Bioscience, Biotechnology, and Biochemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbbb20

Biosynthesis of Branched-chain Fatty Acid in Bacilli: FabD (malonyl-CoA:ACP transacylase) Is Not Essential for In Vitro Biosynthesis of Branched-chain Fatty Acids

Hirosuke OKU[a], Naoya FUTAMORI[a], Kenichi MASUDA[a], Yumiko SHIMABUKURO[a], Tomoyo OMINE[a] & Hironori IWASAKI[a]

[a] Division of Molecular Biotechnology, Center of Molecular Bioscience, University of the RyukyusNishihara, Okinawa 903-0213, Japan

Published online: 22 May 2014.

Biosci. Biotechnol. Biochem., **67** (10), 2106–2114, 2003

Biosynthesis of Branched-chain Fatty Acid in *Bacilli*: FabD (malonyl-CoA:ACP transacylase) Is Not Essential for *In Vitro* Biosynthesis of Branched-chain Fatty Acids

Hirosuke Oku,† Naoya Futamori, Kenichi Masuda, Yumiko Shimabukuro, Tomoyo Omine, and Hironori Iwasaki

Division of Molecular Biotechnology, Center of Molecular Bioscience, University of the Ryukyus, Nishihara, Okinawa 903-0213, Japan

Received March 26, 2003; Accepted July 7, 2003

It was found that the partially purified β-ketoacyl-ACP synthase of *Bacillus insolitus* did not require the addition of FabD (malonyl-CoA:ACP transacylase, MAT) for the activity assay. This study therefore examined the necessity of FabD protein for *in vitro* branched-chain fatty acid (BCFA) biosynthesis by crude fatty acid synthetases (FAS) of *Bacilli*. To discover the involvement of FabD in the BCFA biosynthesis, the protein was removed from the crude FAS by immunoprecipitation. The His-tag fusion protein FabD of *Bacillus subtilis* was expressed in *Escherichia coli* and used for the preparation of antibody. The rabbit antibody raised against the expressed fusion protein specifically recognized the FabD in the crude FAS of *B. subtilis*. Evaluation of the efficacy of the immunoprecipitation showed that a trace of FabD protein was present in the antibody-treated crude FAS. However, this complete removal of FabD from the crude FAS did not abolish its BCFA biosynthesis, but only reduced the level to 50–60% of the control level for acyl-CoA primer and to 80% for α-keto-β-methylvalerate primer. Furthermore, the FabD concentration did not necessarily correlate with the MAT specific activity in the enzyme fractions, suggesting the presence of another enzyme source of MAT activity. This study, therefore, suggests that FabD is not the sole enzyme source of MAT for *in vitro* BCFA biosynthesis, and implies the existence of a functional connection between fatty acid biosynthesis and another metabolic pathway.

Key words: branched-chain fatty acid; biosynthesis; *Bacillus*; malonyl-CoA; ACP

Fatty acids are the most important component of bacterial membrane lipids. The chemical structure of fatty acids occurring in bacteria is classified largely into two types: straight-chain fatty acids (SCFAs) and branched-chain fatty acids (BCFAs) of the iso- and anteiso-series. The SCFA synthesis represented by the *E. coli* system has been studied in detail, and the biosynthesis pathway consists of a set of individual enzyme proteins (type II) that function in concert to produce a variety of fatty acids.[1,2] Although the lower activity of BCFA biosynthesis in the cell-free system of *Bacilli* has severely impeded the efforts to establish a detailed synthetic mechanism by using the individually isolated enzymes, the available evidence strongly supports the view that BCFAs in bacteria are synthesized by a mechanism very similar to that of SCFA synthesis operating in *E. coli*.[3]

The difference between SCFA and BCFA biosynthesis thus appeared to lie in the precursor specificity of the initiation step: the substrate specificity of acyl-CoA:ACP transacylase (step B in Fig. 1)[4] or the first step condensing enzyme of β-ketoacyl-ACP synthase III (KAS III) FabH (steps C, D, and E in Fig. 1). It has actually been shown by using a His-tag fusion protein of FabH that the *Bacillus* FabH (bFabH) is a determining factor in BCFA biosynthesis.[5] There are at least two FabH (KAS III) like protein and one FabB/F (KAS I/II) homologue encoded by the *B. subtilis* genome.

The initiation of the condensing reaction (steps C, D, and E in Fig. 1) usually requires the presence of malonyl-CoA:ACP transacylase (MAT) FabD that catalyzes the conversion of malonyl-CoA to malonyl ACP (step A in Fig. 1). It was also shown that the *B. subtilis* fabD gene complemented the MAT deficiency of an *E. coli* mutant.[6] However, we found in the course of enzyme purification work that partially purified native KAS from *B. insolitus* did not necessarily require the addition of FabD for the activity assay. Thus this study clarified the requirement of FabD for BCFA biosynthesis *in vitro* and discussed the necessity of FabD for the condensation reaction in *Bacilli*.

† To whom correspondence should be addressed. Tel/Fax: +81-98-895-8972; E-mail: okuhiros@comb.u-ryukyu.ac.jp

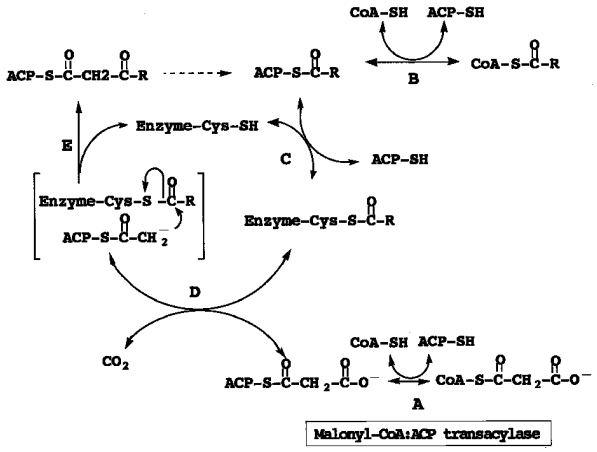

Fig. 1. Schematic Representation of the Condensing Reaction in the Fatty Acid Biosynthesis.

Both malonyl-CoA and acyl-CoA become available to the β-ketoacyl-ACP synthase (KAS) after their conversion into ACP thioesters by steps A and B. FabD is thought to be responsible for charging the ACP subunit with a malonyl group (step A). The enzyme KAS catalyzes the condensation of acyl-ACP and malonyl-ACP to produce β-ketoacyl-ACP. The first step is the transacylation of the acyl-moiety from acyl-ACP to the active site cysteine of KAS. The enzyme next reacts with malonyl-ACP and releases CO_2. The condensing enzyme assay in this study thus measured the release of [^{14}C]-CO_2 from [^{14}C]-malonyl-CoA as described in the methods section.

Materials and Methods

Materials. [^{14}C]-Malonyl-CoA and [^{14}C]-isoleucine were purchased from Muromachi Chemicals Co. [^{14}C]-isoleucine was converted into the corresponding α-ketoacid using L-amino acid oxidase and catalase (both from Sigma-Aldrich Japan.) as described previously.[7] Malonyl-CoA and acyl carrier protein from *E. coli* were from Sigma-Aldrich Japan Co. Other chemicals used were all the highest grade and were obtained from the domestic suppliers.

Construction of expression vectors and purification of His-tag protein. The malonyl-CoA:ACP transacylase gene (fabD) of *B. subtilis* was amplified with a genomic DNA as PCR template. The forward and reverse primers designed based on the FabD gene sequence in the DNA database "SubtiList" respectively created a restriction site for *Nde*I and *Bam*HI at the 5′ terminus, and consisted of 5′-CATGCATATGATTGCATTTTTATTCCCGG and 5′-TTTAGGATCCAGCATTATCATTCTCCTCCT. The PCR products were first ligated into pCRII (Invitrogen) by use of a TOPO-TA cloning kit (Invitrogen), and used to transform TOPO10-competent *E. coli* cells. A plasmid was isolated and digested with *Nde*I and *Bam*HI. The released insert DNA fragment were separated by agarose gel electrophoresis, and ligated into pET-15b digested previously with *Nde*I and *Bam*HI. The ligation mixture was used to transform a BL21(DE3) competent cell strain. The plasmid harboring the fabD gene was isolated, and sequenced to verify the absence of PCR artifacts. Protein expression was induced according to the manufacture's instruction manual. The His-tag fusion protein was purified by use of His Trap chelating affinity column (Amersham Pharmacia Biotech).

Preparation of short-chain acyl-CoA. The branched short-chain fatty acids were converted into their thioesters as described previously.[8] Briefly, the short-chain fatty acid (5 μmol) was reacted with carboxydiimidazole (6 μmol) in 0.2 ml of tetrahydrofuran to give a rise to 1-acylimidazole. After 30 min of reaction at room temperature, the solvent was evaporated off, and the reaction product was dissolved in 0.2 ml tetrahydrofuran-H_2O (2:1 by vol.). The solution was allowed to react with 5 μmol of CoASH in 0.5 ml of tetrahydrofuran-H_2O (2:1 by vol.). The reaction was continued for 4 hours at room temperature with a nitrogen atmosphere. Tetrahydrofuran was evaporated off, and the residual aqueous solution was acidified to pH 3–4 by the addition of Dowex 50 (H^+). Dowex 50 was removed by filtration, and the filtrate was extracted with diethyl ether (0.5 ml × 3 times) to remove unreacted fatty acid. The concentration of acyl-CoA thioester was measured by DTNB (5,5′-dithiobis-2-nitrobenzoic acid) method as described previously.[9] Acyl-CoA thioester synthesized by this method was usually over 90% pure on the basis of free and esterified SH assays.[9]

Bacterial strain and cell culture. The strains used for the preparation of crude enzyme were *Bacillus subtilis* (ATCC23857) and *Bacillus insolitus* (ATCC-23299). Cells were suspended in 15% glycerol and stored at −70°C. The culture medium for *B. subtilis* contained: glucose, 10 g; yeast extract, 1 g; Bacto peptone, 1 g; K_2HPO_4, 1.5 g; NH_4H_2PO4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $ZnSO_4 \cdot 7H_2O$, 10 mg; $CuSO_4 \cdot 5H_2O$, 0.5 mg; $MnCl_2 \cdot 4H_2O$, 4.5 mg; and $FeSO_4 \cdot 7H_2O$, 10 mg, in 1 liter of distilled water.[10] The culture medium for *B. insolitus* was Soybean-Casein-Digest broth supplied by Nissui Pharmaceutical Co. (Tokyo, Japan). The indicated concentration of the medium was peptone 17 g, soybean-peptone 3 g, NaCl 5 g, glucose 2.5 g, and K_2HPO_4 2.5 g per litter.

The seed culture in 20 ml was inoculated with the stock cells, and was incubated 7 hours at 30°C for *B. subtilis*, and 16 hours at 20°C for *B. insolitus*. One litter of medium was inoculated with 10 to 20 ml of seed culture, and cells were harvested at the late exponential growth phase by centrifugation. The yield of cells was usually in the range of 5–6 g wet weight/l culture medium.

Preparation of crude enzyme. A typical procedure for the preparation of crude enzyme was as follows. Pelleted cells were suspended in 3 vol. of 10 mM phosphate buffer (pH 7.0) containing 2 mM 2-mercaptoethanol and 10% glycerol, and were sonicated with an ultrasonic generator (Nihon Seiki Seisakusho Co., Tokyo) for 3×3 min with 1-min cooling intervals. The irradiated cell suspension was centrifuged for 28,000×G for 30 min, and the supernatant was again centrifuged at 156,000×G for 2 hours. To the supernatant was added streptomycin sulfate to make its concentration equivalent to protein in the extract. The precipitate formed was then removed by centrifugation at 28,000×G for 20 min. The supernatant obtained after streptomycin treatment was mixed with 1/10 vol. of 1 M phosphate buffer (pH 7.0) and with $(NH_4)_2SO_4$ to 60% saturation. The precipitate formed was collected by centrifugation, and dissolved in a minimal volume of 10 mM phosphate buffer (pH 7.0). The final protein concentration was usually from 20 to 60 mg/ml.

Purification of the condensing enzyme. Purification of the condensing enzyme was done by a use of fast protein liquid chromatography (FPLC) system (Amersham-Pharmacia Biotech) or perfusion chromatography system (Perceptive Biosystems). The crude enzyme (0–60% $(NH_4)_2SO_4$ precipitate was diluted 5 to 9 times with the starting buffer containing 20 mM histidine-HCl (pH 5.5), 2 mM dithiothreitol (DTT) and 10% glycerol, and was put on a Mono Q HR 10/10 column (Amersham-Pharmacia Biotech) which had previously been equilibrated with the same buffer. The column was washed with 3 ml of starting buffer and was eluted with a linear gradient of 0–0.65 M NaCl in 27 ml of the buffer with the flow rate of 1.5 ml/min.

The fractions containing the condensing enzyme were mixed with 3 M $(NH_4)_2SO_4$ to make 1.8 M, and put on a reversed-phase column PE/M (4.6 mm ID×100 mm, Perceptive Biosystem) equilibrated with 10 mM phosphate buffer (pH 7.0) containing 1.8 M $(NH_4)_2SO_4$, 2 mM DTT and 10% glycerol. The sample was eluted with a negative linear gradient of 1.8–0 M $(NH_4)_2SO_4$ in a 15 ml of eluent. The active enzyme fractions were combined and concentrated by ultrafiltration (Centricon 30 or 50, Amicon Inc.) to 0.2 ml, and put onto Superose 12 HR 10/30 column (Amersham-Pharmacia Biotech) that had been previously equilibrated with the elution buffer containing 50 mM potassium phosphate (pH 6.0), 0.15 M NaCl, 2 mM DTT, and 10% glycerol. The sample was eluted with a flow rate of 0.2 ml/min.

Condensing enzyme assay. [^{14}C]-CO_2 liberated by the action of the condensing enzyme from [1,3-^{14}C]-malonyl-CoA was collected in NaOH, and used for measurement of radioactivity.[11] The reaction mixture contained 50 mM triethanolamine (pH 7.5), 1 mM DTT, 2.5 μM ACP, 40 μM [1,3-^{14}C]-malonyl-CoA (37 kBq/μmol) in 0.5 ml. The reaction was incubated at 30°C for 30 min, and stopped by addition of 0.2 ml 5% $HClO_4$. The [^{14}C]-CO_2 produced was collected into 0.2 ml of 2 N NaOH for 60 min in a closed reaction tube, and was counted with 8 ml of scintillation fluid (ACS II).

Fatty acid synthetase (FAS) activity. Fatty acid synthetase (FAS) activity was measured by incorporation of [U-^{14}C]-α-keto-β-methylvalerate (KMV) (740 kBq/μmol) or [1,3-^{14}C]-malonyl-CoA (37 kBq/μmol) into fatty acid. The radiolabeled fatty acid was isolated from the reaction mixture by a solvent extraction procedure. The standard reaction mixture contained, in mM: triethanolamine, 50 (pH 7.5); DTT, 2; thiaminepyrophosphate, 0.5; NADPH, 0.5; malonyl CoA, 0.1; and [U-^{14}C]-KMV 0.01, in a total volume of 500 μl. In the case where cold acyl-CoA was the precursor, [U-^{14}C]-KMV was replaced with cold acyl-CoA (0.1 mM), and cold malonyl-CoA was replaced with [1,3-^{14}C]-malonyl CoA (0.04 mM). The reaction mixture was incubated at 30°C for 30 min. The enzyme reaction was stopped by the addition of 0.025 ml of 6 N HCl, and the acidified reaction mixture was centrifuged at 600×*g* for 5 min. The supernatant was mixed with 0.35 ml of H_2O, 0.5 ml of methanol and 3 ml of CCl_4 in a glass-stoppered test tube. The lower layer (CCl_4) was washed twice with 2.5 ml of 50% methanol containing 0.05 N HCl. The washed CCl_4 layer was evaporated to dryness by flushing with N_2, and its radioactivity was counted with a toluene scintillator.

Malonyl-CoA:ACP transacylase (MAT) assay. The MAT activity assay was done essentially as described elsewhere.[12] Briefly, the reaction mixture containing 7.5 μM [1,3-^{14}C]-malonyl-CoA (2035 kBq/μmol), 10 μg ACP (23.2 μM), 1 mM DTT, 100 mM imidazole-HCl (pH 7.0), and up to 1 μg of FabD in a final volume of 50 μl was incubated at 30°C for 5 min. The reaction was stopped with 0.4 ml of 5% perchloric acid, followed by the addition of 370 μg of carrier crude ACP prepared from a *B. subtilis* homogenate. The precipitate formed was collected by filtration with a 0.2 μm pore size membrane filter, and was further washed twice with 0.5 ml of 5% perchloric acid. The malonyl-ACP present in the precipitate was re-dissolved in 0.5 ml 0.1 N NaOH, and counted for its radioactivity.

Preparation of antibody. Antibody against the purified His-tag fusion protein FabD was raised in a male Japanese white rabbit with a mixture of equal volume of Freud's complete adjuvant and 203 μg antigen FabD protein. The animal received the injection twice a week for one month, and was bled after the

enough antibody production was confirmed by double immunodiffusion analysis.[13]

Enrichment of IgG was made by $(NH_4)_2SO_4$ fractionation as follows: the rabbit serum was mixed with an equal volume of 50% $(NH_4)_2SO_4$ in 50 mM phosphate buffer (pH 7.0). After 20 min, the precipitate formed was collected by centrifugation (12000 × G, 10 min), and washed with 25% $(NH_4)_2SO_4$ in 50 mM phosphate buffer (pH 7.0). The washed precipitate was dissolved in 20 mM phosphate buffer, (pH 7.0) and dialyzed overnight against the same buffer. The dialyzed solution was centrifuged, and the supernatant was stored at −70°C until needed.

Immunoprecipitation. The crude FAS was mixed with an equal amount of IgG to give the final enzyme concentration of 19.6 mg/ml. Since the crude FAS contained 1.04 μg FabD/mg total protein, the final concentration of FabD in the mixture was 20 μg/ml. The mixture containing 3 mg of enzyme protein was used with or without centrifugation for the BCFA biosynthesis or MAT assay. In the case of control, IgG was replaced by bovine serum albumin to adjust the total protein concentration.

Western blotting. Samples separated on SDS-PAGE were transblotted to nitrocellulose membranes as described elsewhere. The membrane was washed with phosphate-buffered saline containing Tween 20 (PBS-T): 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, 0.1% Tween 20, and subsequently incubated in a 10% skim milk blocking solution for 30 min with occasional shaking. The membrane was washed with PBS-T and incubated with primary antibody (anti FabD) for 60 min at room temperature. The un-bound antibody was washed out with several changes of PBS-T. The anti-FabD IgG bound to the sample was made visible by ECL Western blotting detection reagent (Amersham-Pharmacia Biotech).

Results

Figure 2 shows the SDS-PAGE of the His-tag fusion protein FabD. The expressed His-tag protein was purified to homogeneity by Ni^{2+} chelate affinity chromatography, and thereafter desalted with a PD-10 column (Amersham-Pharmacia Biotech). The estimated molecular mass on SDS-PAGE was 41 kDa, which was slightly higher than the calculated molecular size of 37 kDa. This preparation was used for the condensing enzyme assay and for the preparation of antibody.

In order to choose the bacterial strain for the purification of the condensing enzyme, we first studied *in vitro* biosynthesis of fatty acids by the cell-free homogenate of *B. subtilis* and *B. insolitus* (Fig. 3).

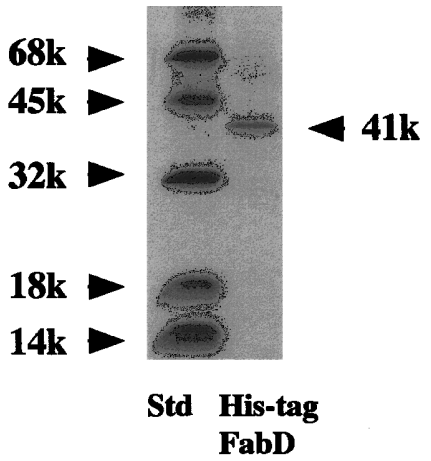

Fig. 2. SDS-PAGE of Expressed His-tag Fusion Protein FabD.
The expressed protein was purified to homogeneity and separated on a polyacrylamide gel.

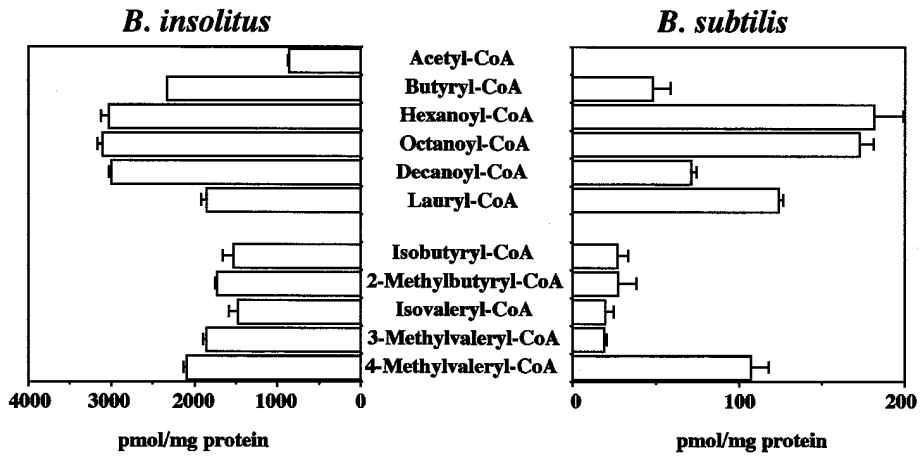

Fig. 3. Fatty Acid Biosynthesis from Short-chain Acyl-CoAs in *B. insolitus* (A) and *B. subtilis*.
The assay mixture contained 50 mM triethanolamine (pH 7.5), 2 mM thiaminepyrophosphate, 0.5 mM NADPH, 0.04 mM [1,3-^{14}C]-malonyl-CoA, 0.1 mM acyl-CoA, and 0.5–2 mg of crude FAS in 500 μl. The reaction was done at 30°C for 30 min, and the reaction product of radiolabeled fatty acid was extracted into CCl_4 for measurement of its radioactivity.

Table 1. Component Requirements of Condensing Reactions

Component omitted	Activity	
	nmol/mg	%
None (complete)	67.3 ± 3.73	100 ± 5.5
FabD	76.8 ± 16.7	114 ± 24.8
ACP	0.0 ± 0.0	0.0 ± 0.0
Hexanoyl-CoA	20.6 ± 12.4	30.6 ± 18.4
DTT	32.1 ± 12.4	47.6 ± 18.4

Data are mean ± SE (n = 3).

In contrast to the case of *E. coli*, the cell-free homogenate of *B. subtilis* and *B. insolitus* accepted a variety of acyl-CoA derivatives as a precursor for fatty acid biosynthesis. Acyl-CoAs of chain-lengths C4 to C12 were good precursors for fatty acid biosynthesis by the crude fatty acid synthetase (FAS) of *B. subtilis* and *B. insolitus*. On the whole, the crude FAS of *B. insolitus* showed a rather broad spectrum of precursor specificity, accepting both straight-chain and branched-chain acyl-CoA (BCAC) to an almost comparable extent. The relatively higher biosynthetic activity of *B. insolitus* was also notable (see the difference in the scale of X-axis). The crude FAS of *B. subtilis* surprisingly manifested a preference for straight-chain acyl-CoA, except for acetyl-CoA, rather than toward the branched-chain counterpart.

Combination of an ion-exchange column (Mono Q), a hydrophobic column (PE/M), and a gel-filtration column (Superose 12), purified the condensing enzyme 5-fold from the cell-free homogenate of *B. insolitus*. On the course of this purification, the assay component requirement of the partially purified condensing enzyme was studied (Table 1). Based on the substrate specificity of the crude FAS, hexanoyl-CoA was the acyl-CoA substrate for the condensing reaction. The reaction unexpectedly did not require the FabD, while was completely dependent on the presence of ACP. Removal of hexanoyl-CoA and DTT respectively lowered the activity to 30.6 and 47.6% of the control value. The incomplete block of the condensation reaction by withdrawal of the substrate hexanoyl-CoA may show that the decarboxylation reaction at the malonyl-ACP binding site partially proceeded even in the absence of acyl-CoAs, but not to the completion of the reaction.

The above observations prompted us to suspect the necessity of the FabD for the condensation reaction and also for the BCFA biosynthesis. There was actually a 5-fold increase in the specific activity of the condensing enzyme with the progress of the purification, even when the assay was done without FabD. Purification of KAS from *B. subtilis* encountered difficulties due to its low activity and instability, as we mentioned in the foregoing section. We therefore prepared the antibodies against FabD, and studied its effect on *in vitro* BCFA biosynthesis by crude FAS of *B. subtilis*. Figure 4 shows the Western blotting profile of the crude FAS from *B. subtilis* and *B. insolitus*. The antibody raised against the His-tag fusion protein FabD recognized essentially a single protein in both crude FASs. The densitometric analysis found that crude FAS from *B. subtilis* and *B. insolitus* respectively contained 1.04 μg and 0.37 μg of FabD/mg total protein. The crude FAS from *B. insolitus* contained the FabD in concentrations from one sixth to one third of *B. subtilis*, and contrasted with its higher BCFA biosynthetic activity compared with that of *B. subtilis*. As was expected, the native FabD in the crude synthetase moved slightly faster than the His-tag fusion protein FabD. It is thus now possible to remove only the FabD protein from the crude FAS by immunoprecipitation.

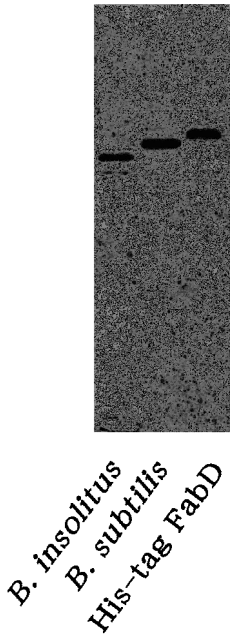

Fig. 4. Western Blotting Profile of Crude FAS from *B. subtilis* and *B. insolitus*.

The sample size was; 0.005 μg for His-tag fusion protein FabD; 5 μg crude FAS from *B. subtilis*; and 10 μg for crude FAS from *B. insolitus*. Densitometric analysis found that the concentration of FabD was 1.04 μg/mg for *B. subtilis* crude FAS, and 0.37 μg/mg for *B. insolitus* enzyme.

A preliminary experiment on double immunodiffusion found that the equivalence was reached with 0.25 mg anti-FabD IgG against 1 mg of crude FAS. To ensure the complete removal of FabD by the immunoprecipitation method, the concentration course of antibody was studied against the His-tag fusion protein FabD (Fig. 5A). In this case, the final concentration of His-tag FabD in the reaction mixture was comparable to the following immunoprecipitation experiment. As shown in Fig. 5A, addition of antibody itself had little effect on FabD activity. However, removal of the FabD-antibody complex by the centrifugation completely abolished the activity in the supernatant. Based on this experiment, an excess of antibody (1 mg against 1 μg FabD) was used

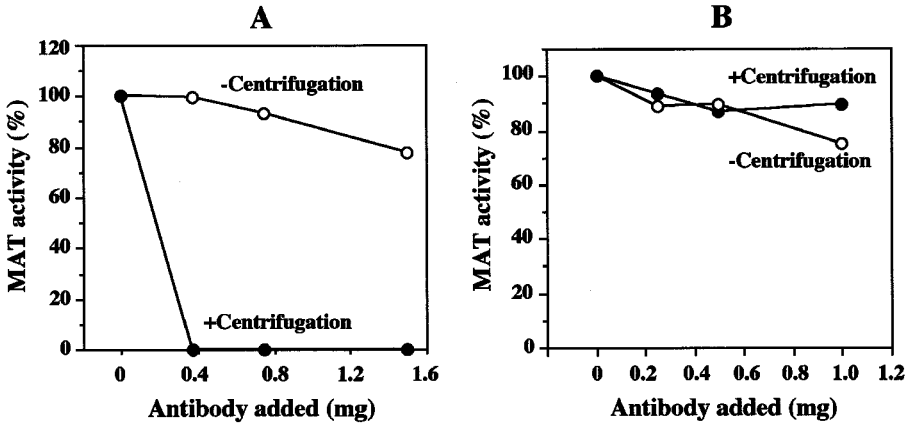

Fig. 5. Effects of Rabbit Anti-FabD on MAT Activity of His-tag Fusion Protein FabD (A) and Crude FAS from *B. subtilis* (B).

The MAT assay contained 100 mM imidazole-HCl (pH 7.0), 1 mM DTT, 10 μg ACP, 7.5 μM [^{14}C]-malonyl-CoA, His-tag fusion protein FabD (A), or crude FAS (B) both non-treated or treated with antibody at 30°C for 30 min. The resultant mixtures with (closed circle) or without centrifugation (open circle) were the enzyme source. The final concentration of FabD in the reaction mixture was adjusted to approximate the following FAS assay experiment shown in Table 2. Data are expressed as the percentages of the control activity (non-treaed enzyme). Control activity was 645 nmol/mg for His-tag fusion protein FabD (A) and was 1.89 nmol/mg for crude FAS (B).

for the later immunoprecipitation experiment to ensure the complete removal of FabD in the crude FAS.

In analogy to the constructed immunoprecipitation experiment shown in Fig. 5A, the effect of antibody on MAT activity of crude FAS was studied (Fig. 5B). The X-axis shows the amount of antibody added against 1 mg of crude FAS containing 1.04 μg FabD. Thus, the final concentration of FabD in the mixture was also similar to the following immunoprecipitation experiment. In contrast to the case of Fig. 5A, removal of FabD from the crude specimen did not significantly reduce its MAT activity.

The removal of FabD by immunoprecipitation was further assessed by Western blotting. Based on the results of concentration course experiments (Fig. 5A), an excess of antibody (1 mg) was used against 1 μg of FabD in the crude enzyme preparation. Figure 6 shows the Western blot profile of crude enzyme treated with the anti-FabD. Consistent with the foregoing observation, only a single band was present in the crude *B. subtilis* enzyme (lane 1). Treatment of the crude enzyme with non-immunized control IgG had essentially no effect on the intensity of the FabD band (lane 2). However, treatment with anti-FabD IgG significantly decreased the band intensity almost to the blank level (compare lanes 3, and 5). As shown in Fig. 6, the background level of the faint band was also detected in the antibody itself at the molecular size of FabD (lanes 4 and 5). Densitometric analysis of the band intensity and subtraction of the background level revealed that the crude enzyme contained a trace of FabD protein. The immunoreactive bands of 44 k and 68 k both originated in the IgG (lanes 4 and 5).

The crude FAS retained more than 80% of its original activity even after removal of FabD protein

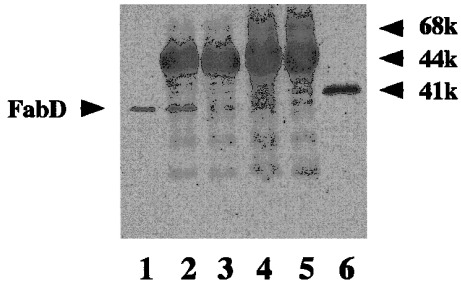

Fig. 6. Western Blotting Profile of Crude FAS Treated with Anti-FabD IgG.

Crude FAS treated with pre-immune IgG or anti-FabD IgG were analyzed by Western blotting. The sample size for crude FAS and IgG was 10 μg, and was 0.03 μg for His-tag FabD. Lane 1, control crude FAS (non-treated); lane 2, crude FAS + pre-immune IgG; lane 3, crude FAS + anti-FabD IgG; lane 4, pre-immune IgG; lane 5, anti-FabD IgG; lane 6, His-tag fusion protein FabD.

by centrifugation, suggesting the presence of another enzyme source for FabD (Fig. 5B). We therefore attempted to identify another MAT source in the crude FAS of *B. subtilis*, and examined the concentration of FabD and MAT activity in the enzyme fractions separated on Mono Q column. The highest concentration of FabD was noted around fraction 25, while the highest MAT specific activity was seen with the fractions of 20 or 21 (Fig. 7). The MAT activity thus did not necessarily correlate with the concentration of FabD in the enzyme fractions, supporting the view that there was an another enzyme source of MAT activity in the cell homogenate of *B. subtilis*. This is particularly true for the fraction 19 which lacked FabD and showed relatively higher specific activity. The gel-filtration chromatography of the combined specimens of fraction 19 and 20 found that the molecular mass for the alternate MAT activity was 58 kDa.

Effects of immunoprecipitation on the *in vitro* BCFA biosynthesis were studied with several precursors. BCFAs are synthesized from branched-chain α-keto acids (BCKAs) of valine, leucine, and isoleucine as the primer source: α-ketoisovalerate (KIV), α-ketoisocaproate (KIC), and α-keto-β-methylvalerate (KMV).[14] These BCKAs are successively decarboxylated either by BCKAs dehydrogenase to give a rise to branched-chain acyl-CoAs (BCACs): isobutyryl-CoA from KIV, isovaleryl-CoA from KIC, and α(2)-methylbutyryl-CoA from KMV, or possibly by BCKAs decarboxylase to give rise to the corresponding aldehyde derivatives.[3,15] Although the crude FAS accepts both BCKAs and BCACs for *in vitro* BCFA biosynthesis, the latter seemed to be a better substrate for the current purpose because these precursors directly react with malonyl-ACP, the reaction product of FabD, to produce fatty acids. As KMV among the BCKAs was the best precursor for crude FAS,[15] this precursor was also included as the primer in this experiment.

Removal of FabD by immunoprecipitation lowered the incorporation of [^{14}C]-malonyl-CoA into fatty acid 50 to 60% of control (none), but did not completely diminish the BCFA biosynthesis from BCAC primers (Table 2). In the case of [^{14}C]-KMV as a precursor, the treatment lowered the activity only to 80%. Thus the removal of FabD from the crude synthetase system did not necessarily eliminate the BCFA biosynthesis from both BCKA and BCAC primers.

Addition of the pre-immune serum IgG slightly elevated the level of fatty acid biosynthesis from BCACs, but not from KMV.

We observed an anti-FabD reactive band in the crude FAS of *B. insolitus* as seen in Fig. 4. However, no precipitation line between anti-FabD and the crude FAS of *B. insolitus* was formed on double immunodiffusion analysis. For this reason, no immunoprecipitation experiment was done with the crude FAS of *B. insolitus*.

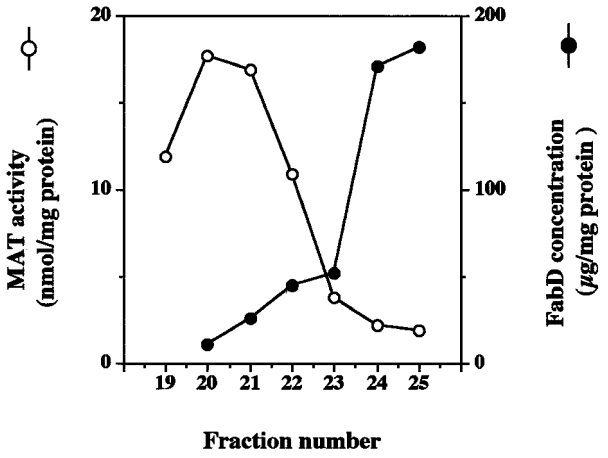

Fig. 7. MAT Specific Activity (open circle) and FabD Concentration (closed circle) in the Mono Q Column Fractions.

The concentrations for FabD were estimated by the densitometric analysis of the band intensity on Western blotting.

Discussion

The finding that FabD was not essential for the assay of the partially purified condensing enzyme from *B. insolitus* prompted us to study the necessity of FabD in the BCFA biosynthesis. This observation may simply be interpreted as that the partially purified KAS contained contaminant FabD. However, the increase in the specific activity of KAS with the progress of purification may argue against this explanation. The purification procedure usually decreases the contaminant concentration in the specimens. Thus, if FabD is essential for the condensing reaction, one can expect that the specific activity of KAS assayed without addition of FabD should be lowered by the purification. However, the purification of KAS increased the specific activity to 5-fold, which may suggest its independence of this enzyme on FabD reaction. This may in turn suggest that KAS of *Bacilli* itself has the intrinsic MAT activity.

It has been proposed that the *B. subtilis* KAS III homolog fabHA (yjaX) or fabHB (yhfB) is a deciding factor for the preference of bulky BCACs as primers of BCFA biosynthesis. His-tag fusion protein of these gene products preferentially accepted iso- and anteiso-BCAC primers as substrates rather than acetyl-CoA, which is the starter unit for the SCFA biosynthesis.[5] The nature of the condensing enzyme from *Bacilli* seemed to differ from that for SCFA biosynthesis. This explanation may further be

Table 2. Effects of Anti-FabD on BCFA Biosynthesis by the Crude Fatty Acid Synthetase of *B. subtilis*

Antibody added	Precursors[a]							
	[^{14}C]-KMV		Isovaleryl-CoA		2-Methylbutyryl-CoA		Isobutyryl-CoA	
	pmol/mg	%	pmol/mg	%	pmol/mg	%	pmol/mg	%
None	4.9±0.3	100.0±6.3	162.7±13.4	100.0±8.21	216.8±3.5	100.0±1.6	186.4±6.6	100.0±3.6
Preimune IgG	4.6±0.6	94.7±13.2	205.6±2.3	126.3±1.44	N.D.[b]	N.D.[b]	N.D.[b]	N.D.[b]
Anti-FabD IgG	4.0±0.2	82.6±3.6	101.9±3.7	62.6±2.27	107.3±2.2	49.5±1	104.8±6.5	56.2±3.5

Data are mean±SE (n=3).

[a] For precusors of CoA derivatives, the radiolabeled tracer was ^{14}C-malonyl-CoA.

[b] Not determined.

supported by their resistance to the antibiotic thiolactomycin, which selectively inhibits dissociated Type II, but not Type I, FAS.[16] Thus the studies on KAS entities in *Bacilli* may unveil the novel and unique features of this enzyme, and may address the questions raised by this study.

We herein first demonstrated that the FabD protein is not essential for *in vitro* BCFA biosynthesis by the crude FASs of *B. subtilis*. We applied the immunoprecipitation method to remove FabD protein from the crude BCFA synthetase. The antibody prepared in this study specifically recognized the FabD protein in the crude FAS (Figs. 4 and 6), and was found to be effective to remove FabD protein from the crude system. By use of a similar method, our previous study has demonstrated the requirement of BCKA decarboxylase for the BCFA biosynthesis, and confirmed the usefulness of the immunoprecipitation method.[15] The supernatant after immunoprecipitation in this study thus specifically lacked FabD protein retaining with activities of all other members of FASs. However, the removal of FabD did not necessarily eliminate the *in vitro* BCFA biosynthetic activity from the crude FAS, but only reduced the level to 50–60% for the case of BCACs, and to 80% for KMV primer. This finding clearly indicates that the FabD in the crude system is not essential for the BCFA biosynthesis.

The point to mention is that there may be an alternate enzyme source that can catalyze the MAT reaction. We attempted to identify another enzyme source in the crude FAS, and detected alternate MAT activity apart from FabD protein (Fig. 7). Thus, the observation that the removal of FabD from the crude FAS did not eliminate the MAT activity in the crude enzyme may be explained by the presence of another enzyme source for FabD. A search of the *B. subtilis* genome database found three MAT homologues described as involved in polyketide synthesis: PksC, PksE, and PksD with the similarity of 56, 54, and 40% to FabD, respectively. The polyketide synthase (PKS) and FAS are related not only in their mechanism of carbon chain extension but also in their primary sequences. It thus appeared hold true that these two systems evolved from a common origin after early gene duplication. As the many biochemical steps of FAS and PKS are similar, it seems quite reasonable that these two system share common enzymatic activities up to the point at which the pathways diverge. In this context, it is noteworthy that FabD has been implicated as functioning to charge the ACP subunit of FAS and PKS prior to the condensation reaction.[17] Thus the gene product of MAT homologues in the crude extract may be a potential alternate enzyme source, and may compensate the deficiency of the FabD activity for BCFA biosynthesis.

The independence of BCFA biosynthesis on FabD protein may alternatively agree in part with the previous observation that the reconstituted polyketide synthase (PKS) from *Streptomyces coelicolor* produced polyketides without MAT when holo ACP is present in excess. The polyketides are synthesized by a similar mechanism that resembles fatty acid biosynthesis. In that case, the PKS-ACP catalyses selfmalonylation, and provides malonyl-ACP for the successive condensing reaction.[18–20] As is the case of PKS, it also may be possible that the ACP of *B. subtilis* undergoes selfmalonylation, and may serve as a chain extension unit of fatty acid biosynthesis. To address this question, we studied the selfmalonylation of *B. subtilis* ACP under the same conditions as the MAT assay, but in the absence of a MAT enzyme source. The purified *B. subtilis* ACP alone, however, showed no selfmalonylation in this study (data not shown), and this may negate the possibility above that the ACP selfmalonylation was responsible for the FabD independent BCFA biosynthesis.

Although this study first demonstrated that BCFA biosynthesis by crude FAS did not necessarily require FabD protein, the current *in vitro* results should be reasonably related to *in vivo* experiments. One experimental approach for this purpose is to study BCFA biosynthesis in an FabD-deficient mutant. The FabD-deficient mutant of *E. coli* was an auxotroph requiring both saturated and unsaturated fatty acid.[1] The authors hope that this paper triggers some studies to clarify the metabolic link between fatty acid biosynthesis and other pathways including polyketide synthesis in *B. subtilis*.

References

1) Magnuson, K., Jackowski, S., Rock, C. O., and Cronan, J. E., Regulation of fatty acid biosynthesis in *Escherichia coli*. *Microbiol. Rev.*, **57**, 522–542 (1993).
2) Rock, C. O., and Cronan, J. E., *Escherichia coli* as a model for the regulation of dissociable (type II) fatty acid biosynthesis. *Biochim. Biopys. Acta*, **1302**, 1–16 (1996).
3) Kaneda, T., Iso- and anteiso-fatty scids in bacteria: biosynthesis, function and taxonomic significance. *Microbiol. Rev.*, **55**, 288–302 (1991).
4) Butterworth, P. H. W., and Bloch, K., Comperative aspects of fatty acid synthesis in *Bacillus subtilis* and *Escherichia coli*. *Eur. J. Biochem.*, **12**, 496–501 (1970).
5) Choi, K.-H., Heath, R. J., and Rock, C. O., β-Ketoacyl carrier protein synthase III (FabH) is a determining factor in branched-chain fatty acid biosynthesis. *J. Bacteriol.*, **182**, 365–370 (2000).
6) Morbidoni, H. R., Mendoza, D., and Cronan, J. E., *Bacillus subtilis* acyl carrier protein is encoded in a cluster of lipid biosynthesis genes. *J. Bacteriol.*, **178**, 4794–4800 (1996).
7) Kaneda, T., Biosynthesis of branched long-chain fatty acids from the related short-chain α-keto acid

substrates by a cell-free system of *Bacillus subtilis*. *Can. J. Microbiol.*, **19**, 87–96 (1973).
8) Kawaguchi, A., Yoshimura, T., and Okuda, S., A new method for the preparation of acyl-CoA thioesters. *J. Biochem.*, **89**, 337–339 (1981).
9) Ellman, G. L., Tissue sulfhydryl groups. *Arch. Biochem. Biophys.*, **82**, 70–77 (1950).
10) Kaneda, T., Biosynthesis of branched chain fatty acids I. Isolation and identification of fatty acids from *Bacillus subtilis* (ATCC 7059). *J. Biol. Chem.*, **238**, 1222–1228 (1963).
11) Heath, R. J., and Rock, C. O., Regulation of malonyl-CoA metabolism by acyl-acyl carrier protein and β-ketoacyl-acyl carrier protein synthases in *Escherichia coli*. *J. Biol. Chem.*, **270**, 15531–15538 (1995).
12) Han, L., Lobo, S., and Reynolds, K. A., Characterization of β-ketoacyl-acyl carrier protein synthase III from *Streptomyces glaucescens* and its role in initiation of fatty acid biosynthesis. *J. Bacteriol.*, **180**, 4481–4486 (1998).
13) Johnstone, A., and Thorpe, R., Precipitation techniques in agar and agarose. In ''Immunochemistry in Practice'', Blackwell Scientific Publications, Oxford, pp. 120–140 (1982).
14) Naik, D. N., and Kaneda, T., Biosynthesis of branched long-chain fatty acids by species of *Bacillus*: relative activity of three a-keto acid substrates and factors affecting chain length. *Can. J. Microbiol.*, **20**, 1701–1708 (1974).
15) Oku, H., and Kaneda, T., Biosynthesis of branched-chain fatty acids in *Bacillus subtilis*. A decarboxylase is essential for branched-chain fatty acid synthetase. *J. Biol. Chem.*, **263**, 18386–18396 (1988).
16) Arimura, N., and Kaneda, T., Type selective inhibition of microbial fatty acid synthases by thiolactomycin. *Arch. Microbiol.*, **160**, 158–161 (1993).
17) Summers, R. G., Ali, A., Shen, B., Wessel, W. A., and Hutchinson, C. R., Malonyl-coenzyme A:acyl carrier protein acyltransferase of *Streptomyces glaucescens*: a possible link between fatty acid and polyketide biosynthesis. *Biochemistry*, **34**, 9389–9402 (1995).
18) Hitchman, T. S., Crosby, J., Byrom, K. J., Cox, R. J., and Simpson, T. J., Catalytic self-acylation of type II polyketide synthase acyl carrier proteins. *Chem. Biol.*, **5**, 35–47 (1998).
19) Matharu, A.-L., Cox, R. J., Crosby, J., Byrom, K. J., and Simpson, T. J., MCAT is not required for *in vitro* polyketide synthesis in a minimal actinorhodin polyketide synthase from *Streptomyces coelicolor*. *Chem. Biol.*, **5**, 699–711 (1998).
20) Zhou, P., Florova, G., and Reynolds, K. A., Polyketide synthase acyl carrier protein (ACP) as a substrate and a catalyst for malonyl ACP biosynthesis. *Chem. Biol.*, **6**, 577–584 (1999).