNA from each strain was digested with *Sph*I and *Pst*I. The hybridization probe was radiolabeled *fabH*. Lane M, λ-*Hin*dIII molecular size standards (sizes are indicated in kilobases); lane 1, M145 (parental strain); lane 2, WP11 (integration of pIJ8155 through event 2); lane 3, WP21 (same as WP11 but with a second copy of *fabH* integrated at the ΦC31 *att* site on pIJ84); lane 4, WP22 (pIJ8155 excised through a reversal of the original integration event); lane 5, WP23 (pIJ8155 excised through event 1 leaving the disrupted copy of *fabH* in the *fab* cluster); lane 6, apramycin-sensitive revertant of WP11 (as M145). Note that irrelevant lanes are unidentified.

introduced into strain WP11 on pIJ84 such that it would be expressed under the control of the thiostrepton-inducible promoter *tipA*p. The resulting strain, WP21, was confirmed by Southern hybridization to contain the second copy of *fabH* integrated at the ΦC31 *att* site (Fig. 4b, lane 3). WP21 was propagated through one round of growth and sporulation on a medium containing thiostrepton at 2.5 μg/ml but lacking apramycin. Southern hybridization showed that of 16 apramycin-sensitive segregants isolated, 10 had reverted to wild type (for an example, see Fig. 4b, lane 4) and the other 6 had undergone the second crossover (event 1) to create an in-frame deletion in *fabH* from the *fab* gene cluster; one segregant of the latter type was named WP23 (Fig. 4b, lane 5). This demonstrated that *fabH* can readily be deleted from the chromosome to yield a viable strain, but only if a second copy of *fabH* is available to complement the deletion. In parallel, apramycin-sensitive segregants from WP21 grown in the absence of thiostrepton (for induction of *tipAp*) were also sought. One out of seven apramycin-sensitive colonies was confirmed by Southern analysis to have undergone deletion of *fabH*, reflecting the known low level of *tipAp* promoter activity even in the absence of the thiostrepton inducer.

It appears that *fabH* can be deleted without causing lethality only when a second *fabH* copy is expressed in the same cells, implying that *fabH* is involved in an essential primary metabolic process, most likely fatty acid biosynthesis. This result does not rule out alternative mechanisms for initiation of fatty acid biosynthesis in *S. coelicolor* (e.g., any of the homologues of FabH that have been identified as part of the *S. coelicolor* genome project; a separate acetyl-CoA:ACP acyltransferase might bypass the action of FabH, as is the case in plant FASs [7]; decarboxylation of malonyl-ACP might provide an acetyl starter unit for straight-chain fatty acid biosynthesis, and a second FAS might also exist [5]). It merely shows that, if they exist, their activities are insufficient to suppress the effect of a deletion of *fabH*. The physiological target of thiolactomycin in *Streptomyces* remains an enigma, but these results suggest that alternative components of the FAS may be targets for thiolactomycin (e.g., FabF, the condensing enzyme thought to be responsible for elongation of fatty acids) and that these too might have some influence on the ratio of branched- to straight-chain fatty acids.

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