The β-ketoacyl acyl carrier protein synthase III (FabH) enzyme is responsible for fatty acid biosynthesis in Streptomyces. In Streptomyces spp., the majority of fatty acids are synthesized from branched starters such as isobutyryl, isovaleryl, and anteisovaleryl units. The enzyme FabH, the product of the \( fabH \) gene, is essential for the initiation of fatty acid biosynthesis in these bacteria. This enzyme is part of a family of enzymes that catalyze the condensation of acyl-CoA and malonyl-ACP to form β-ketoacyl-ACP, which is used as a starter unit in the synthesis of fatty acids.

The \( fabH \) gene is cotranscribed with other genes involved in fatty acid biosynthesis, forming a gene cluster. In vitro studies have shown that FabH is specific for acyl-CoA starter units, particularly for short-chain acyl-CoA derivatives. The enzyme has a broad range of substrates, including branched- and straight-chain units.

In Streptomyces, the \( fabH \) gene is expressed along with other genes involved in fatty acid biosynthesis. This cooperation is essential for the viability of the cells, as disruptions in the \( fabH \) gene can lead to disrupted fatty acid synthesis and affect cell viability.

In summary, the \( fabH \) gene is crucial for fatty acid biosynthesis in Streptomyces, playing a key role in the initiation of the process. Understanding the function and regulation of this enzyme is essential for the study of fatty acid metabolism in these bacteria.
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 acyl-ACP (14). After incubation 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 the fab 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 1 μl was analyzed on an agarose gel. Oligonucleotides were as follows (Fig. 2b): SC4A7.14 forward, 5′-AACATCGCTGATCGGGCCGTTCG-3′; fabD reverse, 5′-CGAGATCGAGTCCGA TGGCGTC-3′; fabD forward, 5′-GGCGAACGTGAACGGC GCCGG-3′; acpP reverse, 5′-TGACGTCCTCGACCGGGAT GCC-3′; and fabF reverse, 5′-CGATCGAGGCGAAACTGCG 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
promoter upstream of fabD and continued through all four fab genes to terminate just 3' of fabF. The gene upstream of and collinear 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 Smal-to-Styl DNA fragment (883 nucleotides [nt], negative strand) encompassing the start of fabD was prepared with a γ32P 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'-CTTGGTGCGGAGAATGCGAGA-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 fabD overlaps the stop codon of fabH 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-
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 disrupted. This was achieved by transferring a second copy of fabH at the F因子 att site on pIJ84, which was then excised through a reversal of the original integration event. The fabH disruptant was then isolated by screening for apramycin sensitivity.

### Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
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<tr>
<td>S. coelicolor A3(2)</td>
<td>Prototrophic SCP1-SCP2 (wild type)</td>
<td>12</td>
</tr>
<tr>
<td>M145</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WP11</td>
<td>M145/pIJ8155</td>
<td>This work</td>
</tr>
<tr>
<td>WP21</td>
<td>WP11/pIJ84 (2nd copy of fabH)</td>
<td>This work</td>
</tr>
<tr>
<td>WP23</td>
<td>ΔfabH (fab cluster) derivative of WP21</td>
<td>This work</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
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<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>General host for cloning</td>
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</tr>
<tr>
<td>ET12567/pUZ8002</td>
<td>For conjugation with Streptomyces</td>
<td>6</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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</tr>
<tr>
<td>pUC118</td>
<td>General cloning vector</td>
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<tr>
<td>pOJ260</td>
<td>Suicide vector for Streptomyces containing oriT RK2 for conjugation from E. coli to Streptomyces</td>
<td>1</td>
</tr>
<tr>
<td>pIJ8600</td>
<td>Integrating vector for inducible expression of genes cloned under control of the tipAp promoter</td>
<td>19</td>
</tr>
<tr>
<td>pIJ8155</td>
<td>Derivative of pOJ260 containing 3.1 kb of S. coelicolor fab DNA (BglII to PstI) spanning a 650-bp in-frame deletion in fabH (EagI-EagI); used for deletion of fabH</td>
<td>This work</td>
</tr>
<tr>
<td>pIJ84</td>
<td>Derivative of pIJ8600, with a SacI-to-SalI fragment encompassing fabH cloned (with BglII linkers) under control of the tipAp promoter and with vph (for viomycin resistance) cloned in place of aacC(IV) (for apramycin resistance); used to complement the deletion of fabH</td>
<td>This work</td>
</tr>
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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 SphI and PstI. The hybridization probe was radiolabeled fabH. Lane M, λ-HindIII 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 F因子 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 tipAp. 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 thiostreton 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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REFERENCES