Crotonyl-coenzyme A reductase provides methylmalonyl-CoA precursors for monensin biosynthesis by *Streptomyces cinnamomensis* in an oil-based extended fermentation

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It is demonstrated that crotonyl-CoA reductase (CCR) plays a significant role in providing methylmalonyl-CoA for monensin biosynthesis in oil-based 10-day fermentations of *Streptomyces cinnamomensis*. Under these conditions *S. cinnamomensis* L1, a derivative of a high-titre producing industrial strain C730.1 in which *ccr* has been insertionally inactivated, produces only 15% of the monensin yield. Labelling of the coenzyme A pools using [^3H]- β -alanine and analysis of intracellular acyl-CoAs in the L1 and C730.1 strains demonstrated that loss of *ccr* led to lower levels of the monensin precursor methylmalonyl-CoA, relative to coenzyme A. Expression of a heterologous *ccr* gene from *Streptomyces collinus* fully restored monensin production to the L1 mutant. Using C730.1 and an oil-based extended fermentation an exceptionally efficient and comparably intact incorporation of ethyl [3,4- $^{13}\text{C}_2$]acetoacetate into both the ethylmalonyl-CoA- and methylmalonyl-CoA-derived positions of monensin was observed. No labelling of the malonyl-CoA-derived positions was observed. The opposite result was observed when the incorporation study was carried out with the L1 strain, demonstrating that *ccr* insertional inactivation has led to a reversal of carbon flux from an acetoacetyl-CoA intermediate. These results dramatically contrast similar analyses of the L1 mutant in glucose-soybean medium which indicate a role in providing ethylmalonyl-CoA but not methylmalonyl-CoA, thus causing a change in the ratio of monensin A and monensin B analogues, but not the overall monensin titre. These results demonstrate that the relative contributions of different pathways and enzymes to providing polyketide precursors are thus dependent upon the fermentation conditions. Furthermore, the generally accepted pathways for providing methylmalonyl-CoA for polyketide production may not be significant for the *S. cinnamomensis* high-titre monensin producer in oil-based extended fermentations. An alternative pathway, leading from the fatty acid catabolite acetyl-CoA, via the CCR-catalysed reaction is proposed.

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INTRODUCTION

Polyketides are a large group of structurally diverse natural products with a wide range of biological activities (Hopwood, 1997), many of which are produced by streptomycetes. The biosynthesis of a polyketide is catalysed by polyketide synthase (PKS) with a mechanism similar to that utilized in fatty acid synthesis. Despite their structural diversity, polyketides are assembled from several common building blocks, such as malonyl-CoA, methylmalonyl-CoA and ethylmalonyl-CoA (Hopwood, 1993; Katz, 1997; Katz & Donadio, 1993). In streptomycetes, malonyl-CoA and ethylmalonyl-CoA are considered to be generated by

carboxylation of acetyl-CoA and butyryl-CoA, respectively (Liu & Reynolds, 1999; Rodríguez & Gramajo, 1999). Numerous pathways can contribute to providing methylmalonyl-CoA, and the role of these pathways in different polyketide biosynthetic processes has been of interest for many years (Hunaiti & Kolattukudy, 1984; Reynolds *et al.*, 1988; Tang *et al.*, 1994; Zhang & Reynolds, 2001; Zhang *et al.*, 1999a).

Methylmalonyl-CoA is one of the most common precursors used by modular type I PKSs. All of the six polyketide extension steps in erythromycin biosynthesis utilize this precursor (Cortes *et al.*, 1990; Donadio *et al.*, 1991), while seven of the steps in monensin B biosynthesis utilize this precursor (Liu & Reynolds, 1999). The availability of sufficient intracellular methylmalonyl-CoA represents a limiting factor for production of significant quantities of polyketide

Abbreviations: CCR, crotonyl-CoA reductase; ICM, isobutyryl-CoA mutase; MCM, methylmalonyl-CoA mutase; PKS, polyketide synthase.

products in fermentation processes, both in natural hosts (Zhang & Reynolds, 2001; Zhang *et al.*, 1999a) and engineered hosts (Dayem *et al.*, 2002). A thorough understanding of the relative roles of pathways which contribute to generation of methylmalonyl-CoA is required if genetic manipulation of precursor pathways to improve polyketide titres in fermentation processes is to become a reality.

Currently, there are three recognized pathways which could contribute to generation of methylmalonyl-CoA (Fig. 1). (i) Isomerization of succinyl-CoA, catalysed by methylmalonyl-CoA mutase (MCM) (Hunaiti & Kolattukudy, 1984; Marsh, 1999; Thomä & Leadlay, 1998); (ii) Carboxylation of propionyl-CoA, catalysed by propionyl-CoA carboxylase (Bramwell *et al.*, 1996; Rodríguez & Gramajo, 1999) or methylmalonyl-CoA transcarboxylase (Hunaiti & Kolattukudy, 1982). The propionyl-CoA is either from the valine catabolism pathway of pseudomonads and mammals, degradation of odd chain fatty acids or other sources. (iii) A multistep oxidation of isobutyryl-CoA, via methacrylyl-CoA, β -hydroxyisobutyryl-CoA and methylmalonyl-CoA semialdehyde (Reynolds *et al.*, 1988). The isobutyryl-CoA is generated either from isomerization of butyryl-CoA via isobutyryl-CoA mutase (ICM), or valine catabolism. Sources of butyryl-CoA include metabolism of even-numbered fatty acids, and reduction of crotonyl-CoA generated from the condensation of two acetyl-CoA molecules. More recently a fourth and as yet undetermined pathway utilizing MeaA has emerged. The gene encoding the MeaA protein was found

in *Streptomyces collinus*, *Streptomyces cinnamomensis* and *Methylobacterium extorquens*. It encodes a MCM-like protein and appears to be involved in methylmalonyl-CoA formation (Smith *et al.*, 1996; Zhang & Reynolds, 2001). Finally, a monensin A labelling study with labelled acetoacetate in *meaA* and *meaA icm* mutants of *S. cinnamomensis* C730.1 (L1) has indicated the presence of potentially another pathway which links acetoacetyl-CoA with methylmalonyl-CoA, but which does not use MeaA or proceed through a butyryl-CoA intermediate (Zhang & Reynolds, 2001).

Numerous studies have attempted to assess the role of various pathways to provide methylmalonyl-CoA for polyketide biosynthesis (Tang *et al.*, 1994; Vrijbloed *et al.*, 1999; Zhang *et al.*, 1999b), and particularly for monensin. In a wild-type *S. cinnamomensis* strain it has been shown that neither ICM nor MCM is essential for monensin production (Vrijbloed *et al.*, 1999). Thus, neither pathway to methylmalonyl-CoA which uses these coenzyme B₁₂-dependent enzymes is important for monensin production by this strain, under the particular fermentation conditions used. It has been shown that loss of both *meaA* and *icm* genes leads to decreased monensin titres, which can be restored by plasmid-based expression of *mutAB* (encoding the *Amycolatopsis mediterranei* MCM) (Zhang & Reynolds, 2001). Thus, under the low monensin titre fermentation conditions used in this study it was concluded that MeaA must contribute to the methylmalonyl-CoA pool (Zhang & Reynolds, 2001).

Fig. 1. Pathways leading to methylmalonyl-CoA, malonyl-CoA and ethylmalonyl-CoA precursors used for monensin biosynthesis in *S. cinnamomensis*. There is a net synthesis of succinyl-CoA from acetyl-CoA by the glyoxylate cycle, but not by the citric acid cycle. A bold bond indicates intact processing of [3,4-¹³C₂]acetoacetyl-CoA into ethylmalonyl-CoA. Dashed arrows indicate alternative pathways which generate propionyl-CoA and likely methylmalonyl-CoA. CCR, crotonyl-CoA reductase; ICM, isobutyryl-CoA mutase; MCM, methylmalonyl-CoA mutase; MeaA, a mutase with sequence similarity to ICM and MCM.

Similar types of study in an early industrial strain of *S. cinnamomensis* C730.1 have indicated that crotonyl-CoA reductase (CCR), which catalyses the conversion of crotonyl-CoA to butyryl-CoA, plays an important role in providing ethylmalonyl-CoA for monensin biosynthesis, but not methylmalonyl-CoA (Liu & Reynolds, 1999). Two major monensin products from fermentations of *S. cinnamomensis* are monensin A and monensin B (Fig. 2a). Monensin A is made using an ethylmalonyl-CoA instead of methylmalonyl-CoA during the fifth elongation step (Liu & Reynolds, 1999). We have previously cloned and sequenced *S. cinnamomensis* *ccr* and showed that insertional inactivation of this gene in the C730.1 strain generates an

L1 mutant which produces a significantly higher monensin B to monensin A ratio. As there was no impact on overall monensin titres, this study suggested that the butyryl-CoA product of CCR was important for producing ethylmalonyl-CoA but not generating methylmalonyl-CoA (Liu & Reynolds, 1999). This conclusion was supported by labelling studies with [1,2- $^{13}\text{C}_2$]acetate which led to significantly higher intact labelling of the ethylmalonyl-CoA-derived than the methylmalonyl-CoA-derived positions of monensin (Liu & Reynolds, 1999).

All of these previous studies in *S. cinnamomensis* have used fermentation conditions which produce low monensin titres ($< 1 \text{ g l}^{-1}$). Chemically defined and glucose-soybean media have been used predominantly (although some fermentations with the inclusion of oil have been carried out) (Liu & Reynolds, 1999, 2001; Reynolds *et al.*, 1988). The fermentations have typically been in shake-flask cultures for 5–7 days. Industrial fermentations of *S. cinnamomensis* are longer, and utilize oil as the primary carbon source. Two different kinds of oil are used in the initial fermentation medium and are provided as a supplement during the fermentation process. Under these conditions monensin titres are significantly higher. The methylmalonyl-CoA used for monensin production must be efficiently generated from acetyl-CoA, the major catabolite from oil degradation under these conditions. This conversion could occur via the glyoxylate cycle (passing through a succinyl-CoA intermediate) or by an alternative anaplerotic process involving butyryl-CoA and isobutyryl-CoA intermediates (Fig. 1) and in which CCR plays a critical role. We have established the latter pathway is important, since loss of *ccr* in the L1 mutant leads to an 85 % decrease in monensin titres under these fermentation conditions. Labelling studies, a complementation study and analysis of intracellular acyl-CoA pools are all consistent with a major role for CCR in providing methylmalonyl-CoA for monensin production. The results from this study are dramatically different from previous analyses of the L1 mutant and underscore the importance of using appropriate fermentation conditions for probing the role of different pathways in providing precursors for polyketide biosynthesis. Equally important, the study unequivocally demonstrates that an unusual and mostly ignored pathway, in which acetyl-CoA can be converted via butyryl-CoA to methylmalonyl-CoA, is critical for monensin production in an oil-based extended fermentation. A similar pathway may play a significant role in providing methylmalonyl-CoA for other polyketide products where oil is the primary carbon source in the fermentation, challenging the current paradigms regarding the origin of this important and ubiquitous precursor.

Fig. 2. Incorporation of ethyl [3,4- $^{13}\text{C}_2$]acetoacetate into monensin A. (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 proton decoupled NMR spectra of monensin A isolated from *S. cinnamomensis* C730.1 (b) and L1 mutant (c) grown with labelled ethyl acetoacetate in an oil-based extended fermentation. A ^{13}C – ^{13}C coupling is indicated by doublets surrounding the corresponding natural abundance carbon signals, and indicates intact incorporation into monensin A.

METHODS

Materials and general methods. *S. cinnamomensis* C730.1 was a gift of Eli Lilly Co. Construction of *S. cinnamomensis* L1 was reported previously by Liu & Reynolds (1999). MOV medium was the same as that described previously by Stark *et al.* (1967). Each litre of MOB medium contains 40 g cerelese, 35.2 g soybean flour,

2.35 g NaSO₄, 2.35 g NaNO₃, 3.2 g CaCO₃, 0.17 g K₂HPO₄ and trace minerals. MOF contains 5.3% methyl oleate in addition to all components of MOB. Ethyl [3,4-¹³C₂]acetoacetate (0.5 g, ¹³C, 99%) was purchased from Cambridge Isotope Laboratories. [³H]-β-Alanine (1 mCi ml⁻¹, 20 Ci mmol⁻¹) was obtained from Moravsek Biochemicals. [2,4-¹³C₂]butyrate (0.5 g, ¹³C, 99%) was obtained from Sigma-Aldrich. Malonyl-CoA, CoA and methylmalonyl-CoA were obtained from Sigma. Polyclonal antibodies to CCR were developed by the University of Maryland antibody development service.

Fermentation of *S. cinnamomensis* in an oil-based medium.

The fermentation was carried out in three stages. First, 50 ml of MOV medium in a 300-ml flask was inoculated with a fresh R2YE agar plate culture and incubated at 32 °C, 300 r.p.m. for 18 h. Then 0.5 ml of this MOV medium culture was transferred into a 300-ml flask containing 50 ml MOB medium and was incubated at 32 °C, 300 r.p.m. for 24 h. Finally 0.5 ml of MOB medium culture was used to inoculate 5 ml MOF medium in a 50-ml flask and incubated for 10 days at 34 °C at 260 r.p.m. On the fifth day, the MOF culture was supplemented with 1.2 ml natural oils. Purification of monensin A and quantification of monensins from the fermentation broth were performed as described previously (Reynolds *et al.*, 1988).

Western blotting analyses. The polyclonal antibodies against CCR were raised using recombinant CCR expressed in *E. coli* and purified by metal-chelate affinity chromatography. Samples at the different time-points of fermentations were washed with water and diluted in a SDS-loading buffer. Subsequently, the samples were heated to 100 °C for 10 min and cooled on ice, before loading onto NuPAGE 10% Bis-Tris gel (Cat. No. NP0301, Invitrogen) with NuPAGE MOPS SDS running buffer (Cat. No. NP0001, Invitrogen). Proteins were then electroblotted on microporous polyvinylidene difluoride (PVDF) membranes. The membranes were blocked for 1 h, washed, and incubated with primary antibodies (1:750) overnight at 4 °C. After a wash step, immunocomplexes were detected using an anti-mouse IgG (Sigma). The membranes were developed with the NBT/BCIP (Roche) according to the manufacturer's protocol.

Transcript analysis. Total RNA was isolated from *S. cinnamomensis* grown in oil-based medium at different times of fermentation. In order to prevent mRNA degradation, two volumes of RNeasy Protect reagent (Qiagen) were added to one volume of the broth. The RNeasy Midi kit (Qiagen) was used for total RNA preparation according to the manufacturer's instructions. Nucleic acid preparations were treated with DNase I (DNA-free kit; Ambion) as recommended by the manufacturer. Two primer sets were used to detect *ccr* and *mcm* transcripts using a One-Step RT-PCR kit (Qiagen) following methods recommended by the manufacturer: *ccr* forward, 5'-CAAGGACGAGACGGAGATGTT-3'; *ccr* reverse, 5'-CGACACAGATGGGGTTGGC-3' and *mcm* forward, 5'-TGCCGCGCTACAAC-TCCATCTCG-3'; *mcm* reverse, 5'-CTCGTCCAGGGCGTTCGTGTGC-3'. Dimethyl sulfoxide (5%, v/v final) was added to the RT-PCR mixture. For RT-PCR, conditions were as follows: an initial DNA strand synthesis with reverse transcriptase, 52 °C for 30 min, followed by 95 °C for 15 min to activate the DNA polymerase, and then 35 cycles of 94 °C for 10 s, 55 °C for 20 s, 72 °C for 45 s. The negative controls were carried out with each experimental reaction using same enzyme mix but without the initial reverse transcription step.

Incorporation of ethyl [3,4-¹³C₂]acetoacetate and [2,4-¹³C₂]butyrate into monensin A. Ethyl [3,4-¹³C₂]acetoacetate (22.5 mM final concentration) and 30 mM (final concentration) of [2,4-¹³C₂]butyrate were added into two separate fermentation broths in three equal portions on days 3, 4 and 5. After 10 days of fermentation monensin A (16–25 mg) was purified and used for NMR analysis.

HPLC analysis of acyl-CoAs. HPLC analyses of acyl-CoAs was performed with a 250 × 4.6 mm 5 μm Luna C₁₈ column (Phenomenex), with only minor modifications to a recently reported method (Dayem *et al.*, 2002). For non-radiolabelled samples, the eluant was monitored at 260 nm with a UV detector. Radiolabelled samples were analysed with β-RAM model 2 Radio flow-through detector (IN/US Systems). The ratio of scintillation fluid: eluant was 1.5:1. Peaks were assigned by comparing elution time with CoA, malonyl-CoA and methylmalonyl-CoA standards.

Analysis of intracellular acyl-CoA pools. [³H]-β-Alanine (3.7 MBq total) was added into the MOF fermentation broth in two equal portions on day 1 and day 5. On days 3, 7 and 10 of fermentation the cells were collected by centrifugation and washed with sterilized deionized water several times until the supernatant was colourless. The cell pellet was resuspended with 4 ml ice-cold 10% TCA, and then sonicated on ice 5 × 30 s at 5 W. Precipitants were removed by centrifugation and supernatants were filtered with a 0.2 μm syringe filter and then used for HPLC analysis (Dayem *et al.*, 2002).

Expression of *S. collinus ccr* in *S. cinnamomensis* C730.1

L1. *S. collinus ccr* was PCR amplified from the expression plasmid pHL18 (Liu & Reynolds, 1999). The DNA primers were 5'-GA-ATTCGAGCTCGGTACCAGC-3' (rightward primer mapping onto upstream sequence of P_{ermE}) and 5'-ATCACAGTTGAATTCCTAAGCCAGG-3' (leftward primer mapping onto sequence downstream of *ccr*). The PCR primers both contained an *Eco*RI site (italicized) to facilitate cloning. The *Eco*RI-digested PCR product was cloned into the integrative shuttle vector pSET152 to give pGF200. pGF200 was introduced by conjugation (Hopwood *et al.*, 1985) into the *S. cinnamomensis* L1 strain and exconjugants were selected in the presence of 50 mg apramycin ml⁻¹, following standard protocols. Monensin production by the strain L1/pGF200 was determined in a standard fermentation procedure (as described above). Monensin production by C730.1/pSET152 was also evaluated.

RESULTS

Time-course of monensin production and ratio of monensin production in an oil-based fermentation medium

Numerous different fermentation conditions have been reported for *S. cinnamomensis*. We have grown the wild-type strain and industrial strain C730.1 in both a glucose-soybean medium (Liu & Reynolds, 1999; O'Hagan *et al.*, 1995; Zhang & Reynolds, 2001) and a chemically defined medium (Liu & Reynolds, 2001). The strain has also been grown in glucose-soybean meal supplemented with soybean oil, lard oil and methyl oleate (Reynolds *et al.*, 1988; Zhang & Reynolds, 2001). These fermentations are all usually for 5–7 days and have been reported to provide yields of monensin as much as 1–1.5 g l⁻¹ (Reynolds *et al.*, 1988). The ratio of monensin A to monensin B in both these strains is typically around 50:50 (Liu & Reynolds, 1999) although higher ratios of monensin A:B have been reported for the wild-type strain in an oil-based medium (Reynolds *et al.*, 1988). Using fermentation conditions for industrial production of monensin we grew the C730.1 strain for 300 h, with an oil supplementation after 120 h. As shown in Fig. 3 monensin continued to be produced throughout this period, and was at significantly higher levels than we

Fig. 3. Time-course of monensin production by the C730.1 (◆) and L1 strain (■).

previously observed. The ratio of monensin A to monensin B under these conditions was approximately 98:2, a dramatic shift from that reported for the C730.1 strain in other media (Liu & Reynolds, 1999, 2001).

Expression of CCR and MCM during a 300 h oil-based fermentation of *S. cinammonensis*

Previous studies have used CCR activity in *S. cinammonensis* C730.1 cell extracts to gauge CCR expression levels (Liu & Reynolds, 1999, 2001). These studies have indicated that the levels can vary dramatically with the fermentation medium. In several different chemically defined media no detectable levels of CCR activity were observed, whereas in YEME or a glucose-soybean medium used for monensin production, significant CCR activity was observed (Liu & Reynolds, 1999, 2001). CCR expression in an oil-based extended fermentation has not been examined. We used transcript analyses (Fig. 4a) and demonstrated that CCR is expressed at all times during the fermentation. While not quantitative these analyses reproducibly suggested lower levels of transcript in the initial seed (MOV medium) and second stage media (MOB medium). Clear *ccr* transcripts were observed for 96 h and 216 h in the oil-based extended fermentation, demonstrating expression of CCR during the maximal time of monensin production. Using the same RNA template from these fermentations, transcript analyses of *mutB* encoding one of the *S. cinammonensis* MCM subunits (Vrijbloed *et al.*, 1999) showed that it was expressed at all stages of the fermentation process. Western analyses (Fig. 4b) confirmed that the CCR protein was present during the fermentation processes, particularly during the time of maximal monensin production.

S. cinammonensis L1 produces 15% of the monensin yield of the wild-type *S. cinammonensis* C730.1 in an oil-based extended fermentation

The role of CCR in providing butyryl-CoA for monensin A biosynthesis has previously been demonstrated by disruption of the *ccr* gene. In a glucose-soybean medium, the loss of CCR led to a dramatic change of ratio of monensin A to

Fig. 4. Analysis of CCR expression during an oil-based 240 h fermentation of *S. cinammonensis* C730.1. (a) Transcript analysis for *ccr* and *mcm* genes. RT-PCR products for *ccr* gene from 24 h MOB, 96 h fermentation medium, 210 h fermentation medium, 240 h fermentation medium. (b) Western blot analysis for CCR from 24 h MOB medium, 96 h fermentation medium, 210 h fermentation medium, 240 h fermentation medium and 240 h fermentation media of *S. cinammonensis* C730.1/pGF200.

monensin B from 50:50 to 12:88, but did not affect the overall monensin yield (Liu & Reynolds, 1999). In our present work, where we used an oil-based extended fermentation for monensin production, almost completely opposite observations were made. The L1 mutant generates only 15% of the overall monensin titre observed for the C730.1 (as shown in Fig. 3, the monensin production was lower throughout the fermentation process) whereas the ratio of monensin A to monensin B for the L1 mutant was 87:13, only slightly less than the 98:2 observed for the C730.1 strain under the same fermentation conditions. The loss of monensin titre is consistent with the role of CCR in providing a precursor common to both monensin A and monensin B, such as methylmalonyl-CoA.

We confirmed that the decrease in monensin titre in the L1 mutant was due specifically to loss of CCR by carrying out a complementation experiment. In this work a pSET152 derivative (pGF200) carrying the *S. collinus ccr* gene under the control of the *ermE*⁺ promoter was conjugated into the L1 strain. The resulting L1/pGF200 strain generated comparable levels of monensin compared to both the C730.1 and C730.1/pSET152 strains. This set of experiments demonstrates that a single copy of a heterologous *ccr* gene expressed from *ermE*⁺ promoter can fully restore monensin titres to the L1 strain. The work also demonstrates that pSET152 and derivatives thereof can be integrated into the ϕ C31-*attB* site in *S. cinammonensis* C730.1 without impacting fermentation titres. It has been shown that in some

strains such site-specific integration can lead to decreased fermentation titres (Baltz, 1998).

The incorporation of ethyl [3,4-¹³C₂]acetoacetate and [2,4-¹³C₂]butyrate into monensin A

It has long been established that dual-labelled acetoacetyl-CoA can be incorporated intact into methylmalonyl-CoA-derived positions of monensin (Reynolds *et al.*, 1988). The intact incorporation is inconsistent with a pathway in which acetyl-CoA generated from acetoacetyl-CoA is converted via the citric acid cycle and succinyl-CoA to methylmalonyl-CoA. Rather, the intact labelling is consistent with a more direct pathway which may involve a butyryl-CoA intermediate (Zhang & Reynolds, 2001). In all experiments reported to date there is incorporation into the acetyl-CoA (malonyl-CoA) methylmalonyl-CoA and butyryl-CoA (ethylmalonyl-CoA)-derived positions suggesting no overwhelming flux in pathways originating from acetoacetyl-CoA (Zhang & Reynolds, 2001). Also, the level of incorporation into the butyryl-CoA (ethylmalonyl-CoA)-derived positions is typically 10-fold higher than either the acetyl-CoA (malonyl-CoA) or methylmalonyl-CoA, suggesting significant dilution of the labelled material by other pathways (Zhang & Reynolds, 2001). It has thus been argued that a pathway passing through acetoacetyl-CoA and potentially butyryl-CoA as an intermediate is not the primary source of methylmalonyl-CoA for monensin production (Zhang & Reynolds, 2001).

A dramatically different series of results were obtained from incorporation studies using the C730.1 *S. cinnamomensis* strain in the oil-based extended fermentation. As shown in Fig. 2(b) comparable intact labelling into both the butyryl-CoA- (ethylmalonyl-CoA) and methylmalonyl-CoA-derived positions was observed. Enriched doublets surrounding the natural abundance signals for C-2, C-4, C-6, C-12, C-18, C-22 and C-24 and the corresponding methyl substituents were observed, consistent with incorporation of [2,3-¹³C₂]methylmalonyl-CoA. The level of labelling was approximately $1.1 \pm 0.12\%$. A similar set of enriched-doublets was observed for C-32 and C-33, consistent with incorporation of [3,4-¹³C₂]ethylmalonyl-CoA. In this case the level of enrichment was $1.35 \pm 0.1\%$. These data suggest that the butyryl-CoA pool used to generate ethylmalonyl-CoA precursor also generates more than 80% of the methylmalonyl-CoA precursor pool. An alternative interpretation is that the labelled acetoacetyl-CoA is converted to methylmalonyl-CoA primarily via a pathway not using a butyryl-CoA intermediate, and that labelled butyryl-CoA generated from this acetoacetyl-CoA is diluted by a large pool of butyryl-CoA generated from the degradation of long chain fatty acids. This possibility was discounted by carrying out additional incorporation studies with ethyl [3,4-¹³C₂]acetoacetate (the L1 mutant, described below) and [2,4-¹³C₂]butyrate (C730.1 strain). In the latter case, a threefold enrichment of both C16 and C33 (derived from C2 and C4 of ethylmalonyl-CoA) and the

methyl substituents of monensin (derived from C3 of methylmalonyl-CoA) were observed (data not shown). These observations clearly support the hypothesis that the major pathway providing methylmalonyl-CoA under these conditions passes through a butyryl-CoA intermediate. The final observation from these incorporation studies was that there was no detectable level of labelling of the malonyl-CoA-derived positions of monensin (see C10 in Fig. 2b), from either labelled ethyl acetoacetate or butyrate. Thus the pathway flux from acetoacetyl-CoA, the last intermediate in the degradation of straight chain fatty acids, is not towards acetyl-CoA, a surprising observation given that fatty acid degradation provides the primary carbon source in the fermentation. The observation indicates that instead the carbon flux is from acetoacetyl-CoA towards butyryl-CoA (which is subsequently efficiently converted to both ethylmalonyl-CoA and methylmalonyl-CoA).

A labelling study with ethyl [3,4-¹³C₂]acetoacetate was also carried out with the *S. cinnamomensis* L1 mutant. Intact labelling of the methylmalonyl-CoA-derived positions decreased to 0.16% (a 95% decrease from that observed in the C730.1 strain). A significant drop in intact labelling of the positions derived from C3 and C4 of butyryl-CoA (ethylmalonyl-CoA) to 0.4% (a 70% decrease) was also observed. Previous analyses of the L1 mutant have shown that labelling of these units by [1,2-¹³C₂]acetate (which would generate [3,4-¹³C₂]acetoacetyl-CoA *in vivo*) also decreases by more than 75% relative to the C730.1, but that intact low-level labelling of the methylmalonyl-CoA-derived positions is unaffected (Liu & Reynolds, 1999). The observations of loss of labelling of ethylmalonyl-CoA- and methylmalonyl-CoA-derived positions in the oil-based extended fermentation is again consistent with the hypothesis that these precursors are obtained from a CCR-mediated pathway in which acetoacetyl-CoA is channelled through a common butyryl-CoA intermediate. Loss of CCR blocks this pathway, leading to the observed decreased monensin titres. The acetoacetyl-CoA intermediate from straight chain fatty acid degradation, unable to generate butyryl-CoA, is thus converted to acetyl-CoA (malonyl-CoA), representing a switch in flux from this pathway intermediate. Consistent with this hypothesis, the decreased levels of labelling of the methylmalonyl-CoA and ethylmalonyl-CoA-derived positions of monensin were matched by a concomitant increase in labelling of the acetyl-CoA (malonyl-CoA)-derived positions of monensin (see Fig. 2c). The level of labelling in the product of the L1 strain was 0.4% (Fig. 2c), in contrast to the lack of any detectable labelling in that of C730.1 strain (Fig. 2b) grown under similar conditions.

Direct analysis of intracellular acyl-CoA pools

The loss of overall monensin titres and labelling studies with the L1 mutant were all consistent with an inability to convert the primary carbon source in the fermentation into methylmalonyl-CoA. Direct evidence for this hypothesis was sought by carrying out fermentations in the

presence of [^3H]- β -alanine. The radiolabelled β -alanine is a biosynthetic precursor of coenzyme A and thus serves to radiolabel all acyl-CoA pools within a cell (Dayem *et al.*, 2002). The ^3H -labelled acyl-CoAs in the crude lysates are separated by HPLC and detected by radioactive detector. Analysis of radiolabelled pools in this way has been described previously for *E. coli* *panD* strains and has led to very efficient labelling of the coenzyme A pools (Cronan, 1980; Dayem *et al.*, 2002; Jackowski & Rock, 1984). Creation of a *panD* equivalent mutant in *S. cinnamomensis* strain L1 would introduce a second mutation and thus cloud any interpretation of the effect of the *ccr* loss to acyl-CoA pools in the C730.1 strain. Despite the associated loss in sensitivity, we were able to compare the relative levels of malonyl-CoA, CoASH and methylmalonyl-CoA (Fig. 5), and observed clear and reproducible differences in the relative ratio of intracellular acyl-CoAs between *S. cinnamomensis* C730.1 and the *ccr* mutant C730.1 L1 on each of days 3, 7 and 10. In both strains, we observed relatively small levels of malonyl-CoA which did not differ significantly either between the two strains, or over the cause of the fermentation. The loss of *ccr*, however, causes a decrease in the level of methylmalonyl-CoA relative to CoASH during the time of monensin production (day 3 and 7). In *S. cinnamomensis* C730.1, the amount of methylmalonyl-CoA is significantly larger than the CoA pool (comprising about 74–82 % of combined pools). The situation is reversed in the L1 mutant, where methylmalonyl-CoA pools represent only 18–26 % of this combined CoA pool. These observations support the role of CCR in providing the monensin biosynthetic pathway with methylmalonyl-CoA. One additional interesting observation is that on day 10 of the fermentation the percentage of CoASH decreased, while methylmalonyl-CoA increased, in both strains. In the C730.1 strain only malonyl-CoA and methylmalonyl-CoA were detectable. In the L1 strain the levels of methylmalonyl-CoA exceeded the CoA levels. While all interpretations of relative levels of acyl-CoA pools should be made cautiously, these observations suggest that the loss of monensin production is not lack of precursor availability. Rather, lower monensin production causes a build up of these precursors.

DISCUSSION

Sources of ethylmalonyl-CoA

Butyryl-CoA, the presumed precursor to ethylmalonyl-CoA, can originate from many pathways. It has long been established that one source is via isomerization of the valine catabolite, isobutyryl-CoA. Indeed numerous incorporation studies have demonstrated that either labelled valine or isobutyric acid can be incorporated into both butyrate-derived straight chain fatty acids and butyryl-CoA (ethylmalonyl-CoA)-derived positions of polyketide antibiotics (Reynolds & Robinson, 1985; Reynolds *et al.*, 1986, 1988; Wallace *et al.*, 1995). More recently it has been shown that CCR plays a critical role in providing butyryl-CoA from

Fig. 5. Partial HPLC spectra of intracellular acyl-CoAs pools. (a), (b) and (c) correspond to days 3, 7 and 10, respectively. The thin line represents *S. cinnamomensis* C730.1 (—). The thick line is *S. cinnamomensis* C730.1 L1 (—). Only trace amounts of malonyl-CoA were observed in both strains. The area of the CoA and methylmalonyl-CoA peaks were calculated as a percentage of their combined area: day 3, C730.1 26 % CoA + 74 % methylmalonyl-CoA, L1 64 % CoA + 36 % methylmalonyl-CoA; day 7, C730.1 18 % CoA + 82 % methylmalonyl-CoA, L1 64 % CoA + 36 % methylmalonyl-CoA; day 10, C730.1 4 % CoA + 96 % methylmalonyl-CoA, L1 37 % CoA + 63 % methylmalonyl-CoA.

acetyl-CoA and acetoacetyl-CoA precursors in many polyketide biosynthetic processes (Liu & Reynolds, 1999). Indeed, many gene clusters which encode antibiotics using an ethylmalonyl-CoA precursor often encode what is assumed to be a second copy of *ccr* (Gandecha *et al.*, 1997; Kakavas *et al.*, 1997; Palaniappan *et al.*, 2003; Rangaswamy *et al.*, 1998; Wu *et al.*, 2000). Surprisingly, the monensin biosynthetic gene cluster does not contain a *ccr* gene (Oliynyk *et al.*, 2003). In an oil-based extended fermentation, butyryl-CoA

is generated as an intermediate during the degradation of even-number fatty acids. This β -oxidation process also generates acetyl-CoA (for palmitate there would be a 7:1 ratio of acetyl-CoA to butyryl-CoA). The very high monensin A to monensin B ratio observed for the C730.1 strain under the oil-based extended fermentation is consistent with β -oxidation providing the majority of the butyryl-CoA for monensin A biosynthesis. This pathway does not require CCR, and thus the L1 mutant, which has significantly reduced monensin production, still makes mostly monensin A. In this case the overall methylmalonyl-CoA titres have been reduced dramatically due to an inability to process the acetyl-CoA, but there is still sufficient butyryl-CoA and thus ethylmalonyl-CoA to support monensin A production at these levels.

Sources of methylmalonyl-CoA

The source of methylmalonyl-CoA production in most fermentation processes has been a source of some controversy. Clearly the predominant carbon source in the fermentation will determine which pathway or pathways play significant roles. Oxidation of the valine catabolite isobutyryl-CoA has been well studied in many streptomycete strains. However, studies have clearly shown that this pathway does not contribute significantly to precursor supply for either doramectin biosynthesis in a *Streptomyces avermitilis bkd* mutant, or monensin biosynthesis in *S. cinnamomensis* C730.1 grown in glucose-soybean meal (Cropp *et al.*, 2000; Zhang & Reynolds, 2001). Valine is also not the predominant carbon source in the complex media used in industrial fermentations for polyketide products, and is thus unlikely to provide the majority of methylmalonyl-CoA precursors. The same is likely to be true for a pathway which involves direct carboxylation of propionyl-CoA, as only a small proportion of the carbon sources present in most industrial fermentations will be catabolized to propionyl-CoA. An isomerization of the citric acid cycle intermediate succinyl-CoA to generate (2R)-methylmalonyl-CoA and a subsequent epimerization step is likely the major source of (2S)-methylmalonyl-CoA in fermentation processes grown in carbohydrate and/or protein-based media. Indeed, it has been shown that MCM can play a significant role in providing methylmalonyl-CoA precursors for monensin biosynthesis for *S. cinnamomensis* C730.1 grown in a glucose-soybean medium (Zhang & Reynolds, 2001; Zhang *et al.*, 1999a). This pathway has been introduced into *E. coli* strains and shown to be essential for production of polyketide products such as 6-deoxyerythronolide B (Dayem *et al.*, 2002).

Fermentations in which the carbon source is primarily fats and oils will generate significant quantities of acetyl-CoA, which needs to be converted to methylmalonyl-CoA in order to generate high titres of the polyketide product. In most bacteria, acetate assimilation is accomplished via the glyoxylate cycle, which catalyses the net synthesis of succinyl-CoA from two molecules of acetyl-CoA. Analysis of the *Streptomyces coelicolor* genome sequence reveals the

presence of genes putatively encoding isocitrate lyase and malate synthase, the two essential enzymes for this process. Malate synthase and isocitrate lyase have recently been studied from several streptomycetes (Chan & Sim, 1998; Goh *et al.*, 2003; Huttner *et al.*, 1997; Loke & Sim, 2000; Loke *et al.*, 2002; Soh *et al.*, 2001). We have cloned and sequenced homologues of these genes from *S. cinnamomensis*. While glyoxylate enzymes appear to be present in a variety of streptomycetes, their role in acetate assimilation or providing precursors such as methylmalonyl-CoA for polyketide biosynthesis remains undetermined. An alternative pathway for acetate assimilation involving CCR has been proposed for streptomycetes (Han & Reynolds, 1997). This pathway, which leads directly to methylmalonyl-CoA, bypassing succinyl-CoA, was found over 15 years ago with the discovery of the coenzyme B₁₂-dependent rearrangement which catalyses the interconversion of butyryl-CoA and isobutyryl-CoA (Reynolds & Robinson, 1985; Reynolds *et al.*, 1986, 1988). To date, the evidence from isotopic labelling studies and gene deletion experiments have indicated that CCR and this butyryl-CoA pathway play a minor role in providing methylmalonyl-CoA for polyketide biosynthesis. However, these analyses were not conducted under conditions where the fermentation titres were optimized using an oil-based extended fermentation process. Under these conditions the labelling studies, the dependence of monensin titre on CCR, and the analysis of the acyl-CoA pools, together indicate that the butyryl-CoA pathway may indeed provide the majority of the methylmalonyl-CoA precursor, despite the observation that the MCM genes are clearly expressed and that genes encoding glyoxylate cycle enzymes are present.

The low-level monensin production and acyl-CoA pool analysis of the L1 strain together indicate that CCR is not essential for generation of all of the methylmalonyl-CoA. Clearly, the butyryl-CoA-derived intermediate from straight chain fatty acid degradation can be converted into methylmalonyl-CoA in the L1 mutant. Presumably other pathways, including MCM, also can contribute. The incorporation of dual-labelled ethyl acetoacetate into the butyryl-CoA (ethylmalonyl-CoA)-derived unit of monensin A in the L1 mutant (albeit at a much lower level than the C730.1 strain) also indicates that the butyryl-CoA pathway may still function. The conversion of acetoacetyl-CoA to butyryl-CoA in the L1 strain has previously been observed in glucose-soybean meal fermentations (Liu & Reynolds, 1999) and may reflect the presence of another enoyl thioester reductase able to catalyse this reaction.

Several studies have indicated the presence of an additional pathway or pathways which link acetoacetyl-CoA with methylmalonyl-CoA, but which do not proceed through a butyryl-CoA intermediate. Labelling studies with [1,2-¹³C₂]acetate in the L1 mutant and C730.1 strain in a glucose-soybean medium have demonstrated comparable low-level intact incorporation into the monensin positions derived from [2,3-¹³C₂] of methylmalonyl-CoA

(Liu & Reynolds, 1999, 2001). In a separate set of studies [$1\text{-}^{13}\text{C}$]-butyrate and ethyl [$1,3\text{-}^{13}\text{C}_2$]acetoacetate were incorporated into the methylmalonyl-CoA derived positions of monensin A in both an *icmA::hyg* mutant and a *mutB::hyg* mutant of *S. cinnamomensis* (Vrijbloed *et al.*, 1999). Thus it has been argued that in addition to the isomerization of butyryl-CoA (by ICM) and succinyl-CoA (by MCM) another pathway can generate methylmalonyl-CoA from acetoacetyl-CoA (Liu & Reynolds, 2001; Vrijbloed *et al.*, 1999; Zhang & Reynolds, 2001). The current studies show that such a pathway or pathways cannot be important for *S. cinnamomensis* grown in the oil-based extended fermentation as both the monensin titres decreased, and there was almost a complete loss of labelling of methylmalonyl-CoA derived positions by acetoacetyl-CoA in the L1 mutant (in fact, the flux from this intermediate switches towards the formation of acetyl-CoA).

Implications for strain improvement

Until now the butyryl-CoA pathway has not been considered as important in providing methylmalonyl-CoA for polyketide biosynthetic processes. Understanding changes that have led to strain improvement and attempts to rationally improve fermentation titres have often focused on the pathway from succinyl-CoA. Carboxylation of propionate and degradation of valine have also been considered as alternative potentially important sources. The present work demonstrates that in a *S. cinnamomensis* fermentation where the carbon source is primarily lipids, the butyryl-CoA pathway produces the majority of the methylmalonyl-CoA. It remains to be determined if this is the case for other oil-based fermentation processes. Nonetheless, it is clear that alternative pathways must be considered to have important roles and that the role of the fermentation medium is critical. Studies that aim to understand the role of precursor pathways involved in high titre production strains cannot be done under alternative fermentation conditions that provide lower titres.

In *S. cinnamomensis* we have demonstrated that the flux of the primary lipid metabolite acetyl-CoA (a precursor to malonyl-CoA) passes through butyryl-CoA (a precursor to ethylmalonyl-CoA) to methylmalonyl-CoA. We have cloned and sequenced many of the genes in this process (including four different acyl-CoA carboxylases). It may therefore be possible to make rational changes in the relative levels of the common polyketide precursors, malonyl-CoA, ethylmalonyl-CoA and methylmalonyl-CoA. Thus it might be possible to alter C730.1 or other monensin-producing strains to be a host for production of a range of different polyketide aglycone structures, regardless of which of these precursors they require/utilize.

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