Malonyl-Coenzyme A:Acyl Carrier Protein Acyltransferase of *Streptomyces glaucescens*: A Possible Link between Fatty Acid and Polyketide Biosynthesis[†,#]

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ABSTRACT: *Streptomyces glaucescens*, a Gram-positive soil bacterium, produces the polyketide antibiotic tetracenomycin (Tcm) C. To study possible biochemical connections between the biosynthesis of bacterial fatty acids and polyketides, the abundant acyl carrier protein (ACP) detected throughout the growth of the tetracenomycin (Tcm) C-producing *S. glaucescens* was purified to homogeneity and found to behave like many other ACPs from bacteria and plants (apparent M_r of 20 000 on gel filtration chromatography, apparent M_r of 3400−4800 on sodium dodecyl sulfate−polyacrylamide gel electrophoresis under reducing conditions, and p$I \approx 3.8$). By using an oligodeoxynucleotide synthesized in accordance with the sequence of residues 25−36 of the ACP, the *fabC* gene encoding this protein was cloned, and expression of this gene in *Escherichia coli* yielded the ACP entirely as the active holoenzyme. Sequence analysis of 4.3 kilobases (kb) of DNA flanking *fabC* revealed the presence of three other genes oriented in the same transcriptional direction in the order *fabD*, *fabH*, *fabC*, and *fabB*. Each of the four genes is predicted to encode proteins with high sequence similarity to the following components of the *E. coli* fatty acid synthase (FAS): the FabD malonyl-coenzyme A:ACP acyltransferase (MAT), FabH 3-oxoacyl:ACP synthase III, AcpP ACP, and FabB 3-oxoacyl:ACP synthase I. Expression of the *S. glaucescens fabD* gene in *E. coli* produced active MAT able to catalyze in vitro the transfer of radioactive malonate from malonyl-coenzyme A to the *E. coli* AcpP and *S. glaucescens* FabC ACPs, as well as to the TcmM ACP component of the Tcm type II polyketide synthase [Shen, B., et al. (1992) *J. Bacteriol 174*, 3818−3821]. Expression of *fabD* also restored the high-temperature growth of the *E. coli fabD89* mutant that bears a temperature-sensitive MAT. The latter finding and the close similarity between the organization of the *S. glaucescens fabDHCB* and *E. coli* FAS-encoding genes (*fabH*/*fabD*/*fabG*/*acpP*/*fabF*) suggest that the *S. glaucescens* genes encode FAS enzymes. Moreover, on the basis of its in vitro activity, it is possible that the *S. glaucescens* FabD MAT is responsible for charging the TcmM ACP with malonate in vivo, a key step in the synthesis of the deca(polyketide) precursor of Tcm C. This implies the existence of a functional connection between fatty acid and polyketide metabolism in this bacterium.

Secondary metabolic pathways most likely arose from the pathways of primary metabolism by genetic duplication followed by differentiation. Consequently, many of the biochemical steps in the elaborate pathways of secondary metabolism commonly are mechanistically analogous to those of primary pathways. Furthermore, both primary and secondary metabolic pathways often utilize the same pool of small-molecule precursors, which implies that some enzymatic activities may be shared up to the point at which the pathways diverge.

Polyketides are one of the largest classes of secondary metabolites, and many of them are biologically active. Some, such as the tetracyclines, doxorubicin, and the erythromycins, are currently in therapeutic use. These compounds are synthesized by a mechanism that resembles long-chain fatty acid biosynthesis: small fatty acid units (acetate, propionate, butyrate, etc.) are sequentially condensed to yield extended linear chains (O'Hagen, 1991). Unlike fatty acid biosynthesis, however, the carbonyl groups of the growing polyketide chain are not always fully reduced during biosynthesis, leaving a reactive 3-oxoacyl thioester intermediate, presumably still attached to the enzyme, that can be elaborated in a variety of ways.

The analogy between polyketide and long-chain fatty acid biosynthesis has been extended to the genetic level by recent studies that have demonstrated similarity between the products of several PKS[1] genes (Hopwood & Sherman, 1990; Katz & Donadio, 1993) and their FAS congeners in *Escherichia coli* (Vanden Boom & Cronan, 1989; Magnuson et al.,

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[1] Abbreviations: Tcm, tetracenomycin; ACP, acyl carrier protein; BSA, bovine serum albumin; CoA, coenzyme A; cpm, counts per minute; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FAS, fatty acid synthase; IPTG, isopropyl β-D-thiogalactopyranoside; kb, kilobase(s); MAT, malonyl-coenzyme A:ACP acyltransferase; nt, nucleotide(s); ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; PKS, polyketide synthase; PMSF, phenylmethanesulfonyl fluoride; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TFA, trifluoroacetic acid.

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1993), yeast (Schweizer et al., 1987), and mammals (Witkowski et al., 1991). Since the polyketide biosynthetic machinery most likely arose in some organism from the enzymes of fatty acid biosynthesis, it would be particularly interesting to compare fatty acid and polyketide biosynthesis within the same species, especially from the point of view of the biochemical and enzymological integration and/or segregation of the divergent pathways. Unfortunately, in the most prodigious producers of polyketides, the Gram-positive, filamentous soil bacteria *Streptomyces*, fatty acid biosynthesis has not been well explored.

Streptomyces glaucescens produces the aromatic polyketide antibiotic tetracenomycin C (Tcm C), and the genes responsible for the biosynthesis of Tcm C, including the PKS genes, have been cloned (Motamedi & Hutchinson, 1987) and sequenced (Bibb et al., 1989; Summers et al., 1992, 1993). Here we report the isolation of the principal ACP of *S. glaucescens*, the cloning and nucleotide sequence of the four-gene cluster that contains the *fabC* gene for this ACP along with *fabD*, *fabH*, and *fabB*, and studies of the expression and activity of FabC ACP and FabD MAT. On the basis of the abundance and pattern of appearance of FabC, it seems likely that this gene cluster encodes several components of a type II FAS in *S. glaucescens*. This idea is supported by the fact that the *S. glaucescens fabD* gene is able to complement a mutation in the corresponding *E. coli fabD* gene for the MAT of its FAS. We find that the FabD MAT of *S. glaucescens* is fully competent to charge TcmM, the Tcm PKS ACP, with malonate, suggesting that this MAT might be shared by both pathways. Revill et al. (1995) reached the same conclusion about the possible role in actinorhodin biosynthesis of a MAT isolated from *Streptomyces coelicolor*.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Bacteriophage, and Plasmids. The bacteriophages M13mp18 and M13mp19 and the plasmid pUC19 are described by Yanisch-Perron et al. (1985). pT7-7 and pGP1-2 used as expression vectors (Tabor & Richardson, 1985) were obtained from Stanley Tabor, Harvard Medical School, and pGEM3Zf and pGEM7Zf were purchased from Promega (Madison, WI). *S. glaucescens* GLA.0 was originally obtained from Ralf Hütter (Eidgenossischem Technischen Hochschüle, Zürich, Switzerland) and was grown in R2YENG medium at 30 °C, as described previously (Motamedi & Hutchinson, 1987). The *E. coli* strains DH5 (Sambrook et al., 1989), DH5α (Sambrook et al., 1989), and K38 (Tabor & Richardson, 1985) were grown in 2× YT broth (16 g of Bactotryptone, 10 g of yeast extract, and 5 g of NaCl per liter) (Sambrook et al., 1989). The *E. coli fabD89* mutant strain (Clark & Cronan, 1981) was obtained from the *E. coli* Genetic Stock Center, Department of Biology, Yale University, through the courtesy of Barbara Bachmann. Dry media and Bacto agar were purchased from Difco Laboratories (Detroit, MI). Salts were purchased from Mallinckrodt (Paris, KY). Malonyl-CoA and the FAS ACP of *E. coli* were purchased from Sigma (St. Louis, MO). [2-^{14}C]Malonyl-CoA (55 mCi/mmol) was purchased from Amersham (Arlington Heights, MA), and D-[1-^{14}C]pantothenic acid sodium salt (59.2 mCi/mmol) was from DuPont-NEN Research Products (Boston, MA). Restriction endonucleases and other enzymes were obtained from either Amersham, Bethesda Research Laboratories (Bethesda, MD), New England Biolabs (Beverly, MA), Promega, or United States Biochemical Corp. (Cleveland, OH).

Isolation of the Principal S. glaucescens ACP. (a) Assay of ACP Activity. ACP content in partially purified fractions was assayed by using its ability to serve as an acceptor of [2-^{14}C]malonyl-CoA, catalyzed by crude MAT of *S. glaucescens*. Fractions (50 μL) were incubated at room temperature with 20 μL of 100 mM Na_xPO_4 (pH 7.2)/10 mM DTT buffer and 20 μL of a MAT solution (5 mg/mL, as prepared in the following). After 10 min, 10 μL of dilute [2-^{14}C]-malonyl-CoA (8000 cpm) was added, and the reaction was allowed to proceed for 1−2 min. The reaction was stopped by adding 100 μL of 10 mg/mL BSA and 400 μL of 20% (vol/vol) TCA. Precipitated proteins were retained on 0.2 μm GF/B filters (Whatman) and washed with 10% (vol/vol) TCA using a vacuum manifold (Millipore, Bedford, MA). TCA-precipitated [2-^{14}C]malonyl-*S*-ACP retained on the filters was counted to determine the ACP activity. Appropriate buffer blanks were also processed and counted to determine the amount of background activity to be subtracted from the experimental values.

(b) Preparation of Crude Extract. S. glaucescens cells, harvested by centrifugation and washed with 0.5 M NaCl followed by 100 mM Na_xPO_4 buffer (pH 7.2), were resuspended in 10 mL of 100 mM Na_xPO_4 buffer (pH 7.2) containing 10 mM DTT, 1 mM PMSF, and 0.5 mM EDTA per gram of cells (wet weight). Lysozyme (Sigma, 2 mg/mL) was added and the mixture was left to incubate at room temperature for 2 h with occasional stirring. To the viscous slurry were added $MgCl_2$ (25 mM final concentration) and DNAse (1 μg/mL, Calbiochem, San Diego, CA), and the incubation was continued for another hour on ice. After centrifugation (24000*g*, 15 min, 4 °C) to remove cellular debris, NaCl was added to a concentration of 300 mM, and a neutral 10% (vol/vol) solution of poly(ethylene imine) (Sigma) was added dropwise with stirring to a 0.2% final concentration; centrifugation as before yielded a clear, straw-colored supernatant. Solid $(NH_4)_2SO_4$ was added to the supernatant to bring it to 50% saturation, and the solution was centrifuged as before. The pellet was discarded and more $(NH_4)_2SO_4$ was added to bring the supernatant to 85% saturation. The solution was centrifuged again and the pellet was stored at −80 °C, while the clear supernatant was acidified by dropwise addition of glacial acetic acid to pH 3.9 and left to stir overnight at 4 °C. After extended centrifugation (30000*g*, 60 min, 4 °C), this pellet was also stored at −80 °C. The MAT was also precipitated in the pellet at 50−80% saturation of $(NH_4)_2SO_4$ and was used in the ACP assay described earlier upon dissolution of the pellet in 20 mM Na_xPO_4 (pH 7.2), 2 mM DTT, and 20% glycerol.

This and the succeeding chromatography were monitored at 280 nm and carried out at 4 °C, except for the brief time when the enzyme was on the fast protein liquid chromatography columns that were at room temperature. All columns were purchased from Pharmacia Biotech (Piscataway, NJ).

(c) Sephacryl S-200 Chromatography. The combined ammonium sulfate and acid pellets were resuspended in a minimum volume of 20 mM Na_xPO_4 buffer (pH 7.2) containing 2 mM DTT and 20% (vol/vol) glycerol and applied to a Sephacryl S-200 HR column (2.6 × 52 cm). Proteins were resolved at a flow rate of 2 mL/min using 20 mM Na_xPO_4 buffer (pH 7.2) containing 2 mM DTT and 150

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          1                                                        50
    AcpP} .......... stIeeRVkkI IgEqlGVkqE EVTDnasFv. ..EDLGaDSL
   SeACP} .......drk eifeeRIeqV laEqlGIPaE qITEEadLr. ..EDLGmDSL
    FabC} .....maatq eeIvagladI VnEiaGIPvE DVqlDksFt. ..DDLdvDSL
    NodF} madqltleii saInklVkae ngErtsValg EITtDteLt. ..s.LGiDSL
    TcmM} ........vp qiglpRlveI IrEcaGdPdE rdlDgdiLdv tyqDLGyDSi
Consensus ---------- --I--RV--I I-E--G-P-E E-TDD--L-- --EDLG-DSL
                                                              *
          51                                                      100
    AcpP} dtVElVmALE EEFdteIPdE EaE...kitT VqaaiDyIng hqA*......
   SeACP} dLVElVsALE DEvGmrVeqs qlE...gieT VGhvmEltld lvArlatasa
    FabC} sMVEVVvAaE ErFdVkIPdE Dvk...nLkT VGdatEyIlk hqA*......
    NodF} gLaDVlwdLE qlYGIkIemn taDawsnLnn IGdvvEaVrg lltkev*...
    TcmM} aLlEIsakLE qDLGVsIPgE E......LkT prhtlhlVnt etAgeva*..
Consensus  -LVEVV-ALE EEFGV-IP-E E-E----L-T VG---E---- --A-------

          101
   SeACP} adkpeaas
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FIGURE 1: Comparison of the predicted protein sequence of FabC with those of other ACPs by the PILEUP method (Devereux et al., 1984). The region with the Ser (*) for the attachment of 4′-phosphopantetheine is underlined. SeACP: *S. erythraea* putative FAS ACP (Revill & Leadlay, 1991). NodF (Geiger et al., 1991).

mM NaCl. Calibration of the column with aldolase (161 000), BSA (68 000), ovalbumin (45 000), carbonic anhydrase (29 000) and cytochrome *c* (12 300) was performed at the same flow rate in the same buffer minus the DTT.

(d) Mono Q Chromatography. Pooled enzymatically active Sephacryl fractions were dialyzed against 1 L of 25 mM Tris-HCl (pH 8.0) overnight at 4 °C and applied to a Mono Q 10/10 column equilibrated in the same buffer. Proteins were eluted by using a linear gradient of 0−0.5 M NaCl in 25 mM Tris-HCl (pH 8.0) over 30 min at a flow rate of 4 mL/min.

(e) Chromatofocusing. Enzymatically active fractions from the Mono Q column were pooled, dialyzed against 1 L of 25 mM *N*-methylpiperazine (pH 5.5) overnight, and loaded onto a Mono P 5/20 chromatofocusing column equilibrated in the same buffer. After the sample was washed through, elution at 1 mL/min was accomplished by switching to Polybuffer 74 (Pharmacia, diluted 1:15) at pH 3.0.

(f) Phenyl Superose Chromatography. Removal of the Polybuffer necessitated the use of a phenyl Superose 5/5 column. Pooled enzymatically active Mono P fractions were brought to 50% saturation with the addition of saturated $(NH_4)_2SO_4$ solution. They were then applied to the phenyl superose column, equilibrated in 50 mM Na_xPO_4 buffer (pH 7.2) and 50% saturated $(NH_4)_2SO_4$, and resolved with a linear gradient over 30 min from 50 to 0% $(NH_4)_2SO_4$ in 5 mM Na_xPO_4 buffer (pH 7.2) at 0.5 mL/min.

(g) N-Terminal Amino Acid Sequencing. Prior to protein sequencing, purified FabC ACP was desalted by a final pass through a Vydac C_4 reversed phase column (Hesperia, CA). Elution was accomplished with a linear gradient from 0 to 90% (vol/vol) CH_3CN in aqueous TFA (0.1%, vol/vol) over 50 min at a flow rate of 1 mL/min. Brief vacuum concentration removed the acetonitrile prior to the ACP enzyme assay. Two samples of 175 and 55 pmol were used for the N-terminal sequence determination by automated Edman degradation at the Harvard University Microchemistry Facility, and the first 44 and 30 amino acids were determined, respectively, to yield the sequence identical to that shown in Figure 1.

Cloning and Sequence Analysis of the Region Containing the fabC ACP Gene. In order to locate the gene that encodes the principal ACP of *S. glaucescens*, an oligodeoxynucleotide probe was designed from the N-terminal amino acid sequence of the purified ACP. Choice of which portion of the N-terminal sequence to use was governed by two considerations: first, the sequence had to be unique in comparison to the DNA-derived amino acid sequence of the TcmM ACP from the Tcm C gene cluster of *S. glaucescens*, and second, reverse translation of the chosen portion of the sequence had to yield a relatively small number of degenerate codons. Thus, residues 25−36 (DVQLDKSFTDDL) of the ACP were selected to be the basis for the oligodeoxynucleotide probe, and the 35-mer 5′-GACGTICAGCTIGACAAGIIITTCACI-GACGACCT-3′ was prepared. Design of the probe took advantage of the fact that codon usage in *Streptomyces* spp. is strongly biased, favoring G or C residues in the third (wobble) position, and inosine (I) residues were incorporated whenever a single nucleotide had less than an 80% chance of populating a specific position based on *Streptomyces* codon usage (Bibb et al., 1984). An interesting feature of this probe that does not seem to have adversely affected its utility is the central triplet of inosine residues representing the highly degenerate serine codon. The probe sequence is also fairly A+T rich for *Streptomyces* spp. (45% A+T, ignoring the six inosine residues), which was predicted to increase specificity.

Total DNA from *S. glaucescens* GLA.0 was prepared by a modification of the rapid small-scale isolation procedure of Hopwood et al. (1985), and this DNA was digested with a variety of restriction enzymes or pairs of enzymes. Following agarose gel electrophoresis and Southern transfer to Hybond-N membranes (Amersham), the blots were hybridized with ^{32}P-end-labeled oligodeoxynucleotide and then washed at a final stringency of 0.5× SSC (Sambrook et al., 1989) at 37 °C. For each restriction enzyme digest the probe illuminated a single DNA species, providing several candidate fragments for cloning (data not shown). Initially, a 2 kb *Xho*I−*Xma*I fragment was cloned into pUC19 from an agarose gel size-fractionated total DNA digest. The

nucleotide sequence of this fragment revealed the ACP gene flanked on both sides by long, incomplete ORFs. To obtain the complete nucleotide sequence of the flanking ORFs, a 4.5 kb *Bgl*II−*Sph*I fragment that encompassed the original 2 kb segment was similarly cloned, and the sequence of the entire 4.5 kb region was determined as described previously (Summers et al., 1992). This sequence was then analyzed using the Genetics Computer Group computer programs (Devereux et al., 1984) as outlined elsewhere (Summers et al., 1992).

Expression of the S. glaucescens fabC Gene in E. coli. The forward 5′-CTAGCGCGCTCCGGAGGTGATCATATG-GCAGCAACTCAGGAAGAGATCGTCGCCGGTC-3′ and reverse direction 3′-GACTCGAGGTCATGCATGGATC-CTCAGGCC TGGTGCTTGAGGATGTACTCG-5′ oligodeoxynucleotides were used to amplify the *fabC* gene by the PCR protocol described previously (Decker et al., 1993), using the 4.5 kb *S. glaucescens* FAS DNA template. The PCR product was purified by using a Gene Clean kit (Bio101, La Jolla, CA), cloned into pGEM3Zf after *Sst*I and *Hin*II digestion, and then moved as an *Nde*I−*Bam*HI segment into pT7-7 to give pWHM192. This construct was used to transform *E. coli* K38 cells carrying pGP1-2 by selection for ampicillin and kanamycin resistance.

E. coli K38 (pGP1-2/pWHM192) cells were grown at 30 °C overnight in 5 mL of 2× YT broth in the presence of 200 μg/mL ampicillin and 75 μg/mL kanamycin, heat induced at 42 °C for 10 min, and then kept at 30 °C for another 90 min. The cells were harvested by centrifugation, resuspended in 200 μL of buffer containing 25 mM Tris-HCl (pH 8), 300 mM sucrose, 25 mM EDTA, and 2 mg/mL lysozyme, incubated on ice for 15 min, and centrifuged at 14 000 rpm for 10 min. To the resulting pellet was added 200 μL of cold Tris−EDTA buffer (Sambrook et al., 1989) containing 0.5% Triton TX-100, and the cells were pipeted back and forth several times. A solution of 10 mM PMSF (15 μL) was then added, and the mixture was incubated on ice for 5 min and centrifuged at 14 000 rpm for 10 min. A 40 μL portion of the resulting supernatant was mixed with 10 μL of 5× Laemmli loading buffer (Laemmli, 1970) and 2 μL of β-mercaptoethanol, boiled for 5 min, and loaded onto an 18% SDS−polyacrylamide gel. Following electrophoresis, the gel was stained with Coomassie Blue.

Purification of the FabC ACP from E. coli (pWHM192) Cells. A 2 L 2× YT culture of *E. coli* K38(pGP1-2/pWHM192) cells was grown as described earlier. Cells were heat-induced at 42 °C for 15 min, transferred to 30 °C for another 90 min, and harvested by centrifugation (8000*g*, 10 min, 4 °C). After sequential washing with 0.5 M NaCl and 0.1 M Na_xPO_4 buffer (pH 7.2), the resulting pellet was suspended (10 mL/g, wet weight) in 100 mM Na_xPO_4 buffer containing 2 mM DTT, 0.1 mM PMSF, 1 mM EDTA, and 10% glycerol. Lysozyme (2 mg/mL) was added, and the mixture was incubated at room temperature for 2 h with occasional stirring. $MgCl_2$ (25 mM) and DNAse (1 μg/mL) were added to the viscous slurry and the incubation continued on ice for another hour. The lysate was centrifuged (25 400*g*, 10 min, 4 °C), the pellet was discarded, and solid $(NH_4)_2SO_4$ was added to the supernatant to 62% saturation. This mixture was again centrifuged as before, the resulting pellet was discarded, and additional solid $(NH_4)_2SO_4$ was added to the supernatant to 82% saturation. The mixture was centrifuged as before to yield an active pellet. Glacial acetic acid was added to the supernatant to adjust the pH to 3.9, and the mixture was stirred overnight at 4 °C and centrifuged (25400*g*, 10 min, 4 °C). The acid pellet thus obtained was combined with the previous $(NH_4)_2SO_4$ pellet. The recombinant FabC ACP was similarly assayed for its ability to serve as the acceptor of [2-^{14}C]malonyl-CoA catalyzed by MAT and was purified to near homogeneity from the combined pellets by similar chromatography on Sephacryl S-200 and Mono Q columns, as described earlier for FabC ACP from *S. glaucescens* GLA.0.

Expression and Enzymatic Assay of the FabD MAT in E. coli. Two oligodeoxynucleotides 5′-TAGGTACCGCGCG-CATTGACTGGAGGTAGTCATGAGAGTACTGGT-ACTCGTCGCTCCC-3′ (forward primer) and 5′-CTC-GAGCTCATGCATGGATCCTCAGGCCTGCTCT-GCGAAGAGC-3′ (reverse primer) were used to mutagenize the N-terminus of the *fabD* gene by the PCR method described earlier to create a *Bsp*HI restriction site, alter the codon usage of the second and third codons, and introduce *Kpn*I and *Bss*HII restriction sites on the 5′-end and *Xho*I, *Sst*I, *Nsi*I, and *Bam*HI sites on the 3′-end outside of the *fabD* coding region for ease of manipulation. The amplified DNA was digested with *Bsp*HI and *Bam*HI and ligated into pTrc99a (Amman et al., 1988) at the *Nco*I and *Bam*HI restriction sites to give pWHM193. This plasmid was introduced into the *E. coli fabD*89 mutant by transformation and selection for ampicillin resistance.

The *E. coli fabD89* (pWHM193) transformant was treated as described by Amann et al. (1988) for expression of the cloned *fabD* gene. Cells were grown at 30 °C in 2× YT medium containing ampicillin (150 μg/mL) for 16 h, harvested and resuspended in fresh 2× YT containing ampicillin (150 μg/mL). Expression of *fabD* was induced by the addition of 1 mM IPTG followed by growth at 42 °C for 90 min. The cells were washed with a chilled solution of 0.5 M NaCl and 50 mM Tris-HCl (pH 8.0) and suspended [10 mL/g (wet wt)] in 50 mM Tris-HCl (pH 8.0), containing 1 mM EDTA, 5 mM DTT, and 0.1 mM PMSF. After lysis of the cells using lysozyme (1 mg/mL) for 30 min at room temperature, the mixture was transferred to ice and $MgSO_4$ (25 mM) and DNAse (1 μg/mL) were added. After 30 min, the cell lysate was centrifuged (25 400*g*, 10 min, 4 °C) and the supernatant was used for the MAT assay.

FabD MAT Assay. The activity of FabD was assayed by its ability to catalyze the transfer of the malonyl group from [2-^{14}C]malonyl-CoA to ACP, as estimated from the radioactivity of acid-precipitated [2-^{14}C]malonyl-*S*-ACP. The 50 μL assay mixture consisted of 100 mM Tris-HCl (pH 8), 5 mM DTT, 25 μM ACP, and 10 μL of the above cell-free extract, and assays were performed similarly to those described before for the FabC ACP.

Autoradiography of FabD MAT Activity. To establish that FabD catalyzed the transfer of malonate to specific ACPs, the FabD assay was performed as described earlier, but the products of the reaction mixture were resolved by a conformationally sensitive native PAGE (12%) method (Shen et al., 1992; Rock et al., 1981). After electrophoresis, the gel was soaked in Amplify solution (Amersham) for 20 min, vacuum-dried without staining, and exposed to Kodak XOmat X-ray film for 72 h at −80 °C.

Protein Analysis. SDS−PAGE was done by the Laemmli (1970) procedure or performed on the PhastSystem (Pharmacia) as directed by the manufacturer. Phast gels were

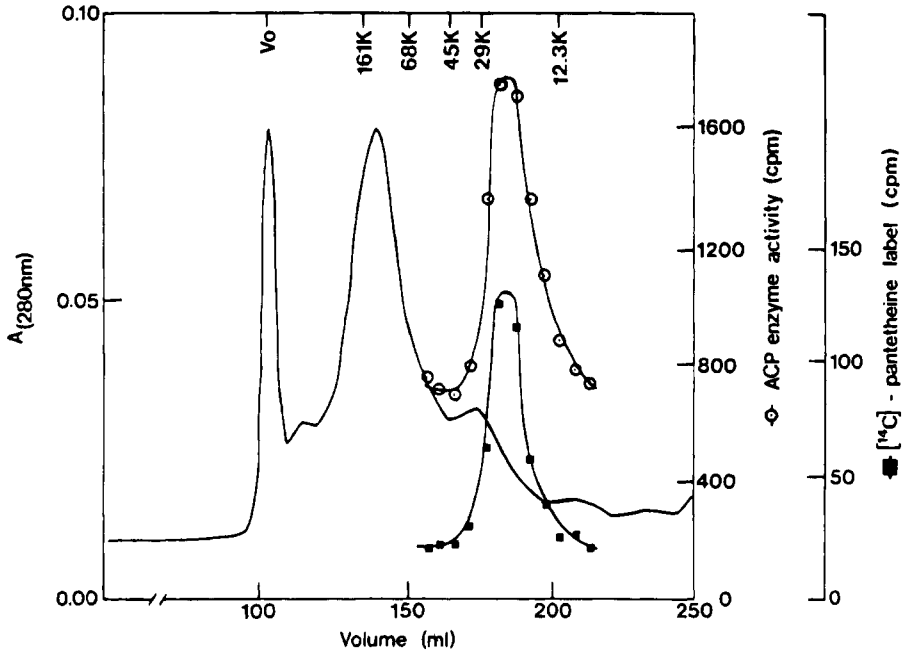


FIGURE 2: Elution profile of FabC during gel filtration, showing the correspondence between ACP activity and radioactive labeling by [1-^{14}C]-pantothenate as a function of the molecular mass of the native protein.

Table 1: Results of FabC Purification from *S. glaucescens*

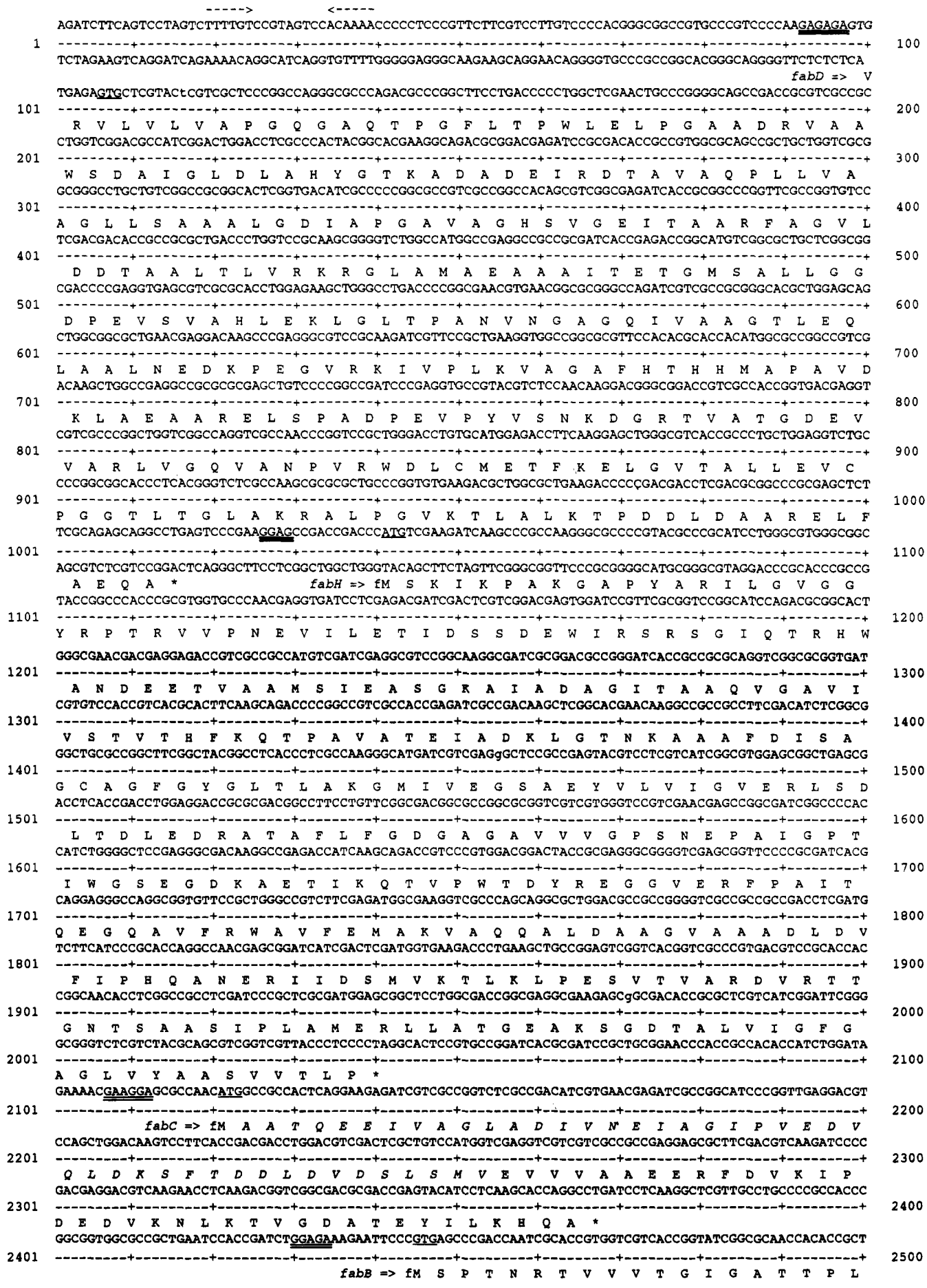
step	late log phase of growth[a] protein (mg)	activity (mmol min^{-1})[c]	yield (%)	specific activity (mmol min^{-1} mg^{-1})	purification (-fold)	early log phase of growth[b] protein (mg)
cell-free extract	1.3×10^3	9.4	100	7.2×10^{-3}		1.2×10^3
$(NH_4)_2SO_4$ + acetic acid pellet	4.3×10^2	0.39	4.1	9.1×10^{-4}		4.0×10^2
Sephacryl S-200	36	17	1.8×10^2	0.47	67	34
Mono Q HR 10/10	4.8	6.9	73	1.4	194	6.0
Mono P HR 5/5	0.80	1.8	19	2.3	319	
phenyl Superose HR 5/5						0.27 (2.9)[d]

[a] From 20 g of 26 h old cells. [b] From 33 g of 12 h old cells, and only the amount of protein from each step is given. [c] Activity is defined as micromoles of ACP malonylated per minute in the presence of the malonyl-CoA:ACP transferase. The abnormal activity at the initial steps of the purification is speculated to be due to the endogenous malonyl-CoA:ACP transferase. [d] Protein concentration determined by the Bradford method with BSA as standard underestimates the amount of FabC due to its acidic nature. The value in parentheses was estimated from the protein concentration found by N-terminal amino acid sequencing.

either silver or Coomassie Blue according to Heukeshoven and Denrick (1988). Protein quantitation was performed by the Bradford method (Bradford, 1976) standardized with BSA.

RESULTS

FabC Is the Most Abundant ACP of S. glaucescens. From our knowledge of the *S. glaucescens* type II PKS genes, which govern the synthesis of Tcm F2 (Shen et al., 1993) and consist of the TcmK 3-oxoacyl:ACP synthase, the TcmL protein, and the TcmM ACP (Bibb et al., 1989; Shen et al., 1992; Shen & Hutchinson, 1993) plus the putative TcmN (Summers et al., 1992) and TcmJ (Summers et al., 1993) polyketide cyclases, we expected to be able to isolate the TcmM ACP from *S. glaucescens* at the time of Tcm C biosynthesis. As a typical component of fatty acid, membrane, or pheromone biosynthesis, ACPs have been isolated from many different bacteria (e.g., Rock & Cronan, 1980; Cooper et al., 1987; Hale et al., 1987) and a few plants (e.g., Høj & Svendsen, 1983; Ohlrogge, 1987) by assaying either for the incorporation of radioactive pantothenate into the 4′-phosphopantetheine prosthetic group in vivo or for the transfer of malonate from labeled malonyl-CoA to the ACP in vitro. Neither of these methods requires prior information about the actual function of the ACP in the organism, which was important at the outset of our work since we had not yet developed a way to assay the Tcm PKS. In preliminary experiments that used *E. coli* FAS ACP as the acceptor and [2-^{14}C]malonyl-CoA as the radioactivity donor, we observed that MAT activity was present in a 50−85% $(NH_4)_2SO_4$ pellet fraction of a cell-free extract of *S. glaucescens*. This MAT activity was then used in the malonyl transfer assay to purify the ACP(s) present in the *S. glaucescens* cell-free extract. A single peak of ACP activity eluted with an apparent M_r of 20 000 during gel filtration, which is consistent with the behavior of other bacterial ACPs (Rock & Cronan, 1980; Cooper et al., 1987; Hale et al., 1987), and this activity coincided with the radioactivity due to [1-^{14}C]-pantothenate (Figure 2), which labels the 4′-phosphopantetheine prosthetic group of ACPs specifically (Shen et al., 1992). The ACP enzyme activity and [^{14}C]pantothenate labeling remained coincident during further purification by anion exchange chromatography, chromatofocusing [where the *S. glaucescens* ACP eluted late in the pH gradient (pH 3.8) as is characteristic of other ACPs (Rock & Cronan, 1980; Høj & Svendsen, 1983; Kuo & Ohlrogge, 1984; Cooper et


-----> <-----
fabD => V
fabH => fM S K I K P A K G A P Y A R I L G V G G
fabC => fM A A T Q E E I V A G L A D I V N E I A G I P V E D V
Q L D K S F T D D L D V D S L S M V E V V V A A E E R F D V K I P
fabB => fM S P T N R T V V V T G I G A T T P L

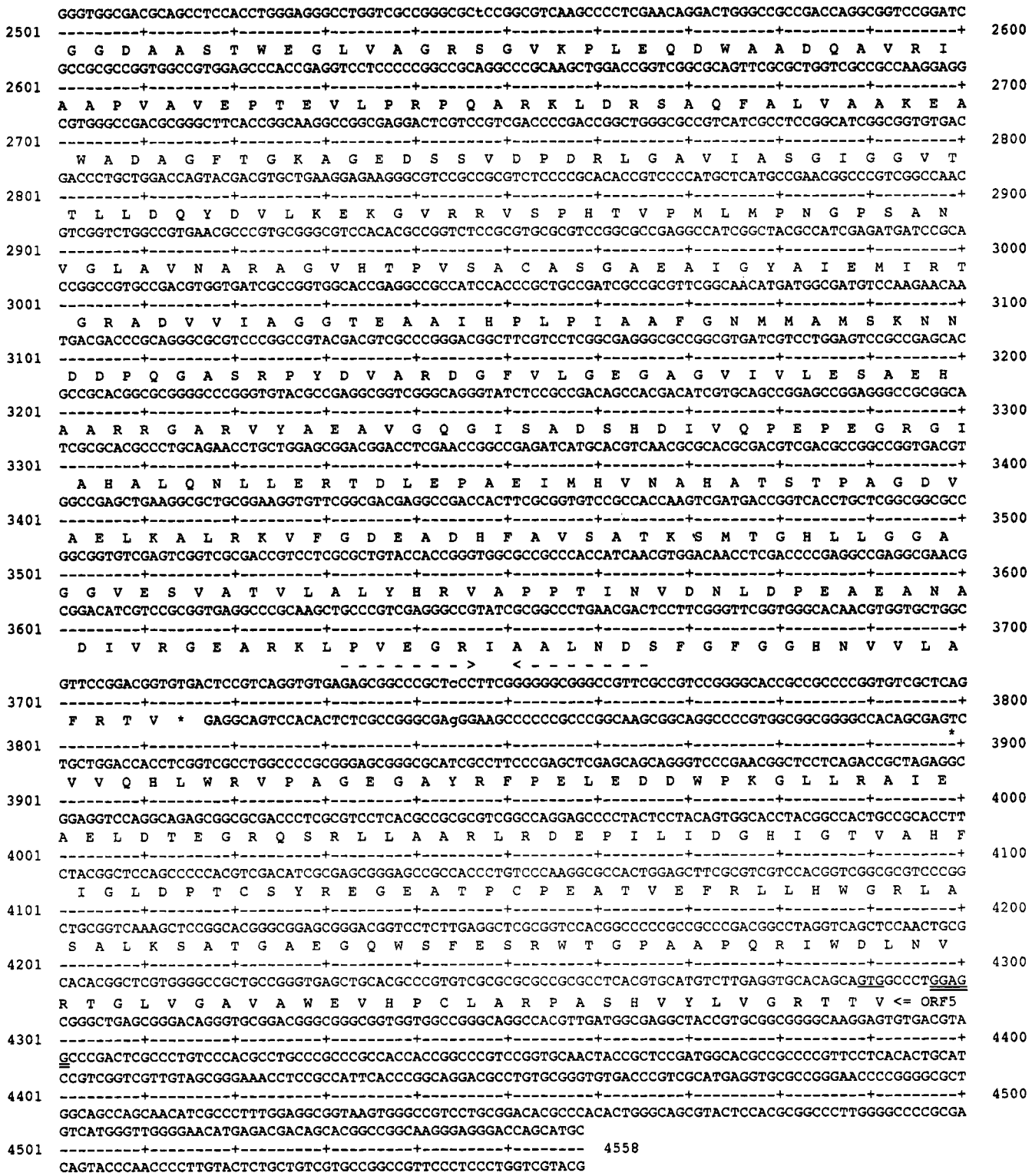


FIGURE 3: Nucleotide sequence and deduced protein sequences of the *S. glaucescens fabC* gene cluster. The predicted translation start sites and ribosome binding sites are singly and doubly underlined, respectively. (The favored of two possible translational start sites for *fabD* is underlined.) The deduced protein sequences are shown below the corresponding genes; the N-terminal portion of FabC that was confirmed by amino acid microsequencing is shown in italics. Inverted repeat sequences discussed in the text are indicated by arrows above the DNA sequence. The DNA sequence data have been deposited in GenBank (accession number L43074).

al., 1987)], and hydrophobic interaction chromatography. The results of purification of the principal ACP from the early log and stationary growth stages of *S. glaucescens* cells are shown in Table 1.

The purified ACP was named FabC and is predicted to have a molecular mass of 8 kDa on the basis of the gene sequence data presented here. It exhibits abnormal mobility in its denatured as well as native forms, running with an apparent M_r of between 3400 and 4800 relative to aprotinin (6500) in SDS−PAGE analysis (data not shown), while it elutes with an apparent M_r of 20 000 during gel filtration as described earlier.

N-Terminal sequence analysis established extensive sequence similarity over the first 44 amino acids between FabC

and several other ACPs, confirming the identity of the purified protein as an ACP (Figure 1). The 4′-phosphopantetheine attachment site is inferred to be Ser_{40} by analogy with the corresponding site, Ser_{36}, in *E. coli* FAS ACP (Vanamann et al., 1968). It is clear from the comparisons in Figure 1 that FabC is not the TcmM PKS ACP (Bibb et al., 1989). In fact, TcmM was not detected in the early log or stationary phase cell extracts by the methods used to detect and isolate FabC, even though its presence at the later time has been confirmed by Western analysis (Shen et al., 1992).

Nucleotide Sequence and Organization of the Gene Cluster That Encodes the FabC ACP. Since FabC is different from TcmM, we characterized the new ACP further by cloning and analyzing the nucleotide sequence of a 4.5 kb fragment of the *S. glaucescens* chromosome that carries the *fabC* gene (Figure 3). Within this segment, five complete ORFs with the characteristic G+C bias of a *Streptomyces* gene were identified by computer-assisted analysis (Devereux et al., 1984). Four of the ORFs, including the *fabC* gene, are transcribed in the same direction in a head-to-tail fashion (Figure 3). Short gaps (≤80 nt) separate these four ORFs, suggesting that they may be transcribed together as part of a single transcript. The fifth ORF lies downstream of the first four ORFs, and this ORF would be transcribed convergently. A large inverted repeat capable of forming a stem−loop structure [calculated $\Delta G = -39.6$ kcal/mol (Tinoco et al., 1973)] resides between the converging fourth and fifth ORFs (Figure 3, nt 3732−3765), and this structure may function as a transcription terminator. Finally, a short, unusually A+T-enriched (for *Streptomyces*) inverted repeat that resembles the *E. coli lac* repressor binding site (Gilbert & Maxam, 1973) is located about 70 nt upstream of the first ORF in this sequence[2] (Figure 3, nt 19−39).

The *fabC* gene is the third and shortest ORF among the cluster of four collinear ORFs, and the DNA-derived amino acid sequence of FabC is identical to the experimentally derived protein sequence, except that residue 42 (alanine = 1, since the initiating methionine is removed after translation) is predicted to be serine instead of leucine. Overall, FabC should comprise 81 amino acids ($M_r = 8782$) and have an acidic isoelectric point (pI = 3.8), which agrees well with the characteristics of other ACPs (Cooper et al., 1987; Rock & Cronan, 1980).

The Four Gene Products Encoded by the S. glaucescens FabC ACP Gene Cluster Have Homologs Involved in E. coli Fatty Acid Biosynthesis. A comparison of the predicted primary structure of the cloned FabC protein with the primary structures of a variety of other ACPs (Figure 1) reveals that the cloned ACP is most similar to the fatty acid biosynthetic AcpP protein of *E. coli* [47% identity by GAP analysis (Devereux et al., 1984)]. Less similarity is observed between FabC and the TcmM PKS ACP of *S. glaucescens* (30% identity) or other PKS ACPs (data not shown), as well as the putative FAS ACP of *Saccharopolyspora erythraea* (Revill & Leadlay, 1991) (27% identity). Consistent with its expression during vegetative growth (Table 1), these findings suggest that the FabC protein may be a component of the *S. glaucescens* FAS.

[2] Sequencing and analysis of ≈1 kb of DNA farther upstream has established the presence of another ORF, apparently transcribed convergent to *fabD*, whose deduced product is not significantly similar to any known protein in the databases as of February 1995 (E. Wendt-Pienkowski and C. R. Hutchinson, unpublished results).

The three ORFs that are found with the *fabC* gene also seem to encode components of the fatty acid biosynthetic machinery in *S. glaucescens* and, thus, were named the same as their individual *E. coli* FAS homologs. The first ORF, *fabD*, should encode a protein of 305 amino acids (M_r = 31 190) whose DNA-derived primary structure bears strong similarity to that of the FabD malonyl-CoA:ACP acyltransferase of *E. coli* fatty acid biosynthesis (Magnuson et al., 1992; Verwoert et al., 1992) (37% identity). The second ORF, *fabH*, would encode a 37 kDa enzyme that resembles the *E. coli* FabH condensing enzyme (Tsay et al., 1992) (39% identity). In *E. coli*, the FabH protein catalyzes the first step in fatty acid biosynthesis, condensing the acetyl-CoA starter unit with the first malonyl-CoA extender unit bound to the FAS ACP. The protein encoded by *fabH* also displays limited similarity to plant chalcone synthases (Reimold et al., 1983) (as does the *E. coli* FabH enzyme), perhaps reflecting a similarity in mechanism (both FabH and chalcone synthases recognize acyl-CoA substrates and ligate them to protein-bound acyl thioester substrates) and/or reflecting the fact that fatty acids in *Streptomyces* are commonly initiated with bulky branched-chain starter units such as isovalerate, isobutyrate, and α-methylbutyrate (Kaneda, 1991), which are sterically similar to the *p*-coumaryl starter unit of chalcone biosynthesis. Finally, the fourth ORF, *fabB*, would encode a protein of 423 residues (M_r = 43 603) that resembles the *E. coli* FabB 3-oxoacyl:ACP synthase I (Kauppinen et al., 1988) (36% identity). The *E. coli* FabB protein catalyzes the condensation between malonyl-*S*-ACP and the acetoacetate formed by FabH as part of the process that elongates the 3-oxobutyrate intermediate to palmitate (Magnuson et al., 1993). Although the deduced products of these four ORFs in the putative *S. glaucescens* FAS cluster strongly resemble their respective *E. coli* homologs, the latter genes are arranged in a different order, *fabH*, *fabD*, *fabG* (a 3-oxoacyl:ACP reductase), *acpP* (the ACP gene), and *fabF* (3-oxoacyl:ACP synthase II), and contain the *fabG* gene not present in the *S. glaucescens fab* cluster (Magnuson et al., 1993) (Figure 4). [This comparison also raises the possibility that the *S. glaucescens fabB* gene may instead encode an *E. coli* FabF homolog.]

The predicted product of the convergent fifth ORF does not resemble the deduced products of any known genes in the GenBank release 81 or EMBL release 37 databases by TFASTA analysis, but the C-termini of Orf5 and a putative protein from an incompletely sequenced region immediately downstream of genes for the β-subunit of a propionyl-CoA carboxylase and a 3-oxoacyl:ACP synthase in a *Rhodoccocus* sp. (GenBank accession number M95713) share 46% identity over 63 residues.

Expression of the fabC Gene in E. coli Produces the Holoenzyme Containing 4′-Phosphopantetheine. The roles of two of the putative *S. glaucescens* FAS genes were examined by determining whether they had the predicted enzymatic properties upon expression in *E. coli*. The *fabC* ACP gene was studied first to determine whether it behaved like the *S. glaucescens tcmM* ACP-encoding gene which, when expressed in *E. coli*, largely produces the apoprotein without the 4′-phosphopantetheine prosthetic group attached to Ser_{40} (Shen et al., 1992). To facilitate expression in the T7 RNA polymerase-dependent pT7-7 expression vector of Tabor and Richardson (1985), *fabC* was modified by PCR-directed mutagenesis to introduce an *Nde*I restriction site at

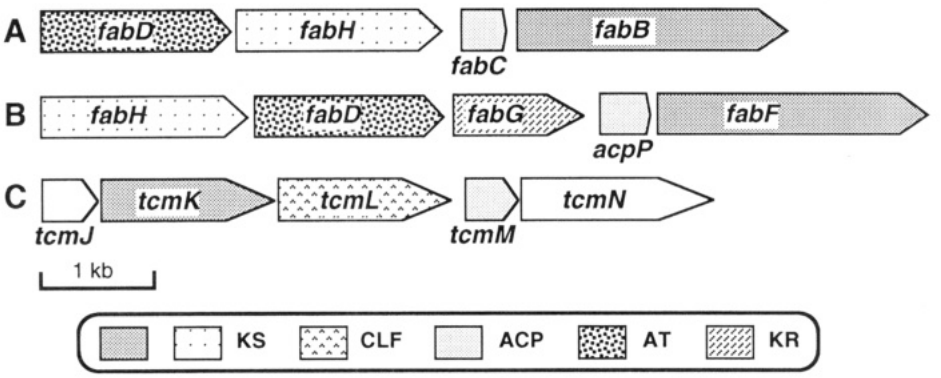


FIGURE 4: Comparison of gene organization among genes encoding FASs and a PKS: (A) *S. glaucescens* putative FAS genes; (B) *E. coli* FAS genes; (C) Tcm PKS genes. The sizes of the wedged boxes indicate the relative sizes of the deduced gene products, whose actual or deduced functions are indicated by the following symbols: KS, β-ketoacyl:ACP synthase; CLF, chain length factor (McDaniel et al., 1993) [a homolog of the KS enzymes from type II PKSs (Hopwood & Sherman, 1990; Katz & Donadio, 1993)]; ACP, as in the text; AT, acyltransferase; KR, ketoreductase [except for *tcmJ* (Summers et al., 1993) and *tcmN* (Summers et al., 1992]. Recent evidence suggests that the *E. coli fabF* gene may instead be the newly discovered *fabJ* (Siggaard-Andersen et al., 1994).

Table 2: Counts per Minute of Acid-Precipitated Radioactivity from the Acylation of Different ACPs by [2-^{14}C]Malonyl-CoA Using the Cell-Free Extract of the *E. coli fabD89* pWHM193 Strain Expressing the *S. glaucescens* fabD Gene *at* 42 °C

		pWHM193	
ACP	vector only	uninduced	induced
No ACP			118
S. glaucescens FabC	86	1410	2150
S. glaucescens TcmM	106	1960	2080
E. coli AcpP	91	1079	1420

the ATG start codon and to change the third bases of the next two codons to correspond to codons used in highly expressed *E. coli* genes and/or to contain either an A or a T in the third position, following our earlier work on *tcmM* expression (Shen et al., 1992). The mutated gene was cloned into pT7-7 to give pWHM192, which was then introduced by transformation into *E. coli* K38 cells already carrying pGP1-2 that provides T7 RNA polymerase expressed from a temperature-controlled gene (Tabor & Richardson, 1985). After overnight growth at 30 °C, *fabC* expression was induced at 42 °C for either 5, 10, 15, or 30 min. Analysis of the proteins in cell extracts by SDS−PAGE indicated that the optimum induction time was 10 min (data not shown).

The FabC protein was purified to homogeneity by the procedure of Shen et al. (1992). This pure protein was then assayed using [2-^{14}C]malonyl-CoA in a filter binding test to measure the stoichiometric attachment of malonate to the pantetheine sulfhydryl of the holoenzyme catalyzed by the crude MAT preparation obtained from *S. glaucescens*, as described previously (Shen et al., 1992; Shen & Hutchinson, 1993). The data in Table 2 show that purified FabC is an efficient acceptor of the radiolabeled carbon from [2-^{14}C]-malonyl-CoA, suggesting that, in contrast to the behavior of the TcmM ACP, the FabC ACP is expressed in *E. coli* as the holoenzyme. This idea is supported by the behavior of FabC on anion exchange chromatography where only the holoenzyme was observed (data not shown).

Expression of the S. glaucescens fabD Gene in the E. coli fabD Strain Complements the Temperature-Sensitive fabD89 Mutation. To determine whether the *S. glaucescens fabD* gene in fact encodes MAT, we tested the ability of *fabD* to complement the *E. coli fabD89* mutation, a mutant allele producing a temperature-senstive MAT that results in the cessation of growth on minimal medium when the temperature is shifted from 30 to >38 °C due to lack of the *fabD*-derived MAT activity (Clark & Cronan, 1981). The *S. glaucescens fabD* gene was cloned as pWHM193 in pTrc99a (Amman et al., 1988), which provides for regulated expression of genes cloned under the control of an IPTG-inducible *trc* promoter. The parent vector and pWHM193 were each introduced into the *fabD89* strain by transformation, and representatives of the two types of transformants were grown at 30, 37, and 42 °C for 2 h following the addition of 1 mM IPTG. Cell-free extracts obtained from these three cultures and analyzed by 12% SDS−PAGE showed distinct bands in all of the lanes from the *fabD89* (pWHM193) transformants, irrespective of the temperature at which they were grown or whether *fabD* expression had been induced by IPTG, indicating a lack of tight control over *fabD* expression (data not shown). The data in Table 2 show that the cell free extracts from *E. coli fabD89*(pWHM193) cultures grown at 42 °C contained similar levels of MAT activity before and after induction of *fabD* expression, confirming the apparent leakiness of the *fabD* gene cloned in pTrc99a. Nevertheless, compared to the vector-only control, it is clear

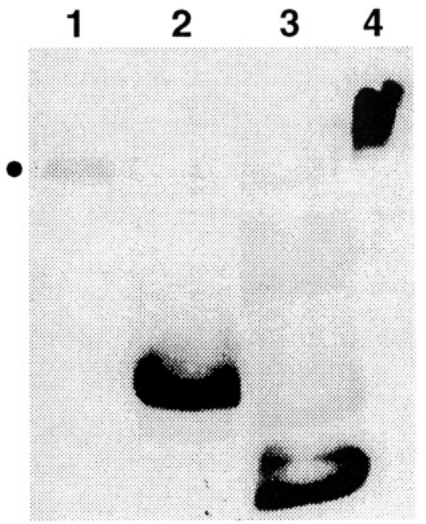


FIGURE 5: Autoradiogram showing the acylation of different ACPs catalyzed by the *S. glaucescens* FabD MAT expressed in the *E. coli fabD89* mutant: lane 1, no ACP added (the • indicates the faint band due to endogenous FAS ACP); lane 2, TcmM; lane 3, FabC; lane 4, AcpP from *E. coli*. Lanes 2−4 contain approximately 25 μg of added ACP.

A

```
          1                                                  50
  EcFabD} mtqFaFVfPG QGsQTvGmLa dmaasyPive EtfAeaSaAl GyDLwaltqq
  SgFabD} vrvLvLVaPG QGaQTpGfLt pwle.lPgaa DrvAawSdAi GlDLahygtk
Consensus ------V-PG QG-QT-G-L- ------P--- ---A--S-A- G-DL------

          51                                                 100
  EcFabD} gpAEElnkTw qtQPaLltAs valyrvwqqq GgkAPammAG HSlGEysAlv
  SgFabD} adADEirdTa vaQPlLvaAg llsaaal... GdiAPgavAG HSvGEitAar
Consensus --A-E---T- --QP-L--A- ---------- G--AP---AG HS-GE--A--
                                                      *
          101                                                150
  EcFabD} cAGViDfadA vrLVemRGkf MqEAvpegtg aMaAiiGlD. DaSIAkacee
  SgFabD} fAGVlDdtaA ltLVrkRGla MaEAaaitet gMsAllGgDp EvSVAh....
Consensus -AGV-D---A --LV--RG-- M-EA------ -M-A--G-D- --S-A-----

          151                                                200
  EcFabD} aaEgqvvsPv NfNspGQVVi AGhkEaverA gaacKaaGak ralPLpVsvp
  SgFabD} .lEklgltPa NvNgaGQIVa AGtlEqla.A lnedKpeGvr kivPLkVaga
Consensus --E-----P- N-N--GQ-V- AG--E----A ----K--G-- ---PL-V---

          201                                                250
  EcFabD} sHcalMkPAa DKLAvelaki tfnaPtVPvV nNvDvkcetn GDaIrdaLVr
  SgFabD} fHthhMaPAv DKLAeaarel spadPeVPyV sNkDgrtvat GDeVvarLVg
Consensus -H---M-PA- DKLA------ ----P-VP-V -N-D------ GD-----LV-

          251                                                300
  EcFabD} QlyNPVqWtk svEymaaqGV ehLyEVgPGk vLTGLtKRiv dtltasALne
  SgFabD} QvaNPVrWdl cmEtfkelGV taLlEVcPGg tLTGLaKRal pgvktlALkt
Consensus Q--NPV-W-- --E-----GV --L-EV-PG- -LTGL-KR-- ------AL--

          301        316
  EcFabD} PsaMaAAlEL *.....
  SgFabD} PddLdAArEL faeqa*
Consensus P----AA-EL ------
```

B

```
          1                                                  50
    SgFabB}.vsptnrtVV VTGIGattPl GgdaAstWEg LvAGRSGVkp leqdwAAdqa
    TcmK}  mtrhaeKRVV ITGIGVraPg GagtAafWDl LtAGRtatrt islFdAApyR
    EcFabB}.....mKRaV ITGlGIvssi Gnnqqevlas LreGRSGItf sqeLkdsgmR
Consensus  ------KRVV ITGIG---P- G---A--W-- L-AGRSG--- -----AA--R

          51                                                 100
    SgFabB}vRIAapVavE P.TEvLpRpQ ARkLDRsaQF ALVaAkEAwA DAGFtgkAge
    TcmK}  SRIAGeIdFD PigEgLsprQ AstyDRAtQL AvVcArEAlk DsGLdPaA..
    EcFabB}ShVwGnVkLD t.TglidRkv vRfMsdAsiY AFlsmeqAiA DAGLsPeAyq
Consensus  SRIAG-V--D P-TE-L-R-Q AR--DRA-Q- A-V-A-EA-A DAGL-P-A--

          101                                                150
    SgFabB}dssVdPDRlG aVIaSGIGgv TtLldqYdVL kEkGvRRvsp HTvpm.....
    TcmK}  ...VNPERIG vsIGtaVGct TgLdreYarv sEgGsRWlvd HTlaveqlfd
    EcFabB}....NnpRVG lIaGSGgGs. ....prFqVF gadamRgprg lkavgpyvvt
Consensus  ---VNP-R-G --IGSG-G-- T-L---Y-V- -E-G-R---- HT--------

          151                                                200
    SgFabB}.LMPnGpSAn VglavnArag VhtpvSACAS GAEAIGYAIE MIRtGRADVV
    TcmK}  yFvPtsIcre VAweagAeGp VtvVStgCtS GlDAVGYgtE LIRdGRADVV
    EcFabB}kaMasGVSAc lAtpfkihGv nysISSACAt sAhcIGnAVE qIqlGkqDIV
Consensus  --MP-G-SA- VA----A-G- V---SSACAS GA-AIGYA-E -IR-GRADVV
                                    *
```

```
          201                                                  250
    SgFabB}IAGGTEAaIh PLpIAaFgnM mAMSkN.NDD PqgASRPYDv aRDGFVLGEG
    TcmK}  VcGaTDApIs PitVAcFDAi kAtSaN.NDD PahASRPFDr nRDGFVLGEG
    EcFabB}fAGGgEelcw eMace.FDAM gsLStkyNDt PekASRtYDa hRDGFViagG
Consensus  -AGGTEA-I- P---A-FDAM -A-S-N-NDD P--ASRPYD- -RDGFVLGEG

          251                                                  300
    SgFabB}aGVIVLEsaE HAaRRGArVY AEaVGqGisa DshDiVqPeP EGRgiAhAlq
    TcmK}  saVfVLEELs aArRRGAHaY AEVrGFatrS nafhMtglkP DGRemAeAIt
    EcFabB}gGmVVvEELE HAlaRGAHIY AEIVGYGatS DgaDMVaPsg EG.....AVr
Consensus  -GV-VLEELE HA-RRGAH-Y AE-VG-G--S D--DMV-P-P EGR--A-A--

          301                                                  350
    SgFabB}nLLErtdlep aEImhVNAHa TSTPaGDVaE LkALRkVFGD eAdhfAVSAT
    TcmK}  aaLDqarrtg dDlhYINAHG sgTrqnDrhE tAAFkrsLGq rAydvpVSsi
    EcFabB}cMkmamhgvd tpIdYlNsHG TSTPvGDVkE LAAiReVFGD ks..pAISAT
Consensus  --L------- --I-Y-NAHG TSTP-GDV-E LAA-R-VFGD -A---AVSAT

          351                                                  400
    SgFabB}KSMTGHlLGg AGgvEsVAtv LALyHrVaPP TINVDnLDPE AEanaDIVrg
    TcmK}  KSMiGHSLGA iGslElaAca LAiEHGVIPP TaNyE..EPD pEcdlDyVpn
    EcFabB}KaMTGHSLGA AGvqEaIysl LmLEHGfIaP sINIEeLDeq AaglniVtet
Consensus  KSMTGHSLGA AG--E--A-- LALEHGVIPP TIN-E-LDP- AE---D-V--
                  *
          401                                        435
    SgFabB}eARkLpVEgr iaaLndSFGF GGhNVvLafR tv*..
    TcmK}  vAREqrVDtv lsVgSgfgGF qsaaVlarpk etrs*
    EcFabB}tdRELt.... .tVMSnSFGF GGtNatLvmR klkd*
Consensus  -AREL-V--- --V-S-SFGF GG-NV-L--R -----
```

FIGURE 6: Protein sequence comparisons of MAT and 3-oxoacyl:ACP synthases done by the PILEUP method (Devereux et al., 1984): (A) *S. glaucescens* FabD and *E. coli* FabD MATs (the predicted active site region is doubly underlined, with the essential Ser indicated by an *); (B) *S. glaucescens* FabB and TcmK and *E. coli* FabB 3-oxoacyl:ACP synthases (the predicted active site regions are singly and doubly underlined, with the essential Cys in the synthase and Ser in the putative acyltransferase motif indicated respectively by *).

that this activity is due to the cloned *fabD* gene. This conclusion was confirmed by observing that the *fabD89* (pWHM193) strain was able to grow on solid minimal medium at 42 °C, a nonpermissive temperature for growth of the control *fabD89* (pTrc99a) strain.

S. glaucescens FabD MAT Acts on Both FAS and PKS ACPs. Type II PKS enzyme complexes such as the one from *S. glaucescens* are believed to require malonyl-*S*-ACP for the polyketide chain extension reaction (Hopwood & Sherman, 1990; Katz & Donadio, 1993). Although type II PKS gene clusters contain specialized ACP genes—TcmM in the tetracenomycin gene cluster (Bibb et al., 1989; Shen et al., 1992)—they lack a clearly recognizable MAT gene. One source of this acyltransferase enzyme could be the FAS in the same organism since fatty acid synthesis also requires an MAT to charge the ACP with malonate. By using the FabC assay described in the Experimental Procedures, the cell-free extract from the *E. coli fabD89* (pWHM193) strain induced at 42 °C was found to catalyze the transfer of the following amounts of radioactivity (cpm) from 5 μM [2-^{14}C]-malonyl-CoA (8000 cpm) to 25 μM *E. coli* AcpP and *S. glaucescens* FabC and TcmM ACPs: no ACP added, 900 cpm due to the endogenous FAS ACP of the host strain; *E. coli* AcpP, 3040 cpm; FabC. 3970 cpm; and TcmM, 2970 cpm. Even though the FabD enzyme appears to have the greatest activity toward FabC, it does not differentiate significantly between the other two ACPs, and it clearly acts on both FAS and PKS ACPs.

To confirm that all three of these ACPs were in fact radiolabeled by malonate as a consequence of the FabD activity, a portion of the assay mixture was analyzed by the conformationally sensitive native PAGE method (Rock et al., 1981). Autoradiography of the resulting gel revealed intense bands for each of the lanes containing one of the three different ACPs, whereas the lane from the sample with no ACP added shows only a faint band due to the small amount of endogenous FAS ACP in the *E. coli* host (Figure 5).

DISCUSSION

Interest in the biochemistry of polyketide biosynthesis, a type of secondary metabolism closely related to fatty acid biosynthesis and found especially among bacteria that make a large number of polyketide-derived metabolites, has undergone a renaissance with the advent of a detailed understanding of the underlying genetics (Hopwood & Sherman, 1990; Katz & Donadio, 1993). Sequence information about type I and type II PKS genes has led to predictions about the structure of the enzyme complexes (Hopwood & Sherman, 1990; Katz & Donadio, 1993) and the mechanisms of the condensation and cyclization reactions characteristic of PKSs (Summers et al., 1993; McDaniel et al., 1993, 1994). The condensation reactions and the attendant reduction and dehydration of poly-(β-carbonyl) substrates have close parallels in the well-known mechanism for the biosynthesis of saturated, long-chain fatty acids (Wakil, 1989). Yet several

questions have arisen, such as what specifies the choice of starter unit (commonly acetyl- or propionyl-CoA) and extender units (usually malonyl- or methylmalonyl-CoA), and what determines the number of cycles of condensation. From fatty acid biosynthesis in *E. coli*, the paradigm often applied to polyketide biosynthesis, it is anticipated that the polyketide starter unit is first loaded onto the 3-oxoacyl:ACP synthase, where it then undergoes reaction with the initial extender unit that has been attached to a PKS-dedicated ACP. (There is as yet no evidence that the starter CoA and extender ACP derivatives can be used directly by a FabH-like activity.) According to this scheme, one or more acyl-CoA transferases are required to charge the 3-oxoacyl:ACP synthase and the ACP with the PKS starter or extender units. The multifunctional type I PKSs contain domains for such an activity (Hopwood & Sherman, 1990; Katz & Donadio, 1993), but discrete acyltransferase genes have not yet been found in the gene clusters encoding type II PKSs. [All of the putative type II 3-oxoacyl:ACP synthases contain a conserved GHSXG motif (Hopwood & Sherman, 1990; Katz & Donadio, 1993), as is typical of many acyltransferases, in which the Ser might be the acceptor for the starter or extender unit from its CoA derivative. However, the NodE 3-oxoacyl:ACP synthase for the formation of the fatty acid-derived nodulation factors in *Rhizobium* spp. (Fisher et al., 1987) lacks this motif, and thus it may not be essential, as discussed below.]

The lack of an apparent acyltransferase function in the majority of type II PKS gene clusters, particularly for the ACP charging reaction, stimulated our interest in finding other sources of the putative acyl-CoA:ACP transferase in *S. glaucescens*. Since this type of enzyme is known to be part of the type II bacterial FAS in *E. coli* (Magnuson et al., 1992; Verwoert et al., 1992), we chose to study the FAS of *S. glaucescens*, focusing first on the principal ACP of this organism with the idea that its gene might be closely linked to ones for 3-oxoacyl:ACP synthase and MAT, as in *E. coli* (Magnuson et al., 1993).

By the two enzymatic assays we have routinely used to detect ACPs in extracts of *S. glaucescens*, only a single ACP, FabC, was detected. TcmM, the ACP for the type II PKS of Tcm C biosynthesis, was present in a much lower amount and detectable only by Western analysis during and after the late log phase of growth, about 6 h later in growth than the appearance of FabC (Shen et al., 1992). On the basis of its abundance and presence early in growth, it seems likely that FabC is the principal ACP for fatty acid biosynthesis in *S. glaucescens*. In support of this notion, the amino acid sequence of FabC is considerably more similar to that of *E. coli* AcpP than it is to the deduced sequences of typical type II PKS ACPs, including those for antibiotic biosynthesis (TcmM, ActI-Orf3), spore pigment production (WhiE-Orf5), or nodulation factor synthesis (NodF) (Figure 1).

Consistent with its proposed role as an FAS ACP, the genes surrounding *fabC–fabD*, *fabH*, and *fabB* (Figure 4A)—encode proteins that exhibit high sequence similarity to components of the type II *E. coli* FAS. The *fabD* gene encodes a MAT, and this gene complements the temperature-sensitive *fabD89* mutation in *E. coli*, indicating that the *S. glaucescens* FabD enzyme is able to interact properly with the components of the *E. coli* FAS to reconstitute fatty acid biosynthesis in this organism. Furthermore, FabD not only catalyzes the efficient transacylation of malonyl-CoA to *S. glaucescens* FabC and *E. coli* AcpP but also converts the TcmM ACP into the respective malonyl-*S*-ACP (Figure 5). The latter finding suggests that FabD may provide the missing transacylase activity required for TcmC production. Similarly, the *S. coelicolor* FabD MAT recently described by Revill et al. (1995), whose sequence is 89% identical to that of the *S. glaucescens* FabD protein, may be necessary for the biosynthesis of actinorhodin, a polyketide that is constructed from acetyl-CoA and malonyl-CoA in nearly the same way as Tcm C. Preliminary sequence data indicate that the *S. coelicolor fabD* gene for this MAT is also clustered with genes encoding FAS-like proteins, as is the gene encoding the abundant ACP of the erythromycin-producing *Saccharopolyspora erythraea* (Revill & Leadlay, 1991). The *fabH* gene encodes a relative of *E. coli* FabH, which catalyzes the initial condensation reaction between starter and extender units (as their CoA derivatives) in fatty acid biosynthesis, and the *fabB* gene encodes an analog of *E. coli* FabB, the principal 3-oxoacyl:ACP synthase.

There are some differences between the FAS genes and enzymes of *E. coli* and *S. glaucescens* that may have some bearing on the questions of their evolution and catalytic mechanism. In the first case, while major portions of the FAS genes are clustered in both organisms, the order of the genes in *E. coli* and *S. glaucescens* is different, and a homolog of *fabG* is not present in the *S. glaucescens* cluster (Figure 4A,B). This is not surprising, perhaps, given the presumed evolutionary distance between Gram-positive and Gram-negative bacteria. With regard to catalytic mechanism, the four FAS enzymes from *S. glaucescens* have strong overall sequence similarities with their congeners in *E. coli*, and the ACP, MAT, and 3-oxoacyl:ACP synthase enzymes contain equivalent active site motifs (Figures 1, and 6A,B). The putative *S. glaucescens* FabB 3-oxoacyl:ACP synthase, however, lacks the putative GHSXG transacylase motif that is present in both the *E. coli* FabB enzyme (Figure 6B) and all of the 3-oxoacyl:ACP synthases of type II PKSs (Hopwood & Sherman, 1990; Katz & Donadio, 1993). The significance of this observation is not yet clear, but we have found that the S351A alteration of this motif in the TcmK 3-oxoacyl:ACP synthase does not alter the ability of *S. glaucescens* to make Tcm F2 in vivo (Meurer & Hutchinson, 1995), suggesting that this motif is not critical to Tcm C production. In a similar study, an S347L mutation in the *S. coelicolor* Act-Orf1 3-oxoacyl:ACP synthase, an analog of the TcmK enzyme, did have an impact on actinorhodin production, although some actinorhodin was still synthesized (Kim et al., 1995). The enhanced severity of the S347L mutation may be a secondary effect resulting from the addition of the large, hydrophobic leucine residue. In any case, in spite of its near ubiquity, it is clear that the central serine residue in this pentapeptide motif is not essential for 3-oxoacyl:ACP synthase function.

Finally, of some interest is a comparison of the genes and enzymes of the putative *S. glaucescens* FAS with those of the same organism's Tcm PKS. It is notable that the two gene clusters have very different organizations, both in terms of the type of genes present and, where similar genes are concerned, their genetic order (Figure 4A,C). While it might be hypothesized that the *tcm* gene cluster originated from the host's FAS gene cluster by duplication, the distinct genetic organization of the two clusters and the divergence in primary sequence of enzymes of similar function suggest

that these two clusters have evolved independently, even though some of their components may interact catalytically. Yet, in contrast to the considerable difference between the PKS gene cluster and the putative FAS gene cluster of *S. glaucescens,* there is substantial similarity in the organization of aromatic PKS gene clusters among *Streptomyces* (Hopwood & Sherman, 1990; Katz & Donadio, 1993). This could be indicative of a common, single origin for the PKS machinery followed by horizontal spread throughout streptomycetes.

Since the lack of a functional *tcmM* gene abolishes Tcm C biosynthesis in vivo (R. G. Summers, E. Wendt-Pienkowski, and C. R. Hutchinson, unpublished results), and Tcm F2 biosynthesis in *S. glaucescens* definitely involves malonyl-*S*-TcmM in vitro (Shen & Hutchinson, 1993), there must be a way to charge the essential TcmM ACP with malonate. The evidence presented here shows that FabD can play this role in vitro and implies that it could also do so in vivo because the timing of FabC appearance suggests that the *fabDHCB* genes are likely to be transcribed and produce FabD at the time of Tcm C biosynthesis. On the other hand, the reported ability of the putative *S. erythraea* FAS ACP and *E. coli fabB* genes to restore biosynthesis of the polyketide-derived blue pigments when introduced into the respective *Streptomyces coelicolor actI*-ORF3 and -ORF1 mutants with defective type II PKS ACP (Khosla et al., 1992) or 3-oxoacyl:ACP synthase genes (Kim et al., 1994), respectively, demonstrates that some FAS and PKS genes are functionally interchangeable in vivo. This fact indicates that the ability of FabD to catalyze the formation of malonyl-*S*-TcmM in vitro does not prove that *fabD* is essential for Tcm C biosynthesis.

The latter question has been addressed by attempting to disrupt the *fabD* gene and determine whether this would affect Tcm C biosynthesis. However, recombination between the chromosomal *fabD* allele and a copy of *fabD* with an in-frame deletion of the active site region, even under conditions of fatty acid supplementation of the growth medium, always maintained the wild-type *fabDHCB* region among the 22 recombinants examined by Southern analysis (E. Wendt-Pienkowski and C. R. Hutchinson, unpublished results). The fact that the organism strongly resisted loss of the *fabD* function (and probably *fabHCB* also) by separating the mutated *fabD* gene from the *fabDHCB* region by vector DNA suggests that *fabD* is essential for growth under the conditions used, as might be expected of an FAS enzyme. Revill et al. (1995) described similar results in an attempted disruption of the *S. coelicolor fabD* gene. Hence, the role of the FabD malonyl-CoA:ACP acyltransferase in Tcm C biosynthesis will have to be determined in some other way.

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