BRANCHED-CHAIN FATTY ACID REQUIREMENT FOR AVERMECTIN PRODUCTION BY A MUTANT OF *Streptomyces avermitilis* LACKING BRANCHED-CHAIN 2-OXO ACID DEHYDROGENASE ACTIVITY

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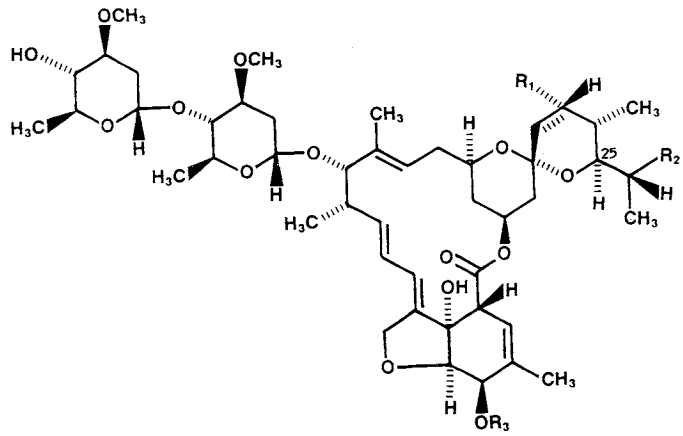
(Received for publication September 10, 1990)

The eight natural avermectins produced by *Streptomyces avermitilis* have the carbon skeleton of either isobutyric or *S*-2-methylbutyric acid incorporated into their structures. A mutant of *S. avermitilis* has been isolated that contains no functional branched-chain 2-oxo acid dehydrogenase activity. The mutant, in contrast to its parent, is unable to grow with isoleucine, valine and leucine as carbon sources. In medium lacking both *S*(+)-2-methylbutyric and isobutyric acid, the mutant is also incapable of making the natural avermectins, while supplementation with either one of these compounds restores production of the corresponding four natural avermectins. These facts indicate that in *S. avermitilis* the branched-chain 2-oxo acid dehydrogenase enzyme functions not only to catabolize the cellular branched-chain amino acids in order to meet energy and growth requirements but also to provide the small branched-chain organic acid precursor molecules necessary for avermectin biosynthesis. Supplementation of the mutant strain with *R*(−)-2-methylbutyric acid yields novel isomeric avermectins unseen in the (unsupplemented) wild-type strain. It was also concluded that acetate and propionate production by branched-chain 2-oxo acid degradation is not absolutely essential for avermectin production.

The synthesis of a polyketide antibiotic during microbial secondary metabolism begins with simple precursor molecules which are often exclusively taken from among acetic, propionic, and *n*-butyric acids[1,2]. Additional structural complexity is introduced when supplementary precursors are used. For example, the synthesis of the avermectins[3] by *Streptomyces avermitilis* incorporates, in addition to acetic and propionic acids, isobutyric and *S*(+)-2-methylbutyric acids as part of the antibiotic structure (Fig. 1—see substituent R_2)[4].

How are these small acidic precursor molecules for avermectins formed? Potential sources include the degradative pathways for the 2-oxo acid analogues of isoleucine, leucine, and valine[1,5,6] (Fig. 2). In order to test whether these reactions[7] provide any or all of these compounds needed for avermectin biosynthesis by *S. avermitilis*, we isolated a mutant of *S. avermitilis* defective in the branched-chain 2-oxo acid dehydrogenase step of isoleucine, leucine, and valine catabolism. We presumed that such a mutant would be unable to produce directly the coenzyme A derivatives of isobutyric, isovaleric, and 2-methylbutyric acids from branched-chain 2-oxo acid substrates, and would be also unable to make the acyl-CoA derivatives of propionic and acetic acids from branched-chain 2-oxo acid degradation, as these acids are formed downstream of the site of the mutation (Fig. 2). We report here, and in the following paper[8], findings that show that the mutant is indeed able to make avermectins only when it is

Fig. 1. The structure of the avermectin family of antibiotics[24].



	R_1	R_2	R_3
A_{1a}		C_2H_5	CH_3
A_{1b}		CH_3	CH_3
A_{2a}	OH	C_2H_5	CH_3
A_{2b}	OH	CH_3	CH_3
B_{1a}		C_2H_5	H
B_{1b}		CH_3	H
B_{2a}	OH	C_2H_5	H
B_{2b}	OH	CH_3	H

Where R_1 is absent, the double bond is present.
Both sugars are α-L-oleandrose.

supplemented with branched-chain fatty acids, and we also infer that branched-chain fatty acid degradation to provide propionic and acetic acids is not necessary for avermectin production.

Materials and Methods

Bacterial Strains

The parent *S. avermitilis* strain ATCC 31272 and its branched-chain 2-keto acid dehydrogenase= branched-chain 2-oxo acid dehydrogenase (*bkd*-11 mutant) ATCC 53567 are deposited in the American Type Culture Collection (ATCC), Rockville, MD 20852, U.S.A. The *bkd*-11 mutant is the subject of pending patent applications.

Mutagenesis and Mutant Isolation

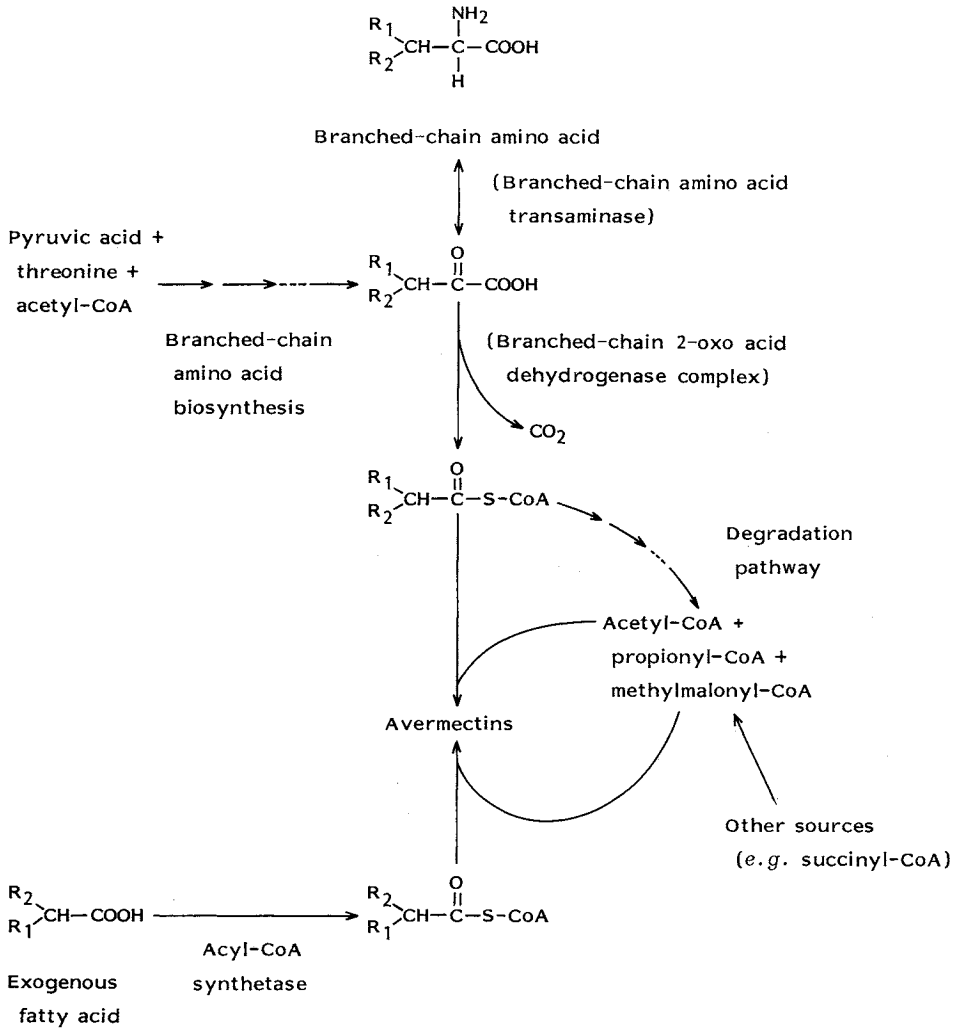
Step 1: 10 ml of a spore suspension of *S. avermitilis* ATCC 31272 in 0.05 M Tris-maleic acid buffer, pH 9, was added to a vial containing 10 mg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG). The vial was incubated and shaken at 28°C for 60 minutes and the spores then washed profusely with 1% NaCl solution.

Step 2: This spore stock was spread on YPD plates to yield ~100 colonies per plate (YPD medium comprises 10 g/liter of each of Bacto-yeast extract, Bacto peptone and glucose; and 15 g/liter of Bacto agar (Difco), adjusted to pH 6.9 before autoclaving).

Step 3: Single colonies were picked from plates after 2~3 weeks of growth at 28°C and placed in individual wells of a standard 96-well microtiter plate. A small quantity of the colony was also patched onto a fresh agar medium to serve as a source of viable cells when mutants were identified.

Step 4: To each well was added ~75 μl of a liquid M9 salts medium[9] containing glucose 1%,

Fig. 2. Branched-chain amino acid degradation and its relationship to avermectin biosynthesis.



Casamino acids 0.1%, and 0.01% of each of isovaleric, isobutyric and 2-methylbutyric acids. After several days of incubation at 28°C, the cells were assayed in Steps 5 and 6 for the presence of branched-chain 2-oxo acid dehydrogenase.

Step 5: A microsuspension of 5% toluene in M9 salts medium was prepared by a brief sonication of the immiscible mixture. To 25 ml of this suspension was added 1.2 ml of a solution containing [1-^{14}C]-2-oxoisocaproic acid, 2.5 μCi/ml and 10.0 μCi/μmol. 50 μl of this overall mixture was added to each of the wells of the microtiter plates containing the colonies to be assayed.

Step 6: The $^{14}CO_2$ produced from each well was trapped and visualized by the procedure described by TABOR *et al.*[10].

An alternative, improved method was used after the *bkd* mutant I-3 was isolated. This method employed single colonies from Step 3 after 7~14 days of growth (rather than 2~3 weeks) and assayed directly by Steps 5 and 6 above.

Mutant candidates identified in the above screen were tested as follows. Isolates were grown in a 300-ml flask containing 50 ml of a liquid medium made up of M9 salts medium with glucose 1%, and "Syncasa — bcaa" (a synthetic mixture of L-amino acids with the approximate composition of commercial Casamino acids, but without the presence of L-valine, L-isoleucine and L-leucine, see below). After growth

at 30°C to early stationary phase (2~4 days), the cells were washed in M9 salts medium and resuspended in cold M9 salts medium containing 1% toluene which had been sonicated to produce a milky-white dispersion of the toluene. The cell/buffer/toluene suspension was incubated for 40 minutes at 30°C in order to permeabilize the cells. The permeabilized cells were then washed in M9 salts medium and finally resuspended in one-fifth the original volume of M9 medium buffer. 180 μl of this suspension were used per assay. A reaction volume of 300 μl contained the toluenized cells, thiamine pyrophosphate (TPP) 0.4 mM, coenzyme A 0.11 mM, nicotinamide adenine dinucleotide (NAD) 0.68 mM, dithiothreitol (DTT) 2.6 mM, $MgCl_2$ 4.1 mM, Tris-HCl 60 mM, pH 7.5, and [1-^{14}C]-2-oxoisocaproate 8,200 dpm (10 μCi/μmol). The reaction was carried out in 15 ml scintillation vials containing a 2×2 cm Whatman No. 4 paper square pressed into the screw cap of the vial. The paper contains 20 μl of 1 M Hyamine Hydroxide (1 M solution of methylbenzethonium hydroxide in methanol) which traps $^{14}CO_2$ evolved in the reaction. After incubation at 30°C for 2 hours, the papers were removed and then immersed in 10 ml of Beckman Aquasol II, and the radioactivity is measured after equilibration in this solvent for 4 hours or more.

The composition of "Syncasa—bcaa", 100-fold concentrate, used in the above procedures is L-alanine (3), L-arginine (4), L-aspartic acid (6), L-cystine (1), L-glutamic acid (20), glycine (1), L-histidine (2), L-lysine (7), L-methionine (3), L-phenylalanine (6), L-proline (10), L-serine (6), L-threonine (4), L-tyrosine (4), L-tryptophan (1), where numbers in parentheses refer to g/liter. The mixture is adjusted to pH 7 and filter sterilized. One volume of concentrate is added to 99 volumes of medium to achieve standard use concentrations.

Avermectin Fermentation

Avermectin fermentations were carried out using a minimal defined medium to which various additions of fatty acids could be made. The fermentation medium is made up as follows: thinned starch (40), potato soluble starch (40), glutamic acid (1.0), arginine (0.168), cystine (0.084), histidine (0.069), leucine (0.798), lysine (0.297), methionine (0.108), phenylalanine (0.168), threonine (0.174), tryptophan (0.048), tyrosine (0.192), K_2HPO_4 (1.0), $MgSO_4 \cdot 7H_2O$ (1.0), NaCl (1.0), $CaCO_3$ (3.5), $FeSO_4 \cdot 7H_2O$ (0.01), $MnCl_2 \cdot 4H_2O$ (0.001), $ZnSO_4 \cdot 7H_2O$ (0.001), where numbers in parentheses refer to g/liter distilled water. The pH is adjusted to 6.8~7.0 with HCl or NaOH.

A 300-ml flask containing 40 ml of this medium was inoculated with 1 ml of cells from a seed culture of the strain similarly grown in a seed medium, which is made up as follows: thinned starch 20 g/liter, Ardamine pH 5 g/liter, Pharmamedia 15 g/liter, $CaCO_3$ 2 g/liter; the pH was adjusted to 7.2 (NaOH). The thinned starch was prepared by hydrolysis of starch with "termamyl", an α-amylase from *Bacillus licheniformis* available from Novo Enzymes, Wilton, CT, U.S.A., to a glucose equivalent of 40%±5%. Ardamine pH is available from Yeast Products, Inc., Clifton, NJ 07012, U.S.A. Pharmamedia is available from Traders Protein, Memphis, TN 38108, U.S.A.

After 8 days of shaking at 29°C, the flask contents were extracted with 4 volumes of an acetonitrile - methanol (7 : 78) solvent mixture and the supernatant was analyzed for avermectins by HPLC.

HPLC Analysis of Avermectins

The assay was carried out using a Beckman 5 μm Ultrasphere ODS C-18 column (4.6 mm × 25 cm), with a flow of 0.75 ml/minute and detection by absorbance measurements at 240 nm. The mobile phase was water (150 ml), acetonitrile (70 ml), and methanol to a final volume of 1 liter.

Chemicals

Unless otherwise indicated, chemicals were obtained from Sigma Chemical Co., [1-^{14}C]pyruvate and [1-^{14}C]-2-oxoisocaproate were from Amersham. Other [1-^{14}C]-2-oxo acids used were prepared from their [1-^{14}C]amino acid analogues (obtained from New England Nuclear Corp.) by the method of MEISTER[11].

Results

Isolation of a Branched-chain 2-Oxo Acid Dehydrogenase Mutant of *S. avermitilis*

The isolation of this mutant was achieved by employing a method that was specifically designed for

Table 1. 2-Oxo acid dehydrogenase activity in mutant I-3.

Expt No.	Strain	Description	% Substrate decarboxylation
1	ATCC 31272	Complete system[a]	42
2	ATCC 31272	−CoA	14
3	ATCC 31272	−TPP	4
4	ATCC 31272	−NAD	27
5	None	Complete system	0.8
6	I-3	Complete system	0.5
7	I-3	[1-^{14}C]-2-Oxoisovalerate[b]	1.7
8	None	[1-^{14}C]-2-Oxoisovalerate[b]	1.8
9	I-3	[1-^{14}C]-2-Oxo-3-methylvalerate[b]	1.7
10	None	[1-^{14}C]-2-Oxo-3-methylvalerate[b]	1.1
11	I-3	[1-^{14}C]Pyruvate[b]	61
12	None	[1-^{14}C]Pyruvate[b]	2.4

[a] The complete system for testing mutants isolated by the mass screening method is described under Materials and Methods. Unless indicated differently, the standard substrate was [1-^{14}C]-2-oxoisocaproate. Radio-labeled 2-oxoisovalerate and 2-oxo-3-methylvalerate gave similar results.

[b] This substrate was used in place of [1-^{14}C]-2-oxoisocaproate in the complete system as described under Materials and Methods.

finding mutants unable to form $^{14}CO_2$ from radio-labeled substrates of decarboxylation reactions[10].

Mutagenized single colonies of *S. avermitilis* strain ATCC 31272 were placed in the wells of 96-well microtiter plates (see Materials and Methods). Colonies showing no $^{14}CO_2$ formation from a [1-^{14}C]-2-oxoisocaproate substrate were retested for branched-chain 2-oxo acid dehydrogenase activity. As can be seen in Table 1, the mutant strain designated as I-3 shows no such activity when compared to the parent strain, which shows an activity partially dependent on expected cofactors for this enzyme[12]. Additional assays similarly show that the two other naturally occurring branched-chain 2-oxo acids, namely, 2-oxoisovalerate (valine analogue) and 2-oxo-3-methylbutyrate (isoleucine analogue) are also not decarboxylated. In contrast, the mutant readily decarboxylates pyruvate. By analogy with the nomenclature introduced to describe similar mutants of *Pseudomonas putida*, the mutation in strain I-3 is designated *bkd*-11[13].

Formation of the Avermectins by Mutant Strain I-3

Consequences of the mutant *bkd* genotype were revealed in experiments measuring its capacity for making the avermectins *in vivo*. Thus, in fermentations where branched-chain and straight-chain fatty acids and their potential metabolic precursors (*e.g.*, fats and oils) were omitted from the fatty acid-free medium, no avermectins were produced (Fig. 3(A)). In contrast, when *S*(+)-2-methylbutyric acid was added to the medium, the "a" forms of avermectin were produced (Fig. 3(B), peaks at 13.3, 14.5, 23.6, and 30.8 minutes); similarly, addition of isobutyric acid in place of 2-methylbutyric acid led only to the formation of the minor "b" forms (Rt's were: oligomycin, 14.1 minutes; "B_{2b}", 9.8 minutes; "A_{2b}", 12.7 minutes; "B_{1b}", 19.4 minutes; "A_{1b}", 25.6 minutes). Interestingly, addition of *R*,*S*-2-methylbutyric acid seemed to lead to the formation of "A_{2a}" avermectins containing both isomeric forms, recognizable as a doublet HPLC peak in the A_{2a} region (Fig. 3(C)). In experiments (not shown) with predominantly *R*(−)-2-methylbutyric acid, the second peak of the doublet A_{2a} HPLC peak was greatly enhanced (2.5 times higher than that of the first peak). UV, NMR and LC-mass spectrophotometric analyses (S. P. GIBSON; unpublished information) of these peaks supported the conclusion that the corresponding isomeric avermectins were formed.

Growth Characteristics of Branched-chain 2-Oxo Acid Dehydrogenase Mutants

The branched-chain 2-oxo acid dehydrogenase-negative mutant strain I-3 was not able to grow with either a mixture of isoleucine, leucine, and valine or their 2-oxo acid analogs as a carbon source. In contrast, good growth was seen for the parent strain. The mutant grew nearly as well as the parent on glucose minimal medium or rich medium. The mutant generally sporulated well on crude media.

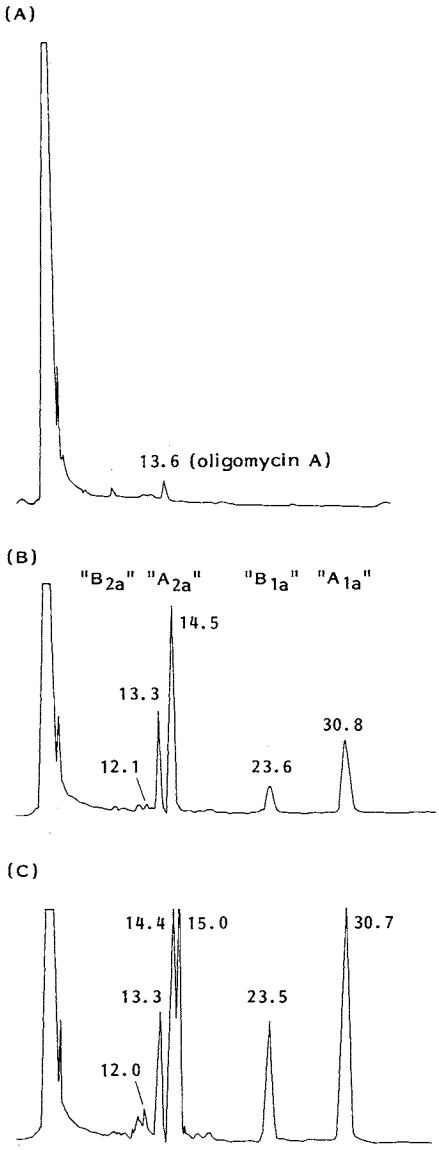
Discussion

The data presented support the conclusion that a branched-chain 2-oxo acid dehydrogenase-negative mutant of *S. avermitilis* has been isolated. Thus, the mutant strain I-3 is unable to grow with either a mixture of the three branched-chain amino acids or their 2-oxo acid analogs as a carbon source. This is expected, since this enzyme activity is an integral part of the catabolic pathway for these compounds (Fig. 2). In this, the mutant is similar to mutants of *Bacillus subtilis*[14,15] and *P. putida*[16] which have been described as negative for branched-chain 2-oxo acid dehydrogenase. The correlation of the mutant defect with the lack of this enzyme activity is most strongly shown by the direct enzymic assay *in vitro* (Table 1). The validity of the assay is demonstrated by the expected cofactor requirements for TPP and CoA shown by the wild-type strain. This mutant differs from the *B. subtilis* mutant in that it retains the ability to decarboxylate pyruvate[15].

The isolation of the mutant strain I-3 allows us to answer part of the question posed earlier as to how the avermectin precursor molecules are made in the cell. Thus, the mutant is essentially incapable of making the natural avermectins ("a" and "b" forms, Fig. 1) unless the free branched-chain fatty acid (either *S*(+)-2-methylbutyric or isobutyric acid) corresponding to the C-25 region of the avermectin molecule is added to the fermentation medium (Fig. 3). This strongly suggests that the 2-oxo acid dehydrogenase enzyme functions uniquely in the wild-type strain to supply these essential natural avermectin precursors. Furthermore, it has been shown that several analogues of the natural branched-chain fatty acids are taken up by a *bkd* mutant strain and used to synthesize new avermectins in the absence of natural avermectin synthesis[8,17]. Since some of these new precursors, in contrast to *S*(+)-2-methylbutyric and isobutyric acids,

Fig. 3. Avermectin formation by mutant I-3, assayed by UV absorption at 240 nm of peaks separated by HPLC.

(A) No precursor fatty acid additions. (B) *S*(+)-2-methylbutyric acid addition. (C) *R,S*-2-methylbutyric acid addition.



Identification of avermectins A_{1a}, A_{2a}, and B_{1a} was made by comparison to the HPLC-analyzed products made by the parent ATCC 31272 strain fermented in the absence of precursor addition (not shown). Confirmation of the assignments of these peaks as avermectins was made by UV diode-array scanning, which revealed the characteristic spectra of the avermectin family.

are unlikely to be degraded to yield acetate and propionate, we conclude that acetate and propionate can be made by the cell by at least one route other than branched-chain 2-oxo acid degradation. Evidence from other investigators in support of this conclusion includes the observation that [2-^{13}C]-valine degradation is not accompanied by appreciable ^{13}C incorporation into the macrocyclic ring of the closely related milbemycin polyketides at positions other than at C-25. Thus, incorporation into the milbemycin D molecule, which has the identical C-25 substituent to the avermectin "b" molecules, occurs only at the C-25 position[18]. More directly, it has been reported that the carbon of avermectin "b" molecules at position 25 (Fig. 1) is markedly enriched for ^{13}C in similar isotope experiments[4,19]. These results are readily explainable if one assumes that significant dilution of the acetate and propionate ^{13}C ring precursors provided by branched-chain 2-oxo acid degradation occurs with unlabeled precursors supplied by another pathway, in keeping with our conclusion above (see also Fig. 2).

Another example of the effect of branched-chain fatty acid uptake influencing antibiotic synthesis is given by the production of paulomycins A and B by *Streptomyces paulus*[20]. These antibiotics are esters of 2-methylbutyric and isobutyric acids, respectively, and their formation, like that of the avermectin "a" and "b" molecules, is presumably from the fatty acyl-CoA products of the branched-chain 2-oxo acid dehydrogenase reaction. It has been shown that the ratio of paulomycins A to B increases dramatically upon addition to the medium of 2-methylbutyric acid. Similarly, this ratio decreases markedly when isobutyric acid is supplied[20].

An interesting finding of the present work is that the mutant strain was able to form what appeared to be a novel avermectin from the "unnatural", *R*(−) isomer of 2-methylbutyric acid. This was recognized as doublet peaks in the A_{2a} (and possibly the B_{2a}) region of elution in the standard HPLC analysis for the avermectins (Fig. 3(C)). The corresponding B_{1a} and A_{1a} forms not seen separately in this analysis have been resolved in other experiments (data not shown). This capacity to form novel avermectins is described more fully in a published patent application[17]; the following paper details these findings[8]. Subsequent to these observations, CHEN *et al.* also described the incorporation of unnatural branched-chain fatty acids by the parent strain of *S. avermitilis* to produce novel C-25-substituted avermectins[21].

The results described here indicate that short α-branched-chain fatty acids are readily taken up by the *S. avermitilis* cell from the surrounding medium. By analogy with what is known for *Escherichia coli*[22], we show the involvement of an acyl-CoA synthetase in this process (Fig. 2). Current thinking regarding the polyketide synthetase substrate requirements presumes that the use of these fatty acids requires their activation to their thio ester forms with either coenzyme A and/or an acyl carrier protein[1]. Similar proposals for fatty acid uptake have been made for *B. subtilis*, based on observations of the effect of exogenously supplied short-chain fatty acids in altering the composition of the long-chain membrane fatty acids[13,23].

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