Engineering precursor flow for increased erythromycin production in *Aeromicrobium erythreum*

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Abstract

Metabolic engineering technology for industrial microorganisms is under development to create rational, more reliable, and more cost effective approaches to strain improvement. Strain improvement is a critical component of the drug development process, yet the genetic basis for high production by industrial microorganisms is still a mystery. In this study, a search was begun for genetic modifications critical for high-level antibiotic production. The model system used was erythromycin production studied in the unicellular actinomycete, *Aeromicrobium erythreum*. A tagged-mutagenesis approach allowed reverse engineering of improved strains, revealing two genes, *mutB* and *cobA,* in the primary metabolic branch for methylmalonyl-CoA utilization. Knockouts in these genes created a permanent metabolic switch in the flow of methylmalonyl-CoA, from the primary branch into a secondary metabolic branch, driving erythromycin overproduction. The model provides insights into the regulation and evolution of secondary metabolism.

Keywords: methylmalonyl-CoA mutase; coenzyme B_{12}; metabolic engineering; strain improvement; *mutB, cobA*; erythromycin

1. Introduction

The large-scale production of antibiotics, anticancer agents, and other important drugs from microbial fermentations has been a cornerstone of the pharmaceutical industry since the development of penicillin in the 1940's. Since microbes from nature do not produce drugs at high enough levels for commercial purposes, strain improvement became a critical part of the drug development process (Vinci and Byng, 1999; Baltz, 2001; Parekh et al., 2000).

Interestingly, the traditional mutate-and-screen method of strain improvement that was developed in the 1940's for the penicillin strain has not changed significantly over the years (Queener and Lively, 1986; Van Nistelrooij, et al., 1998). This is because the method is technically simple to perform and has been successful at generating improved strains. Today's penicillin strain, for example, has been improved 3,000-fold over the strain used in the early 1940's (Van Nistelrooij et al., 1998). However, not all commercial processes have shared in the same high degree of success as penicillin. In the least successful cases, strains cannot be improved enough to generate sufficient material for clinical trials or commercial production. These opportunities for drug development would not be lost, and the current commercial strains might be further improved if a rational alternative to the empirical mutate-and-screen approach was developed.

The goal of this study was to begin to identify the genes involved in a successful mutate-and-screen strain improvement program. It was hoped that this information could be used to better understand the metabolic basis of strain improvement and ultimately to replace the empirical process with rational design (Nielsen, 1998). The model system for this study was polyketide biosynthesis, in particular, the biosynthesis of the macrolide antibiotic erythromycin. Besides being widely studied as a model system for many decades (Hutchinson et al., 1993-1994; Katz and Donadio, 1995; Leadlay, 1997; McDaniel et al., 2001), semisynthetic derivatives of erythromycin are widely used in medicine.

We sought our goal through the method of reverse engineering of improved strains themselves, as opposed to using a hypothesis-driven approach (Thykaer and Nielsen, 2002) or other recently developed approaches (Askenazi et al., 2003; Murli et al., 2003; Zhang et al., 2002a). Unfortunately, we could not reverse engineer the existing high-producing strains used by industry because the relevant genes in these

strains are not tagged and therefore cannot be identified. Furthermore, even if the whole genome could be analyzed, for example, by comparing complete genome sequences between a highly improved commercial strain and its wild-type progenitor, there would be no simple method to distinguish beneficial mutations from silent or deleterious ones in these strains. Therefore, we first had to create improved strains ourselves, in a way that mimicked the traditional mutate-and-screen method, yet allowed the mutations of interest to be analyzed by reverse engineering.

2. Materials and methods

2.1. Bacterial strains, growth media, chemicals, and biochemicals

Aeromicrobium erythreum B-3381 was obtained from the Northern Regional Research Laboratory (Peoria, IL). Cells were cultured on 2xYT(G) agar plates (2xYT: 16 g, tryptone; 10 g, yeast extract; 5 g, sodium chloride; Sambrook et al., 1989), supplemented with 0.4% glucose (G). For liquid culture cells were grown in 2xYT(GMT) broth containing 1.5% glucose (G), 100mM MOPS (M), and 2 ml per liter of trace elements (T; Hopwood et al., 1985). *A. erythreum* was alternatively cultured in modified Soluble Complete Medium (McAlpine et al., 1987; SCM per liter distilled water: 15 g, soluble starch; 20 g, bacto soytone; 0.1 g calcium chloride; 1.5 g, yeast extract; 10.5 g MOPS, pH adjusted to 6.8). Modified SCM contains 2.5x MOPS with pH adjusted to 7.5 before sterilization; after sterilization the following were added, 1.5% glucose, 2 ml/L trace elements (Hopwood et al., 1985), and vitamins (TwinLabs multivitamin, Hauppauge, NY, 5 mg per liter). *Escherichia coli* DH5α-e (Invitrogen, Carlsbad, CA) was grown in Luria Broth (Sambrook et al., 1989) and maintained on LB agar. For plasmid recovery procedures the pir^+-containing *E. coli* cell line EC100D-pir-116 (Epicentre, Madison, WI) was used. For agar-plate bioassays, the thiostrepton-resistant *B. subtilis* PY79 was used as the indicator strain (Weber et al., 1990). When appropriate for growth of drug-resistant *A. erythreum,* solid and liquid media were supplemented with 5 μg/ml kanamycin sulfate (Sigma-Aldrich, St. Louis, MO) and 25 μg/ml thiostrepton (Sigma-Aldrich). *E. coli* media were supplemented with 10 μg/ml tetracycline-HCl (Sigma-Aldrich) for selection and maintenance of the transposon cassette. For selection and maintenance of recombinant plasmids in *E. coli* 50 μg/ml of kanamycin sulfate was used or 100 μg/ml of ampicillin (sodium salt, Sigma-Aldrich). For

experiments with minimal media, AVMM agar was used (Weber and McAlpine, 1992). Where appropriate (Fig. 3), AVMM agar was supplemented with 50 mM methylmalonic acid (Sigma-Aldrich).

2.2. Preparation of A. erythreum tagged mutant library: Library one.

A genomic library of total *A. erythreum* DNA was constructed by partially digesting 10 μg of chromosomal DNA with *Sau*3A1 so that the majority of the fragments were in the 8-20 kb range. After purification from a 0.6% agarose gel, the *Sau3A1* fragments were ligated to *BamHI*-digested pFL2082 that was dephosphorylated by calf intestinal alkaline phosphatase (Roche Molecular Biochemicals, Indianapolis, IN) (Table 1). To construct plasmid pFL2082, the *aph1* gene was released from pUC4K (Amersham-Pharmacia, Piscataway, NJ) by *EcoR1* digestion and cloned into the *EcoRI* site of pBluescript SK+ (Stratagene, La Jolla, CA). The *aph1* gene was released from pBluescript SK+ by *HincII* digestion and cloned into the *SspI* site of pUC19. The *aph1* gene confers kanamycin resistance in both *E. coli* and *A. erythreum* (Miller, 1991). Ligation mixtures were transformed into *E. coli* DH5α-e by electroporation. Transformants were plated on SOB agar plates (Sambrook et al., 1989) containing kanamycin, ampicillin and the color indicator X-gal. Ligation reactions were performed until the ratio of white to blue colonies was 10 to 1. Transformants were grouped into pools numbered 1- 25, and contained 4000 white transformants each. DNA was prepared from each pool separately for creation of Library two.

2.3. Library two: construction of a new transposon derivative Tn5-ottp

A transposon derivative of EZ::TN™<TET-1> (Epicentre, Madison, WI) was generated for *in vitro* transposon mutagenesis. The transposon derivative, designated Tn5-*ottp* was constructed by cloning the 1.7 kb EZ::TN™<TET-1> DNA from Epicentre into the larger (2.3kb) of the two fragments of pUC19 generated by *PvuII* digestion. This plasmid retained the ampicillin resistance gene, and the origin of replication from pUC19, but no polylinker. This construct, designated pFL3010, allowed the transposon to be easily further modified. The first modification was the addition of the thiostrepton-resistance gene (*tsr*) from pIJ487 (Ward et al., 1986). The *tsr* gene was amplified by PCR using forward primer 5'-CGGGGTACCTGATCTGATCAAGGCGAATAC-3' having a *KpnI* site at the 5' end and reverse primer

5'-CGGAATTCACAGAGGCGCTTATCGGTTG-3' having an *EcoRI* site at its 5' end. After *Eco*RI and *KpnI* digestion, the 1.0 kb *tsr* gene was ligated to similarly digested pFL3010, placing *tsr* upstream of the *tet* gene in EZ::TN™<TET-1>. The resulting construct was designated pFL3012. To clone the *ermEp** promoter into pFL3012, pIJ4070 (Bibb et al., 1994) was digested with *MscI* and *PstI*, which released a 280 bp fragment containing the promoter. The *MscI*/*PstI* fragment was ligated to similarly digested pFL3012 to yield pFL2083, placing the ermEp* promoter downstream of the tet gene of Tn<TET-1>. Finally, the 800 bp R6Kγ origin-of-replication (*ori*) fragment from EZ::TN™ <R6Kγ ori/Kan-2> (Epicentre) was amplified by PCR using forward primer 5'-CTGAATTCCACCCTGTGAATGCGCAA-3' and reverse primer 5'-CTGAATTCGAATTGCTTCGTTAATAC-3' both engineered with *EcoRI* sites at their 5' ends. After *Eco*RI digestion the R6Kγ *ori* PCR product was ligated to *EcoRI* digested pFL2083 to yield pFL2087A and pFL2087B, depending on the orientation of the R6Kγ ori. This placed the R6Kγ *ori* at the end of the transposon, downstream of *tsr*. Plasmid pFL2087A was used for the generation of the transposon fragment (Fig. 1C) that was used in the *in vitro* transposition mutagenesis reactions (see below).

2.4. in vitro transposon mutagenesis

Linear transposon DNA was generated through partial digestion of pFL2087A with *PvuII,* followed by gel purification of the 3.74 kb DNA fragment containing the transposon (Fig. 1C). The *in vitro* transposon mutagenesis reaction was performed on each of the 25 pools of DNA from library one according to Epicentre's instructions using DNA in a ratio of four-parts library one DNA to one-part transposon DNA. Following transformation of the 25 individual mutagenesis reactions into *E. coli,* transformants were selected on SOB agar diffusion plates (Fleischmann et al., 1995) containing tetracycline at 10 μg/ml, kanamycin at 50 μg/ml, ampicillin at 50 μg/ml and X-gal indicator. Selecting with all three antibiotics aided in eliminating those plasmids containing transposons that had inserted into the vector backbone. A total of 2000 white transformants were collected from each of the 25 mutagenesis reactions. DNA was prepared from each pool for creation of Library three.

2.5. Library three: protoplast transformations

A. erythreum B-3381 was transformed using a modified protoplast transformation procedure originally described by Roberts et al. (1987). Culture tubes containing 4 ml of 2xYT(G) broth were inoculated with *A. erythreum* and incubated at 32°C overnight. The overnight culture was diluted 1:10 in 25 ml of 2xYT(G) and grown for another 2-4 hours at 32°C with shaking at 350 rpm. Cells were pelleted by centrifugation at 5000 rpm for 10 minutes at room temperature on a Beckman J2-HS centrifuge, JA-17 rotor, followed by a wash in 20 ml of 0.3M sucrose, then resuspended in 5 ml of modified 1x P buffer (Hopwood et al., 1985) containing 5 mg/ml of lysozyme. Our modified P buffer lacks the potassium phosphate cited in the recipe of Hopwood et al. (1985). Cells were incubated for the formation of protoplasts at 32°C for 2 hours. Protoplasts were pelleted as before followed by a wash in 20 ml of modified 1x P buffer. After a final pelleting, protoplasts were resuspended in 1 ml of modified P buffer and either used immediately or stored at –80°C.

Transformations of protoplasts with plasmid DNA from library two were performed using a modification of the procedure described by Roberts et al. (1987) and Miller (1991). Two hundred microliter portions of protoplasts were transferred to eppendorf tubes containing 26 µl of DNA. The DNA was prepared using column chromatography (Qiagen, Midiprep Kit, Valencia, CA) and alkaline denatured using the procedure of Oh and Chater (1997). The contents of the tube were mixed on a vortex mixer for 1 sec, immediately followed by the addition of 800 µl of modified P buffer containing 25% polyethylene glycol (mw 10,000). The protoplasts were dispersed on a vortex mixer for 1-2 seconds. The protoplast mixture was spread evenly over an R2T2 plate, dried to 95% of its original weight. The transformation plate was incubated at 32°C for 22 hours to allow regeneration of cells from protoplasts. The plate was then covered with 1 ml of a thiostrepton solution (800 µg/ml) to select for drug-resistant transformants and incubated at 32°C. After 3-5 days the agar layer was transferred from the original plate and placed on top of a fresh agar layer of 2xYT(G) agar containing 25 µg/ml thiostrepton. The transfer was performed to speed the growth of the primary transformants. Transformants were scored after 5-6 days at 32°C. Each DNA pool from library two was used to create approximately 900 primary *A. erythreum* transformants for micro-fermentation screening. In addition, a selection of *A. erythreum* primary transformants was prescreened (**see Materials and Methods section 2.6**) to eliminate single-crossover recombinants from the group.

2.6. Prescreen for selection of double crossover mutants

To determine the ratio of single to double cross-over strains mutants were analyzed by replicating glycerol stock cultures *via* pin replicator (V&P Scientific, Inc., San Diego, CA) onto agar plates containing 2xYT(G) media supplemented with either Kn-5, Thio-10 or no antibiotic. Mutants that were $Thio^r$ Kn^r were scored as single-crossover and mutants that were $Thio^r$ Kn^s were scored as double-crossover.

2.7. Micro-fermentations.

Fermentation screening of the *A. erythreum* mutant library three (above) was performed in polypropylene microtiter plates (96-deep-wells per plate; 1 ml capacity per well). Microtiter wells were individually stirred using miniature magnetic stirring rods and a rotary tumble stirrer (V&P Scientific, Inc., San Diego, CA). The microtiter plates and stirring apparatus were placed inside an incubator in which temperature and relative humidity were controlled at 33°C and 50-60%, respectively. Each well contained 500 µl of 2xYT(GMT) or modified SCM medium. Wells were inoculated with *A. erythreum* primary transformants or from secondary patches grown on plates containing thiostrepton. To generate master stock cultures for permanent storage of mutants, 100 ul of the deep well 36-hr cultures were transferred to a standard microtiter plate and mixed with an equal volume of 40% glycerol. Master plates were stored at –80°C for later use. For screening, master plates were thawed and 10 µl of the master stock culture were transferred by multichannel autopipet (Matrix Technologies Corp., Hudson, NH) into 500 µl of fermentation medium in the deep well microtiter dishes, and incubated at 33°C for 72 hours. Microtiter dishes were covered with tissue-paper lined lids to minimize well-to-well splashing and evaporation. Shake flask fermentations were performed as described previously (Reeves et al., 2002).

2.8. Plasmid insertion knockout in mutB *by homologous recombination*

To disrupt the *mutB* gene, PCR primers were designed to amplify a 769 bp region internal to *mutB*. The primers used were: forward, 5'GTCGGATCCACGCACAGCCAGACATCG -3' and reverse, 5'-

GTCGGATCCGACGATGTGCACGTCGGCGTC-3'. *Bam*HI sites were engineered at the 5' ends of the PCR primers to facilitate later cloning steps. After amplification, the PCR product was purified and digested with *Bam*HI and ligated to pFL2092 (**Table 1**), an *A. erythreum* integration vector. pFL2092 had been previously digested with *Bam*HI and dephosphorylated with calf alkaline phosphatase (Roche Molecular Biochemicals, Indianapolis, IN). The plasmid used to transform *A. erythreum* protoplasts was designated pFL2106. Protoplast transformations with pFL2106 were performed as described (**Materials and Methods, section 2.5**). The plasmid insertion knockout is designated *mutB*::pFL2106; the Tn5-*ottp* transposon knockout is referred to simply as *mutB*.

2.9. Plasmid insertion knockout in cobA *by homologous recombination*

An internal 275 bp region of *cobA* (from nt 161-436) was amplified using the following primers: (forward) 5'-GTCGGATCCGTGTTCCAGTTCGTGAAG-3' and (reverse) 5'-GTCGGATCCGACCCAGCCCCACTTCATCGG-3' with *Bam*HI sites engineered at the 5' ends. The cycling program consisted of an initial denaturation step for 2 minutes at 95°C followed by 25 cycles as follows: a denaturation step at 95°C for 1 min., an annealing step at 56°C for 1 min. and an extension step at 72°C for 30 sec. After purification from an agarose gel the DNA was digested with *Bam*HI and ligated to *Bam*HI-digested pFL2092 (**Table 1**). pFL2092 had been previously digested with *Bam*HI and dephosphorylated with calf alkaline phosphatase. The plasmid construct used to transform *A. erythreum* protoplasts was designated pFL2093. Protoplast transformations with pFL2093 were performed as described (**Materials and Methods, section 2.5**). The plasmid insertion knockout is designated *cobA*::pFL2093; the Tn5-*ottp* transposon knockouts are referred to simply as *cobA* and *cobA**.

2.10. Stirred jar fermentations

Five-day stirred jar fermentations were performed using LH Fermentation (Emeryville, CA) equipment, Model 500 series III. 2L jars were filled with 1.25 L of modified SCM broth, supplemented with sodium propionate (50mM). Seed inoculum was 50 ml of an overnight culture grown in the same

modified SCM medium as used for the fermentation, except without propionate. Antifoam B (Sigma, St. Louis, MO) was added as needed (approximately 0.75 ml to 1.0 ml/12hrs). Temperature was maintained at 33°C, and stirring was performed at 500 rpm. Sterile-filtered air-flow rate was 1.25 L/min.

2.11. Reverse engineering: plasmid rescue and DNA sequence analysis of the transposon insertion site.

Plasmid rescue was performed on *A. erythreum* transposon mutants by purifying total chromosomal DNA from mutant strains and digesting it with the frequent cutting restriction enzymes *XhoI, Bss*HII, *ApaI, MluI, and StuI*. None of these enzymes cuts within the transposon and therefore could be used for retrieving the intact transposon DNA and sequences adjacent to the insertion site in the chromosome. Following complete digestion of the mutant chromosomal DNA, it was subjected to overnight ligation with T4 DNA ligase (Fermentas, Vilnius, Lithuania). The ligated DNA was transformed by electroporation into the pir^+-containing *E. coli* cell line EC100D-pir-116 (Epicentre). The transformed cells were plated on LB agar (Sambrook et al., 1989) supplemented with 10 μg/ml tetracycline-HCl. Plasmid DNA from two to five Tet^r colonies was analyzed by restriction digestion with the same enzyme used in the rescue procedure. Plasmids that showed an expected restriction pattern were chosen for DNA sequence analysis.

DNA sequencing reactions were performed using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. DNA that was generated through PCR sequencing reactions was precipitated with ethanol and resolved on an ABI 3730 DNA sequencer performed at Davis Sequencing, LLC (Davis, CA). The two sequencing primers used in all reactions were designed from the ends of the modified transposon and were as follows: Forward transposon primer, 5'-GCTGGACAATCGTGCCGGTT –3', and Reverse transposon primer, 5' GGAACACCTACATCTGTATTAACG-3'. All sequences were analyzed by the NCBI BLAST program with default settings.

2.12. Statistical analyses. T-tests and probability values were calculated for 95% confidence intervals using interactive software at http://home.clara.net/sisa/t-test.htm.

2.13. Accession numbers.

The DNA sequences for the *mutB* and *cobA* genes have been deposited in the Genbank database with accession codes AY548464 and AY548465, respectively.

3. Results

3.1. General approach

The strain chosen for this study was the unicellular erythromycin-producing bacterium, *A. erythreum* (**Table 1**), instead of *Saccharopolyspora erythraea*, a mycelial actinomycete and the current industry standard. Compared to *S. erythraea*, *A. erythreum* can be more easily handled, transformed, genetically manipulated (Roberts et al., 1987; Miller, 1991), and screened by micro-fermentation. A mutagenesis method was chosen that presented the opportunity for reverse engineering. The method employed a Tn5-derived transposon for gene tagging (**Fig. 1C**). A similar strategy had been used previously in *Streptomyces coelicolor* to identify genes involved in development (Gehring et al., 2000). Fermentations were performed in a 96-well micro-format, instead of shake flasks to accelerate the screening process. The micro-fermentation growth medium, modified SCM, was modeled after a commercial growth medium that had been developed for high efficiency erythromycin production (McAlpine et al., 1987).

3.2. Isolation of improved mutants

Using an agar plate bioassay method (Reeves et al., 2002), twenty-six mutants with significantly increased erythromycin production (above 50%), and forty-four mutants with low or no erythromycin production were selected for further testing out of a total of 3,049 isolates that were screened in duplicate. Analysis of antibiotic resistance phenotypes of the first 2,267 mutants indicated that 88% were single-crossover insertions of the entire plasmid into the chromosome. The remainder of the transformants were double-crossover (gene replacement) strains in which the transposon was inserted into the genome without plasmid sequences, via homologous recombination. Because of the high frequency of single-crossover

insertions in the library, a prescreen was developed (**Materials and Methods section 2.6**) to increase the frequency of double-crossover mutants. The total final number of double-crossover mutants obtained for the micro-fermentation screen was 1,070. This equals the mutational analysis of approximately 14% of an 8 MB Actinomycete genome, assuming the transposon inserted randomly. Southern blot analysis indicated that the insertions were random (data not shown), except in one case (see below). Also, erythromycin non-producing mutants arose at a frequency of approximately 1%, consistent with the relative size ratio of the *A. erythreum* erythromycin biosynthetic gene cluster (55.4 kb) to the chromosome (Brikun et al., 2004). After the initial fermentation screening program and retesting of higher-producing double-crossover mutants, seven mutants were selected for reverse engineering.

3.3. Reverse engineering of improved mutants

DNA sequence analysis of the transposon insertion site confirmed that all seven mutants contained the predicted double-crossover, gene-replacement insertions. Three insertions were found to be in *mutB*, a gene coding for the alpha subunit of methylmalonyl-CoA mutase (Marsh et al., 1989; Fig. 1A). The remaining four insertions were found to be in *cobA*, a gene coding for cob(I)alamin adenosyltransferase (Escalante-Semerena, 1990; Fig.1B).

Insertions into *mutB* were all found in the same site and in the same orientation; but because all three *mutB* mutants came from the same transformant pool, they were assumed to be merely siblings and not unique insertion events. The insertions into *cobA* were also all in the same site; however, because the *cobA* mutants arose from different transformant pools, and since both orientations of the transposon were found in *cobA* (*cobA and cobA**), the four mutants could not be siblings. The combination of a high frequency and a single site of insertion for the transposon into *cobA* suggested that a transposition hot-spot could be responsible; however, further experimentation, by performing *in vitro* transposition on a cloned *cobA* gene, did not support this hypothesis.

DNA sequence analysis of the *cobA* locus revealed a second gene, *cobB,* that is immediately downstream and translationally coupled to *cobA* (Fig. 1B). The *cobB* gene is a putative cobyrinic acid a,c-diamide synthase (64% identity to the ortholog from *Streptomyces avermitilis* accession number BAC74123.1). The *che* gene (partial sequence), immediately upstream of *cobA*, is a presumptive cobalto-

chelatase with highest identity to the ortholog from *Streptomyces avermitilis* (accession number BAC74125). The *cobB* gene, and other genes downstream, could be affected by the *cobA* knockout.

The *mutAB* gene pair and ORF1 appear as an orthologous cluster of three genes in 10 out of 16 genomes analyzed by Dobson et al. (2002) including *E. coli, Bacillus subtilis,* and more recently, *S. erythraea* (accession number AAM77046.1). ORF1 has been reported to be a coupled ATPase of the lysine-arginine-ornithine transport system in *E. coli* (Celis et al., 1998). Also, human patients with methylmalonic aciduria in the *cblA* complementation group have mutations in their ORF1 orthologs suggesting a specific involvement of ORF1 in vitamin B_{12} utilization (Dobson et al., 2002). Consistent with this is a recent report claiming that ORF1 homologs (referred to as *meaB*) are components of the methylmalonyl-CoA mutase complex required for protection of the enzyme from inactivation (Korotkova and Lidstrom, 2004). ORF1 orthologs have also been suggested to be the methylmalonyl-CoA epimerase, or a transcriptional regulator for methylmalonyl-CoA mutase gene (Korotkova et al., 2002). ORF2 is an ortholog of a putative regulatory gene from the *mazG* family with unknown function (Zhang and Inouye, 2002). In contrast to the *cobA* insertion, the *mutB* insertion is not as likely to generate polar effects by virtue of a large (462 bp) gap of non-coding DNA identified immediately downstream. This gap is characterized by distinctive repeated sequences and potential stem loop structures that might serve a regulatory function (Fig. 1A).

3.4. Growth and antibiotic production in improved mutants

Fermentation studies with *mutB* and *cobA* mutant strains were performed. In three-day modified SCM micro-fermentations the *mutB* mutant strain (strain FL2145, N = 72 replicate cultures) showed an average 74% increase ($t = 12.25$; $P < 0.001$) in erythromycin production over the parent strain (FL262, N = 192 replicates). During logarithmic growth (12 to 34 h) and stationary phase (34- 80 h), in 2xYT(GMT) micro-fermentations, the *mutB* mutant strain maintained an average erythromycin productivity at 7.6 mg per liter per hour. This average productivity was 1.9 and 2.5-fold greater than the average productivity of the parent strain during logarithmic growth and stationary phase, respectively (Fig. 2A and B). The effect was not limited to 2xYT(GMT) media; the same time course analysis was performed in modified SCM broth and the productivity of the *mutB* mutant strain was an average of 2.0 and 1.4-fold greater than the parent strain

during logarithmic growth (12-38 h) and stationary phase (38 – 80 h), respectively. The strain improvement phenotype was not limited to *mutB* mutations created by transposon insertions. Comparable strain improvement phenotypes were obtained using a plasmid insertion knockout (*mutB*::pFL2106 in strain FL2132, **Materials and Methods section 2.8**). The strain improvement phenotype was also seen upon scale-up. Two-fold increases in production ($t = 13.82$; $P < 0.001$) were observed when the *mutB* mutant (FL2145) was compared to the parent (FL262) in replicate modified SCM shake flask fermentations (N = 6 for each strain). Preliminary experiments performed in 2 L stirred-jar fermenters also indicated that the strain improvement phenotype for *mutB* mutants would be maintained at this scale.

In three-day 2xYT(GMT) micro-fermentations, the *cobA* mutant strain (FL2141) showed an average 22% increase in erythromycin production over the parent strain (FL262, N = 24 for both strains; $t = 2.342$; $P < 0.014$). The *cobA** mutation, on the other hand, had a negligible effect on production (strain FL2143, 8% increase; N = 24 for both strains; $t = 0.99$, $P < 0.166$) over the parent strain. Interestingly, when a plasmid insertion was created in *cobA* (FL2177, **Materials and Methods section 2.9**) the effect on production in micro-fermentations was similar to what was seen for the *mutB* mutation (105% increase, $t = 18.59$, $P < 0.001$); 48 *cobA* replicate cultures and 24 replicate parent cultures were compared in 2xYT(GMT).

The *mutB* mutants, both the transposon and plasmid insertion mutants (FL2145, FL2132, respectively) were unable to grow on minimal medium containing methylmalonate as the sole carbon source (Fig. 3, Table 2). Other nutrients that are catabolized to methylmalonyl-CoA such as isoleucine and threonine, were also found to be unable to support the growth of the *mutB* mutant strains on minimal medium (Table 2). The *cobA* and *cobA** strains (FL2141 and FL2143, respectively) did not show the same severe inability to metabolize these nutrients (Table 2).

The *mutB* mutant (FL2145), like the parent, accumulated erythromycin A, and did not accumulate erythromycin biosynthetic intermediates (Fig. 4A). Feeding nutrients that increase pools of propionyl-CoA and methylmalonyl-CoA in the cell boosted erythromycin production in both the *mutB* mutant strain (FL2145) and parent strains; with the mutant strain showing a greater benefit (Fig. 5). For the *mutB* mutant strain, leucine provided the greatest increase in erythromycin production with an average increase of 83% (N = 8 for both strains, $t = 4.69$, $P < 0.001$) over *mutB* strains grown in unsupplemented media and a 223%

increase (N = 8 for both strains, $t = 7.53$, $P < 0.001$) over the parent strain grown under original (unsupplemented) conditions. Sodium propionate gave the next best result with an average increase of 29% (N = 8 for both strains, $t = 2.36$, $P < 0.017$) over the unsupplemented *mutB* mutant strain and a 126% increase (N = 8 for both strains, $t = 6.76$, $P < 0.001$) over the unsupplemented parent strain.

The *cobA* mutant (FL2177), unlike the parent and *mutB* mutant, accumulated small amounts of erythromycin intermediates as could be seen on TLC (Fig. 4B). Nutrient feeding in *cobA* mutant strain fermentations had a positive effect, as it had in *mutB* mutant fermentations, with leucine showing the greatest effect on yield with a 135% increase ($t = 10.02$, $P < 0.001$) over the unsupplemented conditions, and a 156% increase ($t = 11.8$, $P < 0.001$) over the parent strain in unsupplemented conditions. Sodium propionate showed the next largest yield increase (89% increase, $t = 5.59$, $P < 0.001$) over the *cobA* strain grown in unsupplemented conditions and a 106% increase ($t = 6.57$, $P < 0.001$) over the parent grown in unsupplemented conditions. Methionine also had a significant effect on erythromycin production in the *cobA* mutant (FL2141), unlike in the *mutB* mutant and parent strains, boosting production 70% ($t = 5.48$, $P < 0.001$) over the *cobA* mutant grown in unsupplemented conditions.

4. Discussion

The enzyme, methylmalonyl-CoA mutase, coded for by the *mutAB* gene pair, is a heterodimer whose amino acid sequences are highly conserved from bacteria through humans (Valentin and Dennis, 1996; Drennan et al., 1996). With coenzyme B_{12} as its cofactor, it catalyzes the interconversion of methylmalonyl-CoA and succinyl-CoA (Kellermeyer et al., 1964; Hunaiti and Kolattukudy, 1984; McKie et al., 1990). Therefore, both classes of mutants, *mutB* and *cobA,* would diminish or block the activity of methylmalonyl-CoA mutase.

Since methylmalonyl-CoA is a direct precursor of erythromycin biosynthesis, blocking its metabolism would be predicted to have an effect on erythromycin production. Hunaiti and Kolattukudy (1984) reported that methylmalonyl-CoA is derived from succinyl-CoA in *S. erythraea* through the action of methylmalonyl-CoA mutase. Therefore, if *A. erythreum* also derives its methylmalonyl-CoA from

succinyl-CoA, then blocking *mutB* should cause a *decrease* in erythromycin production. Why then, did we see an increase?

One explanation is that the precursor feeding pathways may vary from one actinomycete to another (Cropp et al., 2001; Vrijbloed et al., 1999). However, our metabolic model (Fig. 6) is more consistent with reports that the favored direction of methylmalonyl-CoA mutase is from methylmalonyl-CoA to succinyl-CoA by a ratio of 20:1 (Kellermeyer et al., 1964; Vlasie and Banerjee, 2003). The enzymatically favored direction of methylmalonyl-CoA mutase is consistent with its participation in anaplerotic pathways via succinyl-CoA (Fig. 6). Nutrients such as branched-chain amino acids, odd numbered fatty acids, thymine, methionine and threonine all enter central metabolism through this pathway. These nutrients have also been previously shown to boost the production of some macrolide antibiotics when supplemented into fermentation media (Dotzlaf et al., 1984; Omura et al., 1983; Tang et al., 1994). We therefore postulate that a block in methylmalonyl-CoA mutase leads to an increase in production of erythromycin in *A. erythreum* because methylmalonyl-CoA lies at a metabolic branch-point. The mutation in *mutB* switches the metabolic flow of methylmalonyl-CoA away from the central metabolic branch towards the erythromycin biosynthetic branch (Fig. 6).

Consistent with the metabolic switch model, *mutB* mutant strains were unable to grow on minimal medium containing methylmalonate as the sole carbon source. Isoleucine and threonine, which are metabolized to methylmalonyl-CoA, were also unable to support the growth of *mutB* mutants on minimal medium suggesting that only one route exists to central metabolism down these catabolic pathways. The *cobA* and *cobA** mutant strains did not show the same severe inability to metabolize these nutrients possibly because some mutases do not have a strict coenzyme B_{12} requirement (Hunaiti and Kolattukudy, 1984).

Fortunately for commercial applications, the *mutB* mutant, like the parent, accumulates erythromycin A, and does not accumulate erythromycin biosynthetic intermediates. This result was unexpected because the mutant strains would have only been predicted to show an increase in production of 6-deoxyerythronolide B, which is the polyketide-derived portion of the molecule. Post-polyketide modifications, including biosynthesis and attachment of sugars and other tailoring modifications, should not have been affected by the *mutB* and *cobA* mutations. However, because the entire molecule was made

in greater quantity, methylmalonyl-CoA and its precursor propionyl-CoA must be limiting for the biosynthesis of the entire molecule, with the erythromycin sugars and tailoring reactions available in excess. A similar conclusion has been drawn previously regarding methylmalonyl-CoA as a limiting metabolite for the polyketide antibiotic monensin (Zhang et al., 1999). Consistent with this theory, by feeding specific nutrients that are predicted to increase the pools of propionyl-CoA and methylmalonyl-CoA, erythromycin production was boosted substantially further in both the mutant and parent strains. In these fermentations, the mutant strain shows a greater benefit from these targeted nutrients, as the metabolic switch model would predict.

Interestingly, humans can inherit an analogous inborn error of metabolism where either methylmalonyl-CoA mutase or cob(I)alamin adenosyltransferase are defective (Drennan et al., 1996; Leal et al., 2003). These mutations lead to the accumulation of methylmalonate in the body causing an acidosis that is often fatal. From a biochemical standpoint, erythromycin production protects *A. erythreum* from this harmful metabolic condition, since the organism has evolved a constructive way to dispose of the toxin. This raises interesting issues regarding the evolution of secondary metabolism in bacteria.

Ancestral *A. erythreum* strains may have survived a spontaneous *mutB* mutation in nature using a single enzymatic step to render methylmalonate innocuous. This could have been accomplished using an existing enzyme with a relaxed specificity involved in a related metabolic pathway such as fatty acid biosynthesis. Later, the progeny of this strain could have acquired additional genes to produce a useful bioactive product either by recruitment of endogenous enzymes, or through lateral gene transfers.

Secondary metabolism therefore, could merely be the solution that bacteria devise to first survive, and then later thrive, in the face of “diseases” brought on by “inborn errors of metabolism”. Their survival, where humans cannot, is due to their unique ecological niches and ability to evolve rapidly. Other theories regarding the evolution of secondary metabolism have been proposed (Challis and Hopwood, 2003; Firn and Jones, 2000; Stone and Williams, 1992; Vining, 1992; Demain and Fang, 2000).

A further implication of the metabolic branch model is the opportunity it presents, in the wild type strain, for regulation of metabolic flow down either pathway. Regulation of the expression of the methylmalonyl-CoA mutase or epimerase genes would allow for control of the transition from primary to secondary metabolism. Time course experiments indicate that erythromycin biosynthesis is active during

logarithmic growth (Fig. 2). Therefore, since that metabolic branch does not need to be activated, the primary metabolic branch simply needs to be blocked, for example, through repression of mutase or epimerase gene expression. In the *mutB* knockout strains the metabolic switch is permanent. During the rapid growth phase of the fermentation, the metabolites that would have normally gone into growth and respiration would instead go into erythromycin biosynthesis, thus accounting for the overall increase in erythromycin yield in the mutant strains.

The model of branch-point enzymes controlling flow between primary and secondary metabolic pathways is well established in plants and fungi (Edwards and Gatehouse, 1999). Bacterial secondary metabolic pathways have yet to be described with reference to such a model, and no other specific examples exist, to the best of our knowledge, where strain improvement has been achieved through the operation of a similar metabolic switch between primary and secondary metabolism.

Methylmalonyl-CoA mutase levels in *S. erythraea* have been measured previously by others, and shown to be regulated over time (Hunaiti and Kolattukudy, 1984). Since other changes such as pigment formation and sporulation are regulated similarly during the actinomycete life cycle, it is possible that these developmental functions could be coordinately regulated. Thus a study of regulation of mutase (or epimerase) expression, in this and other actinomycetes, might lead to further insights into global regulation of secondary metabolism and development.

In industry, the mutate-and-screen method often leads to large increases in secondary metabolite production, but typically only in wild-type strains in the early stages of strain improvement. This is similar to what we have found in this study, possibly because both traditional and transposon mutagenesis methods both generate loss-of-function mutations such as the ones described in this report. While this strain improvement concept would be generally applicable to other organisms and natural products, it remains to be seen whether the metabolic switch described in this report is relevant to industrial strains of *S. erythraea*.

In the future we hope to be able to identify additional strain improvement strategies through the completion of the mutational analysis of the *A. erythreum* genome, and through additional rounds of mutagenesis on the *mutB* mutant and subsequent strains.

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Table 1. Plasmids, strains, and transposons used in this study.

Reference	Plasmid or strain	Description
E. coli *plasmids*		
pFL2082	Vector containing the *aph1* gene fron pUC4K (Amersham-Pharmacia, Piscataway, NJ) cloned into the *SspI* site of pUC19. Used to make *A. erythreum* genomic library in *E. coli*. Ap^r $[Kn^r]^a$.	This study
pFL2087A	The plasmid (pUC19-derived) carrying the final modified 3.74 kb transposon, Tn5-*ottp,* (Fig. 1C) used for *in vitro* mutagenesis. Ap^r Tet^r ($Thio^r$).	This study
pFL2092	Useful as an *A. erythreum* integration plasmid by homologous recombination when *A. erythreum* DNA fragments are cloned into it. Contains the thiostrepton resistance gene from pIJ487[2] cloned into the *EcoRI* and *KpnI* site of pFL2082. Ap^r $[Kn^r]$ ($Thio^r$).	This study
pFL2093	A derivative of pFL2092 containing a 276 bp internal *cobA* fragment to generate a single crossover knockout of *cobA* in *A. erythreum.* Ap^r $[Kn^r]$ ($Thio^r$)	This study
pFL2106	A derivative of pFL2092 containing a 769 bp internal *mutB* fragment to generate a single crossover knockout of *mutB* in *A. erythreum.* Ap^r	This study

	[Kn^r] (Thio^r)	
pIJ4070	Ap^r pUC18-derived vector containing the *ermEp** promoter, kindly provided by the John Innes Centre, Norwich, UK.	Bibb et al. (1994)
***A. erythreum* strains**		
FL262	NRRLB-3381. Wild-type strain; erythromycin producer.	French et al. (1970)
FL2132	Single crossover knockout of *mutB* derived by integration of pFL2106. Thio^r Kn^r.	This study
FL2141	Transposon insertion into *cobA* gene (*cobA*) (NP_626119.1) [b]. Thio^r Kn^s. Isolated from Library 3 Pool 3.	This study
FL2143	Transposon insertion into *cobA* gene (*cobA**) insertion in opposite orientation as in FL2141. Thio^r Kn^s. Isolated from library 3 Pool 4.	This study
FL2144	Transposon insertion into *cobA* gene (*cobA**). Thio^r Kn^s. Isolated from Library 3 Pool 5.	This study
FL2145, FL2146, and FL2147	Transposon insertion into *mutB* gene (NP_823216.1)[b]. Thio^r Kn^s. Isolated from library 3, Pool 8 (prescreened for double crossover mutants).	This study
FL2154	Transposon insertion into *cobA* gene (*cobA*); insertion in the same orientation as FL2141. Thio^r Kn^s. Isolated from Library 3 Pool 8.	This study
FL2177	Single crossover knockout of *cobA* derived by integration of pFL2093. Thio^r Kn^r.	This study
***E. coli* strains**		
DH5α-e	Host strain for electroporations to create libraries 1 and 2.	Invitrogen, Carlsbad CA
EC100D *pir*$^+$	Recipient strain for transformations following plasmid rescue. Allows maintenance of plasmids with the R6Kγ origin of replication.	Epicentre, Madison, WI

Transposons		
< EZ::TN™<TET-1>	Tet^r Tn5 derivative used for *in vitro* mutagenesis.	Epicentre,
< EZ::TN™<R6Kγ-ori/Kan-2>	Kn^r Tn5 derivative used for isolating R6Kγ ori and Kanamycin resistance gene.	Epicentre
Tn5-*ottp*	Tn5-derivative transposon constructed for this study containing R6Kγ ***o**ri*, ***t**sr*, ***t**et*, and the *ermE**p**** promotor.	This study

[a] **[]** denotes antibiotic resistance in both *E. coli* and *A. erythreum*; () denotes antibiotic resistance in *A. erythreum,* no brackets denotes antibiotic resistance in *E. coli* only.

[b]Accession numbers indicate best matches from BLASTX search.

Table 2 Growth of *A. erythreum* wild-type, *mutB*, *cobA*, and *cobA** mutants on minimal medium supplemented with different carbon sources.

Carbon source[a]	w.t.[b]	mutB[c]	cobA[d]	cobA*[e]
Glucose[f]	+++	+++	+++	+++
Succinate	+++	+++	+++	+++
Isoleucine[g]	++	-	++	+
Leucine	-	-	-	-
Valine	+	-	-	-
Methionine	-	-	-	-
Threonine	++	-	++	+
Methylmalonate	+++	-	++	+
Propionate	+	-	+	-
Valerate	-	-	-	-
Heptadecanoic acid	+	+	+	+
Cholesterol	-	-	-	-

[a] all carbon sources added to a final concentration of 50mM, except cholesterol and heptadecanoic acid were added to 10 mM. (-) no growth; +, poor growth; ++, moderate growth; +++ excellent growth; growth scored after 7 days at 32°C.

[b] w.t. = FL262 [c] mutB = FL2145 [d] cobA = FL2141 [e] cobA* = FL2143

[f] $(NH_4)_2SO_4$ was added to media in which sugars and fatty acids were used as sole carbon sources.

[g] amino acids acted as the sole carbon *and* nitrogen source.

Fig. 1. A. Physical map of *mutAB* and downstream DNA. A 7281-bp region containing the translationally coupled *mutAB* gene pair is shown (top). Downstream of *mutB,* a presumptive 462-bp untranslated gap occurs. In the center of this gap a 20-bp sequence, GAGCCGATCTCGGTCCGGAC, was found, repeated four times (bottom of panel A). The triangle points to the site of insertion of the transposon in *mutB*, as described in the text. **B.** Physical map of *cobA* and surrounding DNA. The figure shows 1317-bp of DNA in the region around *cobA*. The triangle points to the site of insertion of the transposon in the *cobA* gene, as described in the text. When the transcription of *cobA* is in the same direction as *ermEp** (see Fig. 1C) the mutation is designated simply *cobA*, the opposite orientation is designated *cobA**. **C.** Tn5-derived transposon (Tn5-*ottp*) used for *in vitro* transposon mutagenesis. A derivative of transposon EZ::TN™<TET-1> (Epicentre, Madison, WI) was generated which contained the original *tet* gene and two 19-bp Tn5 transposase recognition sequences at the ends of the transposon. Modifications to this transposon for this study included addition of *tsr*, a thiostrepton resistance gene from pIJ487 (Baltz et al. , 2001), the R6Kγ *ori* from EZ::TN™<R6Kγ-ori/Kan-2> (Epicentre), and the *ermEp** promoter described by Bibb et al. (1994). For construction of this transposon see **Materials and Methods (section 2.3)**.

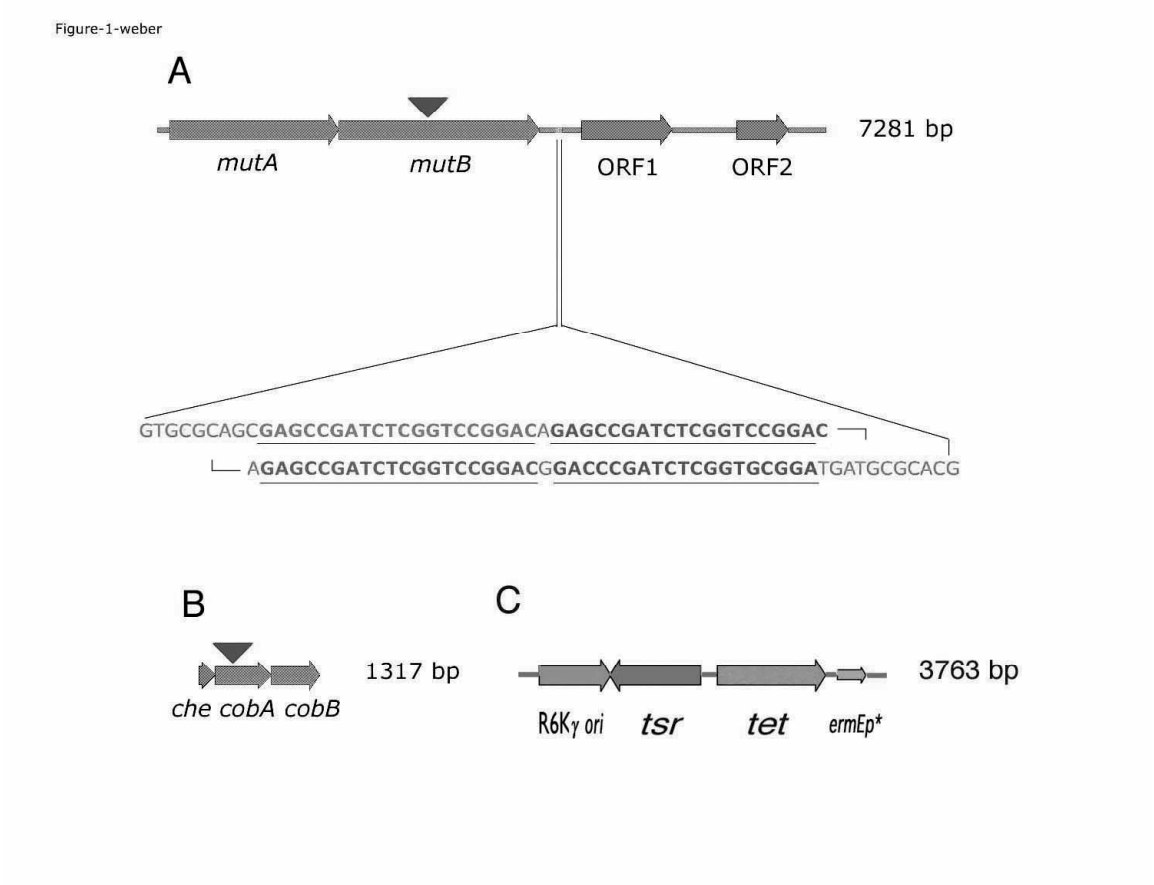
Figure-1-weber
A
7281 bp
mutA
mutB
ORF1
ORF2
GTGCGCAGCGAGCCGATCTCGGTCCGGACAGAGCCGATCTCGGTCCGGAC
AGAGCCGATCTCGGTCCGGACGGACCCGATCTCGGTGCGGATGATGCGCACG
B
1317 bp
che cobA cobB
C
3763 bp
R6Kγ ori
tsr
tet
ermEp*

Fig. 2. Growth and erythromycin production of wild type *A. erythreum* and *mutB* mutant FL2145 in micro-fermentation. (**A**) Growth of parent (wild-type) and FL2145 in supplemented 2xYT(GMT) medium. Values shown are the average absorbance readings of triplicate micro-fermentations measured at 600 nm. **(B)** Erythromycin production by parent and *mutB* strain FL2145 culture broths observed after bioassay of the same samples used to generate the growth curve. Error bars represent one standard deviation for the data obtained from the triplicate fermentations.

Figure-2A-weber

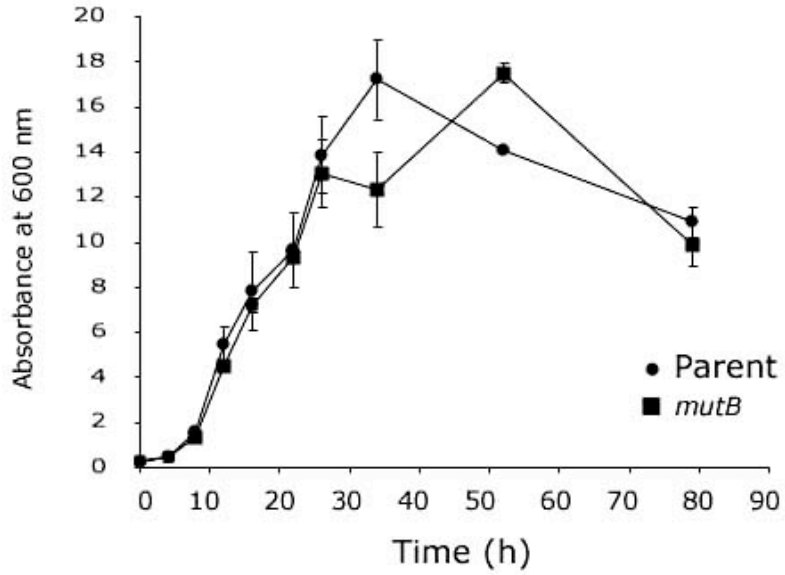

Figure-2B-weber

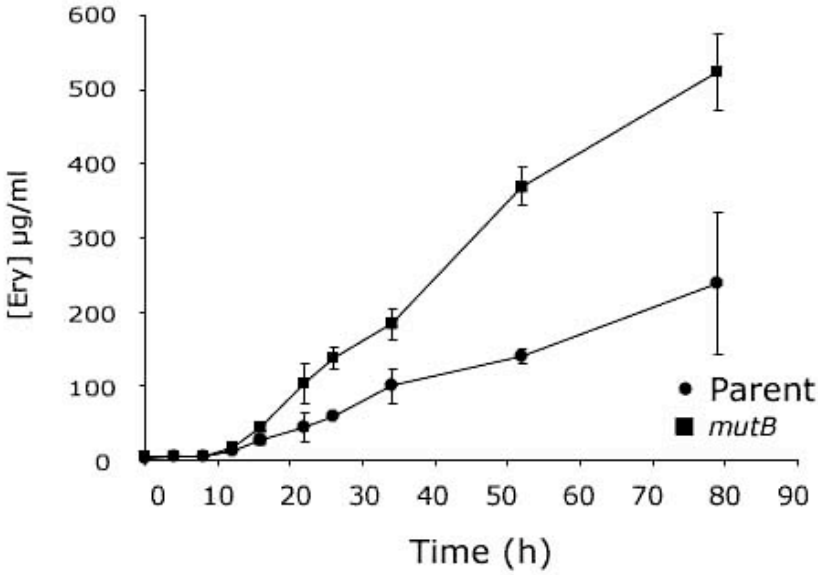

Fig. 3. Growth of *A. erythreum* on minimal medium (AVMM) supplemented with methylmalonic acid (50mM). The parent strain (FL262), *mutB* transposon knockout strain (FL2145), and *mutB* plasmid insertion knockout strains (FL2132-1A&B through FL2132-3A&B) are shown on AVMM minimal medium with 50 mM methylmalonic acid (Sigma-Aldrich, St. Louis, Mo) as the sole carbon source (left) or rich medium, 2xYT(G) (right). Plates were incubated at 33°C for four days. The results show that the *mutB* knockout strains are unable to grow on methylmalonic acid as the sole carbon source. The *cobA* and *cobA** mutant strains showed a less severe growth defect under the same conditions (Table 2).

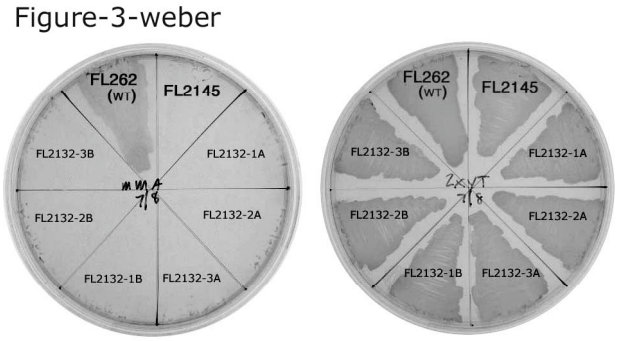

Fig. 4 A. Thin layer chromatography of fermentation broth extracts. The wild-type strain (FL262, lane "wt") and the *mutB* knockout strain (FL2145, lane "*mutB*") were grown in modified SCM medium in 250-ml Erlenmeyer flasks (McAlpine et al., 1987) for five days. The broth was extracted and processed for thin layer chromatography using standard methods (Weber et al., 1990). The results showed that both the parent and mutant culture broths appeared similar except for the intensity of the spot corresponding to erythromycin A. No additional bands were observed in the mutant strain that were not also in the parent strain. The "S" lane contains erythromycin standards (5 ug of each), corresponding to erythromycins A, B and C, and erythronolide B (EB) as labeled. **B.** Analysis of *cobA* mutant (plasmid insertion mutant FL2177), lane "*cobA*", and wild-type strain (FL262, lane "wt") was performed in micro-fermentation in modified SCM medium and extracted as described above. Additional spots (corresponding to erythromycin intermediates) can be seen in the *cobA* mutant strain.

Figure 4

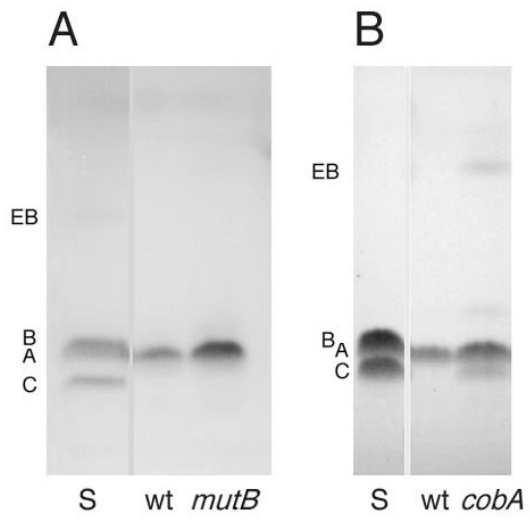

Fig. 5 Nutrient feeding for increased erythromycin biosynthesis. Erythromycin production of wild-type *A. erythreum* (FL262), *mutB* (FL2145) and the *cobA* (FL2141) strains were compared in six-day modified SCM micro-fermentations: lanes 1, unsupplemented; or supplemented with, lanes 2, propionate (50 mM); lanes 3, heptadecanoic acid (10 mM); lanes 4, leucine (50 mM) or, lanes 5, methionine (50 mM). Each strain was tested in 8 replicates for each of the supplemented and unsupplemented media. The average erythromycin yield is shown as a dot within an open circle for each strain in each medium.

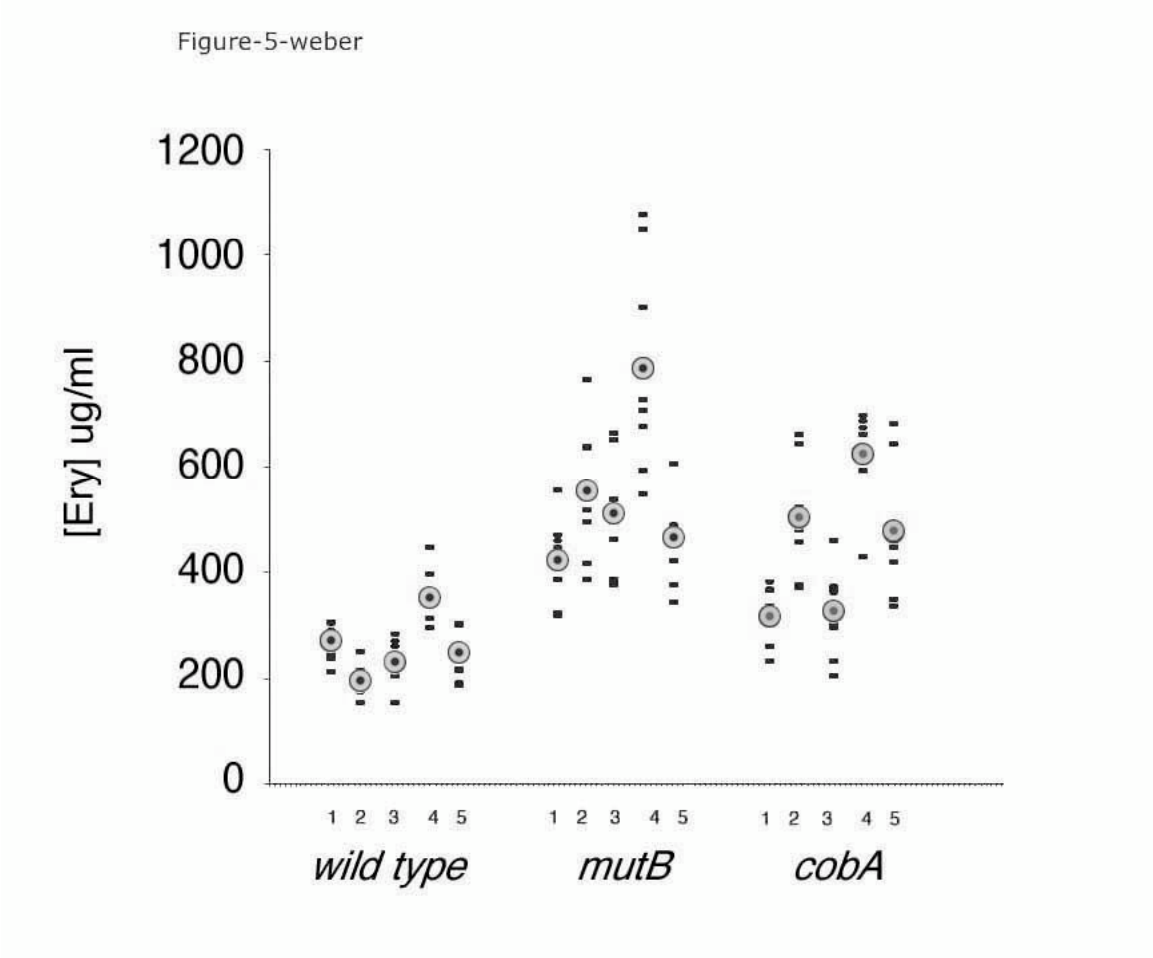

Fig. 6 A metabolic switch for erythromycin strain improvement. Abbreviations: 6dEB, 6-deoxyerythronolide B; carboxylase, propionyl-CoA carboxylase; epimerase, methylmalonyl-CoA epimerase; mutase, (2R)-methylmalonyl-CoA mutase. Not shown is the catabolic pathway for valine which feeds directly into (2S)-methylmalonyl-CoA without going through a propionyl-CoA intermediate. The double-headed arrow on the epimerase indicates a reversible reaction; the mutase is also reversible but favored in the direction towards central metabolism (Drennan et al., 1996; Vrijbloed et al., 1999). The cross in the mutase represents the metabolic switch, and indicates a block in the metabolic pathway leading to central metabolism. According to this model a block in the putative methylmalonyl-CoA epimerase would lead to the same phenotype as a block in the mutase.