In Vivo and In Vitro Effects of Thiolactomycin on Fatty Acid Biosynthesis in Streptomyces collinus

KIMBERLEE K. WALLACE,1 SANDRA LOBO,1 LEI HAN,1 HAMISH A. I. MCArTHUR,2 AND KEVIN A. REYNOLDS1*

Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland at Baltimore, Baltimore, Maryland 21201,1 and Bioprocess Research, Central Research Division, Pfizer Incorporated, Groton, Connecticut 06340

Received 7 February 1997/Accepted 15 April 1997

A stable-isotope assay was used to analyze the effectiveness of various perdeuterated short-chain acyl coenzyme A (acyl-CoA) compounds as starter units for straight- and branched-chain fatty acid biosynthesis in cell extracts of Streptomyces collinus. In these extracts perdeuterated isobutyryl-CoA was converted to isopalmitate (a branched-chain fatty acid), while butyryl-CoA was converted to palmitate (a straight-chain fatty acid). These observations are consistent with previous in vivo analyses of fatty acid biosynthesis in S. collinus, which suggested that butyryl-CoA and isobutyryl-CoA function as starter units for palmitate and isopalmitate biosynthesis, respectively. Additionally, in vitro analysis demonstrated that acetyl-CoA can function as a starter unit for palmitate biosynthesis. Palmitate biosynthesis and isopalmitate biosynthesis in these cell extracts were both effectively inhibited by thiolactomycin, a known type II fatty acid synthase inhibitor. In vivo experiments demonstrated that concentrations of thiolactomycin ranging from 0.1 to 0.2 mg/ml produced both a dramatic decrease in the cellular levels of branched-chain fatty acids and a surprising three- to fivefold increase in the cellular levels of the straight-chain fatty acids palmitate and myristate. Additional in vivo incorporation studies with perdeuterated butyrate suggested that, in accord with the in vitro studies, the biosynthesis of the palmitate from butyryl-CoA decreases in the presence of thiolactomycin. In contrast, in vivo incorporation studies with perdeuterated acetate demonstrated that the biosynthesis of palmitate from acetyl-CoA increases in the presence of thiolactomycin. These observations clearly demonstrate that isobutyryl-CoA is a starter unit for isopalmitate biosynthesis and that either acetyl-CoA or butyryl-CoA can be a starter unit for palmitate biosynthesis in S. collinus. However, the pathway for palmitate biosynthesis from acetyl-CoA is less sensitive to thiolactomycin, and it is suggested that the basis for this difference is in the initiation step.

Despite numerous investigations, a considerable number of questions concerning fatty acid biosynthesis in streptomycetes remain. Initial studies suggested that fatty acids in streptomycetes are biosynthesized by a type I fatty acid synthase (FAS) (6, 28). Such synthases are large, nondissociable, multifunctional complexes which are generally found in yeast and mamalian systems. More recent work has suggested that streptomycetes have a type II FAS (32). The type II FAS is more recent work has suggested that streptomycetes have a type II FAS (32). Such synthases are large, nondissociable, multifunctional complexes which are generally found in yeast and mamalian systems. More recent work has suggested that streptomycetes have both a type I and a type II FAS; such synthases consist of a series of dissociable enzymes (24, 32). The type II FAS is more common in bacterial systems and has been extensively characterized in Escherichia coli (17). The possibility that streptomycetes have both a type I and a type II FAS has not been ruled out (24).

A number of questions concerning the initiation of fatty acid biosynthesis in streptomycetes have also been raised. Streptomycetes produce predominantly branched-chain fatty acids and a small proportion of straight-chain components (28, 32). In vivo analysis has indicated that branched-chain fatty acids are biosynthesized by using the amino acid catabolites methylbutyryl coenzyme A (methylbutyryl-CoA) and isobutyryl-CoA as starter units (27, 38). In vivo experiments have also indicated that butyryl-CoA functions effectively as a starter unit for the biosynthesis of palmitate and myristate, straight-chain fatty acids containing an even number of carbon atoms (27, 38). One source of butyryl-CoA has been shown to be the rearrangement of the valine-derived catabolite isobutyryl-CoA (38). However, in the case of Streptomyces collinus, Streptomyces cinamomons, and Saccharopolyspora erythraea, in vivo analysis clearly demonstrates that the majority of the palmitate is not formed by using butyryl-CoA thus generated. Accordingly, these systems must have either an additional pathway for butyryl-CoA formation or the potential to initiate straight-chain fatty acid biosynthesis from a starter unit other than butyryl-CoA (38). Evidence for the former of these suggestions has been the isolation and characterization of a crotonyl-CoA reductase from S. collinus (37). This enzyme is thought to play a key role in catalyzing the last reductive step in a malonyl-CoA-independent biosynthesis of butyryl-CoA from two acetyl-CoA molecules (37).

Herein, we report the results of a series of in vivo and in vitro studies with S. collinus, aimed at addressing the initiation of straight-chain fatty acid biosynthesis. These studies have demonstrated that (i) a type II fatty acid synthase (FAS) is involved in both straight- and branched-chain fatty acid biosynthesis; (ii) crotonyl-CoA reductase plays no significant role in providing butyryl-CoA for palmitate biosynthesis; (iii) in addition to a pathway using butyryl-CoA as a starter unit for palmitate biosynthesis, a pathway using a different starter unit (likely acetyl-CoA) operates; and (iv) the FAS inhibitor thiolactomycin has differing effects, likely at the initiation point, on branched- and straight-chain fatty acid biosynthesis.

MATERIALS AND METHODS

Materials. Deuterated fatty acids and amino acids were purchased from Cambridge Isotopes. Isobutyric acid with six deuterium atoms (D6-isobutyric acid)
was synthesized from diethyl malonate and deuterated isodomethane by following an established procedure (25). Materials purchased from Sigma included NADPH, CoA, acyl carrier protein (ACP), and malonyl-CoA. Thiolactomycin was provided by Pelzer Incorporated. CoA thioesters were prepared by the mixed-anhydride method as previously described (26).

**Growth of *S. collinus* for assays of FAS activity.** Seed cultures were prepared by inoculating spores from one slant culture of *S. collinus* into a 500-ml flask containing 100 ml of a complex medium consisting of 5 g of glucose, 10 g of succinate, 0.5 g of tryptone, and 2.5 g of yeast extract in 1 liter of distilled water (pH 7.0). Following incubation at 30°C and 250 rpm for 24 h, 35 ml of the seed culture was transferred to a 2-liter flask containing 400 ml of this complex medium. After incubation at 30°C and 250 rpm for 24 h, the cells were harvested by centrifugation at 6,000×g for 30 min. The supernatant was discarded, and the cell pellet was washed with buffer A, containing 250 mM potassium phosphate (pH 7.3), 1 mM diithioerythritol, 0.1 mM phenylmethylsulfonyl fluoride, and 10% (vol/vol) glycerol. Following pelleting of the cells by centrifugation and discardation of the supernatant, the cell pellet was resuspended in 7 ml of buffer A and broken by passage through a French pressure cell at 18,500 to 15,000 lb/in². After centrifugation at 30,000×g, the resulting cell extract was dialyzed for 12 to 15 h against 2 liters of buffer A.

**FAS activity with various starter units.** The FAS assays for determination of the ratio of FAS for straight-chain fatty acid biosynthesis were conducted in 1 ml of dialyzed cell extract of *S. collinus*, 0.13 µmol of ACP from *E. coli*, 0.92 µmol of NADPH, 1 µmol of malonyl-CoA, and a perdeuterated acyl-CoA starter unit in a total volume of 1.4 ml. In one set of experiments, 0.5 µmol of D3-acetyl-CoA, 0.5 µmol of D3-hexanoyl-CoA, and 0.5 µmol of D6-isobutyryl-CoA were used in separate FAS assays. In a separate set of experiments, 0.5 µmol of D3-butyryl-CoA and 0.5, 2.5, and 5 µmol of perdeuterated acetyl-CoA were used in separate FAS assays. An additional FAS assay was conducted with 0.5 µmol of D3-butyryl-CoA and 0.5 µmol of ACP from *E. coli*, 1.84 µmol of NADPH, and 2 µmol of malonyl-CoA. After 2 h of incubation at 37°C, the FAS assays were terminated by treatment with 0.2 ml of 1 N NaOH at room temperature for 15 min (the fatty acid thioesters are hydrolyzed during this step). Supernatants (0.4 ml) of the preincubation mixture were precipitated with an equal volume of solution containing six deuterium atoms, the resulting cell dialyzed for 12 to 15 h against 2 liters of buffer A. This number was calculated by taking the relative abundances of the molecular ions at 271 (methyl isopalmate) and 276 (methyl isopalmitate) and by combining these with the area for the lauric acid standard (control experiments clearly demonstrated that no detectable quantities of endogenous lauric acid were present in the cells).

Determination of the percent labeling of each peak from a deuterated starter unit then allowed the calculation of the labeled and unlabeled fatty acids to be calculated. The determination of the percent labeling of the isopalmate pool from D6-isobutyrate was based on a comparison of the relative intensities of the molecular ions at 270 (unlabeled methyl isopalmate) and 276 (methyl isopalmate) containing six deuterium atoms in the manner described previously (38). Determination of the percent labeling of the palmitate pool from D3-acetate was calculated in an analogous fashion by using the relative intensities of molecular ions at 270 (unlabeled methyl palmitate) and 276 and 277 (methyl palmitate containing six and seven deuterium atoms, respectively). Determination of the percent labeling of the palmate pool from D3-acetate was based on the relative intensities of molecular ions at 273 (methyl palmitate with three deuterium atoms) and 274, after correction for natural abundance of 13C and incorporation of deuterated malonate. Determination of the percent labeling at each of the seven malonate-derived positions in palmitate with one deuterium atom from D3-acetate was calculated based on the ratio of the molecular ion at 271 (methyl palmitate with one deuterium atom) to the molecular ion at 270, after correction for natural abundance of 13C (38). In experiments for labeling of the palmate pool from both D3-acetate and D6-butyrate occurred, the percent labeling from each starter unit was based on the relative intensities of the molecular ions at 276, 273 (after the appropriate corrections), and 270.

This estimation of total amount of each fatty acid (labeled and unlabeled) is based on the assumption that all the long-chain fatty acids extract into hexane-ether (50:50) with the same efficiency and that equal mass quantities of fatty acids give rise to equal-sized peaks in a total ion chromatogram GC-MS analysis. Errors due to these assumptions occur in each experiment set (with and without thiolactomycin) and do not alter the magnitude of an effect of thiolactomycin on fatty acid levels relative to the lauric acid standard (although the reported nanomole quantity is subject to error). A control experiment in which an aqueous solution containing equal quantities of lauric acid, myristic acid, palmitic acid, and isopalmatic acid were converted to their methyl esters, extracted, and analyzed by GC-MS indicated that the errors from these assumptions are small (less than 1%).

In vivo analysis of fatty acid biosynthesis in an *S. collinus ccr* mutant. An *S. collinus ccr* mutant (9) was grown in minimal medium (10 ml) containing either no perdeuterated starter units or 200 mM valine for 24 h in the manner described above. The fatty acid profile was then generated and analyzed in the manner described previously (38).

**RESULTS**

**Assay development.** FAS assays have previously been employed for cell extracts of streptomycetes and other bacteria which produce predominantly branched-chain fatty acids. These methods typically utilize either a radioactive acyl-CoA starter unit or a malonyl-CoA extender and quantitate the radioactivity that extracts into an organic phase (8, 16). Although sensitive, such assays do not permit the unambiguous determination of intact incorporation of starter units into the product. Accordingly, a stable-isotope-based GC-MS assay was developed. This method, based on an in vivo method described for analysis of starter unit utilization in fatty acid biosynthesis (38), employed the direct measurement of intact fatty acids of the starter units into the specific fatty acids in a cell extract. An additional advantage of the assay is that it allows the carbon chain length of the product to be determined.

As shown in Fig. 1, only unlabeled methyl palmitate and methyl isopalmate were obtained from an incubation of the

*Downloaded from http://jb.asm.org/ on October 14, 2017 by guest*
cell extract in the absence of either NADPH, malonyl-CoA, or a perdeuterated starter unit. In contrast, incubation in the presence of ACP, NADPH, malonyl-CoA, and perdeuterated butyryl-CoA resulted in the production of labeled methyl palmitate containing seven deuterium atoms. In the FAS assay the labeled palmitate which formed in the cell extract was readily distinguishable from the unlabeled endogenous palmitate by a shorter retention time in the gas chromatogram and by the mass-spectral analysis (a molecular ion at 277 versus 270). The unlabeled methyl palmitate and palmitate were used as internal standards to compare FAS activities under various conditions.

A FAS assay with a crude cell extract has obvious limitations. Since the enzyme preparation is not pure, numerous competing reactions or degradative enzymes may utilize the substrates and change their apparent concentrations. Such a rationale explains the variation in the magnitude of percent labeling of the individual fatty acid pools between sets of experiments. Accordingly, control assays were conducted for each set of experiments. The relative amounts of unlabeled isopalmitate and palmitate also varied between experiments, possibly due to changes in the dialysis, solvent extraction, and culture conditions. Nonetheless, the trends and observations that are reported were absolutely consistent between the sets of experiments.

**Requirement of FAS activity for malonyl-CoA.** In one set of experiments, 38, 25, 26, and 12% of the palmitate pool was labeled with deuterium atoms when the assays were conducted with 1, 0.5, 0.25, and 0.1 μmol of malonyl-CoA, respectively. In the same experiment, 7 and 5.7% of the myristate pool was labeled in the incubations with 1 and 0.5 μmol of malonyl-CoA, respectively (no detectable level of labeled myristate was observed in the other two assays). Interestingly, only 16- and 14-carbon chain fatty acid products were detected in assays using a butyryl-CoA starter unit, regardless of the ratio of butyryl-CoA to malonyl-CoA. A previous analysis of fatty acid biosynthesis in *Saccharopolyspora erythraea* using an isobutyryl-CoA starter unit showed that under the assay conditions the predominant product appeared to be a terminally branched eight-carbon fatty acid (8).

**Requirement of FAS activity for an ACP.** Addition of the *E. coli* ACP resulted in a marked increase in the production of labeled palmitate from perdeuterated butyryl-CoA. In one experiment, 1, 2.6, and 5.4% of the overall palmitate pool was labeled when the assays were conducted with 0, 0.13, and 0.26 μmol of ACP, respectively. In a separate experiment, 2.2, 4.1, and 6.7% of the overall palmitate pool was isotopically enriched when the assays were conducted in the presence of 0.13, 0.26, and 0.39 μmol of ACP, respectively.

**Isobutyryl-CoA as a starter unit for branched-chain fatty acid biosynthesis.** Incubation of the cell extract in the presence of 0.5 μmol of isobutyryl-CoA and 0.5 μmol of butyryl-CoA resulted in the production of similar levels of labeled isopalmitate (as evidenced by the shoulder peak, adjacent to the unlabeled isopalmitate peak, which contained a molecular ion at 276) and palmitate (Fig. 1). These results suggest that under the assay conditions, butyryl-CoA and isobutyryl-CoA are used comparably for fatty acid biosynthesis. Isobutyryl-CoA has previously been implicated as a starter unit for branched-chain fatty acid biosynthesis by in vivo analyses with *S. collinus* and *Streptomyces avermitilis* (27, 38) and by in vitro analysis with *Saccharopolyspora erythraea* (8).

**Acetyl-CoA, butyryl-CoA, and hexanoyl-CoA as starter units for straight-chain fatty acid biosynthesis.** To determine the preferred starter unit for straight-chain fatty acid biosynthesis in *S. collinus*, in vitro FAS assays were conducted with acetyl-CoA, butyryl-CoA, and hexanoyl-CoA as starter units. Addition of perdeuterated butyryl-CoA results in a greater percent (12% ± 1%) labeling of the palmitate pool with deuterium than either acetyl-CoA (none detectable) or hexanoyl-CoA (4% ± 0.4%), suggesting that it may be the preferred starter unit for straight-chain fatty acid biosynthesis in *S. collinus*. Hexanoyl-CoA was both utilized directly as a starter unit for straight-chain fatty acid biosynthesis and degraded during the assay to butyryl-CoA, which was subsequently incorporated into the palmitate pool (1.4% of the palmitate pool had a molecular ion at 277, corresponding to M+7).

These results suggest that acetyl-CoA, at a concentration of 0.64 mM, is ineffective as a starter unit for straight-chain fatty acid biosynthesis. Increased concentrations of acetyl-CoA clearly permit it to function as a starter unit. However, at the same concentrations (0.37 mM), butyryl-CoA functions 10 times more effectively than acetyl-CoA, as measured by percent labeling of the palmitate pool (26.8 versus 2.5%). Even at high concentrations (1.85 and 3.7 mM), acetyl-CoA does not appear to function as effectively as butyryl-CoA as a starter unit.
In vitro inhibition of fatty acid biosynthesis by thiolactomycin. Thiolactomycin (10 to 250 μg/ml), a known type II FAS inhibitor (10, 11, 20, 21, 31, 35), inhibited palmitate biosynthesis from D7-butyryl-CoA (Fig. 2). A thiolactomycin concentration of 50 μg/ml (240 μM) was estimated to result in a 50\% inhibition of synthase activity under the conditions of this experiment. A similar inhibition of isopalmitate biosynthesis from D6-isobutyryl-CoA by thiolactomycin was observed.

In vivo effect of thiolactomycin on fatty acid biosynthesis. The fatty acid profile of S. collinus cultures grown in the presence of 0.1 mg of thiolactomycin per ml reveals a 5- to 10-fold decrease in the quantities of the branched-chain fatty acids and the odd-numbered straight-chain fatty acid pentadecanoate and a 2- to 4-fold increase in the quantities of straight-chain saturated and unsaturated even-numbered fatty acids (Fig. 3; Table 1). These substantial changes in fatty acid composition were highly reproducible. The overall fatty acid production and bacterial growth were not dramatically affected by thiolactomycin. A Bradford assay (2) of bacterial cells from the two experiments indicated that a small (20\%) decrease was observed for cells grown in the presence of thiolactomycin. A Bradford assay (2) of bacterial cells from the two experiments revealed an approximate decrease of 10\% in the protein concentrations. A higher concentration of thiolactomycin (0.4 mg/ml) did, however, inhibit growth of S. collinus.

Effect of thiolactomycin on the incorporation of butyrate, acetate, and isobutyrate into the palmitate and isopalmitate pool. Addition of thiolactomycin to growing cultures of S. collinus was observed to produce differing effects on the utilization of exogenously supplied starter units for straight- and branched-chain fatty acids (Fig. 4; Table 2). In experiments with D7-isobutyrate, addition of thiolactomycin resulted in a decrease in the production of both unlabeled isopalmitate and labeled palmitate made from D7-butyrate. In the experiments with D6-butyrate, the addition of thiolactomycin resulted in an increase in the amount of palmitate made from D6-butyryl-CoA but not acetyl-CoA. Two additional observations provide support for these suggestions. In the experiments with D7-butyrate, the addition of thiolactomycin resulted in an increase in the amount of palmitate made from D7-acetyl-CoA but not acetyl-CoA. This result suggests that the biosynthesis of palmitate from an acetyl-CoA pool is not inhibited by low levels of thiolactomycin. In contrast to the results of the D7-acetate experiments, the experiments with D6-butyrate demonstrated that addition of thiolactomycin led to a decrease in the levels of labeled palmitate made from D6-butyryl-CoA (although the overall amount of palmitate once again increased). These two sets of experiments suggest that thiolactomycin inhibits palmitate biosynthesis from butyryl-CoA but not acetyl-CoA. Two additional observations provide support for these suggestions. In the experiments with D7-butyrate, the addition of thiolactomycin resulted in an increase in the amount of palmitate made from D7-acetyl-CoA but a decrease in the levels of palmitate made from D6-butyryl-CoA.

In the experiments using D7-acetate, the amount of the palmitate pool labeled by using a D7-acetyl-CoA starter unit ranged from 6 to 28\% (Fig. 4). Substantially lower levels of incorporation of deuterated malonyl-CoA into palmitate and other fatty acids were observed (3 to 5\% labeling at each of the malonate-derived positions with one deuterium atom). This labeling likely reflects the generation of D2-malonyl-CoA from D7-acetyl-CoA and a subsequent incorporation into fatty acids with the loss of one additional deuterium atom during the dehydration step of fatty acid biosynthesis. Lower levels of incorporation as malonate than as acetate likely arise from a washout of deuterium label from either malonyl-CoA or the \(\beta\)-ketoacyl-ACP fatty acid intermediate (30).

**FIG. 2.** Thiolactomycin inhibition of palmitate biosynthesis from D\(_7\)-butyryl-CoA. The FAS activity was calculated based on the fraction of methyl palmitate, in the GC-MS assay, that is deuterated and is expressed as a percentage of that obtained in the control experiment.

**FIG. 3.** Fatty acid profiles of S. collinus. S. collinus was grown in minimal medium in the absence (top) and presence (bottom) of thiolactomycin. Std, lauric acid standard; A, isomyristate (C\(_{14}\)); B, myristate (C\(_{14}\)); C, isopentadecanoate and anteisopentadecanoic acid (C\(_{15}\)); D, pentadecanoic acid (C\(_{15}\)); E, isopalmitate (C\(_{16}\)); F, palmitoleate (C\(_{16:1}\); the position of the double bond is undetermined); G, palmitate (C\(_{16}\)).

CoA. In experiments with D7-acetate, addition of thiolactomycin resulted in an increase in the amounts of both unlabeled palmitate and labeled palmitate made from D7-acetate. This result suggests that the biosynthesis of palmitate from an acetyl-CoA pool is not inhibited by low levels of thiolactomycin. In contrast to the results of the D7-acetate experiments, the experiments with D6-butyrate demonstrated that addition of thiolactomycin led to a decrease in the levels of labeled palmitate made from D6-butyryl-CoA (although the overall amount of palmitate once again increased). These two sets of experiments suggest that thiolactomycin inhibits palmitate biosynthesis from butyryl-CoA but not acetyl-CoA. Two additional observations provide support for these suggestions. In the experiments with D7-butyrate, the addition of thiolactomycin resulted in an increase in the amount of palmitate made from D7-acetyl-CoA but a decrease in the levels of palmitate made from D6-butyryl-CoA.

In the experiments using D7-acetate, the amount of the palmitate pool labeled by using a D7-acetyl-CoA starter unit ranged from 6 to 28\% (Fig. 4). Substantially lower levels of incorporation of deuterated malonyl-CoA into palmitate and other fatty acids were observed (3 to 5\% labeling at each of the malonate-derived positions with one deuterium atom). This labeling likely reflects the generation of D2-malonyl-CoA from D7-acetyl-CoA and a subsequent incorporation into fatty acids with the loss of one additional deuterium atom during the dehydration step of fatty acid biosynthesis. Lower levels of incorporation as malonate than as acetate likely arise from a washout of deuterium label from either malonyl-CoA or the \(\beta\)-ketoacyl-ACP fatty acid intermediate (30).
Effect of thiolactomycin on the fatty acid profile of the S. collinus ccr mutant. It has previously been suggested that crotonyl-CoA reductase may play a significant role in providing butyryl-CoA from straight-chain fatty acid biosynthesis in S. collinus. We have investigated this hypothesis by generating a strain of S. collinus in which the crotonyl-CoA reductase gene (ccr) was disrupted. This ccr mutant and the wild-type strain of S. collinus, grown under identical conditions, exhibited indistinguishable fatty acid profiles. The major straight-chain fatty acid, palmitate, represented 18% of the total fatty acid pool for both the wild type and the ccr mutant. Addition of perdeuterated valine to the fermentation broth resulted in an 83% labeling of the isopalmitate pool with seven deuterium atoms and a 14% labeling of the palmitate pool with deuterium in both strains.

**DISCUSSION**

A type II FAS for straight- and branched-chain fatty acid biosynthesis. Initial reports of fatty acid biosynthesis in Streptomyces coelicolor and Saccharopolyspora erythraea (formerly Streptomyces erythraeus) indicated a type I FAS (6, 28). More recent enzymatic and genetic analyses of fatty acid biosynthesis in S. coelicolor, Streptomyces glaucescens, S. collinus, and Saccharopolyspora erythraea have indicated that these systems have a type II FAS (23, 24, 32, 38). The possibility of both a type I and a type II FAS in streptomycetes has yet to be definitively ruled out (24) but appears to be inconsistent with reports that mutants of either S. glaucescens or S. coelicolor cannot be obtained with a disruption in the putative type II fatty acid biosynthetic genes clusters (23, 32). The clear demonstration that straight-chain fatty acid biosynthesis in dialyzed cell extracts of S. collinus is greatly stimulated by addition of an ACP suggests a type II FAS is at least the major straight-chain FAS activity in these extracts. It has previously been shown that branched-chain fatty acid biosynthesis from isobutyryl-CoA in Saccharopolyspora erythraea was similarly dependent upon addition of the E. coli ACP (8). A background rate of fatty acid biosynthesis observed in both S. collinus and Saccharopolyspora erythraea (8) may be due to the presence of the endogenous fatty acid ACP or even the presence of a type I FAS. Consistent with the suggestion of a type II FAS in S. collinus was the

**TABLE 1. Effect of thiolactomycin on the fatty acid profile of S. collinus**

<table>
<thead>
<tr>
<th></th>
<th>bC14</th>
<th>bC15</th>
<th>bC16</th>
<th>C14</th>
<th>C15</th>
<th>C16:1</th>
<th>C16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>54.8</td>
<td>89.4</td>
<td>72.4</td>
<td>8</td>
<td>11.8</td>
<td>10.2</td>
<td>20.4</td>
</tr>
<tr>
<td>Thiolactomycin</td>
<td>9.8 (18%)</td>
<td>22 (25%)</td>
<td>7.8 (11%)</td>
<td>34.6 (433%)</td>
<td>&lt;3.8 (&lt;32%)</td>
<td>26.6 (262%)</td>
<td>50 (245%)</td>
</tr>
</tbody>
</table>

*Fatty acids were obtained from a 10-ml fermentation and were estimated based on comparison with a lauric acid standard and the caveat that all the fatty acids extract into hexane or ether with the same efficiency. These data are from one experiment. Values in parentheses are percentages of the values obtained in control experiments. In more than 20 additional experiments, an increase in the ratio of straight-chain to branched-chain fatty acids was always observed in the presence of thiolactomycin (the extent of this change was dependent upon experimental conditions, such as inoculum size). Thiolactomycin was added at the time of inoculation to a final concentration of 0.1 mg/ml. The levels of branched-chain C17 fatty acids produced in control experiments were also observed to decrease in the experiments with thiolactomycin (data not shown). Abbreviations: bC14, isomyristate; bC15, iso- and anteisopentadecanoate; bC16, isopalmitate; C14, myristate; C15, pentadecanoate; C16:1, hexadecenoate (an isomer of palmitoleate); C16, palmitate.

**FIG. 4.** In vivo effect of thiolactomycin on the utilization of labeled acetate, butyrate, and isobutyrate for palmitate (A) and isopalmitate (B) biosynthesis in S. collinus.
in vitro and in vivo inhibition of both branched- and straight-chain fatty acid biosynthesis by thiolactomycin, a known type II FAS inhibitor (10, 11, 20, 21, 31, 35). While this in vitro inhibition (50 μg/ml) is substantially less than that reported for *E. coli* (0.4 μg/ml), it contrasts with reports that *Bacillus subtilis* (also a producer of branched- and straight-chain fatty acids) is not significantly inhibited by thiolactomycin at concentrations as high as 200 μg/ml (1). Furthermore, it has been shown that branched-chain fatty acid biosynthesis in a number of *Bacillus* species is largely insensitive to thiolactomycin (1).

**Acetyl-CoA and butyryl-CoA function as starter units for palmitate biosynthesis.** In the majority of FASs studied, straight-chain fatty acids are biosynthesized by elongation of an acetyl-CoA starter unit (16). However, in vivo analysis has suggested that in some systems which generate a mixture of branched- and straight-chain fatty acids, butyryl-CoA can function more effectively than acetyl-CoA as a primer for straight-chain fatty acid biosynthesis (16). Previous in vivo analysis of *S. collinus* has indicated that butyryl-CoA also functions efficiently as a starter unit for straight-chain fatty acid biosynthesis (38). Consistent with these conclusions are in vitro studies of *S. collinus* which demonstrate that at the same concentration butyryl-CoA is a considerably more effective primer for straight-chain fatty acid biosynthesis than either acetyl-CoA or hexanoyl-CoA. However, acetyl-CoA is a common intermediate in cellular metabolism and likely exists at a higher concentration than either butyryl-CoA or hexanoyl-CoA. The present work has demonstrated that acetyl-CoA used at substantially higher concentrations than butyryl-CoA can function effectively as a starter unit for straight-chain fatty acid biosynthesis in an *S. collinus* cell extract. This observation indicates that in vivo, acetyl-CoA may play a significant role in straight-chain fatty acid biosynthesis. Supporting evidence for this conclusion was obtained from the in vivo studies, which clearly showed that D3-acetate was utilized as a starter unit for palmitate biosynthesis.

Analysis of a ccr mutant of *S. collinus* provides additional support for a significant role of acetyl-CoA in straight-chain fatty acid biosynthesis. Previously it has been shown that in *S. collinus* grown in the presence of 250 mM valine, approximately 14% of the total cellular palmitate was generated by using butyryl-CoA generated from the valine catabolite isobutyryl-CoA. Two possible interpretations of this observation are that (i) additional pathways provide butyryl-CoA for straight-chain fatty acid biosynthesis, and (ii) the majority of straight-chain fatty acids are not made by using butyryl-CoA. Evidence for the former of these has been the isolation and characterization of a crotonyl-CoA reductase from *S. collinus* involved in catalyzing the last step in the biosynthesis of butyryl-CoA from two acetyl-CoA molecules. If crotonyl-CoA reductase plays a role in providing butyryl-CoA for fatty acid biosynthesis, an *S. collinus* ccr mutant would be expected to either (i) produce fewer straight-chain fatty acids or (ii) produce a greater proportion of the straight-chain fatty acids by using butyryl-CoA derived from the valine catabolism pathway. A ccr mutant of *S. collinus*, however, exhibited neither of these phenotypes; the percentage of palmitate generated in both the wild type and the ccr mutant of *S. collinus* was unchanged, and a labeling study with perdeuterated valine suggested that in both cases approximately 84% of the palmitate was generated without using the valine-derived butyryl-CoA. Accordingly, the majority of palmitate in *S. collinus* must be made either by using butyryl-CoA derived from a hitherto-unknown pathway or, as suggested above, an alternative starter unit, such as acetyl-CoA. Additional support for the role of acetyl-CoA in initiation of straight-chain fatty acid biosynthesis was provided by in vivo studies with thiolactomycin.

**Thiolactomycin has differing effects in vivo on branched-chain and straight-chain fatty acid biosynthesis.** Thiolactomycin in vivo appeared to inhibit branched-chain fatty acid biosynthesis and activate straight-chain fatty acid biosynthesis. The increase in saturated straight-chain fatty acids was accompanied by an increase in unsaturated straight-chain fatty acids. As the overall levels of fatty acids decreased slightly, it appears that thiolactomycin redirected an inhibited fatty acid synthetic process. A change from branched-chain to straight-chain fatty acids has been observed with an *S. avermitilis* mutant genetically blocked in the formation of isobutyryl-CoA and methylbutyryl-CoA (and therefore unable to produce branched-chain fatty acids) and with *S. cinnamonosensis* grown on a synthetic medium with increasing concentrations of sodium chloride (the changes in the fatty acid profiles in this case were considerably less than reported herein) (5, 18, 22). In all of these cases the bacteria appear to compensate for the loss of the branched-chain anteiso-C15 acids, which are important for reg-

---

**Table 2. In vivo effect of thiolactomycin on utilization of labeled acetate, butyrate, and isobutyrate for palmitate and isopalmitate biosynthesis in *S. collinus***

<table>
<thead>
<tr>
<th>Expt</th>
<th>Unlabeled</th>
<th>D7-acetate</th>
<th>D7-butyrate</th>
<th>Total palmitate</th>
<th>Unlabeled</th>
<th>D6-isobutyrate</th>
<th>Total isopalmitate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>34.8</td>
<td>2.80</td>
<td>37.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiolactomycin</td>
<td>238</td>
<td>67</td>
<td>305</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Addition of D2-acetate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>0</td>
<td>22.1</td>
<td>30.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiolactomycin</td>
<td>90</td>
<td>5</td>
<td>32</td>
<td>127</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Addition of D2-butyrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>34.03</td>
<td>2.27</td>
<td>53.7</td>
<td>90</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiolactomycin</td>
<td>89.1</td>
<td>13.5</td>
<td>25.4</td>
<td>128</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Addition of D6-isobutyrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>97.1</td>
<td>77.5</td>
<td>174.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiolactomycin</td>
<td>14.6</td>
<td>10.6</td>
<td>25.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
ulation of membrane fluidity (15), by producing unsaturated straight-chain fatty acids.

The thiolactomycin-dependent decrease in the levels of isopalmitate and other branched-chain fatty acids was matched by an analogous decrease in the levels of labeled isopalmitate obtained when D₇-isobutyrate was included in the fermentation broth. This in vitro data indicates that thiolactomycin inhibits branched-chain fatty acid biosynthesis from isobutyryl-CoA (in agreement with in vitro data). A decrease in the levels of labeled palmitate obtained from D₇-butyrate also indicates that in vivo, thiolactomycin inhibits straight-chain fatty acid biosynthesis from butyryl-CoA (in agreement with in vitro data). Despite this inhibition, the levels of some straight-chain saturated and unsaturated fatty acids actually increase in the presence of thiolactomycin. This observation suggests that an additional pathway or initiation point for straight-chain fatty acid biosynthesis operates in S. collinus. This process should be less sensitive to thiolactomycin and might, as suggested above, use acetyl-CoA rather than butyryl-CoA as a starter unit. Compelling evidence for this suggestion was the observation that the levels of labeled palmitate made by using a perdeuterated acetate starter unit increased in the presence of thiolactomycin (in sharp contrast to the incorporation results obtained with labeled butyrate and isobutyrate).

**Differential pathways for initiation of straight-chain and branched-chain fatty acid biosynthesis in S. collinus.** One interpretation of the data presented herein is that S. collinus has both a thiolactomycin-sensitive type II FAS (responsible for branched-chain fatty acid biosynthesis) and a thiolactomycin-insensitive type I FAS (responsible for palmitate biosynthesis from acetyl-CoA). Thiolactomycin is known to be selective against type II FASs (10, 11, 20, 21, 31, 35). Although this possibility cannot be excluded, there is no firm evidence of a type I FAS in any streptomycete. Alternatively, the results may indicate that thiolactomycin simply exposed a difference in the initiation of fatty acid biosynthesis from acetyl-CoA and butyryl-CoA, isobutyryl-CoA, or even methylbutyryl-CoA. Two different pathways for initiation of fatty acid biosynthesis have been proposed for E. coli and plants (7, 17), systems which produce exclusively straight-chain fatty acids. In one of these pathways, 3-ketoacyl synthase III (KASIII) catalyzes the condensation of acetyl-CoA with malonyl-ACP to form β-ketobutyryl-ACP (13, 17). In the second pathway, acetyl-CoA is first transacylated to form acetyl-ACP and then condensed with malonyl-ACP by using 3-ketoacyl synthase I (KASI) (17). It is unclear if the transacylation reaction is carried out exclusively by KASIII (which has been shown to have this activity) (4, 34) or by a separate acyl-CoA/ACP transacylase activity (ACAT) (7, 17). Similar roles can be considered for KASIII and an acyl-CoA/ACP transacylase activity in the initiation of fatty acid biosynthesis from the various starter units in S. collinus (Fig. 5). The thiolactomycin results presented herein can be rationalized if the initiation step with butyryl-CoA and isobutyryl-CoA (and presumably methylbutyryl-CoA and propionyl-CoA) is both different from that utilizing acetyl-CoA and more sensitive to thiolactomycin than any other step in the fatty acid biosynthetic process (thiolactomycin is known to have differing effects on the various enzymes involved in fatty acid biosynthesis in plants and E. coli [12, 14, 19, 35]). To date, however, there is no information regarding the individual enzymes involved in initiation of fatty acid biosynthesis in streptomycetes. However, it has been suggested that acetyl-ACP may function as a starter unit for straight-chain fatty acid biosynthesis in streptomycetes (33). While there is no direct data supporting this suggestion, it has been shown that in B. subtilis (which also produces predominantly branched-chain fatty acids) acetyl-ACP, but not acetyl-CoA, is an excellent primer for straight-chain fatty acid biosynthesis (3, 15). In addition, the recent cloning of the putative fatty acid biosynthetic gene clusters of S. coelicolor and S. glaucescens has revealed genes which would encode proteins with high homology to E. coli KASIII (23, 32). It should now be possible to investigate both the role of these putative streptomycete KASIII enzymes in the initiation of

**FIG. 5. Potential pathways for initiation of fatty acid biosynthesis in S. collinus.**
straight- and branched-chain fatty acid biosynthesis and the basis for the unusual in vivo effect of thiolactomycin on the fatty acid profile of *S. collinus*.

**ACKNOWLEDGMENTS**

Financial support for this work was provided by the National Institutes of Health (GM 50541) and Pfizer Incorporated.

**REFERENCES**