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Effects of methylmalonyl-CoA mutase gene knockouts on erythromycin production in carbohydrate-based and oil-based fermentations of *Saccharopolyspora erythraea*

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Abstract In carbohydrate-based fermentations of *Saccharopolyspora erythraea*, a polar knockout of the methylmalonyl-CoA mutase (MCM) gene, *mutB*, improved erythromycin production an average of 126% (within the range of 102–153% for a 0.95 confidence interval). In oil-based fermentations, where erythromycin production by the wild-type strain averages 184% higher (141–236%, 0.95 CI) than in carbohydrate-based fermentations, the same polar knockout in *mutB* surprisingly reduced erythromycin production by 66% (53– 76%, 0.95 CI). A metabolic model is proposed where in carbohydrate-based fermentations MCM acts as a drain on the methylmalonyl-CoA metabolite pool, and in oil-based fermentations, MCM acts in the reverse direction to fill the methylmalonyl-CoA pool. Therefore, the model explains, in part, how the well-known oil-based process improvement for erythromycin production operates at the biochemical level; furthermore, it illustrates how the *mutB* erythromycin strain improvement mutation operates at the genetic level in carbohydrate-based fermentations.

Keywords *Saccharopolyspora erythraea* Erythromycin Methylmalonyl-CoA mutase Knockout Strain improvement Oil-based fermentation

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Introduction

Research into secondary metabolism in actinomycetes offers the opportunity to study metabolic aspects of developmental biology in a prokaryotic system with the potential for the generation of commercial applications. Our interest in this research is for its application to strain improvement where the goal is to develop methods to increase yields of natural products of commercial interest. We have begun by focusing on the macrolide antibiotic erythromycin [6].

In industry, an improved strain is produced through a method of random mutagenesis followed by large-scale shake-flask screening [10]. In our method [12], random mutagenesis and large-scale screening were still used but they were followed by a reverse-engineering step to analyze the genetic basis for the strain improvement effect. The reverse engineering was made possible by adopting transposon-based mutagenesis in place of the classical methods used by industry. The transposon method was first demonstrated in *Streptomyces* coelicolor [5, 15] where improved strains could be easily found among hundreds of other colonies on an agar plate by their darker color. Improved erythromycin-producing strains, however, cannot be as easily found. To simplify the screening process for erythromycin strain improvement, a different erythromycin-producing organism, *Aeromicrobium erythreum*, was used in place of the commercial erythromycin-producing organism, *Saccharopolyspora erythraea*. *A. erythreum* is unicellular, grows faster, is easier to handle and transform, and is more adaptable to a 96-well plate micro-fermentation format than *S. erythraea* [9].

Results from the earlier study in *A. erythreum* [12] led to the finding that knockouts in *mutB* produced significantly higher levels of erythromycin than the parent strain in carbohydrate-based fermentations. These results provided the rationale to perform the analogous experiments in *S. erythraea* as described in this report.

The *mutB* gene codes for the large (beta) subunit of methylmalonyl-CoA mutase (MCM). The *mutA* gene codes for the smaller (alpha) subunit of MCM. The MCM heterodimer is a coenzyme-B12-dependent enzyme that catalyzes the reversible isomerization of methylmalonyl-CoA and succinyl-CoA [1]. The MCM reaction has been previously recognized as a target for strain improvement in other polyketide producing organisms [4, 17, 21]. The results reported here in *S. erythraea* and the previous results in *A. erythreum* show that *mutB* knockouts lead to improved erythromycin production in both of these organisms in carbohydrate-based fermentations. The metabolic model for the erythromycin fermentation now also incorporates results obtained from oil-based fermentations. The new model explains how media composition influences the production of erythromycin by acting, at least in part, through the MCM reaction, and also explains why erythromycin production was found to be reduced in *mutB* knockout strains in oil-based fermentations.

Materials and methods

Strains and growth media

The erythromycin-producing strain used for this study was the wild-type "white" *S. erythraea* FL2267 a derivative of ATCC 11635 (American Type Culture Collection, Manassas, VA). The strains and plasmids used during the course of this study are listed (Table 1). The *Escherichia coli* strain used as a host for cloning experiments was DH5**a**-e (Invitrogen, Carlsbad, CA).

Spores of *S. erythraea* were produced on E20A agar [11]. Seed cultures were prepared in CFM1 broth (Carbohydrate-based Fermentation Medium, a variation of SCM broth described previously [12]). CFM1 is a medium designed for laboratory use where a soluble medium is desired for convenient analysis of growth and chemical analysis of erythromycin production. CFM1 per liter distilled water: Difco™ soluble starch, 60 g; Bacto™-soytone (Difco™), 20 g; $CaCl_2$-$2H_2O$ (Sigma), 0.1 g; Bacto™-yeast extract (Difco™), 1.5 g; MOPS, 26.5 g; pH adjusted to 6.8 with 4 N NaOH (Sigma). CFM1 lacks the glucose, vitamins and trace elements of modified SCM [12]; and has 60 g (4x) of soluble starch. Fermentations were performed in either CFM1 broth or OFM1 broth (Oil-based Fermentation Medium). OFM1 contains insoluble medium components and is meant to closely correlate to an industrial-type fermentation medium. OFM1 per liter: toasted nutrisoy flour (ADM, Decatur, IL), 22 g; Difco™ soluble starch, 15 g; $CaCO_3$ powder (JT Baker, Phillipsburg, NJ), 3 g; $MgSO_4$-$7H_2O$ (JT Baker, Phillipsburg, NJ), 0.5 g; FeSO4-$7H_2O$ (JT Baker, Phillipsburg, NJ), 15 mg; Soy oil, 50 ml (ADM, Decatur, IL). Minimal agar medium, (AVMM, [18]) was supplemented with 50 mM methylmalonic acid (Sigma-Aldrich, St Louis, MO). Pigmentation and sporulation studies were conducted on R2T2 agar. R2T2 is R2T described in Weber et al. [19] minus peptone.

Preparation and screening of *S. erythraea* cosmids containing the *mutAB* region

Total chromosomal DNA was isolated from *S. erythraea* FL2267 cells grown in SCM broth. Partial restriction digest of the DNA by *Mbo*I (Fermentas, Vilnius, Lithuania) was done by varying concentration of the enzyme. Fragments approximately 40 kb in length were separated by pulse field electrophoresis in 1% low melting agarose (Chef DRIII System, BioRad, Hercules, CA). DNA fragments were purified by agarase (Roche Molecular Biochemicals, Indianapolis, IN) treatment according to the supplier's protocol, ligated to a SuperCos cosmid vector (Stratagene, La Jolla, CA), and packaged into phage particles using Stratagene Gigapack III Gold Packaging Extract. Titration and propagation of the packaged extracts were done in *E. coli* strain XL1BlueMR. Approximately 600 clones containing the resulting recombinant cosmids were screened by PCR for the presence of the *mutAB* region using primers based on the available DNA sequence information (Genbank accession no. AY117133). The primer sequences were: gntRF1-5'gtcgaattcGCCGTCACCG TCGACCCCAA3' and gntRR1-5'gtcggatccCAGCAT- CAGCGCTCCCGGA3'. Two cosmids, 5G10 and 6E7, were identified as containing the *mutAB* operon. Cosmid 6E7 was used for DNA sequencing of the *mutAB* flanking regions using a primer-walking technique.

Construction of plasmids used to create *mutB* knockouts in *S. erythraea*

Plasmid pFL2132, used for generation of the polar knockout of *mutB* in strain FL2281, was constructed from four DNA components. The first component was made by opening plasmid pFL8 [11] in the multicloning site using EcoRI and HindIII. The second and third components were made by PCR amplification of the *S. erythraea* chromosome using the primer pairs A1–A2 to generate the 2-kb upstream DNA fragment and the primer pair B1–B2 was used to generate the 1.8-kb downstream DNA fragment (Fig. 1a). The fourth component was made by isolation of the kanamycin-resistance gene from pUC4K (Pharmacia Biochemicals, Piscataway, NJ) by digestion with *Bam*HI. Therefore, in the final construct, pFL2132, the kanamycin-resistance gene was inserted between the two PCR products with the direction of transcription of the kanamycin-resistance gene being opposite to the direction of transcription of the *mutB* gene. The primers used in the formation of the two PCR components were as follows: primer A1, 5'-gt*cga- attc*GCAACACCCAGTCGCTGCTG-3' and primer A2 5'-tgc*ggatcc*TCGATGTAGTGCGAGC CGCC-3' were used to generate the 2-kb PCR product, and primer B1, 5'-tgc*ggatcc*GACGCAGGCG CG CATCGACT-3' and primer B2, 5'-gtc*aagctt*ATTCG ACCTCGTCGAC GATG-3' was used to generate the 1.8-kb product. The complete DNA sequence of *S. erythraea* DNA fragments in pFL2132 was determined to confirm the predicted structure for this construct. The plasmid was first constructed in *E. coli*; it was later integratively transformed into *S. erythraea* using standard protoplast transformation methods [19].

pFL2132 was used for generation of the polar knockout of *mutB* to create strain FL2281. The polar mutation in *mutB* was constructed by replacement of the kanamycin-resistance gene from pUC4K for a 126 bp deleted region of the *mutB* gene. The deleted region falls between primer binding sites A2 and B1 (Fig. 1a). First a single crossover plasmid insertion was performed with pFL2132 by homologous recombination [19] into strain FL2267 to form strain FL2272. Next the plasmid was evicted from FL2272 through a second recombination event, occurring in the absence of drug selection, to form the gene-replacement strain FL2281. Three independent isolates of strain FL2281 were obtained from three independent transformation reactions. The three strains behaved similarly in preliminary testing (data not shown) and strain FL2281-1 was then used in follow-up experiments as the representative FL2281 isolate.

Once the knockout strain was constructed by double crossover gene replacement in *S. erythraea*, a diagnostic PCR reaction was performed on the knockout strain FL2281 to confirm the insertion of the kanamycin- resistance gene into the chromosomal *mutB* gene (Fig. 1b).

pFL2179 was used for generation of the in-frame deletion of *mutB* to create strain FL2302. To generate an in-frame *mutB* deletion mutant, pFL2132 was digested with *Bam*HI to release a unique 1,263 bp fragment consisting entirely of the kanamycin-resistance gene cassette. The remaining,

larger fragment was purified from an agarose gel and religated using T4 DNA ligase (Fermentas, Vilnius, Lithuania). The truncated plasmid was transformed into *E. coli* with selection for ampicillin resistance. Single ampicillin-resistant colonies were replica patched onto SOB agar [14] containing kanamycin and ampicillin. Isolates that were ampicillin resistant but kanamycin sensitive were further analyzed. Ten plasmids from kanamycin-sensitive isolates were digested with *Bam*HI and *Hin*dIII to confirm the loss of the kanamycin-resistance gene cassette. This plasmid, designated pFL2179, contained a 126 bp deletion in *mutB* along with an engineered *Bam*HI site (6 bp) to maintain the reading frame of the gene. pFL2179 was used in a two-step integration and eviction as described above for pFL2132, except that the resulting gene-replacement strains would have a 126 bp deletion in the *mutB* gene instead of an insertion of the kanamycin-resistance gene. The deletion in the *S. erythraea* chromosome was confirmed by diagnostic PCR (Fig. 1b) and DNA sequence analysis of the plasmid insert. Three independent isolates were obtained from three independent transformation reactions. The three strains behaved similarly in preliminary testing (data not shown) and strain FL2302- 1 was used in the follow-up experiments as the representative isolate for FL2302.

Confirmation of *mutB* mutations by PCR

The mutations generated in *S. erythraea* strains FL2281 and FL2302 were confirmed by PCR (Fig. 1b) using primers that spanned the insertion of the kanamycin- resistance gene (in FL2281) and deletion (in FL2302) sites. The primers were: Forward *mutB*F9, 5'-CGAAG TCGCTGTCGCTGCGC-3' and reverse *mutB*R11, 5'- CGATGAGCGGCTGGCGGC-3'. The cycling reaction was as follows. A 2 min denaturation step at 96°C for 1 cycle followed by a 1 min denaturation step at 96°C, a 1 min annealing step at 56°C, and a 1.5 min extension step at 72°C for 25 cycles. A 2x PCR master mix containing recombinant Taq polymerase and MgCl2 at a final concentration of 2 mM was used in all reactions (Fermentas, Vilnius, Lithuania).

Shake-flask fermentation method

Fermentations were performed in unbaffled 250-ml Erlenmeyer flasks with milk-filter closures. The flasks were incubated at 32.5±0.2°C, and 65±3% humidity on an Infors Multitron Shaker having 1-in. circular displacement. Seed cultures containing CFM1 broth were prepared on the same shaker and under the same growth conditions that the fermentations were performed. Seed cultures were inoculated from fresh spores prepared from E20A agar plates. Fermentations were inoculated with 1.25-ml of a seed culture in late logarithmic growth phase (40–45 h) into 25-ml of CFM1 or OFM1 broth. Con

sistency in the preparation of the seed culture was required for each strain to obtain and maintain optimum and reproducible fermentation performance from experiment to experiment. Fermentations were grown for 5 days; their volumes were then corrected for evaporation through the addition of water before being further analyzed.

Bioassay and thin layer chromatography

The analysis of fermentation broths for total antibiotic (erythromycin) content was performed by bioassay. Bioassays for erythromycin were performed using a large plate double-agar layer system (Corning Costar, Cambridge, MA, 245 mm square bioassay dish, catalog number 431111). The bottom agar layer consisted of TSB agar (100-ml). Once the bottom layer was solidified, a top agar layer was poured. Top agar consisted of TSB agar (100-ml) containing 250 ll of 1% triphenyltetrazolium red and a sufficient quantity (6·107) of B. subtilis thiostrepton-resistant spores to produce a confluent lawn of growth. The upper layer was allowed to solidify at room temperature. Broth samples (12-ll) were spotted directly onto 1/4-in. bioassay discs (Schleicher and Schuell, Keene, NH) and allowed to dry. Broth samples from higher producing white strain fermentations were diluted 1:10 in TE pH 7.0 buffer before being assayed. For each flask to be analyzed, two bioassay discs were saturated, dried, and the placed on two separate bioassay plates. Stock erythromycin A solution (100 mg/ml) was prepared in 95% ethanol. Standard erythromycin A solutions to be used for the bioassay were prepared at 10, 25, 50, 100, and 250 lg/ml in TE pH 7.0 buffer. The bioassay plates were incubated overnight at 37°C. After incubation, the diameters of inhibition-zones were measured and converted to concentrations using the standard curve produced for each plate.

To visualize the erythromycin profile of the broths, thin layer chromatography was used. For thin layer chromatography of samples taken from oil-based fermentations, a 1.0 ml sample of fermentation broth was made basic through the addition of 25 ll of 5 N NaOH, then vigorously mixed with a half volume of ethyl acetate in a 1.5 ml Eppendorf microcentrifuge tube. The phases were separated by centrifugation and 400 ll of the top, solvent layer was transferred to a fresh microcentrifuge tube. The solvent layer was evaporated to an oily residue under vacuum and 0.2 ml of acetonitrile was added to the tube to redissolve the extracted material by thorough vortex mixing. Another centrifugation was performed and the upper acetonitrile solvent layer was transferred to a fresh 1.5 ml microcentrifuge tube, leaving behind a small yellow oily pellet that was discarded. The acetonitrile layer was evaporated to dryness under vacuum and the extracted residue was resuspended by vigorous vortex mixing into 12 ll of acetonitrile. Two-microliter portions of the acetonitrile extracts were applied to thin layer chromatography plates and the plates were developed in isopropyl ether and methanol and ammonium hydroxide (75:35:1). Macrolide compounds became visible on plates after spraying with a

mixture of p-anisaldehyde and sulfuric acid and ethanol (3:3:27) and after heating the freshly sprayed plates at 100°C for 5 min. Thin layer chromatography of samples from carbohydrate-based fermentations was performed on concentrated ethyl acetate extracts without acetonitrile extraction.

Statistical analyses

The average percent yield increases and decreases cited in the text are followed in parenthetical text with the range of uncertainty for a 95% confidence interval.

DNA accession numbers

The accession numbers for the two extended DNA sequences of the *S. erythraea mutAB* region are DQ289499 and DQ289500. The accession number for revised DNA sequence of the *mutAB* region of *A. erythreum* is AY548464.

Results

The *mutA*, *mutB*, *meaB*, and SeORF5 gene cluster

The *mutAB* region of the *S. erythraea* chromosome was cloned and its DNA sequence was deposited in Genbank by the Flores laboratory (accession no. AY117133). In this study, DNA sequencing was extended on both ends of the cluster (GenBank accession Nos. DQ289499 and DQ289500) and the entire region was compared to the homologous region of *A. erythreum* (Fig. 1a). The *mutA*, *mutB* and *meaB* genes have identical order and orientation in the two organisms. The general conservation of gene order of these three genes, including in humans, has been reported previously [2, 21]. The *meaB* gene has been reported to be required for protection of MCM from inactivation [7]. The gene downstream of *meaB* in *S. erythraea*, SeORF5, appears to be translationally coupled to *meaB* and encodes a putative GntR-family regulatory protein. The region downstream of *meaB* in *A. erythreum* also contains a regulatory gene, AeORF5, widely separated from *meaB*, by 715 bp (Fig. 1a), and encoding a regulator from the mazG family. Further downstream of SeORF5 in *S. erythraea* a 472-bp gap of presumptive non-coding DNA is followed by a convergent open reading frame, SeORF6, coding for a putative lipoprotein. The closest homologies to this lipoprotein were putative lipoproteins found in *S. coelicolor* (45% identity, accession no. NP_625400) and *Streptomyces* avermitilis (42% identity, accession no. NP_822680).

The genes upstream of *mutA* share no homology between the two organisms, and are oriented in opposite directions. The predicted function of the gene upstream of *mutA* in *S. erythraea*, SeORF1, is unclear; BLAST homology searches show its highest homology to be to hypothetical proteins of unknown function in *S. avermitilis* and *S. coelicolor*. The gene upstream of *mutA* in *A. erythreum*, AeORF1, is as a putative phosphoserine aminotransferase; with high (65 and 68%) identities to putative phosphoserine aminotransferases from *S. avermitilis* (accession no. BAC71595) and *S. coelicolor* (accession no. NP_628536), respectively.

The predicted MutA, MutB, MeaB, and SeORF5 amino acid sequences from *S. erythraea* all show the highest BLAST homology scores to predicted amino acid sequences from the thermophilic actinomycete, *Thermobifida fusca* YX (56, 82, 73, and 56% identities, respectively). The genes from the homologous *T. fusca* cluster follow the same order and close packing arrangement characteristic of the *S. erythraea* genes. One major difference between the two clusters is that the *T. fusca* regulatory gene downstream of *meaB*, is a presumptive anti-sigma factor antagonist; whereas, the gene corresponding to that location in *S. erythraea*, as mentioned above, is a presumptive GntR-family regulatory protein.

Construction of the polar *mutB* knockout strain FL2281 and the non-polar *mutB* knockout strain, FL2302

In this study, two types of *mutB* knockout strains were constructed. Both mutations were in *mutB*, as opposed to *mutA*, to be consistent with previous experiments performed in *A. erythreum* [12]. Knockouts in *mutA* would be predicted to have phenotypes identical to *mutB*, although this has not yet been tested.

In the first knockout strain, FL2281, the kanamycin resistance gene was inserted into *mutB* by a double crossover gene-replacement using plasmid pFL2132 (Table 1 and Materials and methods). The kanamycin-resistance gene insertion replaced a deleted 126-bp region of *mutB* between primer sites A2 and B1 (Fig. 1a). Transcription of the kanamycin-resistance gene was opposite to the direction of transcription of *mutB*. The mutation in strain FL2281 is predicted to be polar on the downstream genes, *meaB* and SeORF5, of the *mutAB* cluster.

In the second *mutB* knockout strain, FL2302, the 126- bp in-frame deletion was replaced by a 6-bp *Bam*HI site. The deletion was designed to be in-frame so as to inactivate the function of MCM but not to affect the transcription of downstream genes (Fig. 1a). The resulting mutated strains were analyzed by PCR to confirm that the mutations were correctly constructed (Fig. 1b).

Comparing carbohydrate-based and oil-based fermentations: effects on erythromycin production in the wild-type strain

Most commercial *S. erythraea* fermentations are performed in an oil-based medium, similar to OFM1 (Materials and methods). For research purposes, and in some commercial situations where the price of oil is prohibitive, a carbohydrate-based fermentation medium lacking oil is used, such as SCM, or in this study we used a modification of SCM called CFM1 (methods). Our results show that the wild-type strain produced an average of 184% (141–236%) more erythromycin in the oil-based fermentation than in the carbohydrate-based fermentation (Fig. 2a and b). This medium effect alone enabled the wild-type strain to produce levels of erythromycin in a range overlapping 1 g/l in a 5-day shake-flask fermentation (corrected for evaporation).

Comparing carbohydrate-based and oil-based fermentations: effects on erythromycin production in the *mutB* knockout strains

The two *mutB* knockout strains, FL2281 and FL2302, were compared to the parent strain in both carbohydrate-based and oil-based 5-day shake-flask fermentations. The soluble carbohydrate-based medium (CFM1) was chosen to be consistent with the fermentation medium used in the previous study with *A. erythreum* [12]. The oil-based medium (OFM1) was included in this study because most commercial producers of erythromycin use an oil-based fermentation.

Both knockout strains showed improved erythromycin production in the carbohydrate-based medium CFM1 (Fig. 2a and b). The non-polar knockout, FL2302, caused a 51% (33–71%) increase in erythromycin production over the parent strain, and the polar insertion knockout strain, FL2281, caused an even greater, 126% (102– 153%) average increase in production over the parent strain. Thin layer chromatography profiles of culture extracts were consistent with the increase in bioactivity being due to erythromycin A. Time course analyses showed the strain improvement effect was evident as early as day 2 and it continued through day 5 of the fermentation (Fig. 2c).

When the strains were compared in oil-based medium (OFM1), both *mutB* knockout strains caused a decrease in erythromycin production. The non-polar knockout, FL2302, caused an average decrease of 39% (25–50%), and the polar *mutB* knockout strain, FL2281, caused an average decrease of 66% (53–76%) in erythromycin production.

Pigmentation and sporulation phenotypes of *mutB* knockout strains

Both *mutB* knockout strains, FL2302 and 2281, were defective in pigment production and sporulation. This was clearly evident on R2T2 agar, where the parent strain developed from white aerial mycelia

to reddish aerial spores. The *mutB* knockout strains, however, stopped their development at white aerial mycelia and did not progress to reddish aerial spores even after extended incubation. Pigmentation by both *mutB* knockout strains was also lacking in liquid cultures, particularly the CFM1 fermentations. Both *mutB* knockout strains could not grow on AVMM agar containing methylmalonate as a sole carbon source, whereas, the parent strain did grow on this medium.

Discussion

Saccharopolyspora erythraea has been in use for over 50 years for the commercial production of erythromycin. Over this time, highly improved strains have been developed, yet no information has been reported regarding the genes and metabolic pathways that are affected during a traditional strain improvement program to create a ‘‘high-producing’’ strain.

In a previous study [12], we showed that inactivation of methylmalonyl-CoA mutase leads to improved erythromycin production in the unicellular model erythromycin-producing organism, *A. erythreum* in a carbohydrate-based medium. In the current study we applied this strategy to the industrial strain, *S. erythraea*, and showed that a similar strain improvement result was obtained when similar fermentation conditions were used. In this case the results from the model strain were predictive of the results from the industrial strain.

Although the magnitude of the strain improvement effect obtained by the polar *mutB* mutation in the carbohydrate-based fermentations was significant at 126% (102–153%), it was outmatched by the 184% (141– 236%) yield increase obtained by simply changing the growth medium for the wild-type strain from carbohydrate-based to oil-based. By combining the benefits obtained with both the polar *mutB* knockout and the more productive oil-based medium, it was thought that an even better erythromycin-producing fermentation would result, but this was not observed. Instead, in the oil-based fermentation, the polar *mutB* knockout strain caused a decrease in production of 66% (53–76%). Despite the negative outcome, the results still provide useful insights into the metabolic pathways affecting erythromycin biosynthesis and create a basis for future experimentation directed ultimately towards strain improvement in the oil-based medium.

An updated metabolic model is presented (Fig. 3) that takes into account the previously reported bidirectionality of the MCM reaction [1] and incorporates the results from the genetic knockout experiments and media manipulations obtained from this study.

The methylmalonyl-CoA metabolite pool is shown at the center of the model, reflecting its assumed importance in determining the production level of erythromycin. The assumption is being made that the propionyl-CoA (the other erythromycin ketide precursor) pool and the methylmalonyl-CoA pool are proportionately linked in order to justify our treatment of the two pools as one for the current model. The size of the precursor pool is determined by the pathways that flow into and out of

the pool, and it is assumed that a net increase in carbon flow into the precursor pool should increase the production level of erythromycin (Fig. 3).

The model shows that the direction of flow of the MCM reaction is reversed in the two different fermentation media. In a carbohydrate-based medium, the MCM reaction acts like a drain on the methylmalonyl- CoA pool. In an oil-based medium, the MCM reaction acts to fill the methylmalonyl-CoA pool. By changing the media from carbohydrate-based to oil-based and reversing the flow of a pathway connected to the methylmalonyl-CoA pool, a doubly-positive process improvement effect is obtained in a single step. Since multiple pathways are assumed to be connected to the methylmalonyl-CoA pool, and may be similarly affected by media composition, the net-positive effect from the media change would be further amplified. We do not mean to imply that MCM or any other gateway enzyme is controlling the direction of flow of metabolites, but rather that the gateway enzymes can be used to monitor the direction of metabolite flow. This is done indirectly by knocking out the corresponding gateway gene and measuring the effect of the knockout on erythromycin production.

Even though oil-based media are known to enhance production of other polyketide natural products, only one other organism, the monensin-producing *Streptomyces cinnamonensis*, has ever been previously analyzed under these two different sets of fermentation conditions. Evidence presented so far from the *S. cinnamonensis* system [8] indicates that the erythromycin and monensin precursor pathways do not share a common metabolic mechanism to explain the process improvement effect seen in oil-based media. In *S. cinnamonensis*, the increase in monensin titers is associated with a metabolic shift from one pathway (utilizing MCM), to a second pathway (utilizing crotonyl-CoA reductase [CCR]) rather than through a metabolic reversal of methylmalonyl-CoA feeder pathways. Furthermore, loss of MCM function in *S. cinnamonensis* does not lead to increased polyketide titers in carbohydrate-based fermentations [17] as it does for both *S. erythraea* and *A. erythreum*.

Another feature of the model (Fig. 3) is that multiple pathways are connected to the methylmalonyl-CoA pool. This is deduced from the observation that the nonpolar *mutB* knockout strain shows only a partial, 39% (25–50%), drop in erythromycin production in oil-based fermentations, and only a partial 51% (33–71%) increase in erythromycin production in the carbohydrate-based fermentation, compared to the wild-type strain. This result implies that the majority of carbon flow into and out of the methylmalonyl-CoA pool occurs through gateways other than the MCM reaction. So far we know that it is unlikely that the CCR pathway [8, 9] plays a role in precursor flow in *S. erythraea* because other laboratories have already investigated this possibility [16] and found no evidence for it. This leaves at least two other pathways that have already been characterized in other organisms that could be involved: the proprionyl-CoA carboxylase pathway [3, 13], and the MeaA pathway [20]. Other pathways yet to be discovered, however, may also be expected to play a role, since this area of metabolism in *S. erythraea* is still relatively uncharacterized.

The model also shows that the effect on erythromycin production caused by the polar *mutB* knockout strain is magnified by comparison to the non-polar *mutB* knockout in both media. Depending on the medium, the magnification effect either further increased or decreased the level of erythromycin production. This effect could be due to the loss of expression of the two downstream genes, *meaB* and SeORF5, that could be directly or indirectly affecting other pathways connected to the methylmalonyl-CoA metabolite pool. The direction of flow of these other pathways may also be under the same medium-induced influence as the MCM reaction (Fig. 3, metabolite "x"). Further studies of the *meaB* and SeORF5 genes should help determine the actual mechanism behind the magnification effect caused by the polar *mutB* knockout.

Finally the model shows pathways that fill the methylmalonyl-CoA metabolite pool regardless of the medium composition (involving metabolites Y and Z, Fig. 3). In the carbohydrate-based medium these pathways represent the main pathway for carbon flow into the methylmalonyl-CoA pool. In the oil-based medium these pathways provide a much lower percentage of the carbon flow.

In conclusion, a metabolic model for precursor flow into erythromycin biosynthesis has been generated from experiments conducted in both low-producing (carbohydrate-based) and high-producing (oil-based) fermentation media. It has been shown that even a carbohydrate-based fermentation medium can be used to generate high levels of erythromycin if a simple *mutB* knockout strain improvement mutation is introduced into the organism. In the oil-based medium, the wild-type strain is naturally suited for high efficiency erythromycin production, and the *mutB* knockout only serves to diminish its capacity for high-level production. The explanation for these differences in erythromycin production between the two media lies, in part, with changes in direction of carbon flow from the methylmalonyl- CoA metabolite pool. Preliminary evidence suggests that additional pathways are connected to the methylmalonyl-CoA pool and that their direction of flow may be controlled by the same medium effect. Further work in this area should lead to a better understanding of the genetics and biochemistry of erythromycin strain improvement.

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Table 1 Bacterial plasmids and strains used in this study

Plasmids	Description	Reference
pFL8	Bifunctional plasmid vector, stable in *E. coli*, but unstably maintained in *S. erythraea*. Derivatives of pFL8 with chromosomal inserts are used to generate stable gene knockouts and gene replacements in the *S. erythraea* chromosome. Carries genes for ampicillin and thiostrepton resistance	Reeves et al. [11]
pFL2132	*S. erythraea* integration vector used to make a polar knockout of *mutB* by gene replacement of a kanamycin-resistance gene cassette, thus creating strain FL2281. Contains two non-contiguous fragments from the *mutAB* region and carries ampicillin, kanamycin and thiostrepton resistance genes	This study
pFL2179	Derivative of pFL2132 that has lost the kanamycin-resistance gene cassette by *Bam*HI digestion followed by religation. Used to make in-frame deletion in *mutB* to create the non-polar *mutB* knockout strain FL2302. Carries ampicillin and thiostrepton resistance genes	This study
Strains		
S. erythraea FL2267	Derivative of *S. erythraea* ATCC 11635. Wild-type (white). Used as host strain in transformations	This study
FL2272	Derivative of FL2267 containing integrated pFL2132 by single crossover insertion. Intermediate strain in the creation of strain FL2281. Thiostrepton and kanamycin resistant	This study
FL2294	Derivative of FL2267 containing integrated pFL2179 by single crossover insertion. Intermediate strain in the creation of strain FL2302. Thiostrepton resistant	This study
FL2281-1 FL2281-2 FL2281-3	Polar *mutB* knockout strains derived by eviction of pFL2132 from strain FL2272-1, -2, and -3, respectively. Kanamycin resistant	This study
FL2302-1 FL2302-2 FL2302-3	Non-polar *mutB* knockout strains derived by eviction of pFL2179 from strain FL2294-1, -2, and -3, respectively	This study
E. coli DH5α-e	Host strain for transformations	Invitrogen, Carlsbad CA

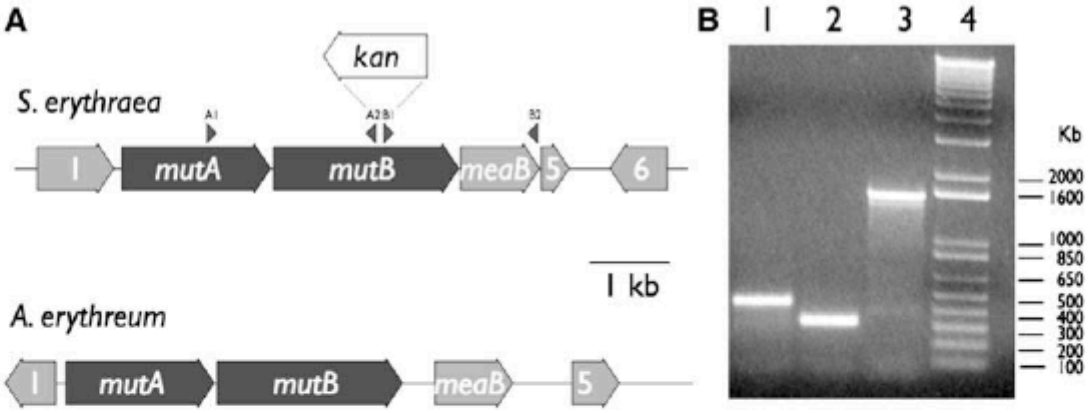

Fig. 1 **a** Map of the *S. erythraea mutAB* region (*top*); *A. erythreum mutAB* region (*bottom*); drawn to scale. The DNA sequence for the *mutAB* region of *S. erythraea* was submitted previously by the Flores laboratory (accession number AY117133). Additional DNA sequence data were added to both ends of this cluster to expand the figure from 5,894 to 8,204 bp (accession numbers DQ289499 and DQ289500). A1, A2, B1, and B2 *triangles* indicate primer binding regions as described (Materials and methods). The genes labeled 1, 5, and 6 are referred to in the text as *Se*ORF1, *Se*ORF5, and *Se*ORF6, respectively. The *arrow* labeled *kan* represents the kanamycin-resistance gene from pUC4K inserted into the *mutB* gene of *S. erythraea* to create the polar knockout strain FL2281 (Materials and methods). New DNA sequence information for the *A. erythreum mutAB* region in this figure (on both ends of the cluster) is deposited as an update to accession number AY548464, which was originally submitted by this laboratory. The genes labeled 1 and 5 are referred to in the text as *Ae*ORF1 and *Ae*ORF5, respectively. **b** PCR analysis of the *mutB* knockout strains and the parent strain. The primers used for PCR analysis are described in Materials and methods. *Lane 1*, strain FL2267 (wild-type): PCR product size is 438 bp; *lane 2*, strain FL2302 (in-frame deletion): PCR product size is 318 bp, fragment is smaller than *lane 1* due to the 120 bp deletion in *mutB*. *Lane 3*, strain FL2281 (polar *mutB* knockout): PCR product size is 1,582 bp, fragment includes a 120 bp deletion, and with the *kan* gene inserted, an additional 1,263 bp. *Lane 4*, DNA reference standard ladder with indicated band sizes

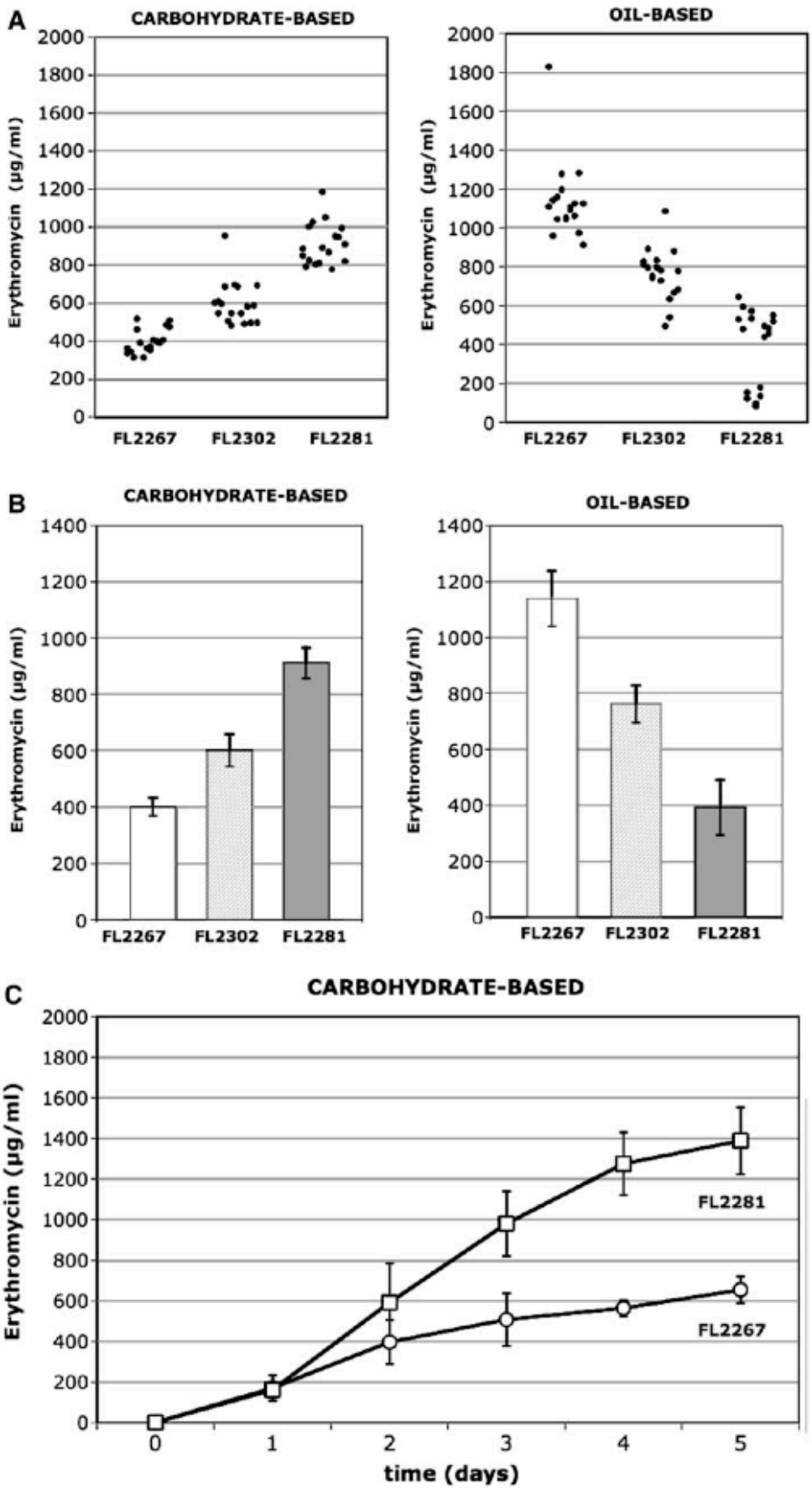

Fig. 2 **a** Scatter plot ($N = 18$) showing the effect of *mutB* mutations on erythromycin production in carbohydrate-based (*CFM1*, *left panel*) and oil-based (*OFM1*, *right panel*) fermentation media. *FL2267*, wild-type strain; *FL2302*, non-polar *mutB* knockout strain; *FL2281*, polar *mutB* knockout strain. Each *circle* represents raw data for one measurement of erythromycin production made by bioassay of diluted fermentation broth (see Materials and methods). Nine flasks were included in the analysis, each flask was measured twice by bioassay on day 5 of the fermentation. **b** *Bar chart* representation of the data presented in **a** showing the average erythromycin production levels of the three strains grown in CFM1 and OFM1 and the standard errors (95% CI) associated with each series of measurements. **c** Time course analysis of CFM1 fermentations ($N = 4$) of the wild-type strain FL2267 (*circles*), and the *mutB* polar knockout strain FL2281 (*squares*). Two flasks were analyzed at each time point; two bioassay measurements were made on each flask. The *error bars* represent standard errors (95% CI) associated with each time point

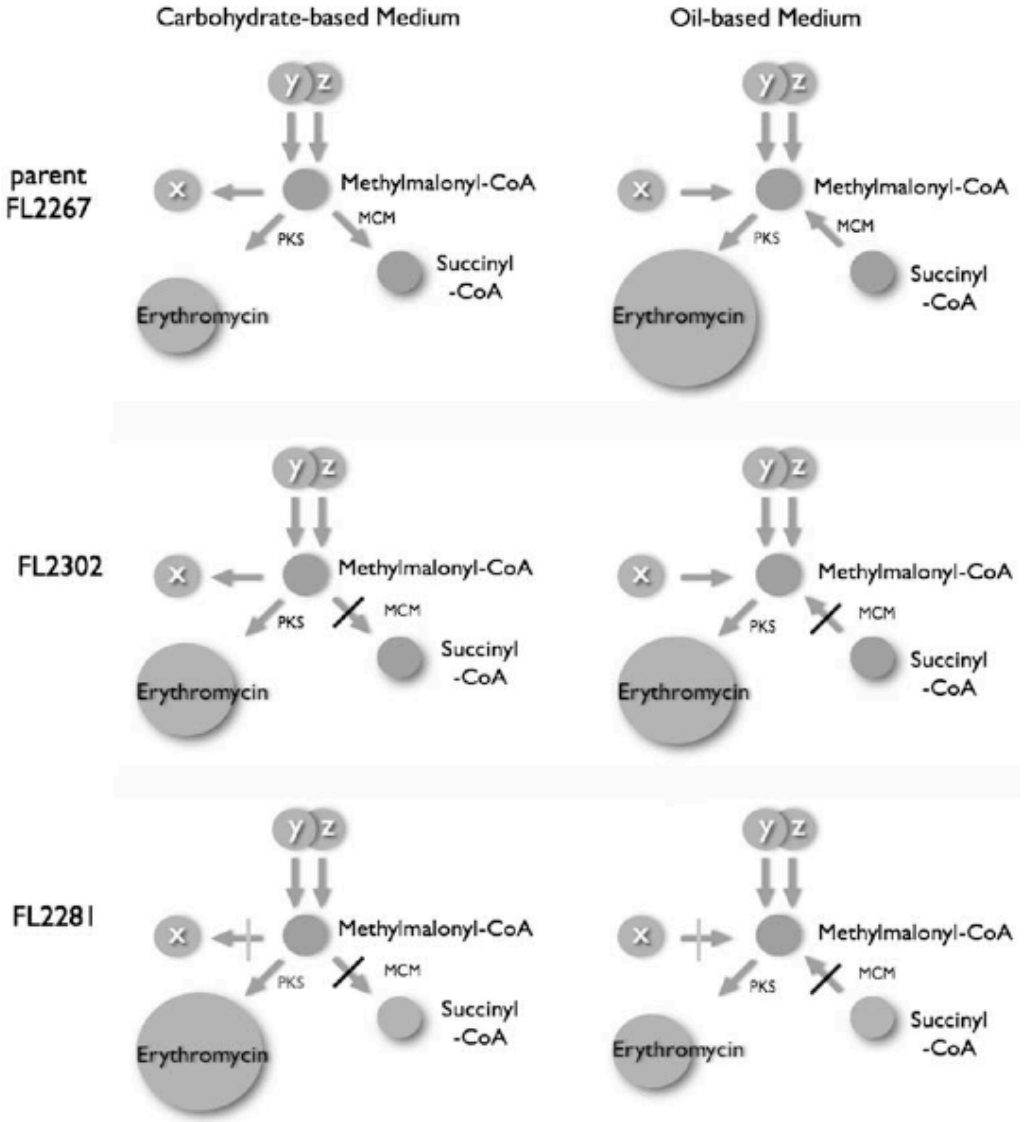

Fig. 3 Metabolic model for erythromycin production by three strains in carbohydrate and oil-based fermentation media. The MCM *arrow* indicates the reaction catalyzed by MCM whose net flow is in the direction shown in the two different media. *MCM*, methylmalonyl-CoA mutase; *PKS*, polyketide synthase. *FL2267*, wild-type strain; *FL2302*, non-polar *mutB* knockout strain; *FL2281*, polar *mutB* knockout strain. *Circles* "y" and "z" represent proposed metabolites that flow, via *arrows* representing unidirectional enzymatic reactions, into the methylmalonyl-CoA pool in both carbohydrate-based and oil-based media. The "x" *circle* represents a proposed metabolite that flows into the methylmalonyl-CoA pool in the oil-based medium, and flows out of the methylmalonyl-CoA pool in the carbohydrate-based medium via a reversible enzymatic reaction represented by the *arrow*. *Black bars* drawn through *arrows* represent genetic blocks due directly to the mutation in *mutB*; *gray bars* are metabolic blocks due to the polar effects of the *mutB* knockout in strain FL2281. The erythromycin *circles* are drawn to scale based on relative experimental production levels (Fig. 2b). The *arrow* to erythromycin represents the multistep biosynthetic reactions required for erythromycin production including the polyketide synthase reactions. The sizes of the circles for other metabolites are not meant to reflect their pool sizes