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Identification and disruptional analysis of the *Streptomyces cinnamonensis msdA* gene, encoding methylmalonic acid semialdehyde dehydrogenase

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Abstract The *msdA* gene encodes methylmalonic acid semialdehyde dehydrogenase (MSDH) and is known to be involved in valine catabolism in *Streptomyces coelicolor*. Using degenerative primers, a homolog of *msdA* gene was cloned and sequenced from the monensin producer, *Streptomyces cinnamonensis*. RT-PCR results showed *msdA* was expressed in a vegetative culture, bump-seed culture and the early stages of oil-based monensin fermentation. However, isotopic labeling of monensin A by [2, 4-$^{13}C_2$]butyrate revealed that this MSDH does not play a role in providing precursors such as methylmalonyl-CoA for the monensin biosynthesis under these fermentation conditions. Using a PCR-targeting method, *msdA* was disrupted by insertion of an apramycin resistance gene in *S. cinnamonensis* C730.1. Fermentation results revealed that the resulting *ΔmsdA* mutant (CXL1.1) produced comparable levels of monensin to that observed for C730.1. This result is consistent with the hypothesis that butyrate metabolism in *S. cinnamonensis* in the oil-based fermentation is not mediated by *msdA*, and that methylmalonyl-CoA is probably produced through direct oxidation of the *pro-S* methyl group of isobutyryl-CoA. The CXL1.1 mutant and C730.1 were both able to grow in minimal medium with valine or butyrate as the sole carbon source, contrasting previous observations for *S. coelicolor* which demonstrated *msdA* is required for growth on valine. In conclusion, loss of the *S. cinnamonensis msdA* neither affects valine catabolism in a minimal medium, nor butyrate metabolism in an oil-based medium, and its role remains an enigma.

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Abbreviations MSDH: Methylmalonic acid semialdehyde dehydrogenase · PKS: Polyketide synthase · ICM: Isobutyryl-CoA mutase · CCR: Crotonyl CoA reductase · PCC: Propionyl CoA carboxylase

Introduction

Polyketides are a group of natural products with diverse structures and a wide range of biological activities [8]. They are synthesized by polyketide synthases (PKSs) in microorganisms and plants, using several common biosynthetic precursors, malonyl CoA (derived from acetyl CoA), methylmalonyl CoA, and to a lesser extent ethylmalonyl CoA (derived from butyryl CoA) [7, 10, 11]. These precursors are mainly obtained from primary metabolic processes such as fatty acid degradation, glycolysis, amino acid degradation, and the citric acid cycle.

Valine catabolism (Fig. 1) has long been considered a potentially important source for precursor supply, and has been studied extensively for a range of different polyketide producing organisms [9, 12, 15, 17, 23, 27, 28]. Valine is first oxidized to isobutyryl-CoA through the actions of valine dehydrogenase and a branched–chain α-ketoacid dehydrogenase. Then isobutyryl-CoA is either isomerized into butyryl-CoA, and further degraded into acetyl-CoA through fatty acid degradation, or converted to 3-hydroxyisobutyryl CoA through a methacrylyl-CoA intermediate [20, 21, 31]. 3-Hydroxyisobutyryl CoA can then be converted to the free acid, oxidized and then metabolized to propionyl-CoA via the action of methylmalonate semialdehyde dehydrogenase (MSDH encoded by *msdA*) (Fig. 1, route A) [1, 4, 25, 31]. Subsequent carboxylation would provide methyl-

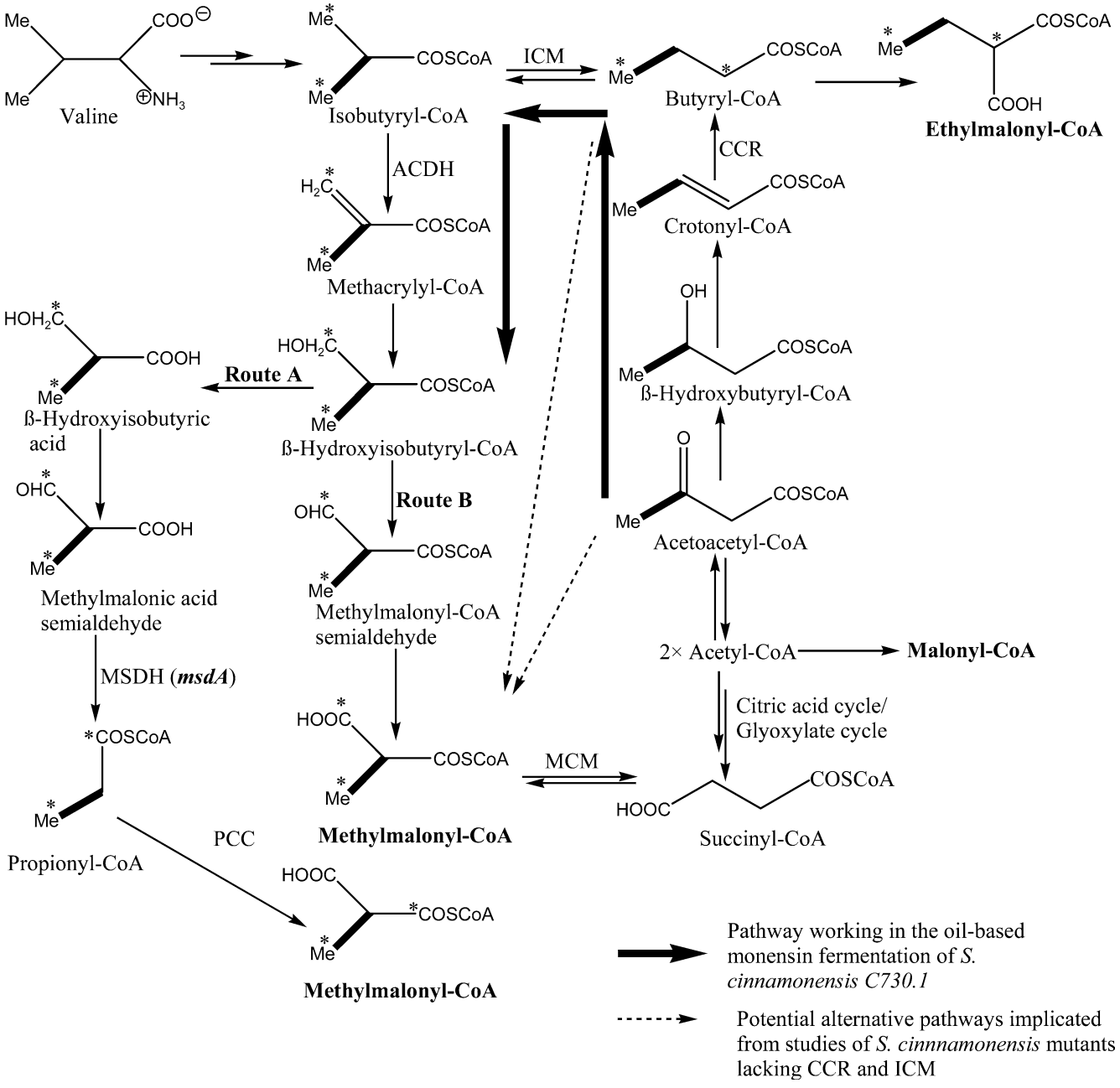

Fig. 1 Pathways leading to methylmalonyl-CoA, malonyl-CoA and ethylmalonyl-CoA precursors used for monensin biosynthesis in *S. cinnamonensis*. The *bold bond* indicates intact processing of [3, 4–$^{13}C_2$] acetoacetyl CoA into methylmalonyl CoA and ethylmalonyl CoA (as determined by intact labeling of carbons in monensin A) as described previously [13]. *Asterisks* indicate labeling of ethylmalonyl CoA and two alternative patterns for labeling of methylmalonyl CoA (route A or route B) from [2, 4–$^{13}C_2$] butyryl CoA. NMR data from monensin obtained in an incorporation experiment with [2, 4–$^{13}C_2$] butyrate are consistent with route B

malonyl CoA. The *msdA* gene of *Streptomyces coelicolor* was previously cloned and expressed in *Escherichia coli* and the recombinant protein was shown to have the predicted MSDH activity. MSDH Activity could be detected in R2YE liquid culture of the *S. coelicolor* wild-type strain, but not the mutant in which this gene had been inactivated by the *hyg* gene [31]. Alternatively 3-hydroxyisobutyryl CoA can be directly oxidized to methylmalonyl-CoA (Fig. 1, route B). Most incorporation studies with isotopically labeled precursors (including methacrylic acid, valine and isobutyric acid) are consistent with streptomycetes utilizing route B as the primary route from 3-hydroxyisobutyryl CoA under the fermentation conditions used. Nonetheless, it has been shown that in *S. coelicolor* the *msdA* gene is essential for growth on valine as a sole carbon source, suggesting that route A might be important under some growth conditions [31].

We have recently demonstrated that when *S. cinnamonensis* was grown in an oil-based extended

fermentation, crotonyl-CoA reductase (CCR) played a significant role in providing methylmalonyl-CoA precursors for biosynthesis of monensins [13]. We proposed that under such conditions acetyl CoA, the major oil catabolite, is converted to crotonyl CoA, via a reversal of fatty acid oxidation, and then reduced to butyryl CoA by CCR. A subsequent isomerization step (catalyzed by ICM, isobutyryl-CoA mutase) provides isobutyryl CoA which is oxidized to methylmalonyl-CoA. An incorporation experiment with ethyl [3, 4-$^{13}C_2$]acetoacetate demonstrated comparable intact labeling of the positions of monensin A derived from C3 and C4 of butyryl CoA/ethylmalonyl CoA and C2-C3 of methylmalonyl CoA. This finding contrasted similar types of incorporation studies using labeled butyrate and acetoacetate into monensin A and other antibiotics. Typically, a significant dilution in the labeling of the methylmalonyl CoA derived positions relative to ethylmalonyl CoA-derived positions is observed, suggesting that other pathways contribute significantly towards providing the methylmalonyl CoA pool [30]. This labeling study did not permit determination of whether route A or route B is used in the conversion of butyryl CoA to methylmalonyl CoA by *S. cinnamonensis* under these conditions (as depicted in Fig. 1 the results from the labeling study would be the same). Herein, we report the results from an incorporation study for [2, 4-$^{13}C_2$]butyrate under these conditions, as well as the cloning of *msdA* from *S. cinnamonensis* and the subsequent generation and analysis of a *ΔmsdA* mutant. The analyses all indicate that route A and MSDH do not play a significant role in either provision of methylmalonyl CoA for monensin production in an oil-based extended fermentation of *S. cinnamonensis*, or for growth on valine or isobutyrate as a sole carbon source. As such, it would appear that the direct oxidation of isobutyryl CoA (route B) is likely the main pathway under both of these growth conditions.

Materials and methods

Strains, plasmids and culture conditions

Escherichia. coli Mach1^{TM}-T1^{R}, obtained from Invitrogen, was used as host for plasmid transformation and grown at 37°C on LB medium. The TOPO TA cloning kit from Invitrogen was used to clone the PCR products. *S. cinnamonensis* C730.1 and C730.7 were gifts of Eli Lilly Co. MOV, MOB and MOF media were the same as those described previously [13, 24]. [2, 4-$^{13}C_2$]Butyrate (0.5 g, ^{13}C, 99%) was purchased from Sigma-Aldrich. Purification of monensin A and quantification of monensin titers were performed as described previously [21]. The fermentation of *S. cinnamonensis* is a three-stage process. First, a fresh R2YE agar plate culture of each *S. cinnamonensis* strain was used to inoculate 50 ml MOV in a 300 ml flask. The cultures were incubated at 32°C, 300 rpm for 18 h and then 0.5 ml of each MOV culture was transferred into 50 ml MOB medium in a 300 ml flask and was grown at 32°C, 300 rpm for 24 h. Finally, 0.5 ml of MOB culture was used to inoculate 5 ml MOF culture in a 50 ml flask and was incubated at 34°C, 260 rpm for 10 days (a total of four flasks and 20 ml of culture were used in the feeding study). On the fifth day of the MOF culture (120 h), the fermentation culture was supplemented with 1.2 ml of natural oils. The minimal medium for solid-phase growth contained 0.05% K_2HPO_4, 0.02% $MgSO_4 \cdot H_2O$, 0.001% $FeSO_4 \cdot H_2O$, 0.1% $(NH_4)_2SO_4$ and 1% UltraPure^{TM} Agarose (Invitrogen) at pH 7.0–7.2. For sole carbon source growth studies, this medium was supplemented with one of the following compounds, 0.1% sodium acetate, sodium propionate, sodium butyrate, sodium isobutyrate, or 0.5% valine.

Incorporation of sodium [2, 4-$^{13}C_2$]butyrate into monensin A

Sodium [2, 4-$^{13}C_2$]butyrate was added into 20 ml of monensin fermentation broth (MOF culture) in three equal portions on days 3, 4 and 5 (to a final concentration of 30 mM). After 10 days of fermentation, monensin A was purified (25 mg) and analyzed by ^{13}C NMR.

DNA preparation and amplification

Plasmid extraction and manipulation was performed with the QIAprep Spin Miniprep Kit and the QIAquick Gel Extraction Kit. The QIAquick PCR Purification Kit was used for PCR product cleanup. *Streptomyces* genomic DNA was isolated with a Wizard Genomic DNA Purification Kit (Promega) with minor modifications. Streptomycetes were cultivated in YEME plus 0.5% glycine for 48 h. Cells were harvested by centrifugation and washed with 10% sucrose twice, and then used for extraction of genomic DNA. All oligonucleotides for PCR primers in this research were synthesized by IDT.

Degenerate primers used for cloning *msdA* from *S. cinnamonensis* were as follows: Forward1 (F1) 5'-ATCACSCCKTTCAACTTCC-3'; Forward2 (F2) 5'-GGCGARCGCT GCATGGC-3'; Reverse1 (R1) 5'-GCCATGCAGCGYTCGCC-3' and Reverse2 (R2) 5'-ACCGGRATCGGCACRTT-3'. The Invitrogen Hifidelity supermix PCR kit was used for the PCR amplification. *S. cinnamonensis* genomic DNA was used as the template. Amplification was performed with a GeneAmp PCR system 2400 (Applied Biosystems) using the following conditions: initial denaturation at 96°C for 3 min, 30 cycles of amplification (30 s denaturation at 96°C, 30 s annealing at 50°C and then 1 min at 72°C for extension), and a final 7 min extension at 72°C. The resulting PCR products were cloned into TOPO-TA vector, sequenced, and compared with the published *msdA* sequence of *S. coelicolor* and *S. avermitilis*.

DNA hybridization, cloning, and sequencing

A Supercos-1 cosmid library of genomic DNA of *S. cinnamonensis* was prepared previously by our research group [30]. Preparation of a digoxigenin-labeled *msdA* probe, colony hybridization, and detection of *msdA*-containing positive clones, were performed following the manufacturer's protocols (Roche Applied Science). A plasmid containing PCR product amplified from *msdA* of *S. cinnamonensis* was used as the template for the PCR preparation of the DIG-labeled *msdA* probe. The positive *E. coli* colony identified in cosmid library screening was cultivated. The cosmid was extracted and digested with different restriction enzymes. The resulting DNA fragments were separated on 0.8% agarose gel and transferred onto positively charged nylon membrane by capillary blotting.

Southern hybridization with the *msdA* probe was used to identify a positive band. The corresponding DNA fragment was isolated, cloned into the pUC19 vector, and sequenced by primer walking. Sequencing was performed on an ABI Prism 3700 DNA sequencer in the DNA core facility of MCV, Virginia Commonwealth University, USA. The DNA sequence was assembled using SeqMan II (DNA star Inc.). The nucleotide sequence and deduced protein sequence were analyzed with DS1.5 software, and compared with other known *msdA* gene sequences in the NCBI database using BLAST.

Transcript analysis of *msdA* in *S. cinnamonensis*

Transcript analysis for *msdA* was carried out at various stages of the *S. cinnamonensis* fermentation process (cells were collected at 18 h of MOV culture and 24 h of MOB culture, 72 h and 168 h and 240 h of MOF cultures). Total RNA purification from *S. cinnamonensis* and RT-PCR were done as described previously [13]. The primers were *msdA*-RTPCR-F 5'-CGTCTCCTTCGTCG GCTCCAC-3' and *msdA*-RTPCR-R 5'-CCGTGACG-TACGA GGCGACCTT-3'. Dimethyl sulfoxide (2.5%, v/v final) was added to the RT-PCR mixture. The RT-PCR reaction conditions were as follows: an initial DNA strand synthesis step with reverse transcriptase, 52°C for 30 min, followed by 95°C for 15 min to activate the DNA polymerase, and then 35 cycles [94°C for 10 s, 59°C for 20 s, 72°C for 45 s]. Negative controls were carried out for all analyses and used the same enzyme mix but without the initial reverse transcription step. Sequencing of the transcript products confirmed that they were the predicted *msdA* product.

Targeted disruption of the *msdA* gene

The *msdA* gene of *S. cinnamonensis* C730.1 was disrupted by a PCR-targeted *Streptomyces* gene replacement method [5]. An apramycin resistance cassette containing the *aac*(3)*IV* resistance gene and *oriT* was amplified from pIJ773 using the long primers: *msdA*-disruption-F 5'-GCCATGGTCCCGATGTGGATGTT CCCGCTGGCCATCGCGATTCCGGGGATCCGTC GACC-3' and *msdA*-disruption-R 5'-CACGCCGAC CATGCCCGCCTCGATCTCCAGCTGGAAGCG*TG TAGGCTGGAGCTGCTTC*-3' (pIJ773 homologous sequence is italicized and the *msdA* homologous sequence is underlined). The PCR product was used for insertional inactivation of *msdA* (removing 774 bp, from position 499 to 1272 of *msdA* of *S. cinnamonensis*), first in cosmid SC4B11, and then in *S. cinnamonensis* C730.1, generating CXL1.1. An analogous experiment with *S. cinnamonensis* C730.7 (a higher monensin titer strain) provided CXL1.7. Both CXL1.1 and CXL1.7 were selected for their resistance to apramycin and sensitivity to kanamycin. Insertional inactivation of *msdA* in each of these strains was confirmed by PCR amplification and sequencing of chromosomal DNA using outside degenerative primers F1 and R2 (primers used previously to amplify an *msdA* fragment from *S. cinnamonensis* C730.1).

Nucleotide sequence accession number

The *msdA* sequence of *S. cinnamonensis* reported here has been deposited at EMBL and GeneBank under accession number DQ005575.

Results

Cloning of an *msdA* homolog from *S. cinnamonensis*

The *Streptomyces coelicolor msdA* was cloned and characterized almost 10 years ago [31]. Sequencing of the *S. avermitilis* genome has identified an *msdA* homolog with approximately 90% identity at both the nucleotide and deduced amino acid sequence levels [18]. We identified highly conserved regions through alignment (Fig. 2) of these two homologs and used this to design degenerative primers to PCR amplify an *msdA* homolog from *Streptomyces cinnamonensis*. We designed two forward primers F1, F2 and two reverse primers R1, R2 (Fig. 2). Three sets of primers: F1/R1, F2/R2 and F1/R2 were used for PCR amplification using *S. cinnamonensis* genomic DNA as template. Each PCR reaction produced a specific DNA band of the predicted size (413nt, 494nt and 890nt, respectively). These three PCR products were cloned with the TOPO TA cloning kit and sequencing revealed that a single gene with 90% nucleotide sequence identity to corresponding regions of the *msdA* from *S. coelicolor* and *S. avermitilis* had been amplified.

The 413nt PCR product was digoxigenin (DIG)-labeled by PCR and used in a hybridization experiment to identify an *msdA*-containing clone SC4B11 from the *S. cinnamonensis* cosmid library. Restriction

Fig. 2 Deduced protein sequence alignment of MSDH (*msdA* gene product) of *S. cinnamonensis* (3), *S. coelicolor* (1) and *S. avermitilis* (2). *F1*, *F2*, *R1* and *R2* indicate conserved regions (*underlined*) on which the PCR primers used to amplify *msdA* from *S. cinnamonensis* were designed

and hybridization analysis identified a 2.3 kb *Bam*HI-*Pst*I fragment as containing the *msdA* homolog, and this was subsequently cloned into pUC19 creating pCXL301 for sequencing.

Nucleotide sequencing of *msdA* from *S. cinnamonensis*

Codon preference analysis and BLAST sequence similarity analysis using the NCBI database were carried out using the DNA sequence of the 2.3 kb *Bam*HI-*Pst*I fragment from cosmid SC4B11. One open reading frame of 1,503 bp (with an ATG start codon at position 406 and a TGA stop codon at position 1908), the same size as *msdA* of *S. coelicolor* and *S. avermitilis*, was revealed. The *msdA* genes of *S. coelicolor* and *S. avermitilis* have a highly conserved RBS core sequence (AGGA), whereas the *msdA* of *S. cinnamonensis* is preceded by a sequence (AGTAGG) with a certain degree of complementarity to the 3'-end of 16S rRNA of *S. coelicolor* which potentially serves as a ribosomal binding site [26].

Comparison of these three streptomycete *msdA* genes showed that they are highly homologous. They share 89% nucleotide identity and 90% amino acid identity (Fig. 2.). Our BLAST results also indicated that the 0.4 kb upstream DNA sequence of the *S. cinnamonensis msdA* gene is homologous to the corresponding region of the *S. avermitilis msdA* gene, but not the *S. coelicolor msdA* gene. The 0.4 kb downstream region of the *S. cinnamonensis msdA* gene did show significant homology to the downstream regions of the *msdA* genes of both *S. coelicolor* and *S. avermitilis*.

Generation and analysis of *ΔmsdA* mutants (CXL1.1 and CXL1.7) of *S. cinnamonensis*

We used insertional inactivation to generate *ΔmsdA* mutants of both *S. cinnamonensis* C730.1 and the higher-titer producer C730.7. The CXL1.1 mutant (108 ± 7.1%) and the C730.1 progenitor (100 ± 6.0%) produced monensin titers which were not statistically different. Similarly C730.7 (100 ± 3.95%) and the corresponding CXL1.7 (99.8 ± 16.7%) mutant produced indistinguishable levels of monensins (the levels of these were higher than with C730.1). If methylmalonyl-CoA production from butyryl CoA is mediated by MSDH (route A in Fig. 1), a decrease in monensin titers would have been observed.

It has previously been shown that in *S. coelicolor* insertion of the hygromycin resistance gene into the *msdA* coding region leads to a mutant no longer able to grow on valine or isobutyrate as a sole carbon source. This *S. coelicolor* mutant retained the ability to grow on propionate as a sole carbon source. In contrast with this result, we observed that the CXL1.1 and CXL1.7 strains grew as well as C730.1 and C730.7 strains on valine or isobutyrate, butyrate or acetate as a sole carbon source. In the absence of any carbon source, there was no significant growth of any of these strains.

Expression of MSDH during a 300 h oil-based fermentation process and its relationship with monensin production

Previously, enzyme assays have been used to confirm both expression of the *S. coelicolor msdA* gene and the

catalytic activity of the corresponding MSDH product [31]. We used transcript analysis to examine *msdA* expression in *S. cinnamonensis* at various stages of an oil-based fermentation (Fig. 3) and compared this to the production of monensin (Fig. 4). As shown, the rate of monensin production was almost linear from day 3 through day 9 of the MOF fermentation. Clear *msdA* RT-PCR products with an expected size of 353 bp were observed at 18 h of MOV culture (the first stage), 24 h of MOB culture (the second stage) and at 72 h (3 days) of MOF culture (the third stage). At 168 h (7 days) and 240 h (10 days) of MOF culture, we could not see *msdA* RT-PCR products. While not quantitative, these results were reproducible and indicate expression of *msdA* through the fermentation process up to the onset of monensin production. These observations suggest MSDH activity is present at this time and possibly further into the fermentation period, depending upon the half-life of the protein. These observations contrast those recently made for the *ccr* gene, where clear transcripts were observed throughout the 10 days of MOF culture (the CCR product plays an important role in providing methylmalonyl CoA precursors under these fermentation conditions) [13].

The incorporation of [2, 4-$^{13}C_2$]butyrate into monensin A

As shown in Fig. 1, butyryl-CoA can be isomerized into isobutyryl-CoA, and can then be converted to methylmalonyl CoA by either route A or route B. Route A (using MSDH) would provide [1, 3-$^{13}C_2$]methylmalonyl-CoA from [2, 4-$^{13}C_2$]butyrate, while route B (direct oxidation of isobutyryl CoA) would provide [1-^{13}C] methylmalonyl-CoA. Labeling studies along these lines have often been carried out using [1-^{13}C]butyrate, [1-^{13}C] isobutyrate, [2-^{13}C] valine and [1-^{13}C]methacrylate and are consistent with processing of the labeled material via route B [16, 17, 19, 21]. However these experiments cannot preclude a contributing role from route A, as this would lead to a complete loss of label. In other cases where compounds such as [^{13}C-*methyl*]isobutyrate have been used, the results are all consistent with route B and do not indicate detectable processing via route A [3, 23]. However, these experiments have not been carried out under oil-based extended fermentation conditions where high titers of the polyketide product are made, and it has now become clear that the role of the butyrate pathway in monensin production in *S. cinnamonensis* varies dramatically with the fermentation conditions [13].

Thus, an incorporation study of monensin A with [2, 4-$^{13}C_2$]butyrate in the oil-based extended fermentation process was carried out. ^{13}C NMR Analysis of the monensin A obtained (Fig. 5) revealed an approximate threefold enrichment for C16 and C33 of monensin A (positions formally derived from C2 and C4 of ethymalonyl CoA) and comparable levels of labeling for C29, C30, C31, C34, C35, and C36 (positions formally derived from C3 of methylmalonyl CoA). This observation is consistent with our previous conclusion that under these conditions most of the methylmalonyl-CoA pool is derived via a butyryl-CoA intermediate [13]. In this study, there was no detectable ^{13}C enrichment for C-1, C-3, C-5, C-11, C-17, C-21 and C-23 of monensin A (positions formally derived from C-1 of methylmalonyl-CoA). This labeling pattern is inconsistent with metabolism of [2, 4-$^{13}C_2$]butyrate via route A, but consistent with route B.

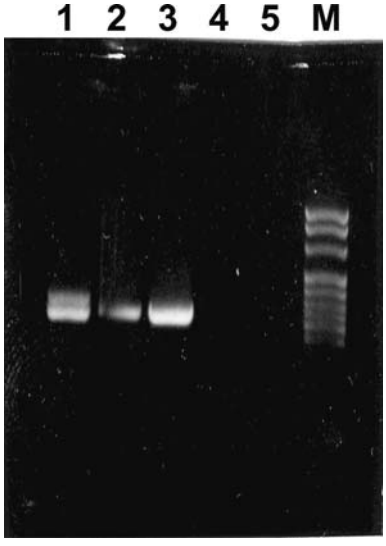

Fig. 3 Analysis of msdA expression with RT-PCR in the oil-based monensin fermentation. *Lane 1*: MOV culture. *Lane 2*: MOB culture. *Lane 3*: 72 h MOF culture. *Lane 4*: 168 h MOF culture. *Lane 5*: 240 h MOF culture

Discussion

We have previously shown that in an oil-based extended fermentation of *S. cinnamonensis*, the methylmalonyl CoA required for the high monensin titers is derived from a butyryl-CoA intermediate. Much of this butyryl CoA was derived from the condensation of two acetyl CoA molecules (the major catabolic product of oil degradation) in a pathway involving CCR (Fig. 1) [13]. In this study, we wished to ascertain if MSDH and route A plays a significant role in the conversion of butyryl CoA to methylmalonyl CoA. We successfully cloned an *S. cinnamonensis msdA* gene which is highly homologous to both *msdA* of *S. coelicolor* (encoding a protein with demonstrated MSDH activity) and *S. avermitilis*. We also demonstrated expression of *msdA* in the fermentation process at the onset of monensin production (but not later), an observation which is not incompatible with its role in methylmalonyl CoA production. However, insertional inactivation of *msdA* had no effect on the monensin titers of either *S. cinnamonensis* C730.1 or C730.7, demonstrating that in neither of these two

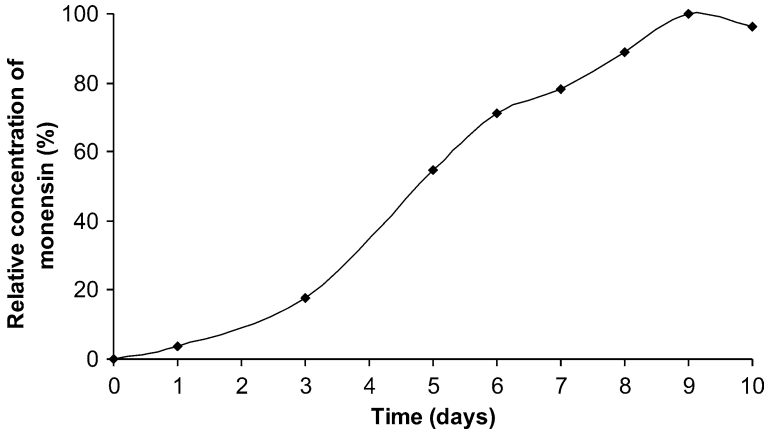

Fig. 4 Time course of monensin production by *S. cinnamonensis* C730.1

industrial strains, taken at different junctures during a strain improvement process, is the *msdA* required for conversion of butyryl CoA to methylmalonyl CoA.

This experiment alone does not prove that the *msdA* gene product does not play a role under normal growth conditions. Another protein with MSDH activity may substitute for loss of *msdA* in CXL1.1 and CXL1.7, or alternatively, a block in route A (from the hydroxyisobutyryl CoA intermediate) may simply lead to greater flux down pathway B (Fig. 1). The deletion of *msdA* in *S. coelicolor* has been shown to result in a mutant where no detectable levels of MSDH activity could be observed after growth in liquid culture [31]. There is also no evidence of any additional *msdA* genes in *S. cinnamonensis*, in fact all PCR reactions using sets of primers (F1, F2, R1 and R2) from chromosomal DNA amplified a single gene. Thus an MSDH activity substituting for loss of the *msdA* in *S. cinnamonensis* seems unlikely, but cannot be ruled out conclusively. However, the incorporation experiment with [2, 4-$^{13}C_2$] butyrate indicates that even when the *msdA* is present and expressed (as in C730.1 strain) the labeled butyryl CoA is processed efficiently to labeled methylmalonyl CoA in a manner entirely consistent with route B, and with no evidence of processing via route A.

The results from both the labeling experiment and disruptional analysis of *msdA* indicate that MSDH and route A are not involved in methylmalonyl-CoA biosynthesis in *S. cinnamonensis* grown under these conditions. It is possible that route A does not function because the other required enzymes are not present. Only propionyl CoA carboxylase (PCC) required for converting propionyl CoA to methylmalonyl CoA has been unambiguously assigned for *S. coelicolor* [2, 22] or *S. cinnamonensis* (Reynolds KA and Florova G, unpublished data). Alternatively, route A may be a functional pathway but not observed in any of these analyses if processing of a 3-hydroxyisobutyryl CoA via the more direct route B is significantly more efficient. In this case, blocking either of the two oxidation steps in this process might permit processing via route A. The genes and corresponding enzymes required for these steps in this pathway have not yet been identified from any streptomycetes.

Route B may also be the major pathway for processing either valine or isobutyrate when *S. cinnamonensis* is grown on solid minimal media containing a single carbon source (as evidenced by the growth of CXL1.1 and CXL1.7 under these conditions). It is curious that the same is not true for *S. coelicolor* where *msdA* is required for growth on a sole carbon source. It is possible that under these growth conditions, expression of *S. coelicolor* genes required for route A and not route B are expressed, whereas in *S. cinnamonensis*, genes for route B are expressed under both these conditions and liquid growth in a series of different media. While this is the simplest interpretation of the observations, it is possible that the reasons may be more complex.

The different observations from these analyses clearly demonstrate that observations drawn from growth of a strain and selected mutants on a sole carbon source are not dependable predictors of the importance of different primary metabolic pathways for growth in a complex media. Indeed, growth on a sole carbon source can often provide quite surprising and contradictory observations. For instance, it has been shown that a *ΔicmA* mutant of *S. cinnamonensis* loses the ability to grow on valine or isobutrate as a sole carbon source (but retains the ability to grow on butyrate). As the isobutyryl CoA mutase encoded by this gene catalyzes the interconversion of isobutryl CoA and butyryl CoA (Fig. 1), this observation suggests that under these growth conditions valine and isobutyryl CoA are not processed by either route A or B but by some other as yet undetermined pathway leading from butyryl CoA [29]. We have also shown that CCR (catalyzing the conversion of crotonyl CoA to butyryl CoA) is required for growth of *Streptomyces collinus* [6] and *S. cinnamonensis* (K. Akopiants, G. Florova and K.A. Reynolds, unpublished data) on acetate as a sole carbon source. Nonetheless, incorporation studies with labeled acetate and acetoacetate into monensin A in liquid fermentation with a *Δccr* mutant of *S. cinnamonesis* have also indicated the presence of a pathway from acetoacetyl CoA to methylmalonyl CoA which passes neither through acetyl CoA or butyryl CoA [13, 14].

In conclusion, the role of *msdA* and the corresponding MSDH activity in both *S. cinnamonensis* and

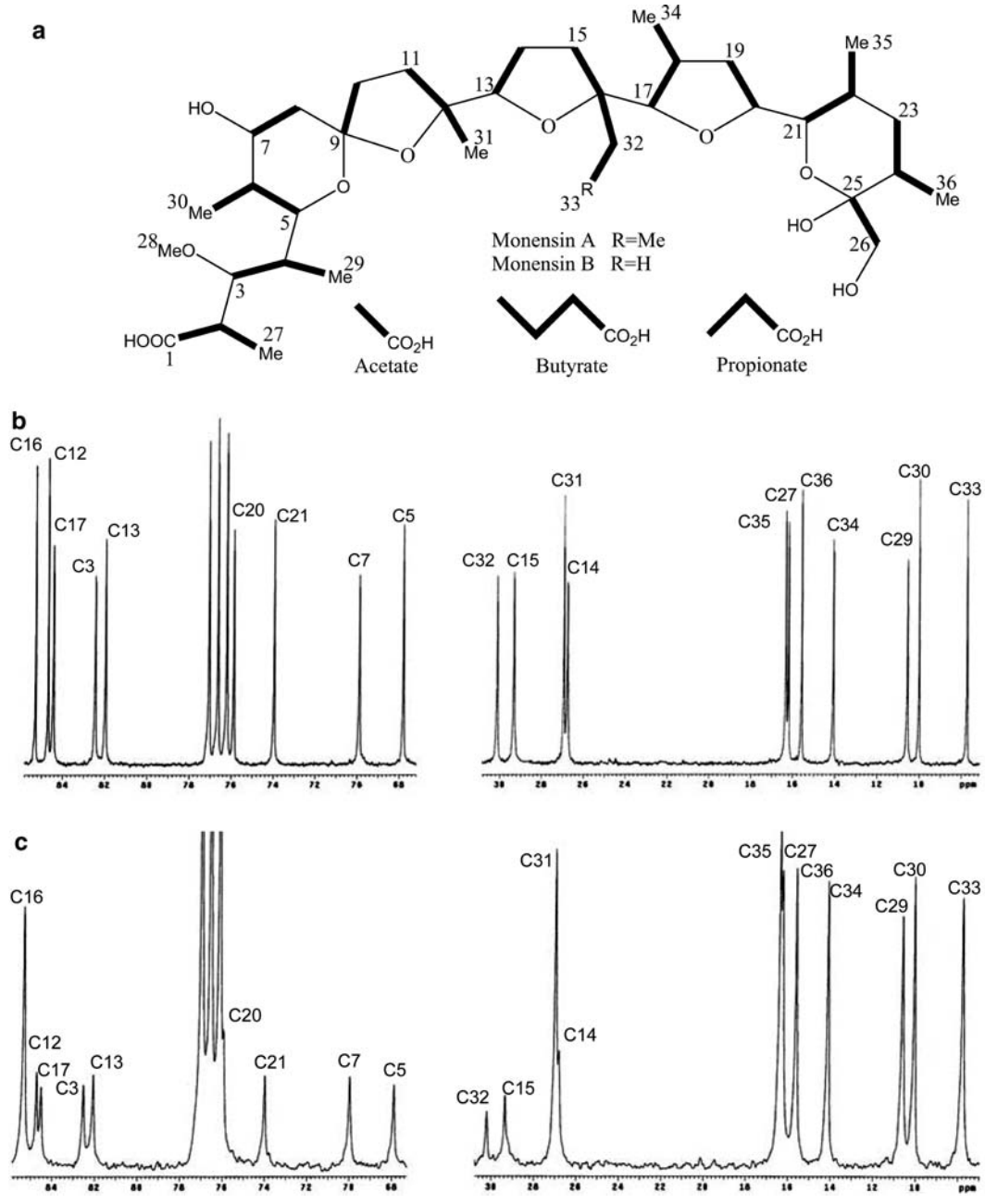

Fig. 5 **a** The structure of monensin A and B. Bonds derived intact from acetate-, propionate-, and butyrate-derived precursors are shown in *bold* **b** and **c**. A portion of the $13_C\{^1H\}$ NMR spectra of monensin A isolated from *S. cinnamonensis* C730.1 grown in the oil-based medium without feeding (**b**) and with feeding of [2, 4–$^{13}C_2$]butyrate (**c**). A threefold enrichment of the monensin A positions formally derived from C3 of propionate and C2 and C4 of butyrate was observed

S. coelicolor remains an enigma. Insights will only be gained through additional analysis, including both identification of genes involved in other steps leading from 3-hydroxyisobutyryl CoA (for both route A and B), and delineation of the additional pathways [13, 14, 29, 30] which appear to link acetoacetyl CoA and butyryl CoA with methylmalonyl CoA.

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