A Metabolic Engineering Approach to Examine Polyketide Production by *Saccharopolyspora erythraea*

A thesis submitted to the University of London for the degree of

DOCTOR OF PHILOSOPHY

by

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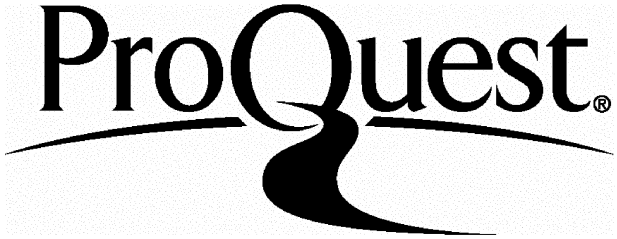

this thesis is dedicated, in loving memory,

to

Shigeki Ushio

ABSTRACT

The objective of this thesis was to use a metabolic engineering and modelling approach to evaluate the effect of genetic modifications in primary metabolism on secondary metabolite production in *Saccharopolyspora erythraea*.

The thesis investigated 1) the physiology of wild-type and genetically modified *S. erythraea* strains studied in batch culture under various nutrient conditions, 2) the physical and antimicrobial properties of red pigments produced by *S. erythraea*, 3) the capabilities of metabolic flux analysis to provide useful predictions of metabolic engineering targets, and 4) the genetic engineering tools to manipulate the primary metabolism of *S. erythraea*. The physiology of *S. erythraea*, in particular the relationship between organic acid overflow metabolism and polyketide synthesis, was studied.

Using *S. erythraea* physiology data and metabolic modelling predictions, over-expression of α-ketoglutarate dehydrogenase was selected as a metabolic engineering target. The aim was to determine if α-ketoglutarate excreted outside the cell could be re-channeled into primary metabolism to increase production of erythromycin and decrease red pigment synthesis. To this end, a new *S. erythraea* strain with an over-expressed heterologous α-ketoglutarate dehydrogenase was constructed and tested. The new strain was able to decrease α-ketoglutarate excretion, but did not produce greater amounts of erythromycin. Even though erythromycin levels were not increased this research could be considered as a first step to rational strain development for erythromycin production. It could also be regarded, in a greater sense, as proof of concept to use of the *S. erythraea* sequence data to identify genes of interest, and to genetically manipulate the central metabolism of *S. erythraea*. The results from this thesis justify research efforts to be continued in three different areas. The first is to continue strain improvement for erythromycin over-production. The second is to understand how to control organic acid excretion. The third is red pigment characterization.

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My husband, Barclay has kept me a clear thinker, a critical scientist, and an enthusiastic student while I have been pursuing my Ph.D. His unwavering love and encouragement has, and will continue to be, the cornerstone of my world.

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LIST OF ABBREVIATIONS

2D	2 dimensional
AA	amino acid
AF	antifoam
ATP	adenosine triphosphte
C/N	carbon to nitrogen ratio
CM	cell maintenance
CoA	coenzyme A
DCW	dry cell weight
DEBS	6-deoxyerythronolide B synthase
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DO	dissolved oxygen
DOT	dissolved oxygen tension
EDTA	ethylene dinitrilotetraacetic acid
ERY	erythromycin
FADH	flavin adenine dinucleotide
GC	gas chromatography
GLY	glycolysis
ICDH	isocitrate dehydrogenase
IPTG	isopropylthiogalactoside
KGDH	ketoglutarate dehydrogenase
MALDI-TOF	matrix assisted laser desorption ionization - time of flight
MCA	metabolic control analysis
MFA	metabolic flux analysis
MOPS	3-morpholinopropanesulfonic acid
MS	mass spectrometery
NADH	nicotinamide adenine dinucleotide

NADPH	nicotinamide adenine dinucleotide phosphate
NS	nucleotide synthesis
PKS	polyketide synthase
PPP	pentose phosphate pathway
PYDH	pyruvate dehydrogenase
RPM	revolutions per minute
RV	red-variant
TCA	tricarboxylic acid
THN	tetrahydroxynaphthalene
TLC	thin layer chromatography
tRNA	transfer ribonucleic acid
UV	ultraviolet
wt	wild-type
WV	white-variant

1 Summary

The objective of this thesis was to use a metabolic engineering and modelling approach to evaluate the effect of genetic modifications in primary metabolism on secondary metabolite production in *Saccharopolyspora erythraea.*

Several Actinomycetes produce commercially valuable polyketide products, however, many of these organisms also produce organic acids and other secondary metabolites that may subvert the carbon source from the desired product. *S. erythraea* produces the commercially important polyketide, erythromycin A. In addition, the strain used in this work (*S. erythraea* wild-type red-variant) produces red pigments, and excretes pyruvate and α-ketoglutarate. The pigment and organic acid products could be perceived as waste products in the context of commercial erythromycin A production.

The thesis investigated 1) the physiology of wild-type and genetically modified *S. erythraea* strains studied in batch culture under various nutrient conditions, 2) the physical and antimicrobial properties of red pigments produced by *S. erythraea*, 3) the capabilities of metabolic flux analysis to provide useful predictions of metabolic engineering targets, and 4) the genetic engineering tools to manipulate the primary metabolism of *S. erythraea*. The physiology of *S. erythraea*, in particular the relationship between organic acid overflow metabolism and polyketide synthesis, was studied.

Using *S. erythraea* physiology data and metabolic modelling predictions, over-expression of α-ketoglutarate dehydrogenase was selected as a metabolic engineering target. The aim was to determine if α-ketoglutarate excreted outside the cell could be re-channeled into primary metabolism to increase production of erythromycin and decrease red pigment synthesis. To this end, a new *S. erythraea* strain with an over-expressed heterologous α-ketoglutarate dehydrogenase was constructed and tested. The new strain was able to decrease α-ketoglutarate excretion, but did not produce greater amounts of erythromycin. Even though erythromycin levels were not increased this research could be considered as a first step to rational strain development for erythromycin production. It could also be regarded, in a greater

sense, as proof of concept to use the *S. erythraea* sequence data to identify genes of interest, and to genetically manipulate the central metabolism of *S. erythraea*.

This work is be the starting point for further research in three areas. The first, is strain improvement for erythromycin over-production. This is important because antibiotics are naturally produced at low levels and progress toward a rational approach to increase yield will be needed for all new and existing products. The second, is to understand how to control organic acid excretion. This is important because several antibiotic producing strains excrete organic acids as wasted carbon. It would be very useful if there is a method to re-direct the wasted carbon to product formation. The third, is red pigment characterization. This is important because the red pigment is a new compound and little is known about it, and it would be useful to re-direct the carbon used to make red pigment to erythromycin production.

Metabolic engineering is a powerful tool that enables the rational design and optimization of organisms that produce important products such as polyketides. With each new piece of research comes information that will contribute to, and advance the field of metabolic engineering. With these efforts, the tools to rapidly and successfully develop efficient and economical biological processes will be increasingly available.

2 Introduction

Polyketides are an important class of natural products that include therapeutic compounds such as antibiotics, antibacterials, antivirals, and antitumor drugs. It is important to have the ability to produce polyketides in large quantities and in an efficient manner because of their therapeutic significance. Additionally, the competitive environment of the pharmaceutical industry necessitates short product development cycle times. Although many research groups are working to discover or synthesize novel polyketides, few are developing methods to efficiently produce these new products at industrially relevant levels. The ability to develop rapidly fermentation processes with high product titers and low analog impurities significantly contributes to minimizing operating costs and maximizing production capacity. Historically, fermentation development has been accomplished by strain optimization using random mutagenesis and screening that did not require a complete understanding of the cell's metabolism. The cell was simply viewed as a black box. A more rational and directed approach to strain optimization may be achieved through the application of metabolic engineering and metabolic modelling, which directs the application of molecular biology to overcome limitations in the metabolic flux of substrate to desired product. With the rapid advances in molecular biology, tools are now available that may increase the effectiveness of applying recombinant DNA techniques to advance process engineering.

Metabolic engineering research is an iterative process proceeding in stages. In such a process a model is first developed of an organism's metabolism, and analyzed to evaluate rationally which pathways to target for modification. Using molecular biology techniques, target pathways are then modified by altering the expression of key enzymes or introducing new enzymes into the system. Finally, well-controlled experiments are performed to determine if the genetic modifications produced the desired results. If the desired results are not obtained, the physiology results obtained from the modified strain can be used to amend the physiological model generated for the organism so that another round of genetic modifications can be sensibly proposed.

The metabolic engineering approach of this thesis research was to manipulate the primary metabolism of *Saccharopolyspora erythraea* to determine if by increasing

flux to the biosynthetic precursors of erythromycin, erythromycin production could be increased. To this end, a metabolic model of the organism was used to evaluate rationally pathways to target for genetic modification. The unique characteristic of metabolic engineering is its perspective of integrated metabolic pathways as compared to individual reactions. The challenge is to understand metabolic fluxes and how to control them under *in vivo* conditions.

2.1 Metabolic Engineering

2.1.1 History

Metabolic engineering was defined in 1991 as the "improvement of cellular activities by manipulation of enzymatic, transport, and regulatory function of the cell with use of recombinant DNA technology" (Bailey, 1991). At that time, metabolic engineering consisted of a series of examples. Most were experiences where a new strain had been developed to investigate a single gene or gene cluster, and the limitations or improvements of the new strain provided additional insight into how best to optimize the strain for a particular objective, e.g. improved product formation. Also, the concept of metabolic network rigidity had been described which illustrated the complexity of metabolic regulation and methods of defining the flexibility of a metabolic node (Stephanopoulos and Vallino, 1991). A metabolic node is a point in a metabolic network where the reaction sequence splits into two or more different pathways. The rigidity of a particular node can be designated and flexible, weakly rigid, or strongly rigid. These categories are based on how much control that node has on the flux to a particular metabolite (Stephanopoulos *et al.*, 1998).

Over the past ten years, metabolic engineering has gained credibility as a codified science, has made several contributions to improving strain development, and has catalyzed the development of many advanced analytical tools. It has been recognized that metabolic engineering is a cyclical or iterative process where the steps consist of synthesis of a new strain based on physiological data, analysis of the new strain compared to the original, and design of the next target for manipulation based on the analysis. These steps have been applied to a variety of different cellular improvement objectives. These objectives or categories of metabolic engineering have been defined as the following: 1) extension of substrate range, 2) elimination or reduction

of by-product formation, 3) improvement of yield or productivity, 4) engineering of cellular physiology for process improvement, 5) pathways leading to new products, 6) pathways for degradation of xenobiotics, and 7) heterologous protein production (Nielsen, 2001). Four of these categories are discussed in more detail below in section 2.1.2.

Successful metabolic engineering requires several tools for the steps of synthesis and analysis of the new strain. With the advent of genome sequencing and bioinformatics, the number of genes that can be cloned and transformed has rapidly increased. In addition, several advancements in molecular biological tools and metabolic pathway analysis tools have been made. Molecular biological tools have improved in the areas of gene disruption and transformation methods, promoter systems, and gene shuffling techniques. Metabolic pathway analysis has been improved by the analytical tools of GC-MS, NMR, 2D-gels electrophoresis, MALDI-TOF MS, and DNA chips (Nielsen, 2001). These tools help to identify the proper metabolic network, quantify the fluxes through the network branch points, and identify control elements with the network. The information can then be used to construct new strains with improved properties.

2.1.2 Examples of Metabolic Engineering

The development of new fermentation biologic processes frequently included a strain improvement program. Some of the objectives of strain improvement typically include, improvement of product yield, elimination of by-product formation, extension of substrate range, and/or improvement of cellular of physiology. These objectives are areas where metabolic engineering could be a useful approach, as described through the following examples. Examples of metabolic engineering approaches to increase erythromycin yield are also discussed.

2.1.2.1 Improvement of Product Yield or Productivity

For most industrial processes, it is important to continually strive for increased yield of the product. This may be achieved through metabolic engineering by increasing the flux through the product biosynthetic pathway, or it may be necessary to increase the availability of precursors to the product pathway. There are difficulties in either case as it may not be simple to determine which enzyme activities to increase, or which precursors are limiting. The task become more difficult if central metabolism

requires modification as it is highly regulated and may involve multiple genetic engineering targets.

Increased biosynthesis of β-lactam antibiotics by *Streptomyces clavuligerus* is one example of increasing product yield through metabolic engineering. β-lactam antibiotics (including penicillin G, cephalosporin C, and cephamycin C) are highly effective, and have low toxicity to humans. Two strategies for increasing product levels have been implemented. In the first, kinetic modelling and metabolic control analysis were performed on the linear cephamycin C biosynthetic pathway (Khetan *et al.*, 1996). The results of the analysis determined that the ACV (α-aminoadipic acid, cysteine, and valine tripeptide) synthase was the rate-limiting step, and that α-aminoadipic acid had the highest control coefficient of the three peptides. A new *S. clavuligerus* strain with increased activity of the LAT (lysine ε-aminotransferase) enzyme was constructed. LAT transfers an amino group from lysine to α-ketoglutarate which is the first of two steps of α-aminoadipic acid synthesis. The new strain produced five-times as much cephamycin C compared to the wild-type (Khetan *et al.*, 1996). In a second approach, it was observed that high levels of penicillin N was accumulating which limited the production of cephalosporin C (Khetan and Hu, 1999). It was decided to over-express the enzyme immediately downstream of penicillin N, DAOC (deacetoxycephalosporin) synthase. The new strain was constructed which had higher levels of DAOC synthase, increased production of cephalosporin C, and did not accumulate penicillin N.

These two examples show that product yield can be improved through different metabolic engineering approaches. This first used mathematical modelling to indicate where to target genetic engineering, and the second used empirical experimental evidence to determine where to target. It should be noted that in these examples the pathway was linear and not strongly regulated. If there had been strong feed-back inhibition, or if the branch point had been a rigid node, these approaches may not have been as successful (Khetan and Hu, 1999).

2.1.2.2 Elimination or Reduction of By-Product Formation

Most strain improvement efforts focus on increasing product yield. However, in the complex system of cellular metabolism, unwanted by-products are frequently formed. By-product formation may cause complications for downstream processing or may be toxic to humans or animals. By-product formation are also undesirable because they may draw carbon away from product formation, thereby, reducing maximum product yield (Nielsen, 2001).

Reduction of oxalic acid formation by *Aspergillus niger* is one example of metabolic engineering implemented to reduce by-product formation. *A. niger* produces glucoamylase which is an industrial enzyme used for the production of high-fructose syrups from starch. This process also produces the undesirable by-product, oxalic acid (Pedersen *et al.*, 2000a). Oxalic acid is unwanted because it reduces carbon availability for product formation, and forms precipitates that interfere with downstream processing. Oxaloacetate hydrolase cleaves oxaloacetate to oxalic acid and acetic acid, and it has been shown that this enzyme is the only pathway to oxalic acid formation in *A. niger* (Kubicek *et al.*, 1988). Subsequently, the gene encoding oxaloacetate hydrolase, *oah*, was cloned and characterized (Pedersen *et al.*, 2000b). The *oah* gene was then disrupted in *A. niger* which resulted in no oxalic acid formation (Pedersen *et al.*, 2000c). However, the glucoamylase yield was reduced by 50% in the *oah* knock-out strain compared to the wild-type even though the growth rates were similar. Flux analysis of the two strains showed that it should be possible to disrupt the *oah* gene without affecting other areas of the metabolism. This example represents successful reduction of by-product formation, but highlights the difficulty of working with complex systems in that overall product yield was sacrificed as a result.

2.1.2.3 Extension of Substrate Range

In several industrial processes it is desirable to extend the substrate range of the organism. This approach is useful if there is an abundant source of raw material available, or if more efficient utilization of a substrate is required. The approach involves inserting a new pathway into the organism and two strategies may be employed. In the first, a gene encoding a transport protein to get the substrate into the cell, and the pathway to convert the new substrate into a compound that can be used

by central metabolism, is introduced into the host organism. In the second strategy, a gene encoding a secreted protein that can convert the new substrate to a compound readily utilized by the host organism is introduced. The potential complications are that the substrate may not be metabolized at a practical rate, and that undesirable by-products may be formed (Nielsen, 2001).

One example of extension of substrate range is engineering *Saccharomyces cerevisiae* to use xylose as a carbon substrate. D-Xylose is the second most abundant sugar in nature, after D-glucose. Xylose is abundantly found in plant material and is inexpensive which makes it an attractive carbon source for reducing production costs. Ethanol is an beneficial fuel from an environmental point of view as no net carbon dioxide is released from combustion. The challenge is to economically produce ethanol so it is competitive with existing chemical processes. *S. cerevisiae* has high ethanol tolerance and has been used to produce ethanol from glucose. Ethanol production by *S. cerevisiae* grown on xylose would be a good approach for economic large-scale ethanol production.

Several attempts have been made to engineer *S. cerevisiae* to grow and produce ethanol with varying degrees of success (Ostergaard *et al.*, 2000). However, promising results reported by Ho *et al.* (1998), showed increased ethanol production and simultaneous growth on xylose and glucose compared to the wild-type. The approach was to express xylose reductase (XR) and xylitol dehydrogenase (XDH), and over-express xylulokinase (XK) (Ho *et al.*, 1998). KR reduces xylose to xylitol. XDH converts xylitol to xylulose. XK, phosphorylates xylulose which can then enter the pentose phosphate pathway. The ethanol yield was 0.32 g of ethanol per g of xylose which is approaching this theoretical yield of 0.51 g of ethanol per g xylose. This example shows that inserting a foreign pathway into *S. cerevisiae* is possible and can have beneficial results. However, these effort have been ongoing for at least 10 years by several groups (Ostergaard *et al.*, 2000).

2.1.2.4 Engineering of Cellular Physiology for Process Improvement

When implementing a process using living cells, some of their inherent properties may be unwanted for the purposes of the process objective. For instance, the organism may be sensitive to low dissolved oxygen concentrations, or the product

formation may be repressed by glucose, or defined morphological characteristics may be desirable but not produced (Nielsen, 2001).

Oxygen limitation can inhibit growth and/or product formation in many organisms. Oxygen limitation may arise because of e.g. poor mixing conditions or high cell densities. One approach to increase tolerance to low oxygen conditions was to express a bacterial *Vitreoscilla* hemoglobin gene (*vhb*) in *Escherichia coli*. The results showed increased protein synthesis at low dissolved oxygen levels (Khosla and Bailey, 1988). Analysis of gene expression showed that the *vhb* gene was up-regulated under low oxygen concentration (Khosla and Bailey, 1989). Although the mechanism is not know in detail, it is thought that VHb scavenges oxygen and is able to provide it for cellular actions (Khosla *et al.*, 1990). Since this study, *vhb* has been cloned into several organism which in most cases resulted in improvements to cellular physiology or product formation (Nielsen, 2001).

2.1.2.5 Improvement of Erythromycin Yield or Productivity

There have been attempts to increase erythromycin production through genetic engineering. Brunker *et al.* (1998), cloned and expressed the *Vitreoscilla* hemoglobin gene (*vhb*) in *Saccharopolyspora erythraea*. The result was a 60% increase in erythromycin presumably due to increased oxygen uptake by the active hemoglobin (VHb) and an oxygen dependent reaction in the erythromycin biosynthesis pathway (Brunker *et al.*, 1998). This reaction was proposed to be the C-6 hydroxylation of 6-dexoyerythronolide B by EryF or the final hydroxylation step by EryK (Stassi *et al.*, 1993; Lambalot *et al.*, 1995). Rowe *et al.* (1998) implemented the promoter system *actII-ORF4* from *Streptomyces coelicolor* to over-express the erythromycin PKS genes in *S. erythraea*. This approach successfully increased total erythromcyins from 10 mg/L to 100 mg/L (Rowe *et al.*, 1998). A genetically engineered PKS (DEBS1-TE) was also over-expressed in this system and production of the triketide lactone product was increased from 10 mg/L to 150 mg/L. In a different approach, Dayem *et al.* (2002) cloned the erythromycin polyketide synthase genes into *E. coli*. The new organism was able to produce 6-deoxyerythronolide B, the polyketide moiety of erythromycin, to levels that compare with industrial processes (Pfeifer *et al.*, 2001). This group was also able to engineer *E. coli* to contain the methylmalonyl-CoA

mutase-epimerase pathway, which enabled the organism to supply the CoA precursors required for erythromycin synthesis (Dayem *et al.*, 2002).

2.2 Polyketides

Over the past 50 years, thousands of microbial metabolites have been found to have a broad range of pharmaceutical activity (Cane, 1997). Therapeutic applications of natural products include antibacterial, antiviral, antitumor, immunosuppressant, antihypertensive and antihypercholesterolemic activity. Polyketides, a class of natural products, comprise a large fraction of the pharmaceutically active metabolites with high commercial value including avermectin, daunorubicin, rapamycin, mevinolin, monensin, tetracycline, FK506, and erythromycin.

In a manner similar to fatty acid biosynthesis, polyketides are synthesized by a series of condensation reactions assembling acyl-CoA precursors, which are activated forms of acetate, propionate, and butyrate. Polyketide synthesis is initiated with a primer unit followed by elongation of the polyketide chain by incorporation of the activated building blocks one at a time. The oxidation level and stereochemistry of the beta-carbonyl are then adjusted through varying degrees of ketoreduction, dehydration and enoylreduction (Cane, 1994). There are several steps in polyketide synthesis at which variations can be incorporated to give specificity to each compound, such as choice of primer unit, number and type of elongation units, control of reduction cycle, stereochemistry, and cyclization pattern (Hopwood, 1997). Due to the many variables associated with polyketide synthesis, the structure and properties of polyketides can vary significantly, however, they can be classified into two categories based on their mode of assembly: aromatic and complex (Katz, 1997).

Aromatic polyketides, e.g. tetracycline and daunorubicin, are assembled mainly with acetate groups resulting in unreduced beta-keto groups during chain elongation. Folding facilitates aldol condensation resulting in 6-membered rings as chain elongation proceeds or completes. These rings are then reduced through dehydration to produce the final product (Katz, 1997). Aromatic polyketide synthesis is accomplished through Type II polyketide synthases (PKS). Type II PKS consists of discrete enzymes whose activities can be purified. Aromatic polyketide synthesis is

achieved through an acyl carrier protein that chaperones the assembling polyketide between enzymes (O'Hagan, 1992).

Complex polyketides, a more diverse group of compounds as compared to aromatic polyketides, can be formed by incorporation of activated acetate, propionate and butyrate units into the chain structure. Other differences include the inability of complex polyketides to undergo folding and aromatization due to structural constraints (methyl side chains), beta-carbonyl reduction, and synthesis chemistry. They instead cyclize through lactonization or remain as long acyl chains (Katz, 1997). Some examples of complex polyketides are erythromycin, avermectin, and rapamycin. Complex polyketide synthesis is carried out by Type I PKS. Type I PKS consists of a single, large multifunctional protein with individual activities located in catalytic domains of the protein. These Type I polyketide synthase genes are organized into modules. Each module codes for the catalytic domains responsible for incorporation of the correct building blocks into the chain and the successive reduction reactions (Cortes *et al.*, 1990; Donadio *et al.*, 1991).

2.2.1 Erythromycin

Erythromycin is an effective antibiotic used to treat infections caused by gram-positive bacteria (Staunton and Wilkinson, 1997). Erythromycin is also used to treat Legionnaire's disease and mycoplasma infections (Staunton and Wilkinson, 1997). Erythromycin was first isolated in 1952 from *Saccharopolyspora erythraea*, which was formerly *Streptomyces erythraeus* (McGuire *et al.*, 1952). It is a macrocyclic complex polyketide with a 14-membered ring and two attached sugars.

2.2.1.1 Erythromycin Biosynthesis

The biosynthesis of erythromycin consists of three parts. The first is the synthesis of the polyketide moiety, 6-deoxyerythronolide, which is formed my a type I polyketide synthase. The second is the synthesis of the two sugars, mycarose and desosamine. And, the third is the assembly and modifications to make the final erythromycin A product. Figure 1 shows the precursors and major reactions of erythromycin A biosynthesis.

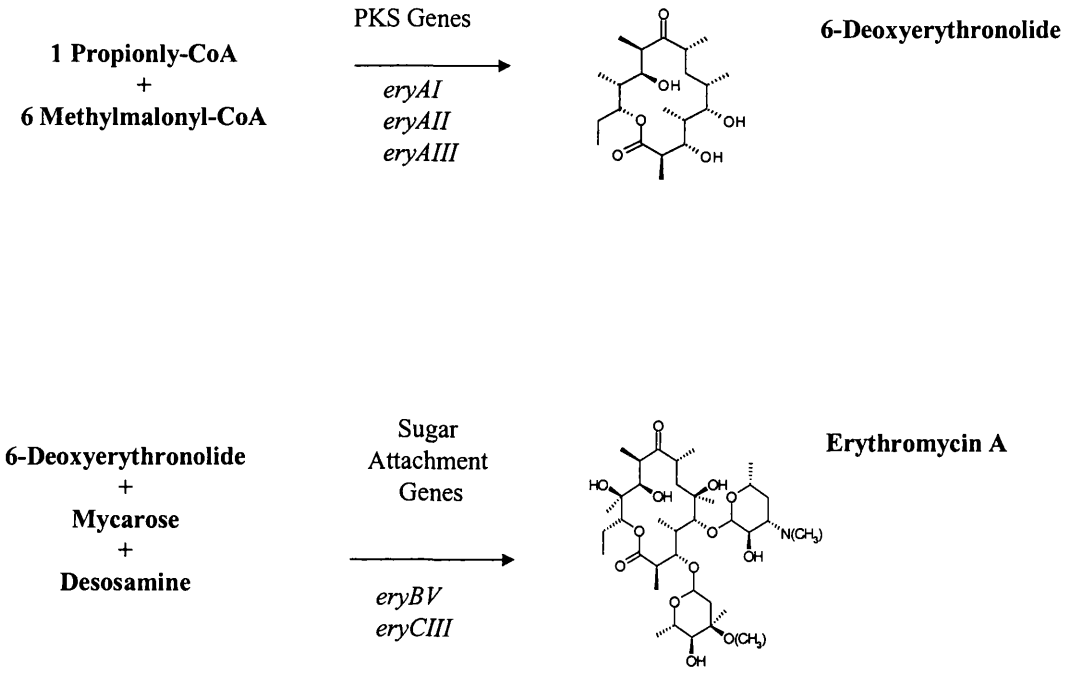


Figure 1: Schematic diagram of the major steps involved in erythromycin biosynthesis.

The first part of erythromycin synthesis is the formation of 6-deoxyerythronolide which is controlled by the erythromycin polyketide synthase. It has been shown that polyketide synthases consist of many catalytic domains where each domain recruits and incorporates the correct building block into the growing chain and controls the successive reduction reactions (Cortes *et al.*, 1990; Donadio *et al.*, 1991). Precursor feeding studies show that the carbon backbone of erythromycin is synthesized from one propionyl-CoA and six S-methylmalonyl-CoA precursors (Kaneda *et al.*, 1962; Martin and Rosenbrook, 1967; Cane *et al.*, 1981; Corcoran and Vygantas, 1982).

It is possible that both precursors to erythromycin can be produced from succinyl-CoA (Bermudez *et al.*, 1998). Bermudez *et al.* (1998) proposed the possible routes to erythromycin precursors as seen in Figure 2. Succinyl-CoA can be converted into R-methylmalonyl-CoA by methylmalonyl-CoA mutase (Hunaiti and Kolattukudy, 1984a), and then converted to the S-methylmalonyl-CoA form by methylmalonyl-

CoA epimerase. S-methylmalonyl-CoA can also be derived from valine catabolism (Tang *et al.*, 1994) and propionyl-CoA carboxylation (Hunaiti and Kolattukudy, 1982; Hunaiti and Kolattukudy, 1984b). However, it has been shown that *S. erythraea* does not require an active propionyl-CoA carboxylase to produce erythromycin (Donadio *et al.*, 1996). Methylmalonyl-CoA mutase and methylmalonyl-CoA decarboxylase are, therefore, most likely the important enzymes for erythromycin precursor synthesis (Bermudez *et al.*, 1998). This makes succinyl-CoA an important intermediate and potential regulatory point in erythromycin biosynthesis because of its effect on the pool sizes of methylmalonyl-CoA and propionyl-CoA. Malonyl-CoA decarboxylase also influences erythromycin biosynthesis, most likely by supplying propionyl-CoA (Hsieh and Kolattukudy, 1994).

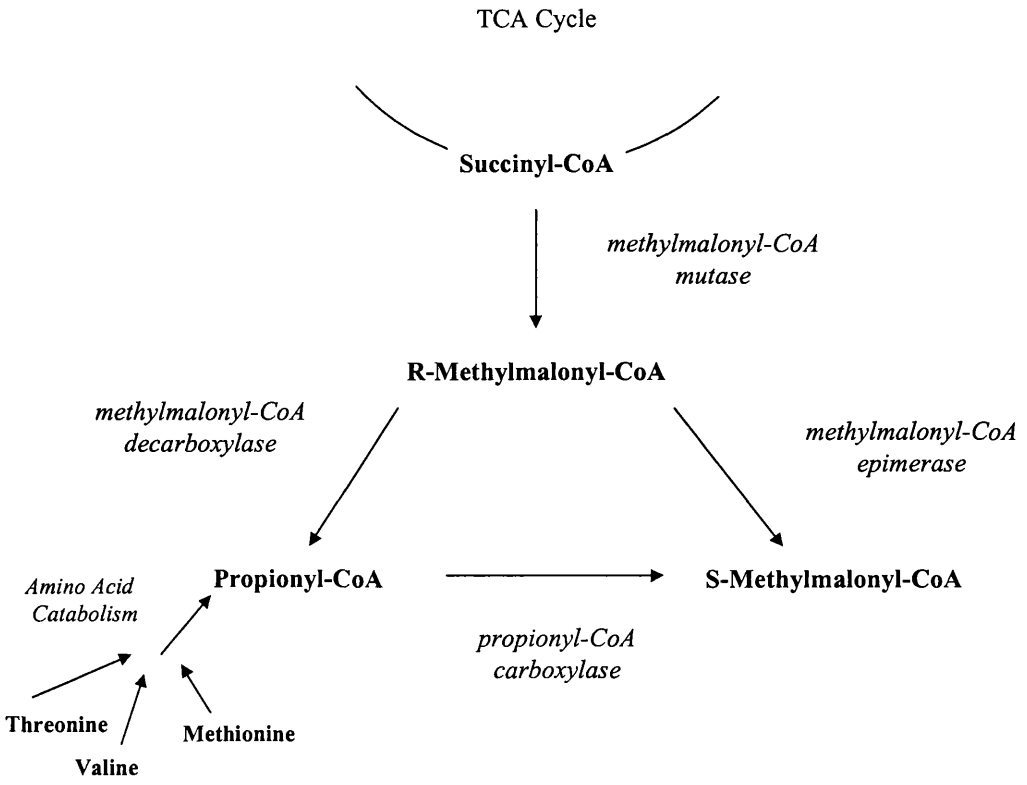


Figure 2: Metabolic pathway routes for erythromycin precursor supply.

The erythromycin gene cluster has been elucidated and includes the genes for three polyketide synthases designated as *eryAI*, *eryAII*, and *eryAIII* (Caffrey *et al.*, 1992; Leadlay *et al.*, 1993). The polypeptide gene product DEBS1, encoded by *eryAI*, controls the loading domain and synthesis of the first two elongation steps. DEBS2, encoded by *eryAII*, incorporates the next two components of chain elongation. And, DEBS3, encoded by *eryAIII*, controls the final two polyketide elongation steps of erythromycin biosynthesis and cyclization to 6-deoxyerythronolide. Figure 3, reproduced from (Staunton and Wilkinson, 1997), shows the gene and enzyme modular organization for synthesis of 6-deoxyerythronolide.

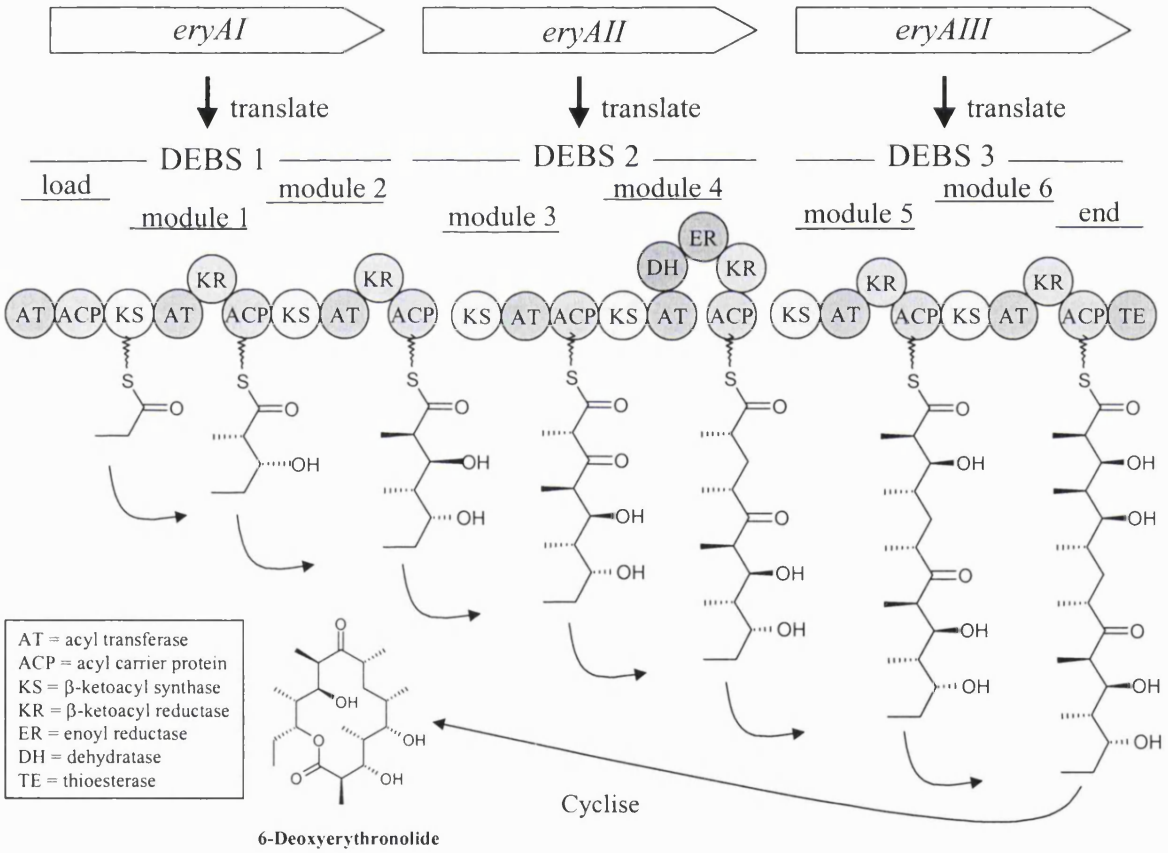


Figure 3: Erythromycin PKS gene and enzyme module organization.

6-Deoxyerythronolide synthesis is initiated when propionyl-CoA is attached to the DEBS 1 loading domain. Propionyl-CoA can be formed by several metabolic routes. Some of these pathways include amino acid catabolism of methionine, threonine or valine and rearrangement of succinyl-CoA as shown in Figure 2 (Tang *et al.*, 1994; Donadio *et al.*, 1996; Bermudez *et al.*, 1998). Six methylmalonyl-CoA units are then linked together by modules 1 through 6 of DEBS 1, 2, and 3. Methylmalonyl-CoA, the activated form of propionyl-CoA, can be produced from succinyl-CoA from the citric acid cycle via a mutase reaction (Hunaiti and Kolattukudy, 1982; Hunaiti and Kolattukudy, 1984a; Bermudez *et al.*, 1998). Methylmalonyl-CoA can also be produced from the catabolism of certain amino acids such as valine as shown in Figure 2 (Tang *et al.*, 1994).

The second part of erythromycin biosynthesis consists of the synthesis of two sugars, mycarose and desosamine. Mycarose and desosamine synthesis are governed by the *eryB* family and *eryC* family of genes, respectively (Gaisser *et al.*, 1997; Gaisser *et al.*, 1998; Salah-Bey *et al.*, 1998). Both sugars are synthesized through a series of reactions originating from glucose-1-phosphate.

The final part of erythromycin synthesis begins with hydroxylation of 6-deoxyerythronolide at the C-6 position to make erythronolide B (Hung *et al.*, 1965; Weber *et al.*, 1985). Mycarose and desosamine are then attached by glycosyltransferases to form erythromycin D (Martin *et al.*, 1966; Majer *et al.*, 1977; Weber *et al.*, 1990). Erythromycin D can be methylated or hydroxylated to form erythromycin B or erythromycin C, respectively. And finally, erythromycin B and erythromycin C are then methylated or hydroxylated, respectively, to form erythromycin A (Corcoran and Vygantas, 1977), as seen in Figure 4.

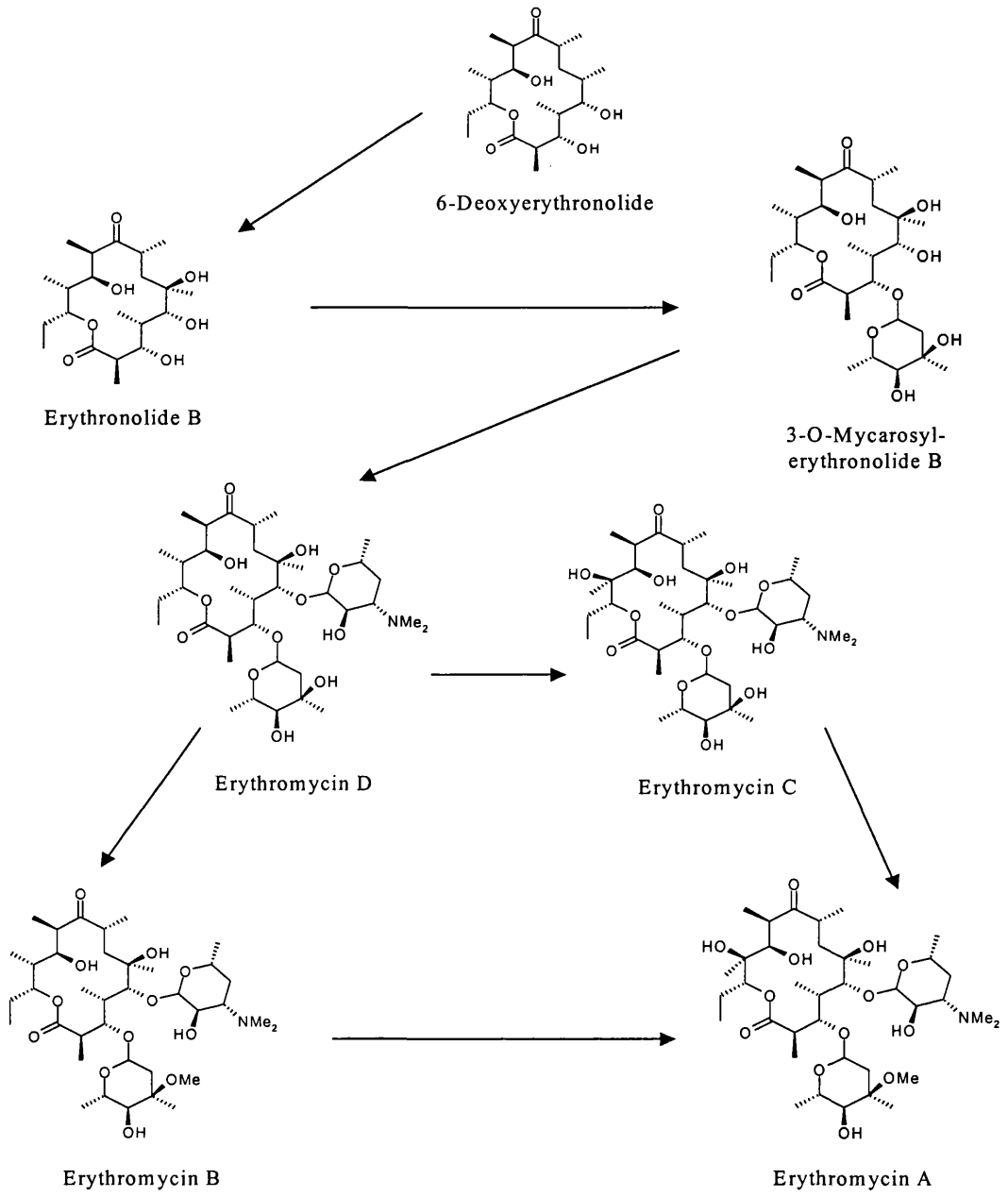


Figure 4: Biosynthesis of erythromycin A from 6-deoxyerythronolide.

2.3 Specific Aims of Thesis

The overall goal of the proposed research is to evaluate the effect of precursor supply on the production of polyketides by increasing the availability of its biosynthetic precursors using metabolic engineering methodology. Specifically, the over-flow metabolism of organic acids was studied to determine if carbon lost to organic acids (pyruvate and α-ketoglutarate) could be re-channeled to primary metabolism which in turn could increase erythromycin precursors. To this end, the physiology of *S. erythraea* was studied to determine factors influencing, growth, organic acid excretion, and product formation (Chapter 3). Activities of the TCA cycle enzymes pyruvate dehydrogenase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase were measured to determine their importance on organic acid excretion (Chapter 4). The flux to erythromycin precursors, through the network of primary metabolic pathways, was analyzed using a metabolic model of *S. erythraea*. The aim was to develop a rational procedure for optimizing *S. erythraea* for maximal production of these precursors (Chapter 5). A hypothesis was presented which proposed that increasing expression of the α-ketoglutarate dehydrogenase gene would decrease α-ketoglutarate excretion and increase erythromycin production (section 5.6.1). The hypothesis was tested by constructing a new *S. erythraea* strain with over-expressed α-ketoglutarate dehydrogenase (Chapter 6) and comparing the growth and product formation to that of the wild-type (Chapter 7). Characterization of a second polyketide (red pigment) produced by *S. erythraea* was also conducted (Chapter 8). Each chapter in this thesis describes the research conducted for each of the topics listed above and includes appropriate background information. Chapter 9 summarizes and discusses the work as a whole, draws conclusions, and suggests future work to explore further the results found this thesis research.

This research attempted to correlate the production of polyketides to the availability of primary metabolites, which was varied using a rational metabolic engineering approach. This thesis constitutes one full cycle of the metabolic engineering process. It included analysis (metabolic characterization of wild-type), design (proposed modifications to organism), synthesis (genetic modification to organism), and analysis (metabolic characterization of new strain) (Nielsen, 2001).

This research addressed the following four specific aims:

Aim 1. To study the physiology of *Saccharopolyspora erythraea* and use the data to develop a metabolic pathway model of *S. erythraea* that contains the metabolic pathways necessary for the synthesis of the erythromycin precursors.

Aim 2. To perform flux analysis to study the synthesis of the erythromycin precursor units using the model developed in Aim 1 so as to rationally establish target pathways for modification which will increase the flux of carbon and/or nitrogen substrates to the erythromycin biosynthetic precursors. Furthermore, to perform enzyme activity analysis to help validate the metabolic network.

Aim 3. To modify genetically the metabolic pathways of *Saccharopolyspora erythraea*, as determined in Aim 2, to increase production of the erythromycin precursors, using recombinant DNA techniques.

Aim 4. To determine experimentally how the genetic modifications of Aim 3 impact the physiology of the organism, including any changes in the production of erythromycin, and to use experimental results from the new *Saccharopolyspora erythraea* strains to validate the model developed in Aim 1.

Many complex polyketides are comprised of similar precursor units, therefore, if efficient erythromycin production is achieved though the efforts of the proposed research, the system could be applied to improve production of other polyketide products of interest. The proposed research will generate the tools necessary to design and produce microorganisms optimized to overproduce secondary metabolites. In addition, it will demonstrate how the rapid advances in molecular biology can be effectively applied to process engineering.

3 *Saccharopolyspora erythraea* Physiology Studies

3.1 Summary

This chapter discusses the physiological studies conducted with *Saccharopolyspora erythraea*. The *S. erythraea* wild-type red-variant was the primary strain used because of its ability to produce high levels of red pigment and because it is easier to genetically manipulate as discussed in Chapter 6. However, several other strains were studied including the white-variant wild-type, and genetically engineered strains with gene knock-outs in the erythromycin biosynthesis pathways (see section 2.2.1.1 for review). These organisms were grown under several nutrient conditions, including carbon, nitrogen, and phosphate limitation. The majority of the studies were performed under nitrogen-limited conditions as these exhibited the greatest differences between strains. The differences in physiology were also critical for the metabolic modelling portion of the thesis as described in Chapter 5.

The main focus of the physiological studies was to evaluate the effect of difference nutrient-limitations on growth and product formation. The primary metabolic parameters of interest were growth, substrate uptake, and organic acid excretion. The secondary metabolic parameters of interest were red pigment production and erythromycin production. Several studies were performed, however, four time course analyses comprise the majority of this chapter. The first study aimed to determine the effect of varying nitrate levels on the physiology of *S. erythraea* wild-type red-variant (wt RV). The second main study was a comparison between the *S. erythraea* wt RVand *S. erythraea* Δ*eryA* grown under carbon and nitrogen limitation. The third study was a comparison between several genetically engineered strains with gene knock-outs in the erythromycin biosynthesis pathway grown under nitrate-limited conditions. The fourth was a two part study to compare the *S. erythraea* wt RV to the *S. erythraea* wild-type white-variant (wt WV). Several other smaller studies considered the effect of the addition of potential precursors on secondary metabolite biosynthesis.

Particular attention of these studies was placed on the relationship between organic acid excretion and secondary metabolite production. It was found that nitrogen-limited growth conditions had the greatest influence over organic acid excretion and red pigment production. An explanation for this observation suggested that organic acid excretion may be caused by metabolic flux imbalance between glycolysis and the TCA cycle where nitrogen-limitation causes a reduction in the flux through the TCA cycle and as a result pyruvate and α-ketoglutarate are excreted from the cell as overflow metabolites. However, it could also be hypothesized that α-ketoglutarate is excreted because it is not needed for glutamate formation once nitrate is depleted.

3.2 Background

3.2.1 Streptomycete Physiology

Streptomycetes are filamentous organisms, which are responsible for the majority of commercial secondary metabolite production. Many secondary metabolites are of industrial importance for their antibiotic, anti-cancer, and anti-fungal properties. Streptomycetes can be grown on solid substrates or in liquid culture. In liquid culture, the organisms can be found as dispersed mycelia or compact pellets. Dispersed mycelia is considered to be a homogeneous suspension of branched hyphae with no diffusional limitations to the exchange of components in the medium. Pellets are believed to be formed when interactions occur between hyphae, solid particles and hyphae, and spores and hyphae and are believed to restrict exchange with media components and dissolved gas, such as oxygen (Prosser and Tough, 1991). It is desirable, in the work presented here, that a dispersed mycelia culture is produced to allow free exchange with media components.

3.2.1.1 Antibiotic Production by Streptomycetes

Streptomycetes produces a broad range of secondary metabolites. Although secondary metabolites are naturally produced, the quantities are typically low. Many years of research has been conducted to attempt to understand the mechanisms of secondary metabolite production and control. As shown here, it is recognized that secondary metabolite production is under strict control and there remains much to understand about the synthesis and control of these molecules.

Nutrient limitation is one of the most common factors associated with onset of secondary metabolism (Doull and Vining, 1990). Most experimental data support the theory that a inhibitor must be depleted before antibiotic synthesis can occur (Bushell, 1988). However, there is another theory suggesting that an activator must be synthesized before antibiotic synthesis can occur (Martin and Demain, 1980). Carbon catabolite repression has been observed with glucose as the carbon source for several antibiotic producing organisms (Martin and Demain, 1978). It has been shown that repression of antibiotic biosynthesis is caused by increased growth rate due to rapid uptake of carbon (Martin and Demain, 1978), and glucose may have a transient repressive effect on erythromycin synthesis (Escalante *et al.*, 1982). Nitrogen assimilation by Streptomycetes can occur by two mechanisms depending on ammonia availability. Ammonia-limited condition employs the high-affinity glutamine synthetase-glutamate oxoglutarate aminotransferase (GS-GOGAT) system. When ammonia is in excess, the glutamate dehydrogenase (GDH) system is utilized (Aharonowitz, 1980). It is possible that one or both of these systems are linked to secondary metabolite suppression (Shapiro and Vining, 1983). Phosphate appears to have a repressive effect on secondary metabolite production (Liras *et al.*, 1990). Varying levels of phosphate have been shown to inhibit antibiotic formation in several Actinomycetes (Lubbe *et al.*, 1985; Obregon *et al.*, 1994).

Classical secondary metabolite production is typically non-growth related where cultures are grown in batch fermentations and secondary metabolite production is initiated by a nutrient limitation. In this respect, there have been several reports on the relationship between growth rate and secondary metabolite production through the use of fed-batch and chemostat fermentation processes. It has been shown, using chemostat fermentation, that a slower growth rate increased clavulanic acid production (Ives and Bushell, 1997). Furthermore, it has also been reported that a slower growth rate induced production of rapamycin and erythromycin in *S. hygroscopicus* and *S. erythraea*, respectively, in batch and fed batch processes (Wilson and Bushell, 1995; Bushell *et al.*, 1997a). However, actinorhodin production by *S. coelicolor* peaked at the mid-range of dilution rates investigated in chemostat experiments (Melzoch *et al.*, 1997).

Media components have been studied to determine the best combination to produce a given product. In *S. hygroscopicus*, which produces rapamycin, over thirty carbon sources were studied and it was found that 2% sucrose and 0.5% mannose was the best combination to produce rapamycin. By contrast, acetate and propionate which are known to contribute to the carbon ring of rapamycin were not suitable for growth or rapamycin production (Kojima *et al.*, 1995). It appears that high phosphate levels are required for exclusive methylemomycin production by *S. coelicolor*, (Hobbs *et al.*, 1992), which is contrary to other reports suggesting that phosphate inhibits secondary metabolite formation (Lubbe *et al.*, 1985; Obregon *et al.*, 1994). Various studies have shown that the effect of ammonia may be important to understand because it appears to have a different effects in different systems. For example, ammonia inhibits growth in *S. clavuligerus* (Ives and Bushell, 1997), and inhibits erythromycin production by *S. erythraea* (Flores and Sanchez, 1985). In a separate study, it was reported that high levels of ammonium sulfate reduced erythromycin production while ammonium nitrate increased production (Potvin and Peringer, 1994a), which may be due to differences in uptake and utilization of the ammonia form.

Dissolved oxygen (DO) is another factor that appears to influence secondary metabolite production. High levels of dissolved oxygen are important for production of tylosin by *S. fradiae* (Chen and Wilde, 1991) as well as for vancomycin produced by *A. orientalis* (Clark *et al.*, 1995). However, it has been reported that erythromycin is produced under both oxygen limited (unbaffled shake-flask with %DO depleted to 0 during growth phase) and oxygen sufficient (baffled shake-flask with %DO not dropping below 78%) conditions (Clark *et al.*, 1995; Heydarian *et al.*, 1996).

3.2.1.2 Nitrogen Regulation of Secondary Metabolites in Streptomycetes

There are several reports on nitrogen regulation of secondary metabolite production which speculate that slowly metabolized nitrogen sources result in prevention of nitrogen catabolite repression of antibiotic biosynthesis (Drew and Demain, 1977; Martin and Demain, 1980). Streptomycin production by *S. griseus* is a classic example where proline, which is slowly metabolized, was determined as the best nitrogen source (Dulaney, 1948). Studies with *S. erythraea* producing erythromycin, showed that product titers were reduced as the ammonium concentration in the medium was increased (Flores and Sanchez, 1985), and that ammonium sulfate to a

greater extent reduced erythromycin production compared to ammonium nitrate (Potvin and Peringer, 1994a; Potvin and Peringer, 1994b). Ammonium has been shown to decrease production of other secondary metabolites, including β-lactam antibiotics produced by *S. clavuligerus* (Fang and Demain, 1995), actinorhodin by *S. coelicolor* A3(2) (Melzoch *et al.*, 1997), and rapamycin by *S. hygroscopicus* (Cheng *et al.*, 1995). However, subsequent studies with *S. hygroscopicus* showed that ammonium sulfate promoted rapamycin production (Lee *et al.*, 1997). The physiological level, i.e genetic or enzymatic, at which nitrogen is controlling metabolism has not been completely elucidated. In a study using *S. coelicolor*, it was found that the glutamine synthetase activity of a *glnE* (adenylyltransferase gene) mutant was not down-regulated compared to the wild-type after excess ammonium was added to the cultures (Fink *et al.*, 1999) suggesting that the control can be at the level of the enzyme. These studies emphasize the difficulty of establishing a set procedure for media optimization.

3.2.1.3 Organic Acid Excretion by Streptomycetes

Organic acids, namely pyruvate and α-ketoglutarate, are excreted from streptomycetes as overflow metabolites (Bormann and Herrmann, 1968; Grafe *et al.*, 1975; Ahmed *et al.*, 1984; Hobbs *et al.*, 1992; Madden *et al.*, 1996), similar to acetate excretion in *E. coli* (Holms, 1986; el Mansi and Holms, 1989; Luli and Strohl, 1990). Bormann and Herrmann (1968) found that pyruvate and α-ketoglutarate were excreted by *S. rimosus* in defined medium containing glucose and casamino acids. It was also found that carbonate increased the amount of acid excreted and cultures free of complex nitrogen sources reduced amounts of acids excreted. Increased amounts of ammonia decreased pyruvate excretion, however pyruvate excretion was observed in the presence of ammonia plus succinate. They concluded that re-consumed pyruvate was converted to α-ketoglutarate, thus maintaining a constant amount of excreted organic acids (Bormann and Herrmann, 1968). It was shown that production of secondary metabolites in *S. hygroscopicus* interfered with the accumulation of α-ketoglutarate. In *S. venezuelae,* decreased activity of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase resulted in increased amounts of excreted acids (Ahmed *et al.*, 1984). In *S. lividans*, it has been shown that the level of pyruvate and α-ketoglutarate excretion was dependent on the nitrogen source when glucose was the

carbon source (Madden *et al.*, 1996). Pyruvate and α-ketoglutarate production has also been observed in *S. coelicolor* (Hobbs *et al.*, 1992).

3.2.1.4 Pigments Produced by Streptomycetes

It has been well documented that *Streptomyces* spp. produce various pigmented secondary metabolites, such as rubrolone produced by *S. echinoruber* sp. nov. (Palleroni *et al.*, 1978), metacycloprodigiosin produced by *S. longisporus ruber* (Wasserman *et al.*, 1969), roseoflavin produced by *Streptomyces* strain No. 768 (Otani *et al.*, 1974), undecylprodigiosin and actinorhodin produced by *S. coelicolor* A3(2) (Rudd and Hopwood, 1980; Bermudez *et al.*, 1998), a melanin-like pigment produced by *S. aureofaciens* (Nakano *et al.*, 2000), and unidentified sporulation pigment by *S. venezuelae* S13 (Scribner *et al.*, 1973). These pigments are considered secondary metabolites and, in some cases, exhibit antimicrobial activity (Otani *et al.*, 1974; Gerber and Lechevalier, 1976; Rudd and Hopwood, 1980). In some *Streptomyces* strains, a mixture of pigments is produced in conjunction with the major pigment product, as determined by thin layer chromatography (Wasserman *et al.*, 1969; Palleroni *et al.*, 1978; Tsao *et al.*, 1985).

There have been a few reports on the physiological factors influencing pigment production in Streptomycetes. The nutritional requirement for pigment production was studied in *S. venezuelae* (Scribner *et al.*, 1973), for which higher amino acid concentrations and added $FeCl_3$ promoted pigment production. In *S. coelicolor* A3(2), the influence of phosphate and ammonium on the production of undecylprodigiosin and actinorhodin was examined (Hobbs *et al.*, 1990). Although undecylprodigiosin was not sensitive to either, actinorhodin was inhibited by ammonium and phosphate to varying degrees depending on the ammonium to phosphate ratio.

3.2.1.5 *Saccharopolyspora erythraea* Physiology

Literature on *Saccharopolyspora erythraea* physiology can be discussed in two main topics and both are presented below. The first topic is the effect of media components on growth and erythromycin production, and the second is the effect of fermentation parameters on growth and erythromycin production.

3.2.1.5.1 Effect of Media Components on Growth and Erythromycin Production

The differences in the physiology of *S. erythraea* when grown on complex or defined carbon and nitrogen sources was observed by Smith *et al.* in 1962. They found that when *S. erythraea* was grown on a complex nitrogen source (soybean oil) erythromycin biosynthesis exhibited a non-growth related secondary metabolite profile and production continued until the substrate was depleted. Addition of sucrose caused renewal of erythromycin production, however, if nitrogen was added with sucrose then production was not renewed. It was presumed that the processes of growth consumed the intermediates, energy, or cofactors that would otherwise be used for erythromycin production. Using a defined nitrogen source (glycine) erythromycin production followed a growth related profile. They observed limited growth which may have been due to the inability to fully use media components and, therefore, allowed for simultaneous erythromycin synthesis. Addition of sucrose and glycine caused renewal of both growth and erythromycin biosynthesis (Smith *et al.*, 1962). It was also suggested that pyruvate, generated internally from sucrose or glycine, was only available for erythromycin biosynthesis when it was not used for growth. The situations where pyruvate may not be needed for growth were nitrogen limitation or inhibitory concentration of erythromycin.

When *S. erythraea* was grown on glucose, a strong but temporary suppression of antibiotic formation was observed. During this antibiotic suppression phase, growth still occurred and the maximum erythromycin suppression occurred at 20 g/L glucose. Glucose only suppressed antibiotic formation when added before the production phase (Escalante *et al.*, 1982). Glucose repression of antibiotic production has also been observed in other Actinomycetes (Martin and Demain, 1978).

The effect of ammonium on erythromycin production was studied and it was found that total erythromycin inhibition was obtained with 100 mM ammonium chloride. Ammonium nitrate and ammonium sulfate also repressed erythromycin formation, however, organic nitrogen sources (glycine, leucine, glutamine) did not reduce erythromycin production. Addition of ammonium after the onset of erythromycin synthesis inhibited production after a 6 hour delay (Flores and Sanchez, 1985). In a separate study, erythromycin production was higher when grown with ammonium nitrate than ammonium sulfate (Potvin and Peringer, 1994a). Erythromycin

production was growth related with ammonium sulfate and growth dissociated with ammonium nitrate. A hypothesis was proposed suggesting that glutamate produced from ammonium could be converted to glutamine via nitrate induced glutamine synthetase where glutamine could then be used as an erythromycin precursor (Potvin and Peringer, 1994b).

Erythromycin A production increased with growth rate in phosphate-limited defined medium chemostat culture. Erythronolide B, 3-α-L-mycarosyl-erythronolide B, erythromycin D, and erythromycin B+C followed this same relationship. The relative level of each compound decreased thorough the progression of the synthesis reactions (see section 2.2.1.1), where erythronolide B had the greatest amount (~7 μmoles/gDCW/h), then 3-α-L-mycarosyl-erythronolide B (~5.5 μmoles/gDCW/h), erythromycin D and B+C (~3 μmoles/gDCW/h), and finally erythromycin A (~2 μmoles/gDCW/h) (Trilli *et al.*, 1987).

In different nutrient-limiting media it was observed that erythromycin production was growth related with nitrogen-limited medium and followed a classical secondary metabolite or growth dissociated pattern with carbon and phosphate-limited media (McDermott *et al.*, 1993). In a separate study, the onset of antibiotic synthesis was coordinated with the minimal protein synthesis rate and minimum ratio of charged to uncharged tRNA. Antibiotic synthesis and increased uncharged tRNA are generally considered stringent responses and it was proposed that there may be some connection between the rate of protein translation and antibiotic synthesis (Wilson and Bushell, 1995). Bushell *et al.* (1997) extended these observations of nutrient limitation and proposed a model suggesting that glucose is a high affinity limitation which means that uptake rates are high until substrate is exhausted. At this point the supply of ATP needed for amino acid activation becomes limiting causing the protein synthesis rate to decrease. For nitrate, which is considered a low affinity limitation, uptake rates are decreased early in growth cycle. The supply of amino acids becomes limiting and the protein synthesis rate decreases earlier in the growth cycle compared to glucose limited cultures. Once the protein synthesis rate decreases, the uncharged tRNA accumulates and binds to the ribosome. Guanosine tetraphosphate (ppGpp), which is

produced upon the reduction of the charged/uncharged tRNA ratio, may then initiate a cascade that results in antibiotic production (Bushell *et al.*, 1997a).

3.2.1.5.2 Effect of Fermentation Parameters on Erythromycin Production and Morphology

Oxygen supply is an important parameter in fermentation. It was found that erythromycin was produced in oxygen limited and oxygen sufficient cultures. The maximum biomass and erythromycin production were the same, although, growth rates and erythromycin production rates were reduced in the oxygen limited cultures (Clark *et al.*, 1995). In a separate study, the maximum biomass and erythromycin production were somewhat decreased when DOT was reduced from 65% to 10%. When the agitation rate was increased from 500 RPM to 750 RPM, the biomass and erythromycin production was further reduced presumably due to mycelial breakage (Heydarian *et al.*, 1996). In another study, it was shown that the amount of initial rapeseed oil did not affect the growth rate or specific erythromycin production in a 2 L fermentation volume. However, at a 7 L fermentation volume, the growth was not affected but the specific erythromycin production increased with increased oil concentration. It is believed that the oxygen limitation at the 2 L scale may have been masking the effect of the varying oil concentrations and that the 7 L fermentation shows the actual effect of increased oil concentration (Mirjalili *et al.*, 1999).

Erythromycin production was greater using an oil based medium compared to a soluble complex medium but required longer fermentation times and was more difficult to recover with membrane filtration technology. Overall, the increased erythromycin from the oil based medium in the feed stream to filtration did not benefit erythromycin recovery (Davies *et al.*, 2000). Erythromycin production was increased using a cyclic fed batch culture. Cyclic fed batch culture produced higher levels of erythromycin than chemostat culture. This was likely due to the high dilution rates typically used in chemostat cultures which do not simulate the low growth rates typical of erythromycin production (Lynch and Bushell, 1995).

There has been several studies on the hyphal strength of *S. erythraea* and the hyphal size at which erythromycin is produced. It was found that hyphal fragments greater than 88 micron exhibit erythromycin production (Martin and Bushell, 1996). This finding was consistent for stirred flasks and for baffled or unbaffled shaken flasks

(Bushell *et al.*, 1997b). *S. erythraea* mutants which showed decreased branching frequency also had increased hyphal strength. This increased hyphal strength increased the number of particles with diameters greater than 88 micron and hence increased erythromycin production (Wardell *et al.*, 2002). The strength of hyphae were found to be about 60% greater during growth phase compared to stationary phase. This is thought to be due to a thinner cell wall during stationary phase as the tensile strength was the same for both phases (Stocks and Thomas, 2001).

3.2.1.5.3 Red Pigments Produced by *Saccharopolyspora erythraea*

Saccharopolyspora erythraea produces the commercially important macrolide antibiotic, erythromycin A, in addition to an unidentified red pigment from which the organism obtained it name (Labeda, 1987). Hsieh and Kolattukudy (1994) investigated the effect of malonyl-CoA decarboxylase disruption on erythromycin production. They observed that the mutant strain lost the capacity to produce erythromycin and a red pigment, while the *S. erythraea* CA 340 parent strain produced erythromycin and 'became red as the culture grew dense'. They also reported that upon addition of exogenous propionate to the growth medium, the mutant was able to re-establish erythromycin production but was not able to restore red pigment production. Clark *et. al.* (1995) examined the effect of controlled oxygen limitation on secondary metabolite formation and observed that *S. erythraea* wt NRRL 2338 produced an 'unidentified soluble red pigment' under oxygen-limited conditions.

Recently, the genes involved in the production of a red pigment produced by *S. erythraea* wt RV have been identified and characterized (Cortes *et al.*, 2002). Their work also shows that a variety of pigments derived from tetrahydroxynaphthalene are produced. Consistent with these results, Udea *et. al.* (1995) identified a water-soluble red-pigment produced by *S. griseus* and determined that the 2 genes *rppA* and *rppB* were necessary for pigment production (Ueda *et al.*, 1995). Funa *et. al.* (1999) identified the red-pigment product of *rppA* produced by *S. griseus* as 1, 3, 6, 8-tetrahydroxynaphthalene (THN). They proposed that a chalcone synthase-like enzyme incorporates five malonyl-CoA units into a pentaketide before cyclizing to THN, which can be oxidized to form flaviolin. Flavolin can form various pigmented compounds which was evident when *rppA* was subcloned into *E. coli* a mixture of

pigmented compounds was produced (Funa *et al.*, 1999). Even though we have not yet structurally characterized the pigments discussed in this report, it is possible that they are similar or identical to the compounds reported above derived from THN. However, very recently in a poster presentation, Weber and colleagues (2002) found that one of the red pigments produced by S. *erythraea* was pyomelanin, a shunt product of tyrosine metabolism. This result presents a new possible metabolic route to red pigment biosynthesis to compare to the THN hypothesis.

3.3 Materials and Methods

3.3.1 *Saccharopolyspora erythraea* Strains

Six strains of *S. erythraea* were obtained from Prof. P. F. Leadlay (Cambridge University, Cambridge, UK). The strains consisted of two wild-type strains, *S. erythraea* red-variant and *S. erythraea* white-variant, and four deletion mutants. The mutations were made in the polyketide synthase genes (Δ*eryA* genes), in the genes governing the sugar attachment steps (Δ*eryBV* and Δ*eryCIII* genes), or in a combination (Gaisser *et al.*, 1998). Table 1 shows the name and description of the strains used in this thesis.

Table 1: *S. erythraea* strains used for physiological studies.

Literature Name	Name Used in Thesis and Description
NRRL 2338	*S. erythraea* wild-type red-variant (wt RV)
NRRL 2338	*S. erythraea* wild-type white-variant (wt WV)
JC2/Del60	*S. erythraea* Δ*eryA*
SGT2	*S. erythraea* Δ*eryA*Δ*eryBV*Δ*eryCIII*
DM	*S. erythraea* Δ*eryBV*Δ*eryCIII*
Δorf14	*S. erythraea* Δ*eryBV*

Liquid and spore stocks were generated for each strain. All manipulations were performed in a biosafety cabinet. The liquid stocks were prepared by inoculating Oxoid nutrient broth with a scrape of bacterial cells from an agar plate (made with Oxoid nutrient agar throughout). The liquid culture was allowed to grow in an orbital shaker for 48 hours at 28°C. After 48 hours, the culture was centrifuged and the

supernatant was removed. One volume of concentrated culture was combined with one volume of 50% glycerol in water. The mixture was then aliquoted into 1 mL volumes and stored at –80°C.

Spore stocks were prepared by streaking liquid culture onto agar plates. The plates were incubated at 28°C for about 3 weeks. Five mL of 20% glycerol/0.1% tween in water was added to plates. The spores were scraped from the plate and collected into a tube. The suspension was aliquoted into 1 mL volumes and stored at –80°C.

3.3.2 Shake Flask Experiments

3.3.2.1 Media

The shake flask experiments were performed with defined minimal media as described previously (McDermott *et al.*, 1993). Table 2 lists the components and concentrations used for carbon-limited, nitrogen-limited, and phosphate-limited media used for the shake-flask experiments described in this chapter. The media was prepared in different parts to avoid degradation of nutrient sources. The phosphate and nitrate salts and MOPS were prepared and autoclaved together. The glucose solution was prepared and autoclaved separately from the other components. The trace elements were prepared at a 100-fold concentration and sterile filtered. Prior to inoculation, the salts, glucose and trace elements were aseptically combined to the correct concentrations.

Table 2: Media composition for shake-flask experiments.

Component	Carbon-Limited Concentration, g/L (mM)	Nitrogen-Limited Concentration, g/L (mM)	Phosphate-Limited Concentration, g/L (mM)
Glucose	15 (83)	30 (167)	30 (167)
K_2HPO_4	7 (40)	7 (40)	0.1 (0.6)
KH_2PO_4	3 (22)	3 (22)	0 (0)
$NaNO_3$	11 (130)	2.4 (28)	11 (130)
MOPS	21	21	21
TRACE ELEMENTS			
$MgSO_4 \cdot 7H_2O$	0.25	0.25	0.25
$FeSO_4 \cdot 7H_2O$	0.025	0.025	0.025
$CuCl_2$	0.00053	0.00053	0.00053
$CoCl_2$	0.00055	0.00055	0.00055
$CaCl_2 \cdot 2H_2O$	0.0138	0.0138	0.0138
$ZuCl_2$	0.0104	0.0104	0.0104
$MnCl_2$	0.0062	0.0062	0.0062
$NaMoO_4$	0.0003	0.0003	0.0003

Nitrate was used as the nitrogen source over ammonia because it has been shown that ammonia suppresses erythromycin production (Flores and Sanchez, 1985; Potvin and Peringer, 1994a).

3.3.2.2 Growth Conditions

Each shake-flask experiment was prepared in the same manner. One 1 mL liquid stock was used to inoculate 50 mL of nutrient broth autoclaved in a 500 mL baffled flask. The nutrient broth culture was allowed to incubate at 28°C for 48 hours in a rotary shaker. Five mL of the nutrient broth was used to inoculate 45 mL of defined medium autoclaved in a 500 mL baffled flask. The culture was allowed to incubate at 28°C for 48 hours in a rotary shaker. Forty mL of the culture was then used to inoculate 360 mL of defined medium autoclaved in a 2 L baffled flask. The culture was allowed to incubate at 28°C for 96-144 hours in a rotary shaker. Forty mL samples were aseptically removed from the 2 L flask at various time points which are

indicated for each experiment in the appropriate section. The pH and biomass of each sample was determined, the sample was immediately centrifuged, and the supernatant was removed from the solids and stored at -20°C until further analysis was required.

3.3.3 Analytical Methods

3.3.3.1 Dry Cell Weight

Biomass was measured using a Mettler Toledo halogen moisture analyzer (model #HG53) as previously described (Davies *et al.*, 2000). Pre-dried Whatman GF/F filters were weighed using an analytical balance. Ten mL of culture was filtered onto the pre-dried filter. The filter plus cells was then dried at 95 °C until a constant weight was achieved. The filter plus cells were weighed on an analytical balance and the DCW was determined by the difference of the weight of the cells plus filter and weight of the filter alone.

3.3.3.2 Glucose

Glucose in the culture supernatants was measured using a previously described method (Dubois *et al.*, 1956). One-half mL of sample or standard was mixed with one-half volume assay reagent (16 g/L sodium hydroxide, 10 g/L dinitro-salicylic acid and 300 g/L sodium potassium tartrate prepared in dH_20). The mixture was incubated at 100°C for 5 minutes, cooled on ice, and quenched with 4 mL of water. The A_{540} of each sample or standard was then measured. Figure 5 shows the typical standard curve range for the glucose assay.

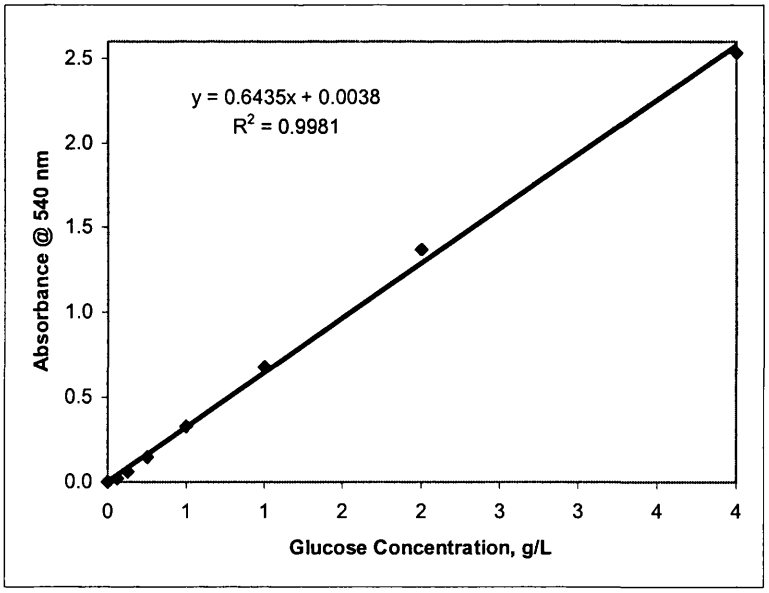


Figure 5: Glucose assay standard curve.

3.3.3.3 Nitrate

Nitrate in the culture supernatants was measured using a previously described method (Cataldo, 1975). Forty μL of sample or standard was mixed with 160 μL salicylic acid (5% w/v in concentrated sulfuric acid). The mixture was incubated at room temperature for 20 minutes, cooled in a water bath, and quenched with 3.8 mL sodium hydroxide (2M). The A_{410} of each sample or standard was then measured. Figure 6 shows the typical standard curve range for the nitrate assay.

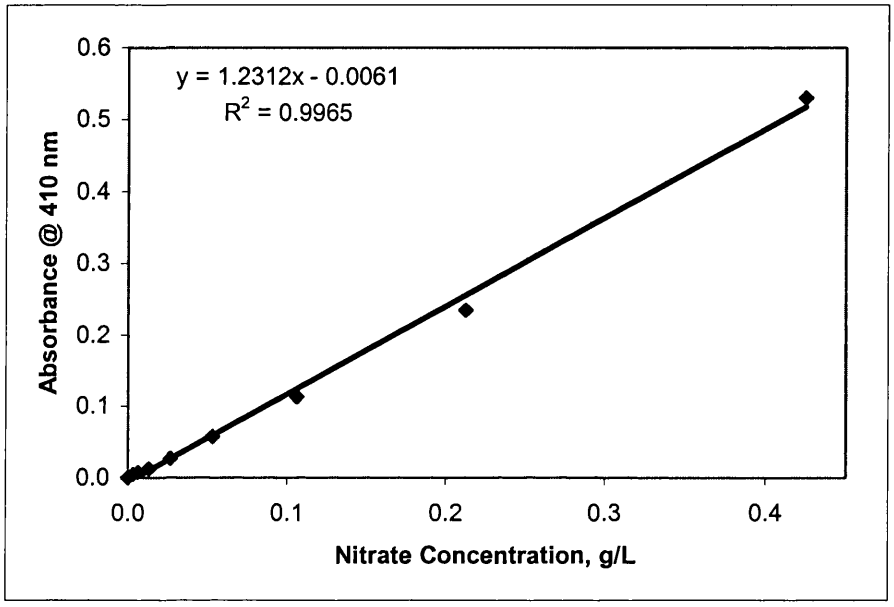


Figure 6: Nitrate assay standard curve.

3.3.3.4 Organic Acid

Organic acids in the culture supernatants were measured by HPLC. Twenty μL of filtered sample or standard was analyzed on an Aminex HPX-87H organic acids column with 5 mM sulfuric acid at a flow rate of 0.6 mL/minute and a column temperature of 50 °C. The organic acids were detected at A_{210} using a UV detector. Figure 7 and Figure 8 show the typical standard curve range for α-ketoglutarate and pyruvate assays, respectively.

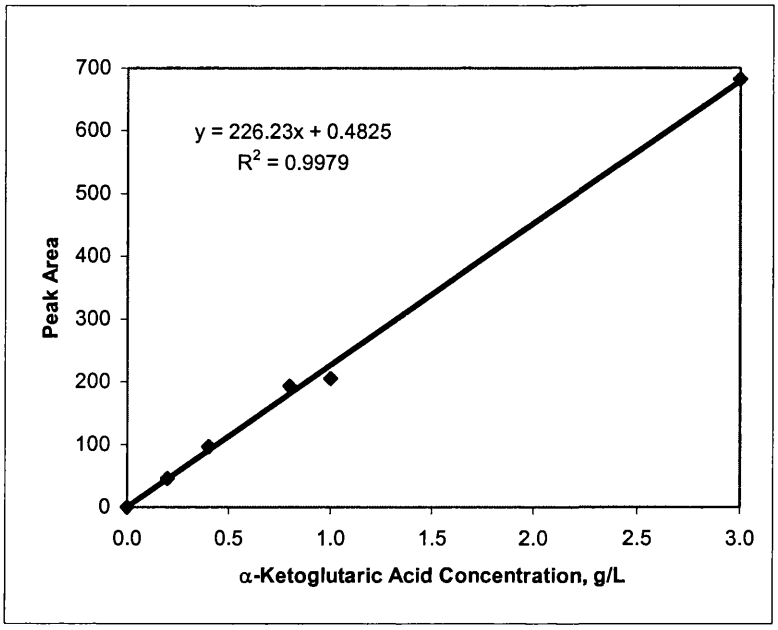


Figure 7: α-Ketoglutarate HPLC assay standard curve.

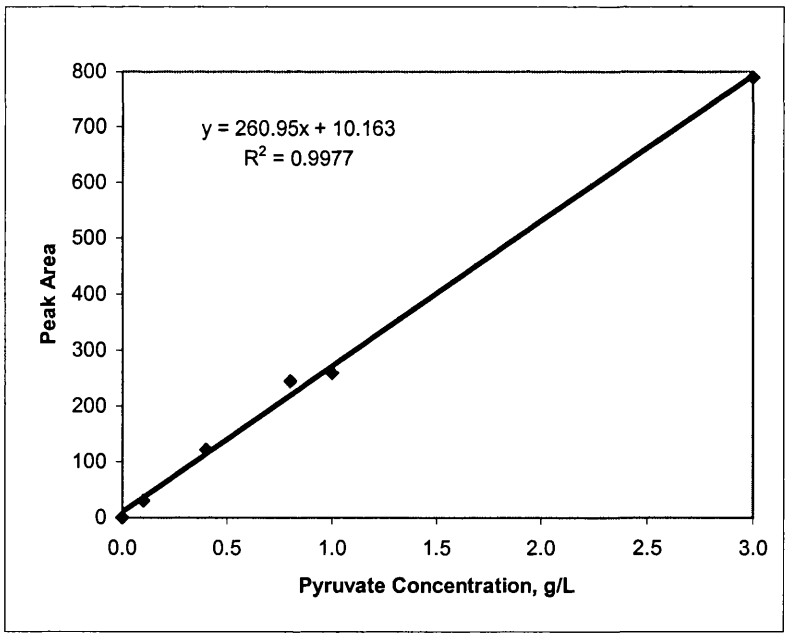


Figure 8: Pyruvate HPLC assay standard curve.

3.3.3.5 Red Pigment

The red pigment was analyzed by absorbance at 486 nm. The A_{486} was determined for filtered culture supernatants. The concentration of pigment was determined from the equation: $A_{486} = 1.0681$ * [pigment, g/L]. The linear range extends from $A_{486} = 0.05$ to $A_{486} = 2.80$. The absorbance/concentration relationship was determined using the isolated pigment described in section 8.3.1.1.

3.3.3.6 Erythromycin

The erythromycin assay consisted of two procedures. First the samples were concentrated five to ten fold using solid phase extraction. Second the concentrated samples were analyzed by HPLC. Experiments were performed to assure that the red pigment did not interfere with erythromycin quantification in these methods.

3.3.3.6.1 Solid Phase Extraction

Prior to HPLC analysis the samples were concentrated using a Phenomenex C18 bond elute solid phase extraction cartridge. The erythromycin extraction was performed by conditioning the cartridge with 5 mL diethylamine (5% v/v in methanol), followed by priming the cartridge with 5 mL sodium phosphate (10 mM, pH 7). Next, 10 mL of filtered culture supernatant was added to the cartridge and rinsed with 5 ml sodium phosphate (10 mM, pH 7). The erythromycin was eluted with 1 mL of methanol, filtered, and stored at –20 °C until analyzed on HPLC.

3.3.3.6.2 HPLC

Erythromycin in the culture supernatants was measured using a previously described method (Heydarian *et al.*, 1998). Erythromycin quantitation was performed by HPLC using a Phenomenex LUNA 3μ phenyl-hexyl column. The samples and standards were analyzed using acetonitrile and 10 mM KH_2PO_4 (pH 7) at a 45:55 ratio at 1.0 mL/minute flow rate, and a column temperature of 70 °C. Erythromycin was detected at A_{215} using a UV detector. Figure 9 shows the typical standard curve range for the erythromycin assay.

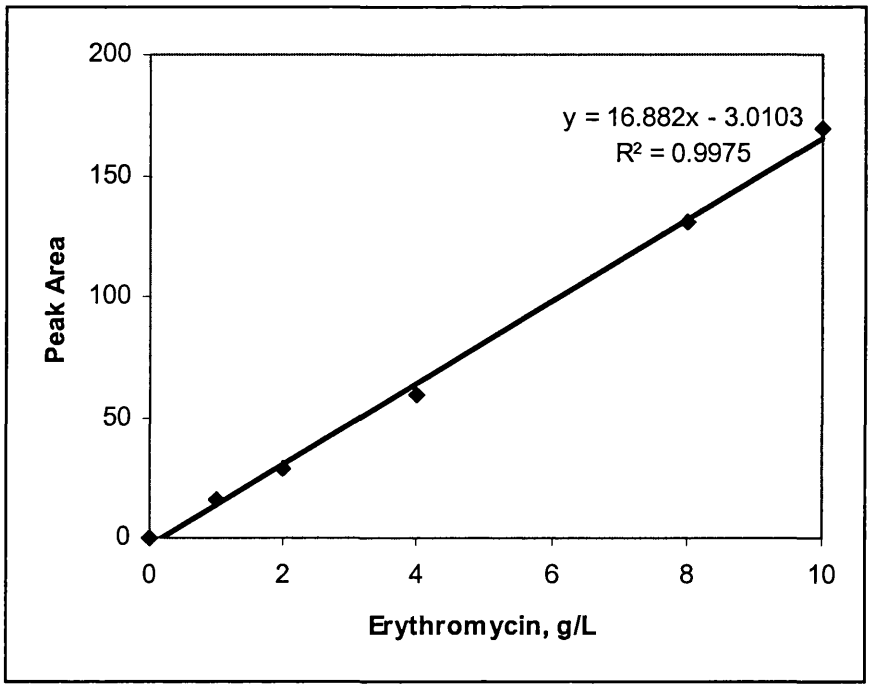


Figure 9: Erythromycin HPLC assay standard curve.

3.3.3.6.3 Assay Selectivity of Erythromycin versus Red Pigment

The published assay procedure for the erythromycin HPLC assay does not evaluate samples with red pigment present (Heydarian *et al.*, 1998). As the majority of the samples tested in this thesis contained erythromycin as well as red pigments, experiments were performed to assure that the red pigment did not interfere with the existing assay procedure.

S. erythraea wild-type red-variant (wt RV) supernatant samples with no pigment (time = 0) and samples with high levels of pigment (~1 g/L, time = 96h) were either spiked or not spiked with erythromycin. The samples were then split and one half was concentrated 9-fold using the bond-elut procedure as described in section 3.3.3.6.1. Several controls were also evaluated. The test samples and control samples are listed in Table 3.

Table 3: Experimental samples to test specificity of erythromycin assay.

Sample #	Sample Description	Red Pigment	Erythromycin Spike	Concentrated by bond-elut
1	*S. erythraea* wt RV time = 0 h	no	no	no
2	*S. erythraea* wt RV time = 0 h	no	1 g/L	no
3	*S. erythraea* wt RV time = 96 h	yes	no	no
4	*S. erythraea* wt RV time = 96 h	yes	1 g/L	no
5	*S. erythraea* wt RV time = 0 h	no	no	yes
6	*S. erythraea* wt RV time = 0 h	no	1 g/L	yes
7	*S. erythraea* wt RV time = 96 h	yes	no	yes
8	*S. erythraea* wt RV time = 96 h	yes	1 g/L	yes
9	dH_2O	no	1 g/L	no
10	Methanol	no	9 g/L	no
11	dH_2O	no	no	no
12	Methanol	no	no	no

After sample preparation and concentration (where appropriate), each of the twelve samples were run on the erythromycin HPLC assay as described in section 3.3.3.6.2 above. The erythromycin concentration of each sample are shown in Table 4.

Table 4: Erythromycin concentrations obtained to confirm specificity of erythromycin assay.

Sample #	Red Pigment	Erythromycin Spike, g/L	Concentrated by bond-elut	Erythromycin Concentration, g/L
1	no	no	no	0
2	no	1	no	1.0
3	yes	no	no	0
4	yes	1	no	0.9
5	no	no	yes	0
6	no	1	yes	8.0
7	yes	no	yes	0
8	yes	1	yes	9.8
9	no	1	no	0.9
10	no	9	no	14.2
11	no	no	no	0
12	no	no	no	0

The results show that all samples spiked with 1 g/L or 9 g/L erythromycin showed acceptable erythromycin recovery regardless of the presence of red pigment. The conclusion of this study was that the red pigment was not interfering with the concentration step of the erythromycin assay.

3.4 Physiological Effect of Initial Nitrate Levels in Media

3.4.1 Objective

The objective of this study was to determine the effect of initial nitrate levels in the medium on organic acid and secondary metabolite (red pigment) production. The nitrate levels spanned the medium range from nitrogen-limited to carbon-limited.

3.4.2 Experimental Conditions

The experimental conditions for this study are shown in Table 5.

Table 5: Experimental conditions for testing physiological effect of initial nitrate levels in media on *S. erythraea* wt RV.

Strains	*S. erythraea* wt RV (see section 3.3.1)
Media	Nitrate-limited medium (see section 3.3.2.1) with the initial nitrate levels varied from 1.89 g/L to 9.44 g/L to obtain carbon to nitrogen ratios (C/N) of 45, 36, 27, 18, and 9, respectively, with the initial glucose concentration remaining 30 g/L.
Growth Mode	Batch 2L Shake-Flask
Temperature	28° C
Shaker RPM	200
Sampling Times, hours	0, 16, 21, 26, 40, 50, 69, 93, and 118.
Experimental Duplication	Each strain grown with each medium was tested independently two times.

3.4.3 Results and Discussion

3.4.3.1 Growth and Substrate Uptake

The effect of nitrate on red pigment production was studied in shake-flask cultures over 118 hours with the range of nitrate concentrations covering both nitrogen-limited and carbon-limited regimes. At carbon to nitrogen ratios (C/N) of 45, 36, and 27, the

cultures were nitrogen-limited. At a C/N of 9, the cultures were carbon-limited. At a C/N of 18, carbon and nitrogen were limiting as nitrogen was depleted by 50 hours and carbon by 70 hours. The dry cell weight, substrate uptake, and pigment production for cultures with C/N of 45, 27, and 9, are shown in Figure 10, Figure 11, and Figure 12 respectively. The maximum dry cell weight, maximum specific growth rate, maximum specific substrate uptake rates, and limiting substrate for all C/N studied are shown in Table 6. As the C/N increases, the maximum dry cell weight, maximum specific growth rate, and maximum specific nitrate uptake rate decrease. However, the maximum specific glucose uptake rate (Table 6) and the maximum specific pigment production (Table 7) increase as the C/N increases.

Table 6: Specific growth and substrate uptake rates from physiological studies with *S. erythraea* wt RV using different C/N ratios.

C/N	Maximum Dry Cell Weight (g/L)	μ_{max} (h^{-1})	Max. Specific Glucose Uptake Rate (g/g·h)	Max. Specific Nitrate Uptake Rate (g/g·h)	Limiting Substrate
45	4.06	0.029	0.082	0.013	Nitrogen (N)
36	4.73	0.037	0.062	0.019	Nitrogen
27	5.45	0.042	0.058	0.022	Nitrogen
18	7.79	0.046	0.050	0.023	N then C
9	10.00	0.049	0.049	0.022	Carbon (C)

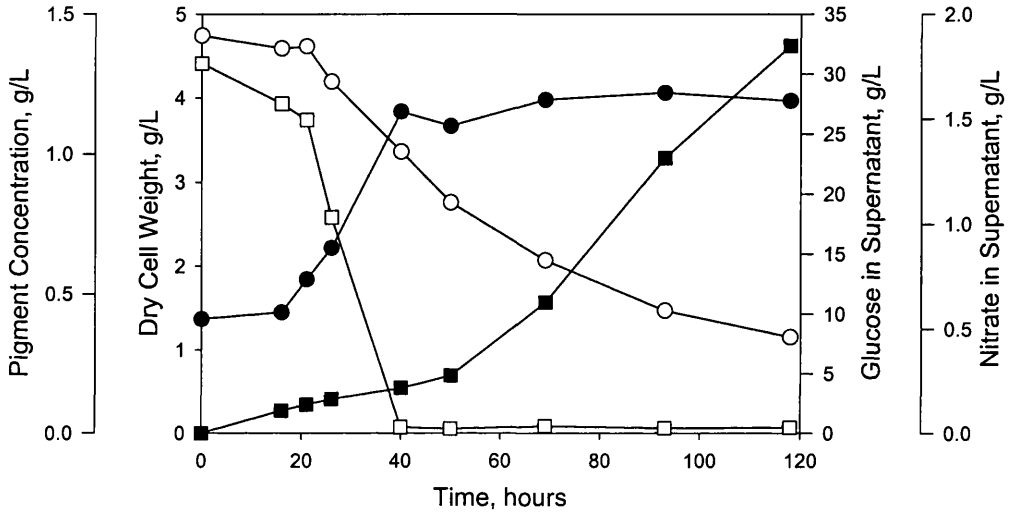


Figure 10: Growth and product kinetics of *S. erythraea* wt RV nitrate-limited batch culture with C/N = 45.

(●) Dry cell weight concentration, (○) Glucose in supernatant, (□) Nitrate in supernatant, and (■) Pigment concentration.

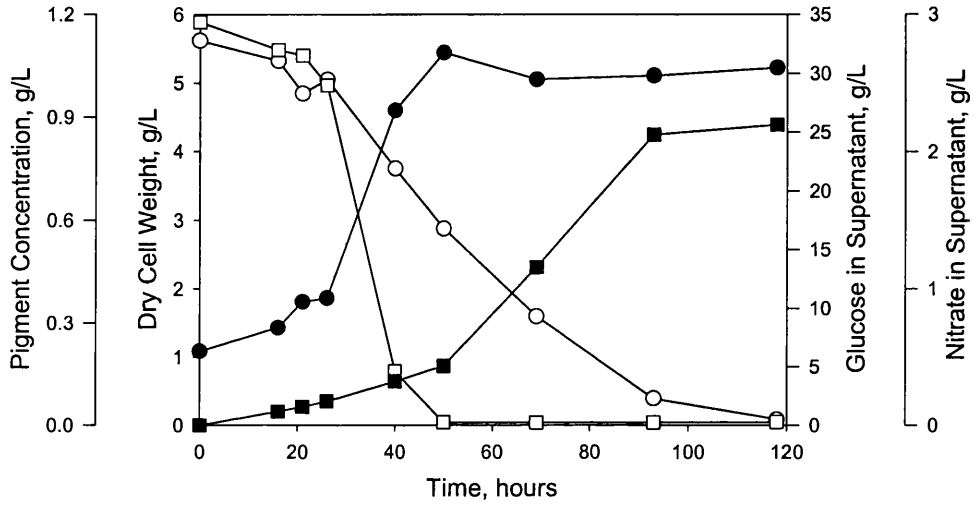


Figure 11: Growth and product kinetics of *S. erythraea* wt RV nitrate-limited batch culture with C/N = 27.

(●) Dry cell weight concentration, (○) Glucose in supernatant, (□) Nitrate in supernatant, and (■) Pigment concentration.

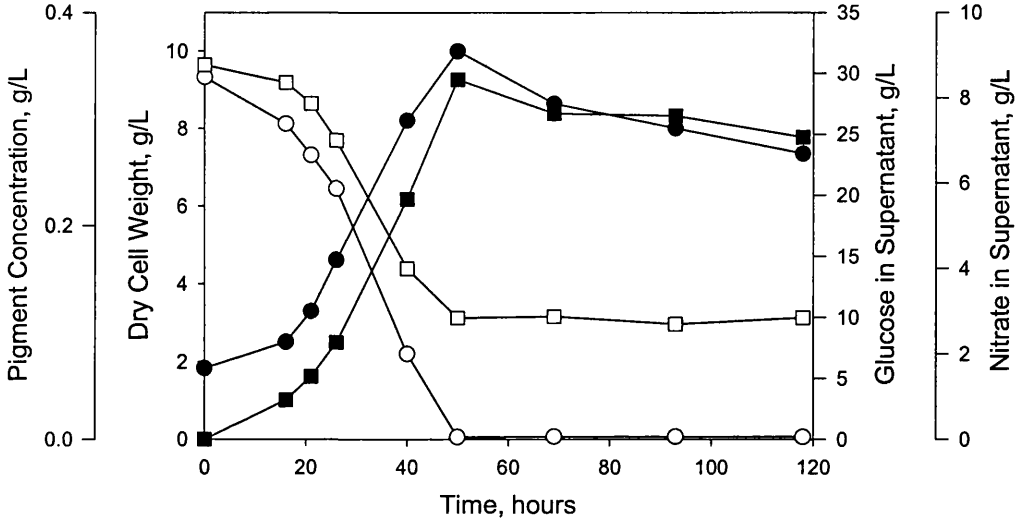


Figure 12: Growth and product kinetics of *S. erythraea* wt RV nitrate-limited batch culture with C/N = 9.

(●) Dry cell weight concentration, (○) Glucose in supernatant, (□) Nitrate in supernatant, and (■) Pigment concentration.

3.4.3.2 Red Pigment Production

Specific red pigment production for each C/N is shown in Figure 13. Under nitrogen-limited conditions, the red pigment was not growth related and was produced in a manner typical for secondary metabolite production as seen in Figure 10. Pigment production slowly increased until nitrate was depleted at which time production was rapidly increased. For cultures with C/N of 36 and 45, which are nitrogen-limited, pigment production steadily increased until the end of the experiment (118 hours). For the culture with C/N of 27, which is nitrogen limited, the pigment concentration increased until 93 hours at which time pigment production remained constant. Cessation of pigment production appears to be due to insufficient available carbon, as the glucose level drops below ~2.5 g/L at 93 hours, as seen in Figure 11. Under carbon-limited conditions (C/N = 9), the pigment production was growth-associated

as seen in Figure 12. At a C/N of 18, which was limited first in nitrogen and then in carbon, the red pigment was also growth-associated. These results indicate a minimum requirement of carbon availability for pigment production. Pigment production was discontinued when the glucose concentration was depleted to approximately 2.5 g/L suggesting that insufficient carbon may be the first step in a more complicated metabolic switch controlling pigment production.

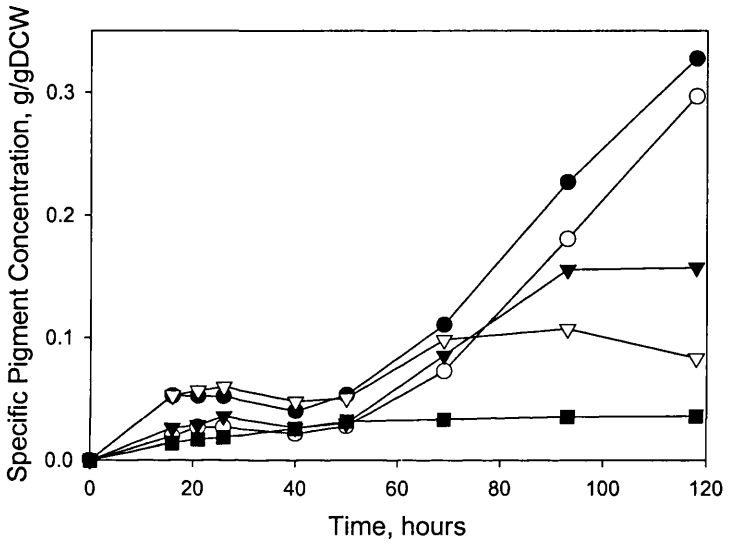


Figure 13: Specific pigment concentration over time produced by *S. erythraea* wt RV grown under different C/N ratios in batch culture.

(●) C/N = 45, (○) C/N = 36, (▲) C/N = 27, (△) C/N = 18, and (■) C/N = 9.

3.4.3.3 Organic Acid Excretion

Specific organic acid production, namely pyruvate and α-ketoglutarate, decreased with increased initial nitrate concentration. The maximum specific pyruvate and α-ketoglutarate production for each C/N are shown in Table 7. The initial nitrate concentration has a greater effect on α-ketoglutarate excretion than on pyruvate excretion. For C/N of 45, 36, 27, and 18, the maximum specific pyruvate production decreased from 0.115 g pyruvate/g DCW to 0.068 g pyruvate/g DCW (~1.7 fold decrease), in contrast to the specific α-ketoglutarate production which decreased from

0.367 g α-ketoglutarate/g DCW to 0.024 g α-ketoglutarate/g DCW (~15.3 fold decrease). Under carbon-limited conditions (C/N = 9), little to no organic acids were excreted. For each C/N studied, the excreted pyruvate was re-consumed by the cell while α-ketoglutarate was not re-consumed. The organic acid production and pigment production over time for C/N = 45, which produced the most organic acids, is shown in Figure 14. At the maximum levels of organic acid excretion, pyruvate and α-ketoglutarate consume about 20 % (on a molar basis) of the total carbon taken up by the cell. This is carbon that could instead be used for product formation. Compared with previous reports, it appears that the profile of pyruvate and α-ketoglutarate excretion by the *S. erythraea* wt RV is similar to other Streptomycetes. Cultures of *S. lividans*, *S. venezuelae*, *S. coelicolor* A3(2), *S. rimosus* also excrete and re-consume pyruvate while α-ketoglutarate is excreted but not re-consumed (Bormann and Herrmann, 1968; Ahmed *et al.*, 1984; Hobbs *et al.*, 1992; Madden *et al.*, 1996).

Table 7: Summary of specific organic acid and pigment production from physiological studies with *S. erythraea* wt red-variant grown under different C/N ratios.

C/N	Maximum Specific Pigment Production (g/g DCW)	Maximum Specific Pyruvate Production (g/g DCW)	Maximum Specific α-Ketoglutarate Production (g/g DCW)
45	0.351	0.115	0.367
36	0.297	0.083	0.287
27	0.158	0.080	0.100
18	0.098	0.068	0.024
9	0.029	0.002	0.005

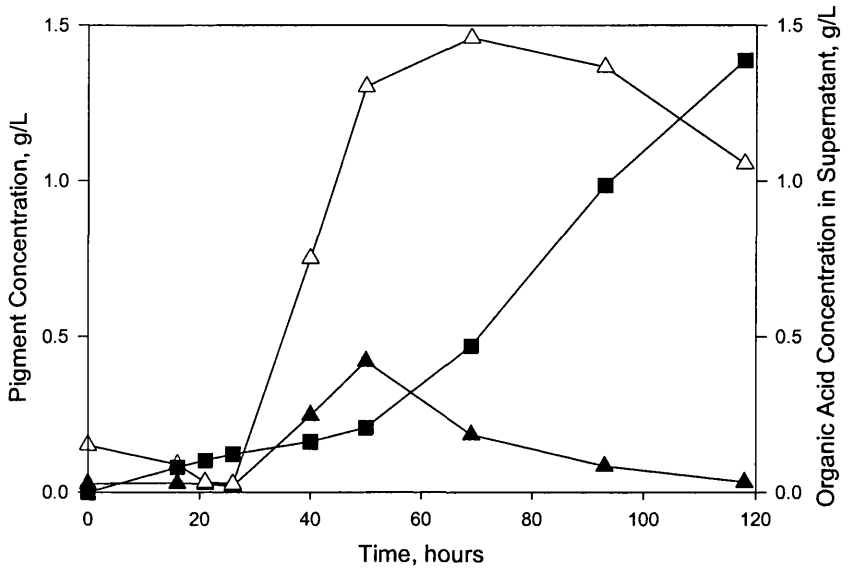


Figure 14: Organic acid excretion kinetics of *S. erythraea* wt RV nitrate-limited batch culture with C/N = 45.

(■) Pigment concentration, (▲) Pyruvate in supernatant, and (△) α-Ketoglutarate in supernatant.

Viollier et. al. (2001) reported that *S. coelicolor* with disrupted citrate synthase and/or aconitase accumulate organic acids, and exhibit deficiency in morphological differentiation and antibiotic production, suggesting that decreased flux though the tricarboxylic acid (TCA) cycle directly effects secondary metabolism (Viollier *et al.*, 2001a; Viollier *et al.*, 2001b). The pigment production by *S. erythraea* wt RV appears to be correlated in a complex manner with organic acid excretion and nitrate availability. The results suggest that organic acid excretion is controlled by the amount of available nitrate such that as the nitrate is depleted, organic acid excretion is increased. However, the rate of pigment production is increased at the same time pyruvate is re-consumed. In contrast, α-ketoglutarate is excreted before pigment production and is not re-consumed by the cell. These results suggest that the TCA cycle enzymes may perform an important role in the interactions between primary and secondary metabolism.

3.4.3.4 Erythromycin Production

Under the minimal media conditions tested, the *S. erythraea* wt RV did not produce measurable amounts of erythromycin A (minimum limit of detection ≤5 mg/L). Unfortunately, it was therefore not possible to compare the production between erythromycin and red pigment in the described experiments. *S. erythraea* wt RV has been reported to produce 10-15 mg/L of total erythromycins as measured by biological assay (Rowe *et al.*, 1998). The analytical method used in this report is based on HPLC and is specific for erythromycin A which may not be sensitive enough to quantify the portion of erythromycin A which may be included in the reported 10-15 mg/L of total erythromycins. *S. erythraea* wild-type NRRL 2338, the strain from which the red-variant strain was derived, produced up to 100 mg/L of erythromycin A in the same nitrate-limited medium described here (data not shown). These results indicate that *S. erythraea* may have the ability to spontaneously mutate to a state where the organism increases the ability to produce the red pigment and decreases its ability to produce erythromycin A and that the *S. erythraea* wt RV is such a mutant.

3.4.3.5 Oxygen Availability

An observed stress condition is oxygen limitation (Clark *et al.*, 1995). To determine if the cultures in these studies were oxygen limited, the DOT was measured in each flask at 40 hours and 50 hours when oxygen demand would be greatest which is near the end of the exponential phase (see Figure 10 and Figure 12). In each culture the first DOT reading (taken within 5 sec. of probe submersion) was above 90 % at 40 hours and 50 hours. It is, therefore, assumed that the none of the cultures were under oxygen limitation during the course of the experiment.

3.4.3.6 Secondary Metabolite Production and Nitrate Levels in Media

It is generally considered that secondary metabolites are produced under various stress conditions, one of which is nutrient limitation (Demain, 1992; Nisbet, 1992). Consistent with the observations, we have found that once nitrate is depleted in the medium, synthesis of the pigment occurs rapidly if there is sufficient carbon available. Conversely, high levels of nitrate in the medium limits the pigment production. These results are consistent with other reports of nitrogen control of secondary metabolites (Smith *et al.*, 1962; Flores and Sanchez, 1985; Cheng *et al.*, 1995; Melzoch *et al.*,

1997). It is shown here that the levels of pigment production by *S. erythraea* wt RV can be controlled by the nitrate concentration. However, our results do not reveal if the influence of nitrate operates at the gene or enzyme level.

3.5 Physiological Effect of Erythromycin Poiyketide Synthase Gene Deletion Under Nitrate and Glucose Limitation

3.5.1 Objective

The purpose of this study was to investigate the physiology of *S. erythraea* wild-type RV compared to the *S. erythraea* Δ*eryA* mutant under carbon (glucose) and nitrogen (nitrate) limited nutrient conditions, and to determine the conditions that yield the greatest differences in growth and product formation kinetics between the *S. erythraea* wild-type RV and Δ*eryA* mutant for the purposes of developing a metabolic model for *S. erythraea.*

3.5.2 Experimental Conditions

The experimental conditions for this study are shown in Table 8.

Table 8: Experimental conditions for testing the physiological effect of erythromycin polyketide synthase gene deletion under nitrate and glucose-limitation.

Strains	*S. erythraea* wt RV *S. erythraea* Δ*eryA* (see section 3.3.1)
Media	Glucose-limited Nitrate-limited (see section 3.3.2.1)
Growth Mode	Batch 2L Shake-Flask
Temperature	28° C
Shaker RPM	200
Sampling Times, hours	0, 16, 20 24, 40, 48, 72, and 96.
Experimental Duplication	Each strain grown with each medium was tested independently two times. The error bars represent ± 1 standard deviation of mean.

3.5.3 Results and Discussion

3.5.3.1 Growth Rate and Substrate Utilization

The wild-type RV and Δ*eryA* strains of *S. erythraea* were grown in defined media under carbon-limiting and nitrogen-limiting conditions with glucose and nitrate as the carbon and nitrogen sources, respectively. The results show that growth, glucose and nitrate uptake are similar for both strains under nitrate-limiting conditions. Growth and glucose uptake are similar for both strains under carbon-limiting conditions, however, nitrate uptake is slower for the Δ*eryA* mutant. The maximum specific growth rate for the wild-type under carbon-limited (0.032 h^{-1}) and nitrogen-limited (0.038 h^{-1}) are similar. For the Δ*eryA* mutant, the growth rate of under carbon-limited (0.035 h^{-1}) is slightly slower than for nitrogen-limited (0.048 h^{-1}) conditions. For both carbon-limitation and nitrogen-limitation the wild-type had a longer lag phase. Figure 15 and Figure 16 show the biomass and limiting-nutrient concentrations for both strains.

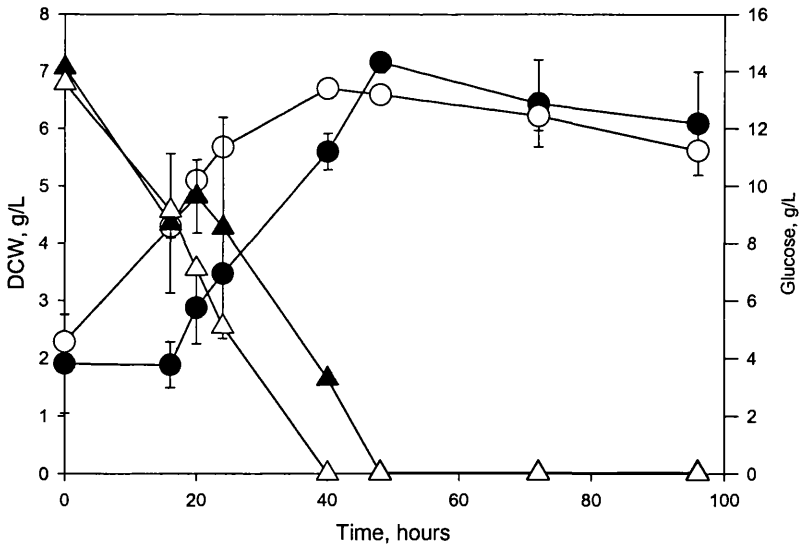


Figure 15: Growth and glucose uptake kinetics of *S. erythraea* wild-type RV and Δ*eryA* mutant grown under carbon-limited conditions in batch culture.

(●) wt RV-Dry Cell Weight, (○) Δ*eryA*-Dry Cell Weight, (▲) wt RV-Glucose, and (△) Δ*eryA*-Glucose.

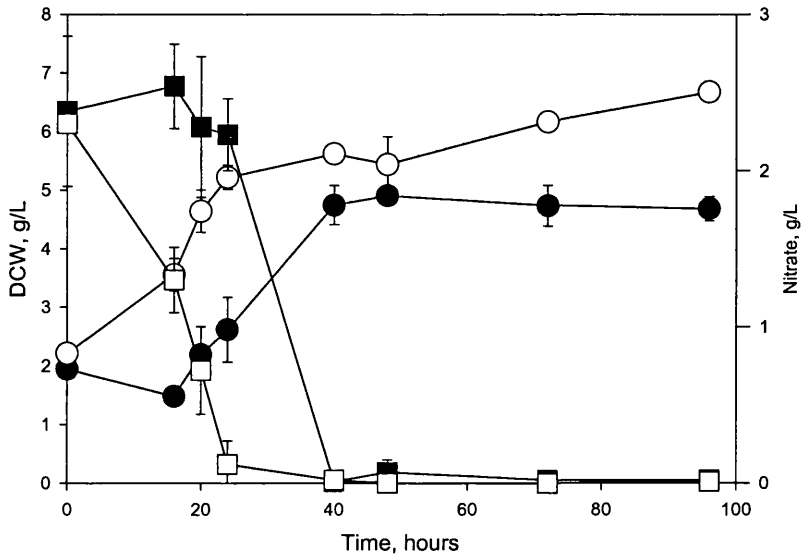


Figure 16: Growth and nitrate uptake kinetics of *S. erythraea* wild-type RV and Δ*eryA* mutant grown under nitrogen-limited conditions in batch culture.

(●) wt RV-Dry Cell Weight, (○) Δ*eryA*-Dry Cell Weight, (■) wt RV-Nitrate, and (□) Δ*eryA*-Nitrate.

3.5.3.2 Organic Acid Excretion

Organic acids are produced by both the *S. erythraea* wild-type and the Δ*eryA* mutant. The results show that pyruvate and α-ketoglutarate production is greater in the Δ*eryA* mutant compared to the wild-type in both carbon and nitrogen-limited conditions. The Δ*eryA* mutant under nitrogen-limiting conditions produces ~4 times greater pyruvate and ~2 times greater α-ketoglutarate than the wild-type. Figure 17 and Figure 18 show organic acid production for the wild-type and Δ*eryA* mutant under carbon and nitrogen limited growth.

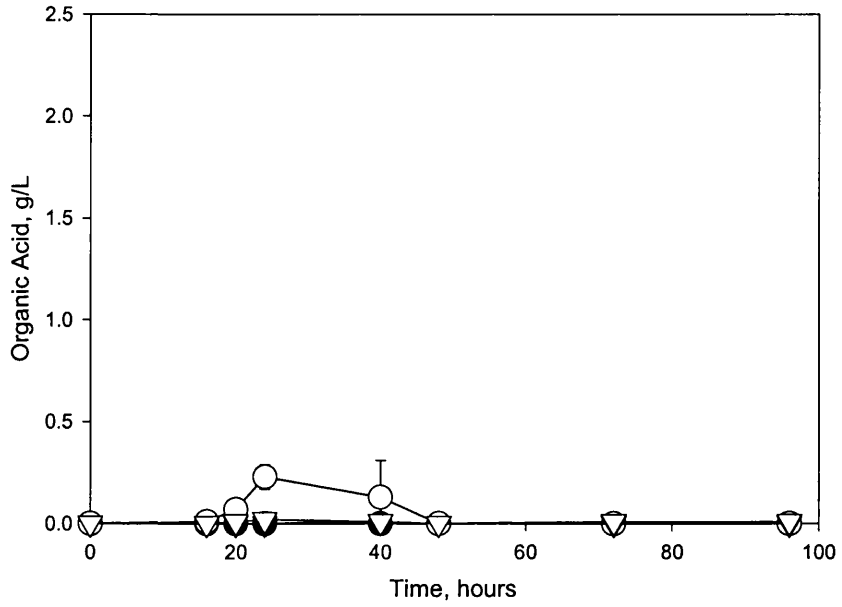


Figure 17: Organic acid excretion of *S. erythraea* wild-type RV and Δ*eryA* mutant grown under carbon-limited conditions in batch culture.

(●) wt RV-Pyruvate, (○) Δ*eryA*- Pyruvate, (▲) wt RV-α-Ketoglutarate, and (△) Δ*eryA*- α-Ketoglutarate.

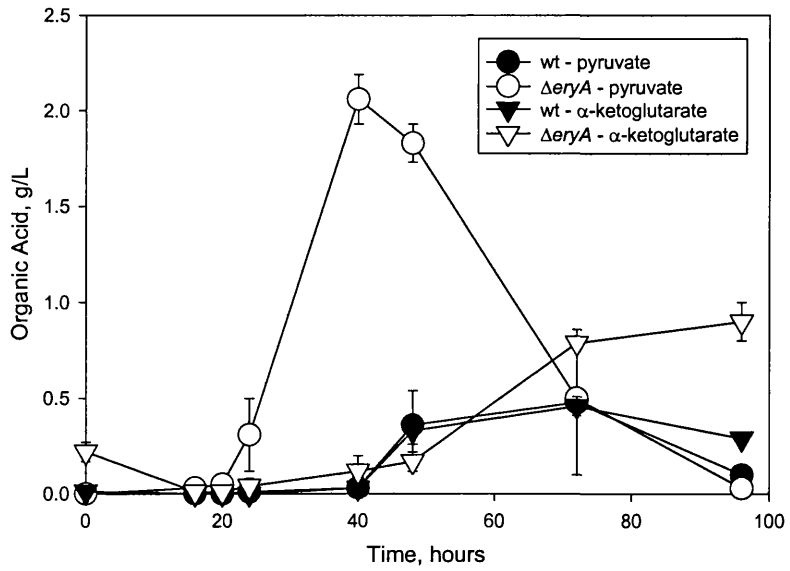


Figure 18: Organic acid excretion of *S. erythraea* wild-type RV and Δ*eryA* mutant grown under nitrogen-limited conditions in batch culture.

(●) wt RV-Pyruvate, (○) Δ*eryA*- Pyruvate, (▲) wt RV-α-Ketoglutarate, and (△) Δ*eryA*- α-Ketoglutarate.

3.5.3.3 Red Pigment and Erythromycin Production

The red-variant wild-type produces a red pigment which is growth dependent under carbon-limiting conditions and is produced at the onset of stationary phase in nitrogen-limiting conditions. The nitrogen-limiting conditions produces a three to four times greater red pigment compared to the carbon limiting-conditions. The Δ*ery A* mutant does not produce any red-pigment under carbon-limited conditions. Under nitrogen-limiting conditions red pigment is produced at the onset of stationary phase but to a lesser extent compared to the wild-type. Erythromycin was not produced to detectable levels. Figure 19 and Figure 20 show the red pigment concentration for the wild-type and Δ*eryA* mutant.

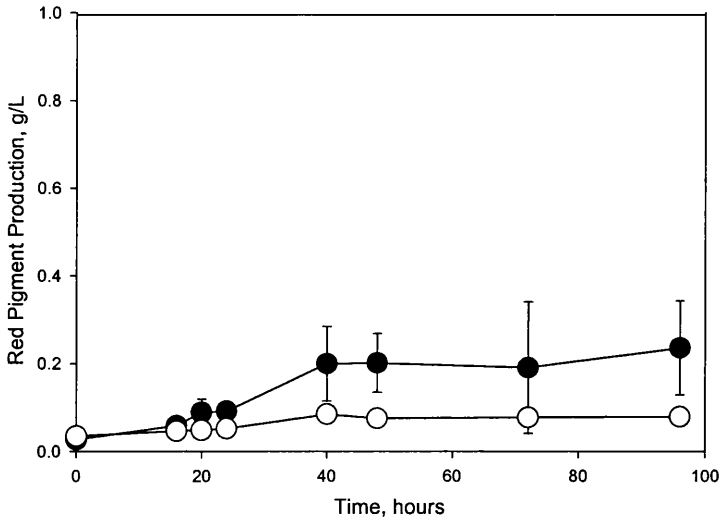


Figure 19: Red Pigment Production of *S. erythraea* wild-type RV and Δ*eryA* mutant grown under carbon-limited conditions in batch culture.

(●) wt RV and (○) Δ*eryA*.

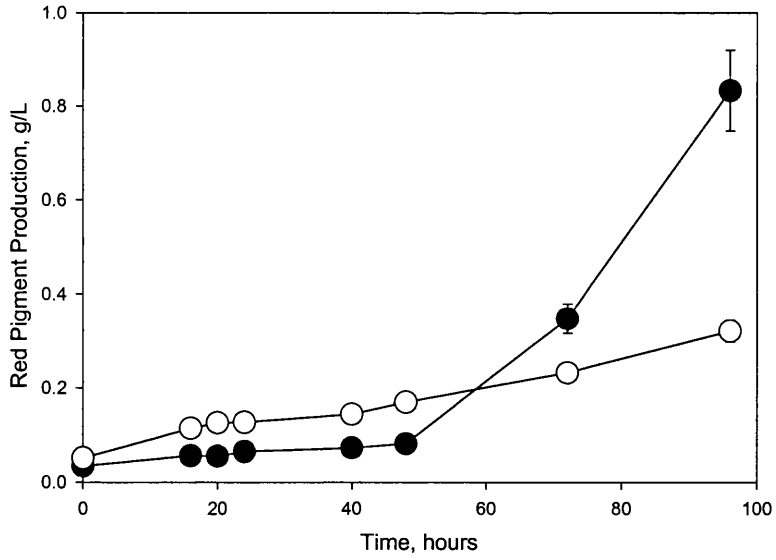


Figure 20: Red pigment production of *S. erythraea* wild-type and Δ*eryA* mutant grown under nitrogen-limited conditions in batch culture.

(●) wt RV and (○) Δ*eryA*.

3.5.3.4 Differences between Carbon and Nitrogen-Limited Growth

In this study, carbon and nitrogen limited growth was investigated for *S. erythraea* wild-type and Δ*eryA* mutant. It was found that larger differences were observed under the nitrogen-limited conditions compared to the carbon-limited. Under nitrogen-limited condition, higher levels of organic acids were produced for both strains and higher levels of red pigment were formed in the wild-type. The production of these metabolites occur at the end of growth phase or during stationary phase which is when the limiting-substrate is depleted. It is considered that as the carbon is depleted the cells do not have enough nutrient to produce these metabolites and this is why we do not observe similar metabolite accumulation in the nitrogen-limited case. Therefore, it was decided to used only nitrogen-limitation for further experiments.

3.6 Physiological Effect of Multiple Genetic Manipulations Under Nitrate Limitation

3.6.1 Objective

The purpose of this experiment was to study if the process of genetic manipulation itself has an effect on growth and product formation under nitrogen limited growth conditions. The strains used in this experiment varied in the number of genetic manipulations. This means that if one gene was deleted the process was performed once, and if two genes were deleted the process was performed twice, etc.

3.6.2 Experimental Conditions

The experimental conditions for this study are shown in Table 9.

Table 9: Experimental conditions for testing the physiological effect of multiple genetic manipulations under nitrate limitation.

Strains	*S. erythraea* wt RV *S. erythraea* Δ*eryA* *S. erythraea* Δ*eryABC* *S. erythraea* Δ*eryBC* *S. erythraea* Δ*eryB* (see section 3.3.1)
Media	Nitrate-limited (see section 3.3.2.1)
Growth Mode	Batch 2L Shake-Flask
Temperature	28° C
Shaker RPM	200
Sampling Times, hours	0, 16, 20, 24, 40, 48, 72, and 96.
Experimental Duplication	Each strain grown with each medium was tested independently two times. The error bars represent ± 1 standard deviation of mean.

3.6.3 Results and Discussion

3.6.3.1 Growth Rate and Substrate Utilization

Five *S. erythraea* strains, wild-type red-variant, Δ*eryA*, Δ*eryABC*, Δ*eryBC*, and Δ*eryB*, were studied under nitrogen-limited conditions. The carbon uptake and nitrogen uptake for each strain was different. The Δ*eryA* and Δ*eryBC* strains grew the faster that the wild-type and the Δ*eryB* and Δ*eryABC* grew slower. The strains that grew faster consumed more nitrogen than the slower growing strains. Figure 21 through Figure 25 show the growth, glucose uptake and nitrate uptake results for the five strains.

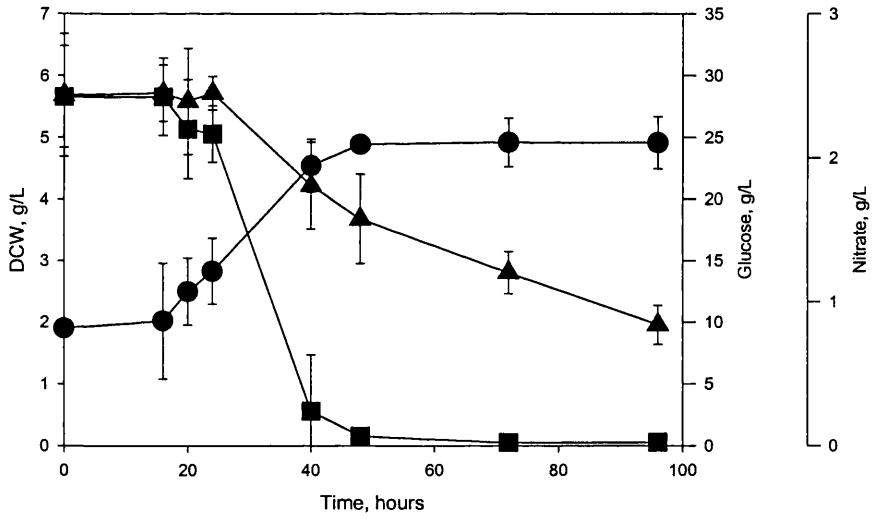


Figure 21: *S. erythraea* wt RV growth, glucose uptake, and nitrate uptake grown in nitrogen-limited batch culture.

(●) Dry Cell Weight, (▲) Glucose, and (■) Nitrate.

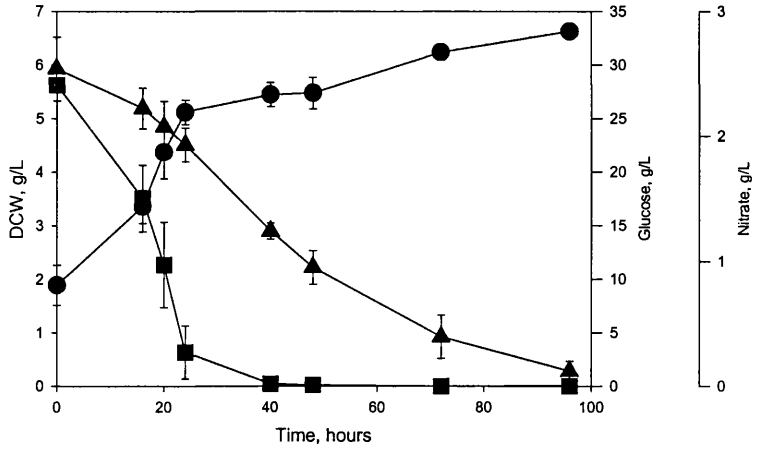


Figure 22: *S. erythraea ΔeryA* growth, glucose uptake, and nitrate uptake grown in nitrogen-limited batch culture.

(●) Dry Cell Weight, (▲) Glucose, and (■) Nitrate.

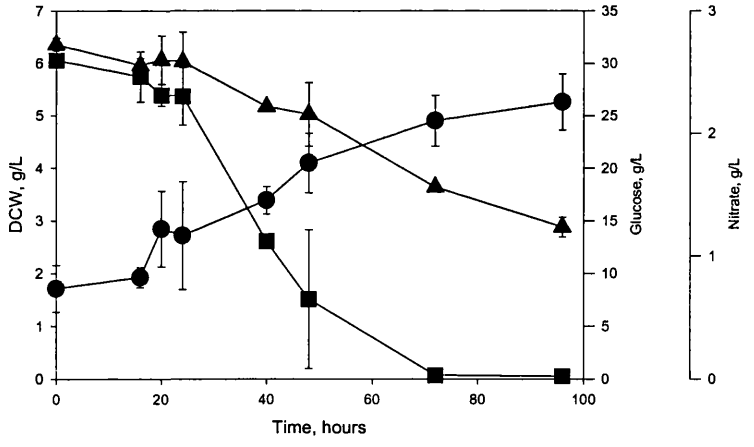


Figure 23: *S. erythraea ΔeryABC* growth, glucose uptake, and nitrate uptake grown in nitrogen-limited batch culture.

(●) Dry Cell Weight, (▲) Glucose, and (■) Nitrate.

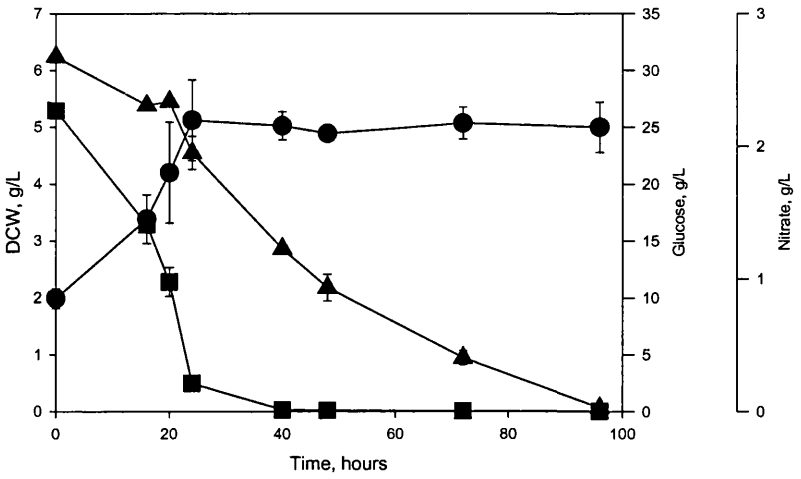


Figure 24: *S. erythraea* Δ*eryBC* growth, glucose uptake, and nitrate uptake grown in nitrogen-limited batch culture.

(●) Dry Cell Weight, (▲) Glucose, and (■) Nitrate.

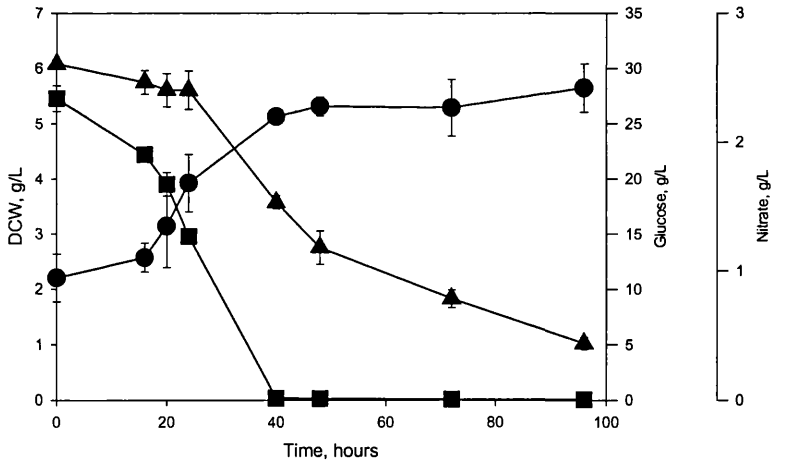


Figure 25: *S. erythraea* Δ*eryB* growth, glucose uptake, and nitrate uptake grown in nitrogen-limited batch culture.

(●) Dry Cell Weight, (▲) Glucose, and (■) Nitrate.

Table 10 lists the maximum specific growth rate and the nitrate uptake rate for each strain under nitrogen limited growth conditions. The data shows that a high maximum specific growth rate correlated with a high nitrate uptake rate.

Table 10: Maximum specific growth rate and nitrate uptake rates.

Strain	Max. Specific Growth Rate (h^{-1})	Nitrate Uptake Rate(g/L/h)
Δ*eryA*	0.053	0.160
Δ*eryBC*	0.052	0.150
wild-type RV	0.032	0.083
Δ*eryB*	0.027	0.080
Δ*eryABC*	0.019	0.070

3.6.3.2 Organic Acid Excretion

All of the strains produced pyruvate and α-ketoglutarate at the end of the growth phase and beginning of the stationary phase. The pyruvate was re-consumed over the remainder of stationary phase while the α-ketoglutarate was not re-consumed. Different amounts of pyruvate and α-ketoglutarate were produced by each strain. The wild-type produced maximum specific amounts of pyruvate and α-ketoglutarate of ~0.03 g/g and ~0.32 g/g, respectively. The Δ*eryA* and Δ*eryBC* produced ~5-fold more pyruvate, Δ*eryB* ~2-fold, and Δ*eryABC* producing about the same as the wild-type. The Δ*eryBC* produced ~1.5-fold more α-ketoglutarate, Δ*eryABC* ~2-fold less, and Δ*eryA* and Δ*eryB* about the same as the wild-type. The amount of pyruvate produced ranged from 0.03 g/g to 0.16 g/g, which was an average 4-fold lower than the range for α-ketoglutarate, 0.15 g/g to 0.45 g/g. Figure 26 and Figure 27 show the levels of organic acids produced for the five strains studied.

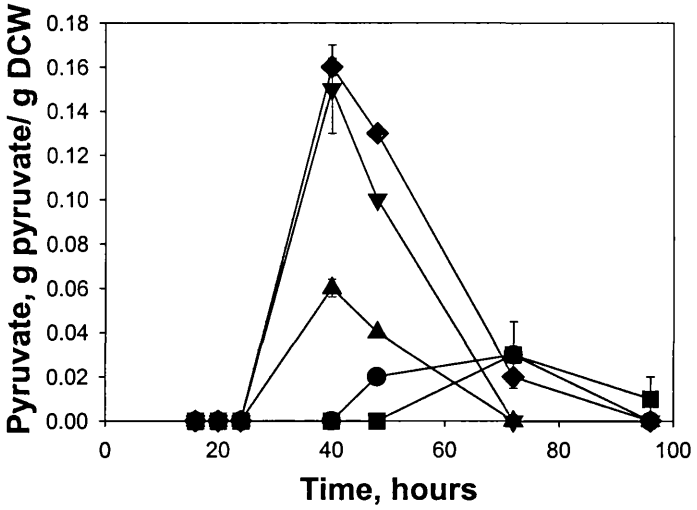


Figure 26: Specific pyruvate excretion of *S. erythraea* wild-type RV, Δ*eryA*, Δ*eryABC*, Δ*eryBC*, and Δ*eryB* mutants grown under nitrogen-limited conditions.

(●) wt RV, (▼) Δ*eryA*, (■) Δ*eryABC*, (◆) Δ*eryBC*, and (▲) Δ*eryB*.

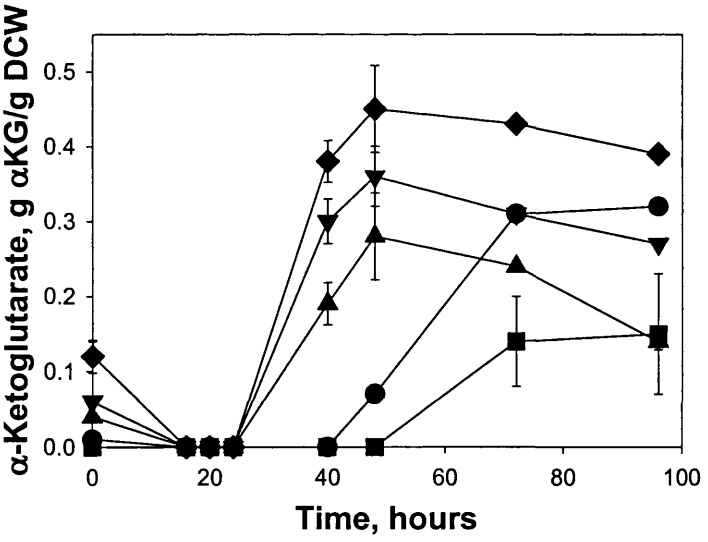


Figure 27: Specific α-ketoglutarate excretion of *S. erythraea* wild-type RV, Δ*eryA*, Δ*eryABC*, Δ*eryBC*, and Δ*eryB* mutants grown under nitrogen-limited conditions.

(●) wt RV, (▼) Δ*eryA*, (■) Δ*eryABC*, (◆) Δ*eryBC*, and (▲) Δ*eryB*.

3.6.3.3 Effect of Genetic Manipulation on *S. erythraea* Physiology

In this study, several *S. erythraea* strains were investigated that have varying levels of genetic manipulation. The five strains studied had between 0 to 3 genes deleted which means the process of manipulating the genetics was performed 0 to 3 times. The process of gene deletion is a long series of genetic manipulation, screening, and selection. It was questioned if the level of genetic manipulation had an effect on the amount of organic acid production or red pigment production as stated in the objective section 3.6.1. The results from this study showed that the level of genetic manipulation does not correlate with the amount of organic acid production, red pigment production, or growth rate. These results provide some confidence that the process of gene deletion does not interfere with physiological studies. Figure 28 and Figure 29 show the relationships between the maximum specific organic acid production and maximum specific growth rate as a function of the number of genetic manipulations.

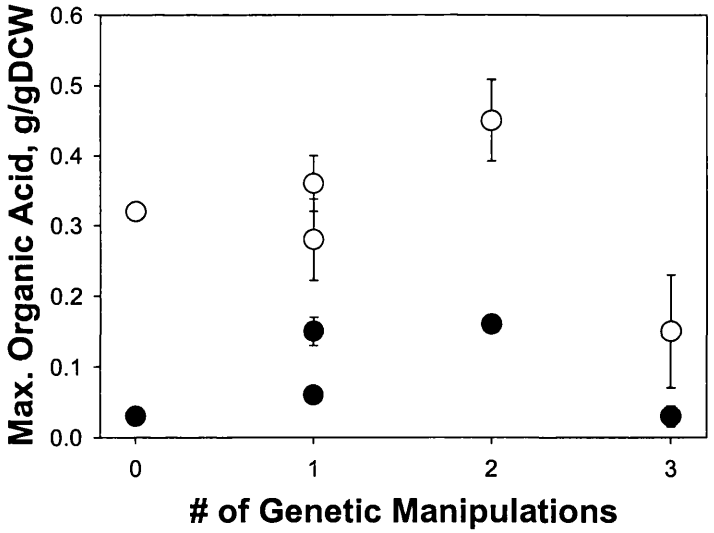


Figure 28: Maximum specific organic acid excretion as a function of the number of genetic manipulations.

(●) Maximum Pyruvate and (○) Maximum α-Ketoglutarate.

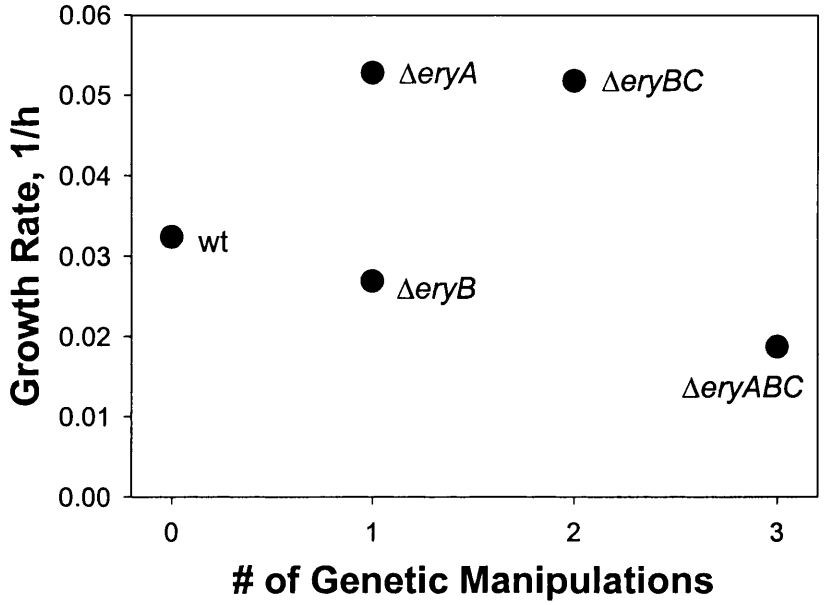


Figure 29: Maximum specific growth rate as a function of the number of genetic manipulations.

It should be noted that even though these is no trend to indicate that gene deletion has a systematic effect on *S. erythraea* physiology, there does appear to be difference between strains which may be due to clonal variation.

3.6.3.4 Effect of Growth Rate on Organic Acid Excretion

The results show that as the growth rate increased the amount of organic acids increased. Figure 30 and Figure 31 show the linear relationship between the maximum specific organic acid production and maximum specific growth rate. It is possible that the branch points at pyruvate and α-ketoglutarate help control the rate of primary metabolism. Therefore, if the rates of glycolysis or the TCA cycle are progressing too rapidly, perhaps the activity of pyruvate dehydrogenase or α-ketoglutarate dehydrogenase decreases which slows down metabolism and causes accumulation of the acids, or perhaps the activities stay constant but cannot keep up with flux through glycolysis. It has been shown, in *S. venezuelae*, that the inability to re-consume excreted α-ketoglutarate corresponded with loss of α-ketoglutarate dehydrogenase activity (Ahmed *et al.*, 1984).

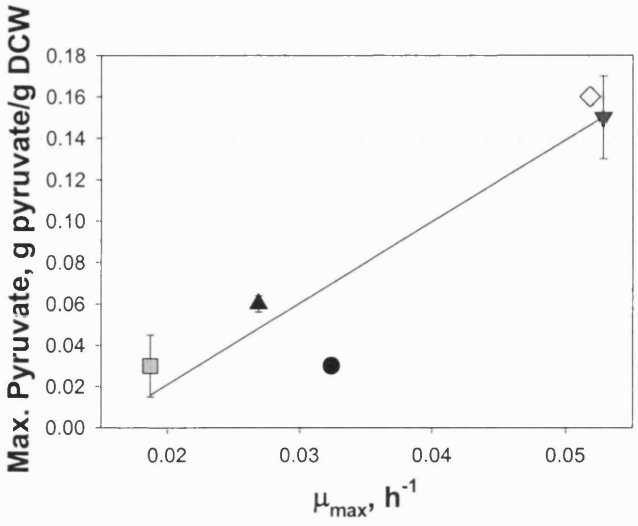


Figure 30: Maximum specific pyruvate production as a function of maximum specific growth rate.

(●) wt RV, (▼) Δ*eryA*, (■) Δ*eryABC*, (◆) Δ*eryBC*, and (▲) Δ*eryB*.

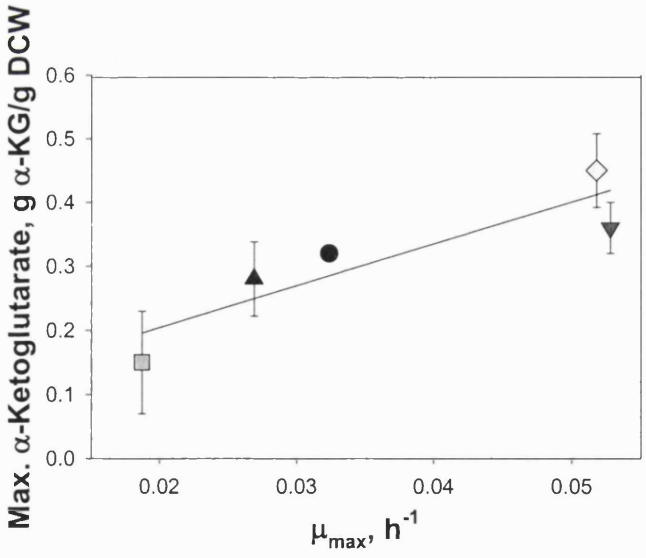


Figure 31: Maximum specific α-ketoglutarate production as a function of maximum specific growth rate.

(●) wt RV, (▼) Δ*eryA*, (■) Δ*eryABC*, (◆) Δ*eryBC*, and (▲) Δ*eryB*.

3.6.3.5 Red Pigment Production

The red pigment was produced in varying amounts by each strain in this study. The wild-type produced the most red-pigment. The Δ*eryABC* and Δ*eryBC* produced similar amounts of pigment as the wild-type while the Δ*eryB* and Δ*eryA* produced lower amounts than the wild-type. Figure 32 shows the specific red pigment concentration for each of the five *S. erythraea* strains studied.

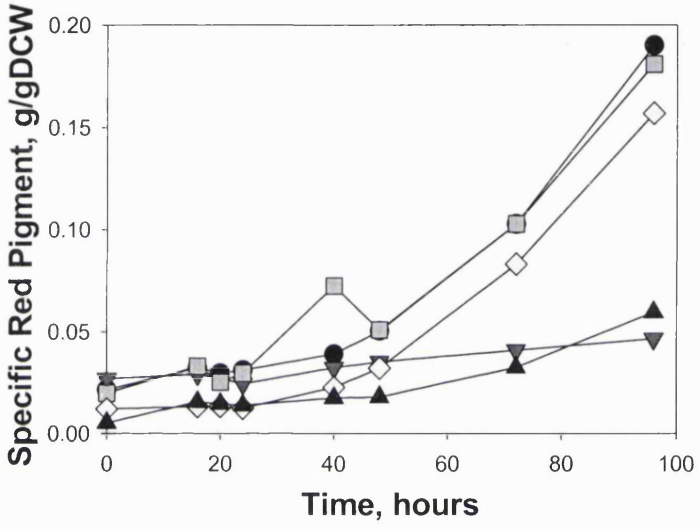


Figure 32: Specific red pigment production of *S. erythraea* wild-type RV, Δ*eryA*, Δ*eryABC*, Δ*eryBC*, and Δ*eryB* mutants grown under nitrogen-limited conditions. (●) wt RV, (▼) Δ*eryA*, (■) Δ*eryABC*, (◆) Δ*eryBC*, and (▲) Δ*eryB*.

The production of the red pigment and the undetectability of erythromycin in the case of the wild-type may suggest that the two secondary metabolites are competing for precursors, which was supported by the observation that when malonyl-CoA decarboxylase was deleted, erythromycin and the red pigment were not produced (Hsieh and Kolattukudy, 1994). The observation of production of the red pigment and no production of erythromycin is an example of the sensitivity the secondary metabolite production.

3.7 Physiological Differences Between *S. erythraea* wt red-variant and *S. erythraea* wt white-variant

Two shake-flask experiments were performed to study the differences in growth and product formation between the red-variant (RV) and white-variant (WV) *S. erythraea* wt strains, where the white-variant is the parent strain of the red-variant. The red-variant produces red pigments while the white-variant produces erythromycin under defined minimal media conditions. The first experiment was a time course study with the *S. erythraea* wt white-variant conducted under the same nitrate varying conditions as with the red-variant in section 3.4. The second study was an end-point study comparing the red and white *S. erythraea* wt variants under nitrate-limited and phosphate-limited defined media.

3.7.1 Influence of Initial Nitrate Level in Media

3.7.1.1 Objective

The objective of this experiments was to study the influence of the initial nitrate level on growth and product formation by *S. erythraea* wild-type white-variant.

3.7.1.2 Experimental Conditions

The experimental conditions for this study are shown in Table 11.

Table 11: Experimental conditions for testing physiological effect of initial nitrate levels in media on *S. erythraea* wt WV.

Strains	*S. erythraea* wt WV (see section 3.3.1)
Media	Nitrate-limited medium (see section 3.3.2.1) with the initial nitrate levels varied from 1.89 g/L to 4.58 g/L to obtain carbon to nitrogen ratios (C/N) of 45, 36, 27, and 18, respectively, with the initial glucose concentration remaining 30 g/L.
Growth Mode	Batch 2L Shake-Flask
Temperature	28° C
Shaker RPM	200
Sampling Times, hours	0, 16, 21, 26, 40, 50, 69, 93, and 118.
Experimental Duplication	Each strain grown with each medium was tested independently two times.

3.7.1.3 Results and Discussion

The *S. erythraea* wt WV results show that as initial nitrate concentration in the medium increased, maximum dry cell weight, maximum specific erythromycin production, and maximum specific organic acid production increased. However, as initial nitrate concentration in the medium increased, substrate uptake and maximum specific growth rate were approximately the same for each C/N (see Table 12). Little to no red pigment was produced for each C/N.

Table 12: Physiological data for *S. erythraea* white-variant.

C/N	Nitrate in Media, g/L	Maximum Specific Growth Rate, h^{-1}	Maximum Dry Cell Weight, g/L
45	1.84	0.031	4.857
36	2.29	0.026	5.128
27	3.04	0.035	6.203
18	4.58	0.037	7.705

The maximum specific organic acid and polyketide production as a function of the initial nitrate concentration in the media are shown for *S. erythraea* wt WV and *S. erythraea* wt RV in Figure 33 and Figure 34, respectively. The *S. erythraea* wt white-variant produced lower amounts of polyketide (pigment or erythromycin) and organic acids compared to *S. erythraea* wt red-variant. Also, the trends were different with respect to the C/N. For the WV, as the nitrate in the media increased, the maximum specific organic acid and polyketide (erythromycin) production increased. However, for the RV, as the nitrate in the media increased, the maximum specific organic acid and polyketide (red pigment) production decreased.

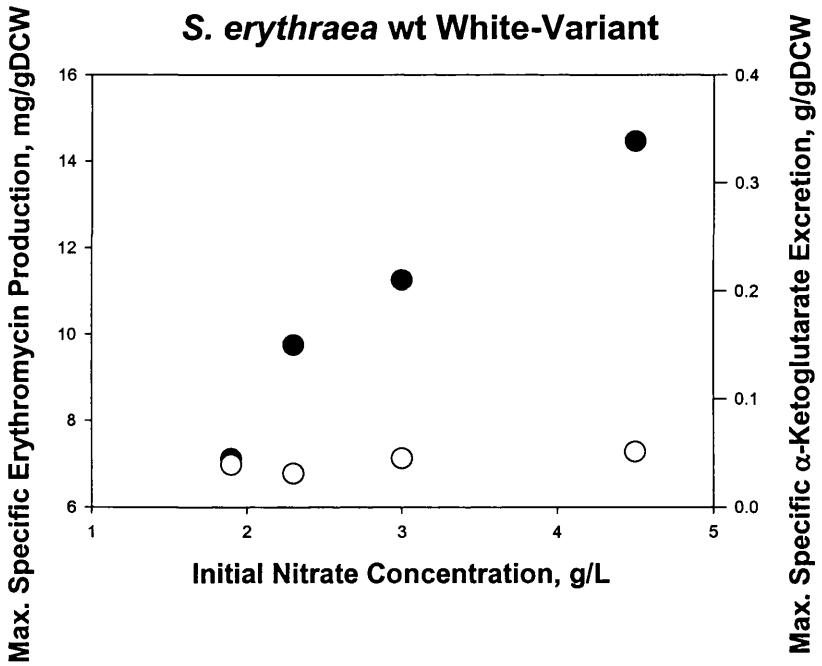


Figure 33: Maximum specific erythromycin and organic acid production as a function of initial nitrate concentration for *S. erythraea* wt white-variant.

(●) Erythromycin and (○) α-Ketoglutarate.

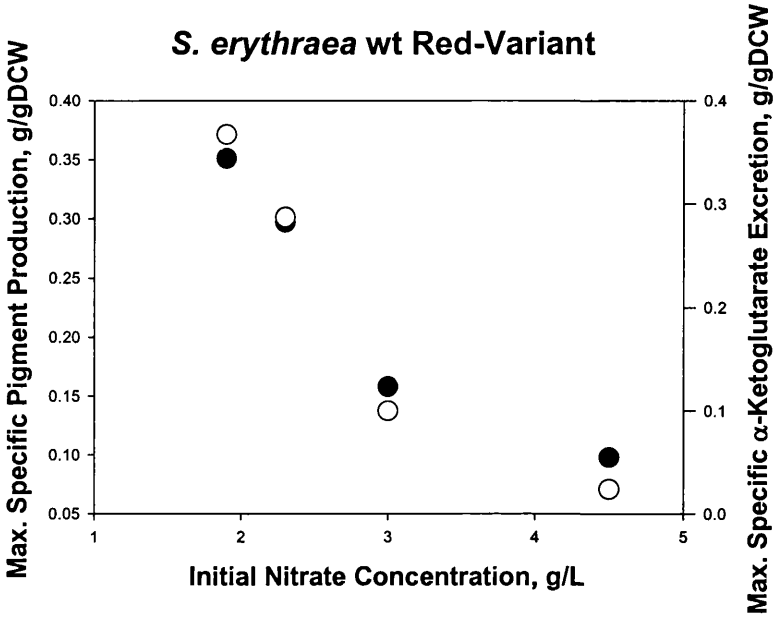


Figure 34: Maximum specific pigment and organic acid production as a function of initial nitrate concentration for *S. erythraea* wt red-variant.

(●) Red Pigment and (○) α-Ketoglutarate.

It is unclear why the trends in organic acid and polyketide production are different, however, it may be due to the demand of α-ketoglutarate and the influence of nitrate on α-ketoglutarate supply (see Figure 35). The RV produces high levels of red-pigment when low levels of nitrate are supplied to the system. The precursors of the red-pigment may be supplied by acetyl-CoA (Cortes *et al.*, 2002), which is upstream of α-ketoglutarate. When nitrate in the medium is low, high levels of α-ketoglutarate are accumulated which may imply that there is a reduction (or block) of carbon flux downstream of α-ketoglutarate. This may also cause more carbon availability upstream of α-ketoglutarate which may then be able to supply higher levels of acetyl-CoA precursors to red pigment production.

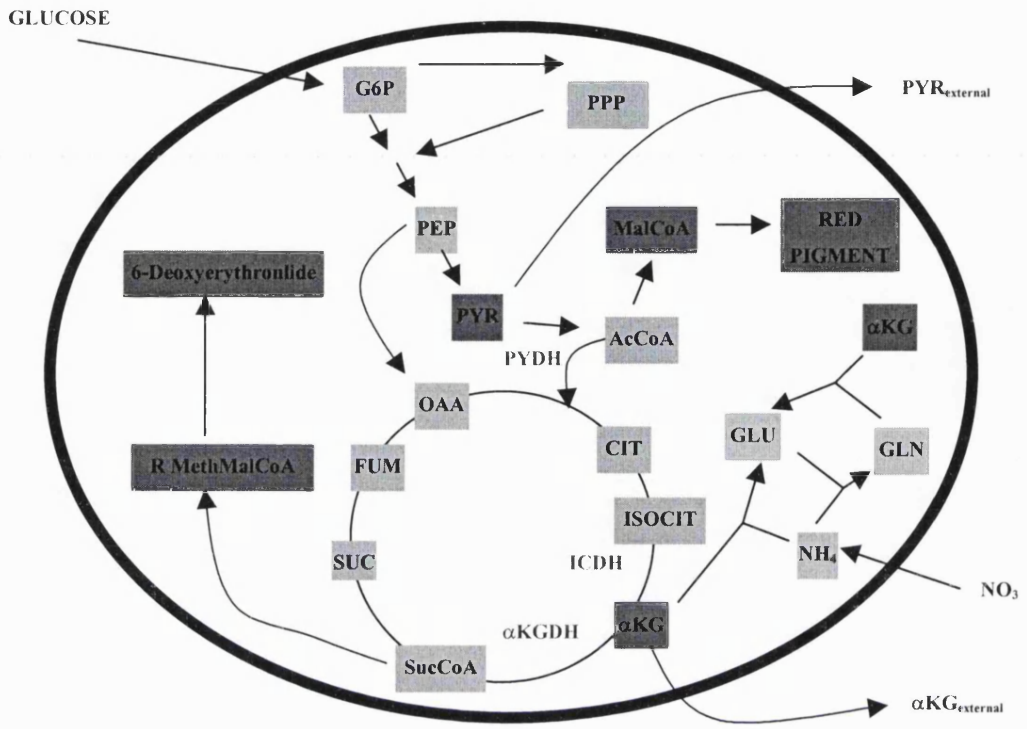


Figure 35: Simplified metabolic reaction network diagram.

For the WV, higher levels of erythromycin are produced when higher levels of nitrate are supplied to the medium. The increase in specific erythromycin production could simply be due to increased amounts of nitrate. There is an accumulation of α-ketoglutarate at the high levels of nitrate, however, these levels are four times lower than the RV. It is still unclear why the RV does not produce erythromycin at high levels of nitrate and low levels of organic acid accumulation.

3.7.2 Difference Between Nitrate-Limited and Phosphate-Limited Growth Conditions

3.7.2.1 Objective

The objective of this experiment was to study the differences in growth and product formation between *S. erythraea* wild-type white-variant and *S. erythraea* wild-type red-variant grown under nitrate-limited and phosphate-limited conditions.

3.7.2.2 Experimental Conditions

The experimental conditions for this study are shown in Table 13.

Table 13: Experimental conditions for testing physiological effect of nitrate-limited and phosphate-limited media on *S. erythraea* wt RV and *S. erythraea* wt WV.

Strains	*S. erythraea* wt RV *S. erythraea* wt WV (see section 3.3.1)
Media	Nitrate-limited medium Phosphate-limited medium (see section 3.3.2.1)
Growth Mode	Batch 500 mL Shake-Flask
Temperature	28° C
Shaker RPM	200
Sampling Times, hours	96
Experimental Duplication	Each strain grown with each medium was tested independently two times.

3.7.2.3 Results and Discussion

The results shown in Table 14 illustrate the differences between the RV and WV *S. erythraea* wt strains. The WV produces about 15 % more biomass than the RV. Under nitrate-limited conditions, the RV produces ~ 4 times more pyruvate and ~2 times more α-ketoglutarate than the WV. However, under phosphate-limited conditions neither strain produced organic acids. Low levels of erythromycin were produced by the WV strain under nitrate and phosphate-limited conditions. Under nitrate limited conditions, the RV produced ~10 more red-pigment than the WV. However, under phosphate-limited condition both strains produced similar amounts which was about one-half the RV nitrate-limited pigment levels.

Table 14: Physiology results of *S. erythraea* wt WV and RV grown under nitrate-limited and phosphate-limited conditions.

	***S. erythraea* wild-type WV**		***S. erythraea* wild-type RV**	
	N-limited	**P-limited**	**N-limited**	**P-limited**
DCW	6.41	6.21	5.23	5.44
pH	7.41	7.46	7.30	7.36
Pyruvate (g/L)	0.27	0.00	1.16	0.01
α-Ketoglutarate (g/L)	0.10	0.00	0.24	0.01
Erythromycin (mg/L)	5.6	6.0	0.0	0.0
Red Pigment (g/L)	0.135	0.575	1.280	0.587

These results substantiate the idea that the red-variant produced from the parent *S. erythraea* wt WV strain has surprisingly different physiological characteristic compared to the parent strain. This could result in difficulties to assure the same variant strain is used in comparable experiments.

3.8 Ancillary Physiological Studies

3.8.1 Effect of Added Propionate or Acetate to Growth Media on Polyketide Production by *S. erythraea* wt RV

3.8.1.1 Objective

The objective of this study was to determine if higher levels polyketides (red pigment or erythromycin) are produced if precursors for polyketide synthesis are added to media. Propionate is a 3 carbon molecule used to make propionyl-CoA and methylmalonyl-CoA which are both used for erythromycin biosynthesis. Acetate is a 2 carbon molecule used to make malonyl-CoA which may be utilized for red pigment biosynthesis (Cortes *et al.*, 2002).

3.8.1.2 Experimental Conditions

The experimental conditions for this study are shown in Table 15.

Table 15: Experimental conditions for testing effect of added propionate or acetate on polyketide biosynthesis by *S. erythraea* wt RV.

Strains	*S. erythraea* wt RV (see section 3.3.1)
Media	Nitrate-limited medium with 1 g/L of either propionate or acetate added. (see section 3.3.2.1)
Growth Mode	Batch 500 mL Shake-Flask
Temperature	28° C
Shaker RPM	200
Sampling Times, hours	96
Experimental Duplication	Each strain grown with each medium was tested independently two times.

3.8.1.3 Results and Discussion

The results of Hsieh and Kolattukudy (1994) showed that *S. erythraea* CA340, with a disrupted malonyl-CoA decarboxylase gene, could not produce erythromycin or the red pigment, and that exogenous propionate could restore erythromycin but not red pigment production. Their results imply that propionate is not a crucial precursor to the red pigment but that propionate is important for erythromycin synthesis. To explore possible precursors to red pigment synthesis, 1 g/L of either sodium acetate (as precursor to malonyl-CoA) or sodium propionate (as precursor to methylmalonyl-CoA) were added to nitrate limited medium (C/N = 36).

Table 16: Average specific red pigment production by *S. erythraea* wt RV with added propionate or acetate.

Additions to N-limited media	pH	Dry Cell Weight, g/L	Red Pigment, g/L	Specific Red Pigment, g/g	Average Specific Red Pigment, g/g
1 g/L Acetate	7.38	4.91	1.13	0.23	0.229 ± 0.0013
1 g/L Acetate	7.37	5.17	1.18	0.23	
1 g/L Propionate	7.39	4.38	0.55	0.13	0.122 ± 0.0046
1 g/L Propionate	7.42	4.62	0.55	0.12	
control	7.23	5.24	1.31	0.25	0.257 ± 0.0106
control	7.30	5.36	1.42	0.27	

The results, shown in Table 16, suggested that acetate (0.229 g pigment/g DCW) does not affect the amount of red pigment produced compared to the control (0.26 g pigment/g DCW) while propionate (0.12 g pigment/g DCW) appears to inhibit synthesis of the red pigment. The maximum specific pigment concentration produced in cultures with propionate added was ~50% of the cultures with no propionate added. These results imply that propionate is not a direct precursor of the red pigment, and may cause carbon to be directed away from red pigment synthesis to other secondary metabolites, or may stress the cells non-specifically. Our results agree with the results of Hsieh and Kolattukudy (1994) in that propionate does not contribute to red pigment production.

3.8.2 Effect of Added Pyruvate or α-Ketoglutarate to Growth Media on Polyketide Production by *S. erythraea* wt RV

3.8.2.1 Objective

The objective of this study was to determine if higher levels polyketides (red pigment or erythromycin) are produced if organic acids are added to media. Pyruvate is the substrate used to make acetyl-CoA which in turn is the substrate to make malonyl-CoA which is used for red pigment biosynthesis. α-Ketoglutarate is the substrate used to make succinyl-CoA which in turn is used to make propionyl-CoA and

methylmalonyl-CoA which are both used for erythromycin biosynthesis. Pyruvate and α-ketoglutarate are both excreted by *S. erythraea* wild-type as waste carbon.

3.8.2.2 Experimental Conditions

The experimental conditions for this study are shown in Table 17.

Table 17: Experimental conditions for testing effect of added pyruvate or α-ketoglutarate on polyketide biosynthesis by *S. erythraea* wt RV.

Strains	*S. erythraea* wt RV (see section 3.3.1)
Media	Nitrate-limited medium with 1 g/L of either pyruvate or α-ketoglutarate added. (see section 3.3.2.1)
Growth Mode	Batch 500 mL Shake-Flask
Temperature	28° C
Shaker RPM	200
Sampling Time, hours	90
Experimental Duplication	Each strain grown with each medium was tested independently two times.

3.8.2.3 Results and Discussion

Pyruvate and α-ketoglutarate are excreted by *S. erythraea* wt RV. To determine if the addition of these organic acids to the medium affected polyketide synthesis, 1 g/L of either was added to the medium. This study also addressed the question if the equilibrium of pyruvate and α-ketoglutarate between the cell and the supernatant had an effect on the levels of polyketide production.

Table 18: Average specific red pigment production by *S. erythraea* wt RV with added pyruvate or α-ketoglutarate.

Additions to N-limited media	pH	Dry Cell Weight, g/L	Red Pigment, g/L	Specific Red Pigment, g/g	Average Specific Red Pigment, g/g
1 g/L Pyruvate	7.28	4.92	1.06	0.22	0.203 ± 0.0183
1 g/L Pyruvate	7.35	5.17	0.98	0.19	
1 g/L α-Ketoglutarate	7.35	4.25	1.22	0.29	0.295 ± 0.0119
1 g/ L α-Ketoglutarate	7.29	4.41	1.34	0.30	
control	6.98	4.33	0.87	0.20	0.215 ± 0.0205
control	7.02	4.87	1.12	0.23	

The results, shown in Table 18, indicated that α-ketoglutarate in the medium may increase the production of red pigment compared to the control. This may occur because α-ketoglutarate is in too much excess and forces the flow of carbon away from the α-ketoglutarate node. This would cause carbon to potentially seek an outlet upstream of α-ketoglutarate. Acetyl-CoA is the substrate for many reactions and could be an appropriate outlet for excess carbon. Acetyl-CoA is a precursor for malonyl-CoA which may be used for red pigment biosynthesis. Pyruvate does not appear to affect the production of red pigment.

3.8.3 Effect of Added Succinic Acid or Valine to Growth Media on Polyketide Production by *S. erythraea* wt WV

3.8.3.1 Objective

The objective of this study was to determine if the addition of succinic acid or valine to the medium increases the production of red pigment or erythromycin by *S. erythraea* wt WV. Both succinic acid and valine are precursors to methylmalonyl-CoA which is utilized in erythromycin biosynthesis (Tang *et al.*, 1994).

3.8.3.2 Experimental Conditions

The experimental conditions for this study are shown in Table 19.

Table 19: Experimental conditions for testing effect of added succinic acid or valine on polyketide biosynthesis by *S. erythraea* wt WV.

Strains	*S. erythraea* wt WV (see section 3.3.1)
Media	Nitrate-limited medium with 1 mM, 10 mM, or 100 mM of either succinic acid or valine added. (see section 3.3.2.1)
Growth Mode	Batch 500 mL Shake-Flask
Temperature	28° C
Shaker RPM	200
Sampling Time, hours	96
Experimental Duplication	Each strain grown with each medium was tested one time.

3.8.3.3 Results and Discussion

Succinic acid and valine are both precursors to methylmalonyl-CoA which is the major building block for synthesizing erythromycin. To determine if these compounds would increase red pigment or erythromycin, varying levels of each were added to the media. The results are shown in Table 20.

Table 20: Specific red pigment and erythromycin production by *S. erythraea* wt WV with added succinic acid or valine.

Additions to N-limited media	pH	Dry Cell Weight, g/L	Specific Red Pigment, g/gDCW	Specific Erythromcyin, mg/gDCW
control	7.56	6.23	0.012	0.719
1 mM Succinic Acid	7.61	7.49	0.013	0.688
10 mM Succinic Acid	7.65	7.80	0.014	0.661
100 mM Succinic Acid	7.84	7.35	0.014	0.701
1 mM Valine	7.53	7.78	0.012	0.575
10 mM Valine	7.63	8.44	0.024	0.732
100 mM Valine	7.79	10.47	0.034	0.979

The results show that the biomass increased when grown with succinic acid as compared to the control. However, the amount of succinic acid did not appear to affect the accumulated biomass. Valine added to the medium also increased biomass compared to the control and as the amount of valine added to the medium increased the biomass increased. This is most likely due to the culture using valine as a nitrogen source after the nitrate had been depleted. Very little red pigment was produced in all cases. There appears to be a slight increase in red pigment as valine is increased however these levels are still very low. It is not expected that the *S. erythraea* wt WV would produce levels of red pigment comparable to the red-variant. Erythromycin production was not effected by the addition of succinic acid. However, the maximum specific erythromycin production increased with increased valine. It appears that *S. erythraea* uses succinic acid added to the medium for biomass and not erythromycin production. Valine appears to be used for both biomass and erythromycin production. It may be that it is more difficult for S. *erythraea* to take up and/or utilize succinic acid compared to valine.

3.8.4 Influence of Anti-foam on *S. erythraea* Growth

3.8.4.1 Objective

The objective of this experiment was to study which anti-foams did not inhibit growth under nitrate-limited minimal medium conditions. It had been previously found that the antifoam polypropylene glycol was inhibiting growth of *S. erythraea* wt RV grown under nitrate-limited conditions in a 20 L bio-reactor.

3.8.4.2 Experimental Conditions

S. erythraea wt RV, were grown in 500 mL shake-flasks for 96 hours. Nitrate was the nitrogen source and was the limiting nutrient (2.4 g/L) and glucose was the carbon source (30 g/L). Five anti-foams were studied as well as the influence of MOPS (used as a buffer) and trace elements on growth. The control consisted of nitrate-limited medium (glucose, nitrate, phosphate, and trace elements) plus MOPS buffer. Table 21 lists the anti-foams tested and if MOPS or trace elements were included in the medium. One sample was removed at 96 hours and analyzed for dry cell weight, pH, and red pigment production.

Table 21: Anti-foams tested for growth inhibition.

Sample	Anti-foam	MOPS	Trace Elements
1	None	Yes	Yes
2	Polypropylene glycol (PPG)	Yes	Yes
3	Antifoam A: 100 % silicone, no emulsions	Yes	Yes
4	Antifoam C: 30 % aqueous emulsion of antifoam A, non-ionic emulsifier	Yes	Yes
5	Antifoam 204: non-silicone, organic, polyol dispersion	Yes	Yes
6	Antifoam 289: silicone and non-silicone	Yes	Yes
7	None	No	Yes
8	None	No	No

3.8.4.3 Results and Discussion

The results, shown in Table 22, showed that the different anti-foams affected the amount of biomass and red pigment produced. Polypropylene glycol inhibited growth but produced amounts of pigment similar to control, as observed previously. The silicone based anti-foams (AFA and AFC) produced slightly lower amounts of biomass but produced high levels of red pigment. The anti-foams that contained non-silicone components (AF0204 and AF289) produced higher levels of biomass compared to the silicone based anti-foams, however they produced low amounts of red pigment. Upon visual inspection, foaming was qualitatively evaluated. All samples containing anti-foam, with the exception of AFC, showed no foaming. The AFC is a dilution of AFA and therefore may not be concentrated enough to eliminate all foaming. The results of this study led to the use of silicone based anti-foams for *S. erythraea* fermentations grown under minimal medium conditions.

Table 22: Influence of various anti-foams on biomass and red pigment production grown under nitrate-limited medium.

Sample	Biomass, g/L	Red Pigment, g/L	% Biomass Compared to Control	% Red Pigment Compared to Control	Visual Inspection of Foaming
1: control	5.55	0.481	100	100	++
2: PPG	3.01	0.416	54	86	-
3: AFA	4.49	0.643	81	134	-
4: AFC	4.19	0.673	75	140	+
5: AF204	4.95	0.313	89	65	-
6: AF289	5.04	0.328	91	68	-
7: -MOPS	4.65	0.735	84	153	++
8: -MOPS, -TE	1.46	0.016	26	3	+

3.9 Discussion and Conclusions

This chapter describes the physiological studies conducted with *S. erythraea* wild-type and genetically engineered strains. Growth, substrate uptake, organic acids production, red pigment production, and erythromycin production were studied under various nutrient limitations.

3.9.1 Growth and Substrate Uptake

The maximum specific growth rate and substrate uptake varied due to the amount of initial nitrate in the media. This may occur only because as the nitrate in the media increased there was more substrate available. The maximum specific growth rate also varied between different strains. The genetically engineered strains were mutated in secondary metabolic pathways and, therefore, it may be thought that primary metabolism may not be affected. However, the genetically engineered strains used in this chapter were prepared by protoplast fusion which may have led to non-specific changes in growth and substrate uptake. Overall, the maximum specific growth rate and substrate uptake for all *S. erythraea* strains tested were sensitive to the media composition.

3.9.2 Organic Acid Excretion

Organic acid excretion (pyruvate and α-ketoglutarate) was affected by nitrate-limited media conditions. Under glucose-limitation, the cells did not produce high levels of organic acids due to lack of carbon availability. Under phosphate-limitation, no organic acids were produced most likely due to sufficient carbon and nitrogen. It appears that nitrogen limitation has the greatest effect on organic acid excretion. As nitrate was depleted pyruvate was excreted and then in mid-stationary phase the pyruvate was re-consumed. The pyruvate excretion may be due to a flux imbalance in the glycolysis and TCA cycle where the flux through glycolysis is still high because of sufficient glucose availability and the TCA cycle flux decreases because of nitrate depletion. α-Ketoglutarate was excreted after nitrate was depleted and then generally not re-consumed. The end of the growth phase reduces the need for α-ketoglutarate to be used for assimilation of ammonia and production of glutamine and glutamate. This may support why α-ketoglutarate excretion does occur until growth ceases. In summary, nitrogen depletion from the cell may create an excess of α-ketoglutarate

inside due to reduced requirements by the cell which then excretes the α-ketoglutarate.

This organic acid excretion pattern was observed to varying degrees with different *S. erythraea* strains all grown under nitrate-limited conditions. In general, as the maximum specific growth rate increased the levels of organic acid excretion increased. This is consistent with the flux imbalance hypothesis because faster growth rates would correspond to increased flux through glycolysis. This would cause a greater imbalance if the TCA cycle flux ceases, resulting in higher levels of excess pyruvate and α-ketoglutarate.

3.9.3 Red Pigment Production

Red pigment production was primarily found with nitrate-limited medium. There is about one-fifth the pigment produced in glucose-limited medium compared to nitrate-limited. This occurs presumably because once carbon availability is depleted, the precursors used for secondary metabolite synthesis are not produced and hence secondary metabolite production stops. This is emphasized by the fact that red pigment production is growth associated under carbon-limited conditions and growth unassociated under nitrogen-limited conditions. Lower levels of red pigment are also found with phosphate-limited conditions compared to nitrogen-limited conditions.

Nitrate-limitation may cause high levels of red pigment because *S. erythraea* uses the pyruvate excreted outside the cell to then make red pigment. As discussed above, pyruvate is excreted and then re-consumed. Re-consumption of pyruvate corresponds with red pigment production. Pyruvate is converted to acetyl-CoA which may then be converted to malonyl-CoA. The red pigment may be formed using five malonyl-CoA units. Carbon and phosphate limited condition produce low levels of pyruvate excretion and low levels of red pigment. Pyruvate may be utilized by the cell to make red pigment under nitrate-limited medium.

3.9.4 Erythromycin Production

Erythromycin was produced at very low levels or not produced in the studies presented here. It may be that the minimal media was too low in nutrients to produce erythromycin by the *S. erythraea* wt RV strain. The *S. erythraea* wt WV produces

erythromycin at low levels 5 mg/L - 15 mg/L and little red pigment. It may be that since the red-variant produced gram per liter quantities of red pigment, there are not enough precursor units because of the minimal media to produce the precursors for erythromycin.

3.9.5 Addition of Potential Precursors

The addition of compounds that may be directed to secondary metabolite precursor synthesis did not appear to increase red pigment or erythromycin production. Pyruvate and acetate were added to increase the availability of malonyl-CoA which is used to synthesize red pigment. Neither appeared to influence red pigment production. Propionate, α-ketoglutarate, succinic acid, and valine were added to increase methylmalonyl-CoA which is used to synthesize erythromycin. Propionate had the effect of decreasing red pigment production but it did not increase erythromycin production. Valine added did appear to increase erythromycin production. However, this may be due to valine being utilized as a nitrogen and/or carbon source.

3.9.6 Conclusions

In conclusion, the physiological studies conducted for this thesis focused on the levels of organic acid excretion, red pigment production, and erythromycin production. Particular attention was on the relationship between organic acid excretion and secondary metabolite production. It was found that nitrogen-limited growth conditions had the greatest influence over organic acid excretion and red pigment production. A hypothesis was suggested that organic acid excretion may be caused by metabolic flux imbalance between glycolysis and the TCA cycle where nitrogen-limitation cause a reduction in the flux through the TCA cycle and before the flux through glycolysis can adjust, pyruvate and α-ketoglutarate are excreted from the cell. However, it could also be suggested that α-ketoglutarate is excreted because it is not needed for glutamate formation once nitrate is depleted.

4 Citric Acid Enzyme Activity Analysis

4.1 Summary

The objective of developing enzyme activity assays was to have the ability to study further why pyruvate and α-ketoglutarate are excreted and accumulated outside the cell as seen in several of the experiments described in this thesis (see sections 3.4.3.3, 3.5.3.2, and 3.6.3.2). To this end, activity assays were developed and utilized for pyruvate dehydrogenase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase because these are some of the key enzymes involved in formation or utilization of pyruvate and α-ketoglutarate.

The results showed that the enzyme activities correlated with the levels of pyruvate and α-ketoglutarate excretion. Pyruvate dehydrogenase activity increased in parallel with growth. After growth cessation, pyruvate dehydrogenase activity decreased in parallel with increased pyruvate accumulation outside the cell, and decreased further as pyruvate concentration outside the cell remained constant. Pyruvate dehydrogenase activity appeared to increase again later in stationary phase and pyruvate outside the cell decreased, presumably, because pyruvate was taken up and utilized by the cell.

As isocitrate dehydrogenase activity increased, α-ketoglutarate outside the cell increased. However, little to no α-ketoglutarate dehydrogenase activity could be detected which may explain the high levels of α-ketoglutarate accumulated which was not re-consumed by the cell as with pyruvate. *S. erythraea* was grown on glycerol to assure that glucose was not causing inhibition of α-ketoglutarate dehydrogenase.

The low enzyme activity results of α-ketoglutarate dehydrogenase suggest that this enzyme may be causing the α-ketoglutarate excretion under nitrate-limited conditions. Increasing activity of α-ketoglutarate dehydrogenase may be a useful approach to prevent excretion and perhaps re-channel the carbon to product formation pathways.

4.2 Background

Organic acid excretion could be considered a waste of carbon when the aim is to produce secondary metabolites by Actinomycetes. Understanding how to control, or prevent, organic acid excretion could be the first step in channeling the excess carbon to secondary metabolic pathways. The organic acids produced by *Saccharopolyspora erythraea*, pyruvate and α-ketoglutarate, are products of the TCA cycle. Three reactions that are involved in the consumption or production of pyruvate or α-ketoglutarate are investigated in this chapter.

4.2.1 TCA Cycle

The TCA (tricarboxylic acid) cycle is an important component of primary metabolism. It oxidizes acetyl-CoA to carbon dioxide. Two cycles (oxidation of 2 acetyl-CoA) completely oxides glucose to carbon dioxide. Over the sequence of reactions the cycle also generates the important cofactors, NADH, NADPH , and FADH. In contrast to glycolysis which is a rapid source of a small amount of ATP per glucose, the TCA cycle is a slower but richer source of energy (Voet and Voet, 1990). There are eight enzymes in the TCA cycle which reduce acetyl-CoA to generate energy, and produce several important metabolites that are used in a variety of pathways including amino acid production, nitrogen assimilation, and secondary metabolite production (Voet and Voet, 1990).

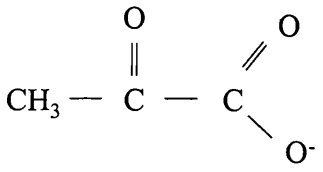
To assure that the cycle continues to run efficiently, there are several regulatory features in place. The main regulatory reactions are considered to be citrate synthase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase. Pyruvate dehydrogenase is also an important regulatory feature which is the reaction that produces acetyl-CoA for entry into the TCA cycle. The results of physiology studies showed that the organic acids, pyruvate and α-ketoglutarate, were excreted from the cell (see Chapter 3). It is interesting that these metabolites are also products or substrates of important regulatory reactions in the TCA cycle. This chapter investigates the enzyme activities of pyruvate dehydrogenase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase to understand what may be controlling or causing organic acid excretion. These three reactions all involve

oxidative decarboxylation and release the three carbons from pyruvate as carbon dioxide.

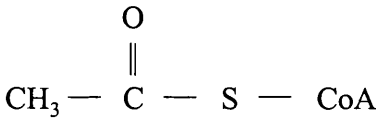
4.2.1.1 Pyruvate Dehydrogenase

Pyruvate dehydrogenase (PYDH) performs oxidative decarboxylation of pyruvate to produce acetyl-CoA.

Pyruvate + CoA + NAD ⟶ **Acetyl-CoA** + CO_2 + NADH



Pyruvate



Acetyl-CoA

Pyruvate dehydrogenase is a multi-enzyme complex composed of pyruvate dehydrogenase (E_1), dihydrolipoate transacetylase (E_2), and dehydrolipoate dehydrogenase (E_3) which catalyze five reactions. It also requires five cofactors and produces carbon dioxide. Pyruvate dehydrogenase, in *E. coli*, is inhibited by high levels of NADH and acetyl-CoA (Gottschalk, 1986). A diagram of the components of the pyruvate dehydrogenase reaction complex is shown in Figure 36 (Gottschalk, 1986).

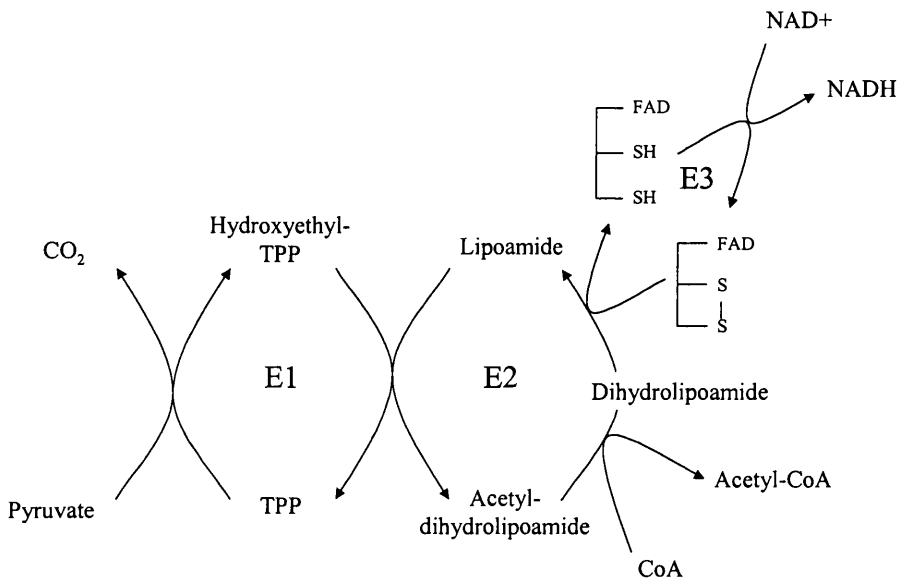
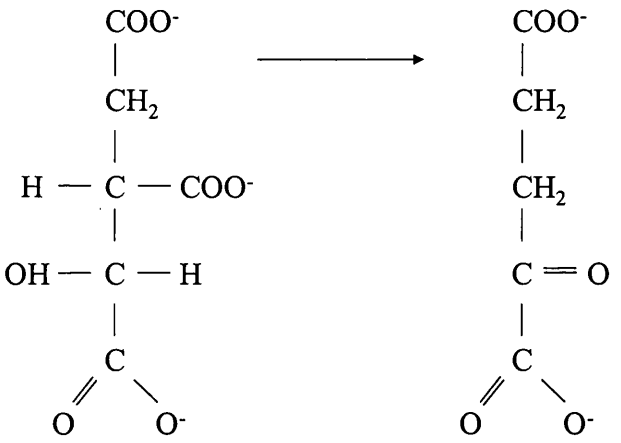


Figure 36: Reactions of the pyruvate dehydrogenase multi-enzyme complex.

4.2.1.2 Isocitrate Dehydrogenase

Isocitrate dehydrogenase (ICDH) performs oxidative decarboxylation of isocitrate to produce α-ketoglutarate. It requires NAD and manganese, and produces one carbon dioxide (Gottschalk, 1986).

Isocitrate + NAD ⟶ **α-Ketoglutarate** + CO_2 + NADH



Isocitrate α-Ketoglutarate

4.2.1.3 α-Ketoglutarate Dehydrogenase

α-Ketoglutarate dehydrogenase (KGDH) oxidatively decarboxylates α-ketoglutarate to succinyl-CoA in a manner very similar to that of pyruvate dehydrogenase. The α-ketoglutarate dehydrogenase is also a multi-enzyme complex made up of three components, α-ketoglutarate dehydrogenase (E1), dihydrolipoyl transsuccinylase (E2), and dihydrolipoyl dehydrogenase (E3). In *E. coli*, the E3 component of α-ketoglutarate dehydrogenase is identical to that of the pyruvate dehydrogenase complex. The complex mechanism is very similar to that illustrated in Figure 36 (Gottschalk, 1986).

α-Ketoglutarate + CoA + NAD → **Succinyl-CoA** + CO_2 + NADH

α-Ketoglutarate Succinyl-CoA

4.3 Materials and Methods

The assay procedure was the same for each enzyme unless otherwise specified. The methods were modified from SIGMA quality control test procedures. The principle of each assay is as follows:

PYDH: Measure formation of Diformazan by the following reaction:

Pyruvate + P_i + mPMS (ox) → PYDH→ Acetyl Phosphate + CO_2 + mPMS (rd)
mPMS (rd) + NTB → 2 mPMS (ox) + Diformazan

where mPMS is 1-Methoxy-5-Methylphenazinium Methyl Sulfate and NTB is Nitro Blue Tetrazolium.

ICDH: Measure formation of β-NADPH by the following reaction:

Isocitrate + β-NADP → ICDH → α-Ketoglutarate + CO_2 + β-NADPH + Mn^{2+}

αKGDH: Measure formation of β-NADH by the following reaction:

α-Ketoglutarate + CoA + β-NAD → αKGDH → Succinyl-CoA + β-NADH + CO_2

4.3.1 Sample Preparation

Each sample taken from the culture to be analyzed for enzyme activity was immediately placed on ice and then the sample was aliquoted into 1 mL tubes for analysis (~ 1 mL per assay). The sample was centrifuged at 13000 RPM for 10 minutes in microcentrifuge. The supernatant was collected and 50 mM TRIS pH 7.4 was added back to pellet to a final volume of 1 mL. The wash step was repeated twice. The pellet was sonicated for 10 seconds "on" and 10 seconds "off" for 8 cycles at 0° C and then frozen at –20° C until all samples from a given experiment had been prepared.

A separate experiment was performed to assure that samples that have been frozen yield the same result as samples that have not been frozen.

4.3.2 Assay Reagents

The reagents for each assay are tabulated in Table 23, Table 24, and Table 25. Each assay reagent was prepared immediately prior to sample analysis as several of the chemicals used in these reagents degrade rapidly.

Table 23: Pyruvate dehydrogenase activity assay reagent.

Reagent	Concentration, mM (except where stated)
Potassium Phosphate	50 .00
Triton X-100	0.05% (v/v)
Pyruvate	50.00
Flavin Adenine Dinucleotide (FAD)	0.01
Thiamine Pyrophosphate	0.20
EDTA	1.00
Magnesium Sulfate	10.00
1-Methoxy-5-methylphenazinium methyl sulfate	0.10
Nitro Blue Tetrazolium	1.00

Table 24: Isocitrate dehydrogenase activity assay reagent.

Reagent	Concentration (mM)
Glycylglycine	67.00
Isocitric Acid	0.44
β-NADP	1.00
Manganese Chloride	0.60

Table 25: α-Ketoglutarate dehydrogenase activity assay reagent.

Reagent	Concentration (mM)
MOPS	50.00
Magnesium Chloride	0.20
Calcium Chloride	0.01
Cocarboxylase	0.30
CoA	0.12
β-NAD	2.00
Cysteine	2.60
α-Ketoglutaric Acid	5.00

4.3.3 Enzyme Activity Measurement and Analysis

For each sample, the specified amount of assay reagent was added to plastic cuvette as indicated in Table 26. The cuvette was placed in spectrophotometer to allow the reagent to equilibrate at specified temperature and wavelength. The sample was then added to the assay reagent and mixed with a pipette. The absorbance was measured as a function of time and the maximum slope is recorded. The specific assay parameters are found in Table 26. A control of purified enzyme was run with each assay.

Table 26: Enzyme activity assay parameters.

	PYDH	ICDH	αKGDH
Temperature	37 °C	37 °C	30 °C
Wavelength	570 nm	340 nm	340 nm
pH	6.3	7.4	7.4
Path Length	1 cm	1 cm	1 cm
Reagent volume	2.7 mL	2.7 mL	2.7 mL
Sample volume	0.3 mL	0.3 mL	0.3 mL

To determine the enzyme activity from the maximum slope, the following equation was used:

Units/mL enzyme = [(Max. slope)*(moles converted)*(V_t)*(Df)]/[(ext. coeff.)*(V_e)]

where moles converted is 2 for PYDH and 1 for ICDH and αKGDH, V_t is the total reaction volume (3 mL), Df is the dilution factor of sample, and V_e is enzyme volume or sample volume (0.3 mL). The extinction coefficient is 43.8 for diformazan at 540 nm (PYDH assay), 6.22 for β-NADPH at 340 nm (ICDH assay), and 6.22 for β-NADH at 340 nm (αKGDH assay).

4.4 Enzyme Activity Analysis of *Saccharopolyspora erythraea* 20 L Fermentation

4.4.1 Objective

The objective of this experiment was to test enzyme activity levels of pyruvate dehydrogenase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase of *S. erythraea* grown under nitrate-limited conditions in a 20L fermentation.

4.4.2 Experimental Conditions

The experimental parameters for testing enzyme activity under nitrate-limited conditions are shown in Table 27.

Table 27: Experimental conditions for testing enzyme activity of *S. erythraea* grown under nitrate-limited conditions.

Strains	*S. erythraea* wt RV (see section 3.3.1)
Media	Nitrate-limited medium (see section 3.3.2.1).
Growth Mode	Batch 20L Fermentation.
Temperature	28° C
Impeller RPM	500
Sampling Times, hours	0, 6, 20, 25, 30, 44, 49, 54, 68, 73, 78, 92, and 116.
Experimental Duplication	Each strain grown with each medium was tested independently two times.

4.4.3 Results and Discussion

A 20 L fermentation of *S. erythraea* wt RV was run under nitrate-limited growth conditions. Figure 37 shows the biomass, substrate uptake, and organic acid excretion data for the fermentation.

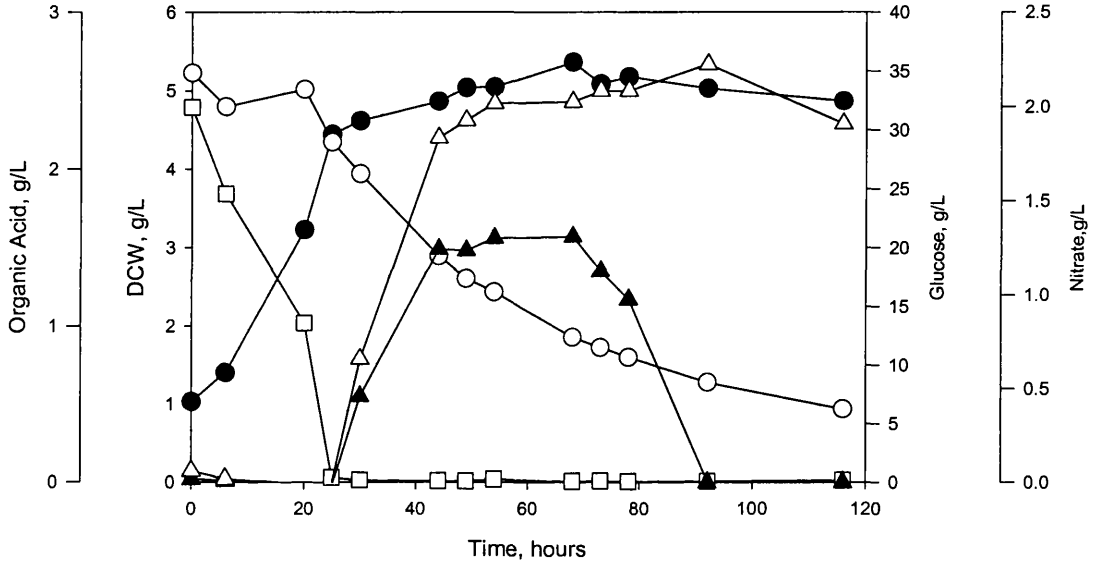


Figure 37: Batch fermentation kinetics for *S. erythraea* wt RV grown under nitrate-limited conditions.

(●) Dry cell weight concentration, (○) Glucose in supernatant, (□) Nitrate in supernatant, (▲) Pyruvate in supernatant, (△) α-Ketoglutarate in supernatant.

Enzyme activities were measured for pyruvate dehydrogenase (converts pyruvate into acetyl-CoA), isocitrate dehydrogenase (converts isocitrate to α-ketoglutarate), and α-ketoglutarate dehydrogenase (converts α-ketoglutarate to succinyl-CoA). Enzyme activity results are shown in Figure 38 and Figure 39.

Figure 38 shows pyruvate accumulation and intracellular pyruvate dehydrogenase activity. Pyruvate dehydrogenase activity increased in parallel with growth. At ~45 hours, after growth cessation, pyruvate dehydrogenase activity decreased in parallel with increasing pyruvate accumulation outside the cell and decreased further as pyruvate concentration outside the cell remained constant. Pyruvate dehydrogenase activity appeared to increase again at ~70 hours and pyruvate outside the cell decreased presumably because pyruvate is taken up and utilized by the cell.

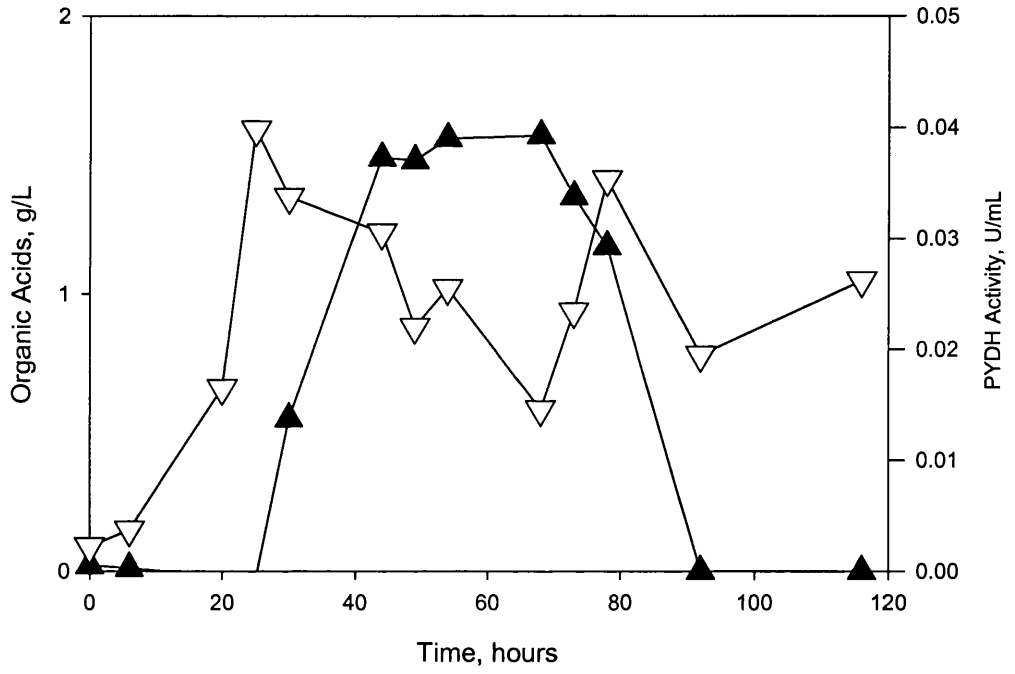


Figure 38: Pyruvate dehydrogenase activity and pyruvate formation for *S. erythraea* wt RV grown under nitrate-limited conditions.

(▲) Pyruvate in Supernatant and (▽) Pyruvate Dehydrogenase Activity.

Figure 39 shows α-ketoglutarate accumulation outside the cell and the enzyme activities of isocitrate dehydrogenase and α-ketoglutarate dehydrogenase. As isocitrate dehydrogenase activity increased, α-ketoglutarate outside the cell increased. However, no α-ketoglutarate dehydrogenase activity could be detected which may explain the high levels of α-ketoglutarate accumulation, which was not re-consumed by the cell as with pyruvate.

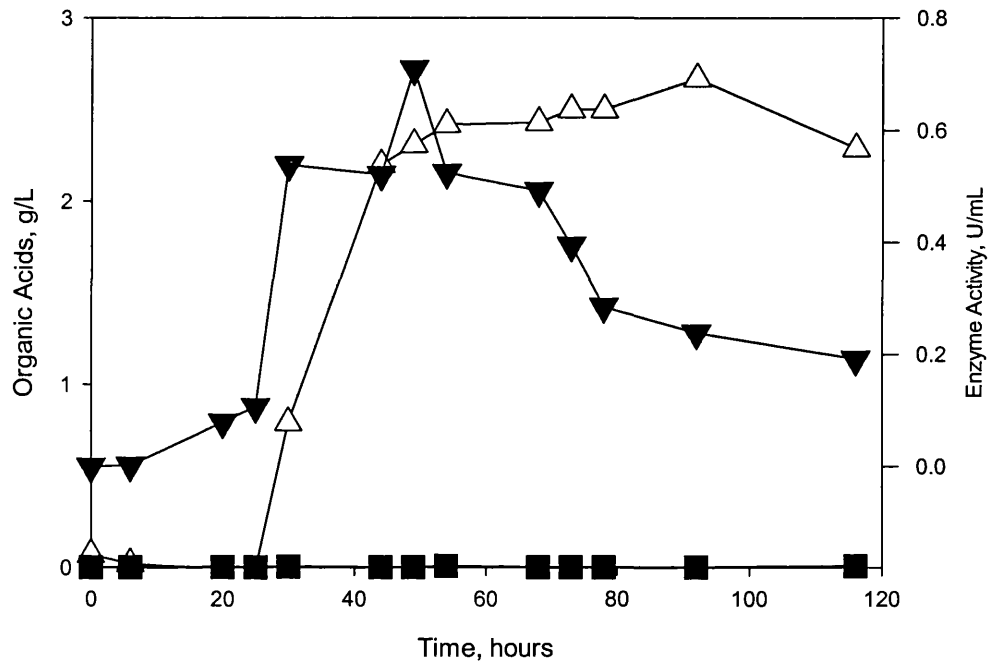


Figure 39: α-Ketoglutarate dehydrogenase activity, isocitrate dehydrogenase activity, and α-ketoglutarate accumulation for *S. erythraea* wt RV grown under nitrate-limited conditions.

(△) α-Ketoglutarate in Supernatant, (▼)Isocitrate Dehydrogenase Activity, and (■) α-Ketoglutarate Dehydrogenase Activity.

These results suggested a correlation between the enzyme activity of PYDH and ICDH and the levels of pyruvate and α-ketoglutarate excreted by the cell. Re-channeling the carbon back into the TCA cycle may increase availability of polyketide precursors which are derived from acetyl-CoA (product of pyruvate dehydrogenase) and succinyl-CoA (product of α-ketoglutarate dehydrogenase). By increasing α-ketoglutarate dehydrogenase activity, higher levels of succinyl-CoA may be produced which could be used to make methylmalonyl-CoA and propionyl-CoA which are direct precursors for erythromycin biosynthesis.

4.5 Effect of Carbon Source and Carbon to Nitrogen Ratio on Enzyme Activity

4.5.1 Objective

To determine if glucose had a repressive effect on α-ketoglutarate dehydrogenase activity the physiological characteristics of *S. erythraea* wt RV grown on glucose was compared to growth on an alternative carbon source (glycerol) at C/N of 45 and 15.

4.5.2 Experimental Conditions

The experimental conditions for this study are shown in Table 28. The media was designed to study two carbon sources (glucose and glycerol) and two C/N (45 and 15).

Table 28: Experimental conditions for testing enzyme activity of *S. erythraea* grown on glucose or glycerol.

Strains	*S. erythraea* wt RV (see section 3.3.1)
Media	Nitrate-limited medium with following changes: 1) no change, 2) 30 g/L glycerol and no glucose, 3) 5.7 g/L nitrate, 4) 30 g/L glycerol and no glucose and 5.7 g/L nitrate.
Growth Mode	Batch 2L Shake-Flask
Temperature	28° C
Shaker RPM	200
Sampling Times, hours	0, 24, 48,72, 96, and 144.
Experimental Duplication	Each strain grown with each medium was tested once.

4.5.3 Results and Discussion

The maximum biomass, maximum specific growth rate, maximum specific carbon and nitrate uptake rate, and maximum specific red pigment production are shown in Table 29. In general, the trends between C/N = 45 and C/N = 15 with glucose as the carbon source were similar to results obtained in previous experiments 3.4 on the effect of nitrate on red pigment and organic acid production. The results also showed that no erythromycin was produced and there was no α-ketoglutarate dehydrogenase enzyme activity. This indicated that glucose repression did not appear to be occurring with respect to enzyme activity or erythromycin production.

Table 29: Physiological data for *S. erythraea* grown on glucose or glycerol.

C/N	Carbon Source	Max. Biomass, g/L	Max. Specific Growth Rate, h^{-1}	Max. Specific Carbon Uptake, g/g DCW	Max. Specific Nitrate Uptake, g/g DCW	Max Specific Red Pigment, g/g DCW
45	Glucose	4.704	0.023	0.061	0.009	0.359
45	Glycerol	4.964	0.013	0.024	0.005	0.188
15	Glucose	9.509	0.031	0.050	0.013	0.137
15	Glycerol	9.939	0.022	0.028	0.009	0.169

The maximum biomass reached the same concentration for glucose and glycerol for the same C/N with about double the level of biomass for C/N = 15 compared to C/N = 45. The maximum specific growth rate was greater for C/N = 15 than C/N = 45 and greater for glucose than for glycerol. Furthermore, the maximum specific glucose uptake rate was greater for C/N = 45 than C/N = 15. Whereas, the maximum specific glycerol uptake rate was about the same for each C/N = 45 and 15. Maximum specific nitrate uptake rate was greater for C/N = 45 than C/N = 15 and greater for glucose than for glycerol. With glucose as the carbon source, more red pigment was produced at C/N = 45 than C/N = 15. With glycerol as the carbon source, about the same levels of red pigment were produced, which were about the same level as the glucose C/N = 15.

Maximum specific pyruvate excretion was greater for C/N = 45 than C/N = 15 as shown in Figure 40. This could be due to an imbalance in flux between the TCA cycle and glycolysis when the low levels of nitrate become limiting. For the C/N =15 the nitrate levels are higher compared to the C/N = 45 and, therefore, more of the carbon source was consumed before the nitrate becomes limiting. This may be reflected in lower amounts of pyruvate excretion.

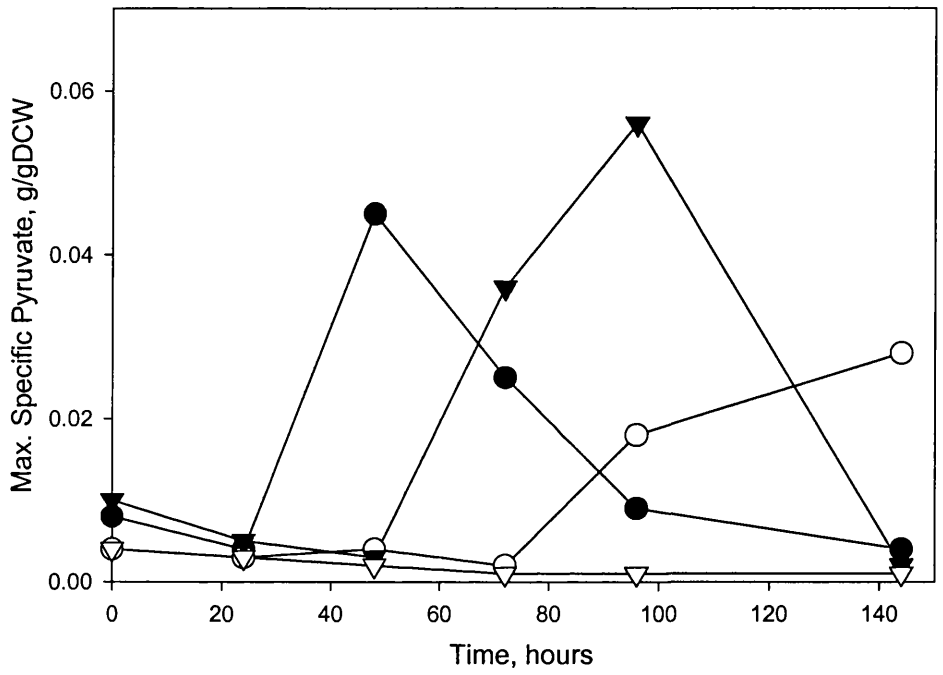


Figure 40: Maximum specific pyruvate excretion for *S. erythraea* grown on glucose or glycerol at C/N of 45 or 15.

(●) C/N = 45 and carbon source is glucose, (○) C/N = 45 and carbon source is glycerol, (▼) C/N = 15 and carbon source is glucose, (▽) C/N = 15 and carbon source is glycerol.

Pyruvate dehydrogenase activity was higher for *S. erythraea* grown on glucose than on glycerol as shown in Figure 41. The pyruvate dehydrogenase activity was higher for the C/N = 45 compared to the C/N = 15. This is the same trend observed for pyruvate excretion. The activity may be increased in response to excess pyruvate.

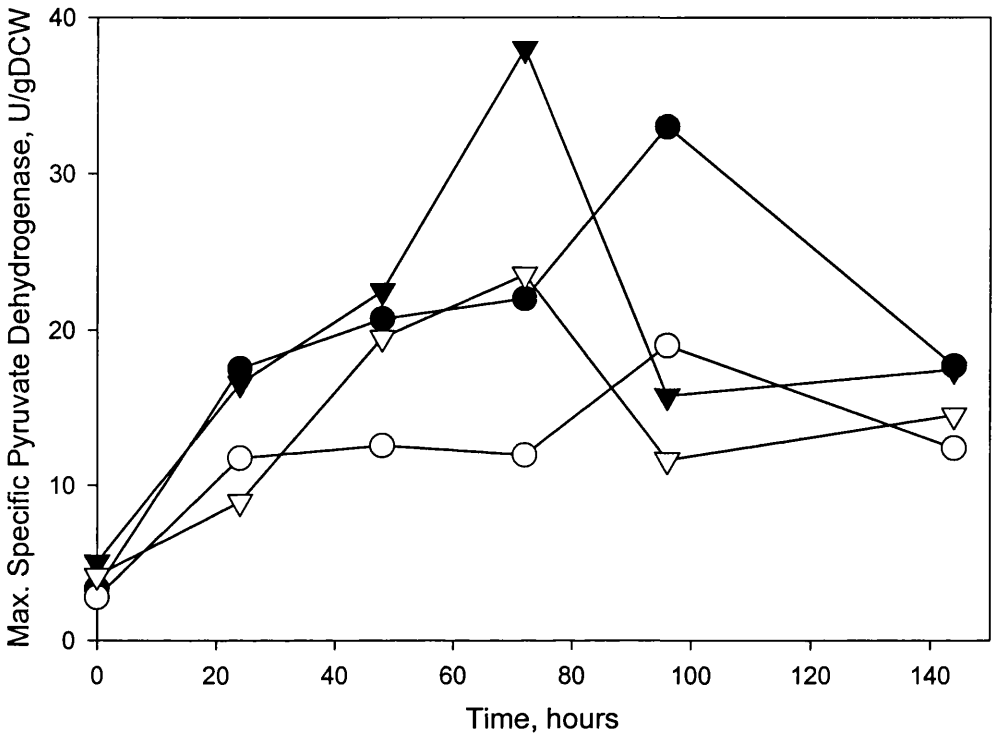


Figure 41: Maximum specific pyruvate dehydrogenase activity for *S. erythraea* grown on glucose or glycerol at C/N of 45 or 15.

(●) C/N = 45 and carbon source is glucose, (○) C/N = 45 and carbon source is glycerol, (▼) C/N = 15 and carbon source is glucose, (▽) C/N = 15 and carbon source is glycerol.

The maximum specific α-ketoglutarate excretion was greater for C/N = 45 than C/N = 15 and was greater for glucose than for glycerol as with pyruvate excretion as shown in Figure 42. Furthermore, greater levels of α-ketoglutarate were produced at C/N = 45 than C/N = 15 with *S. erythraea* wt RV grown on glucose.

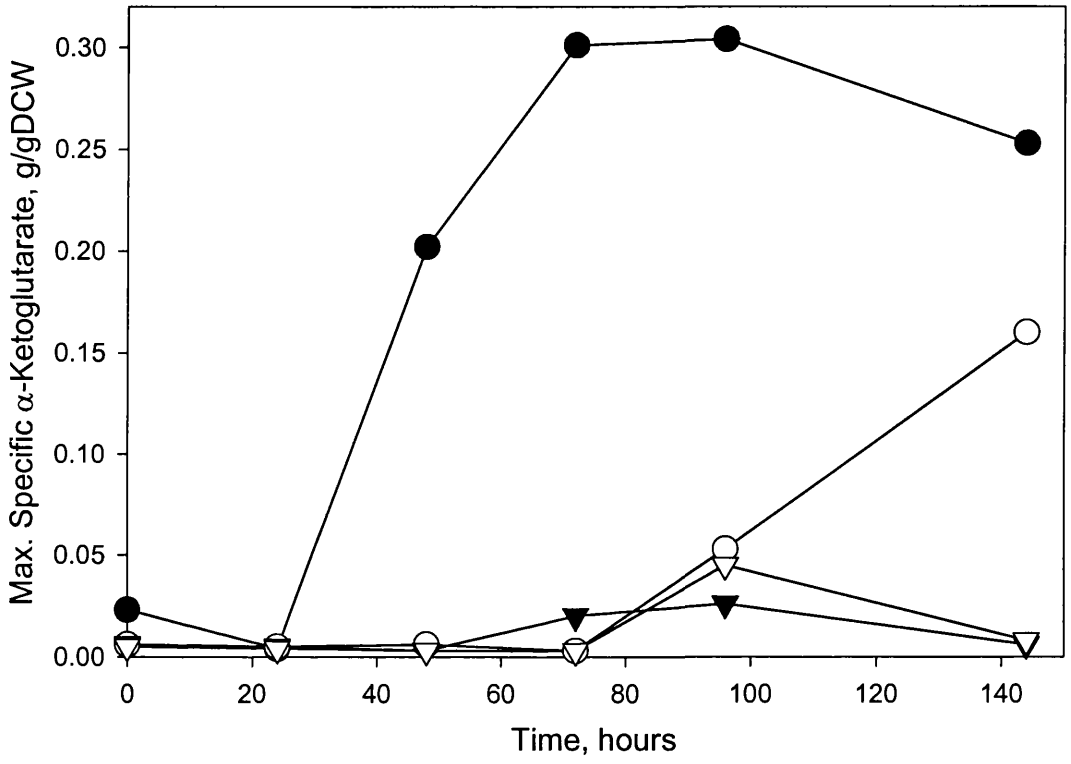


Figure 42: Maximum specific α-ketoglutarate excretion for *S. erythraea* grown on glucose or glycerol at C/N of 45 or 15.

(●) C/N = 45 and carbon source is glucose, (○) C/N = 45 and carbon source is glycerol, (▼) C/N = 15 and carbon source is glucose, (▽) C/N = 15 and carbon source is glycerol.

Isocitrate dehydrogenase activity was about 3-fold greater for *S. erythraea* wt RV grown on glucose than on glycerol as seen in Figure 43. No α-ketoglutarate dehydrogenase activity was detected for either glucose or glycerol as the carbon source suggesting that the enzyme is not suppressed by the carbon source.

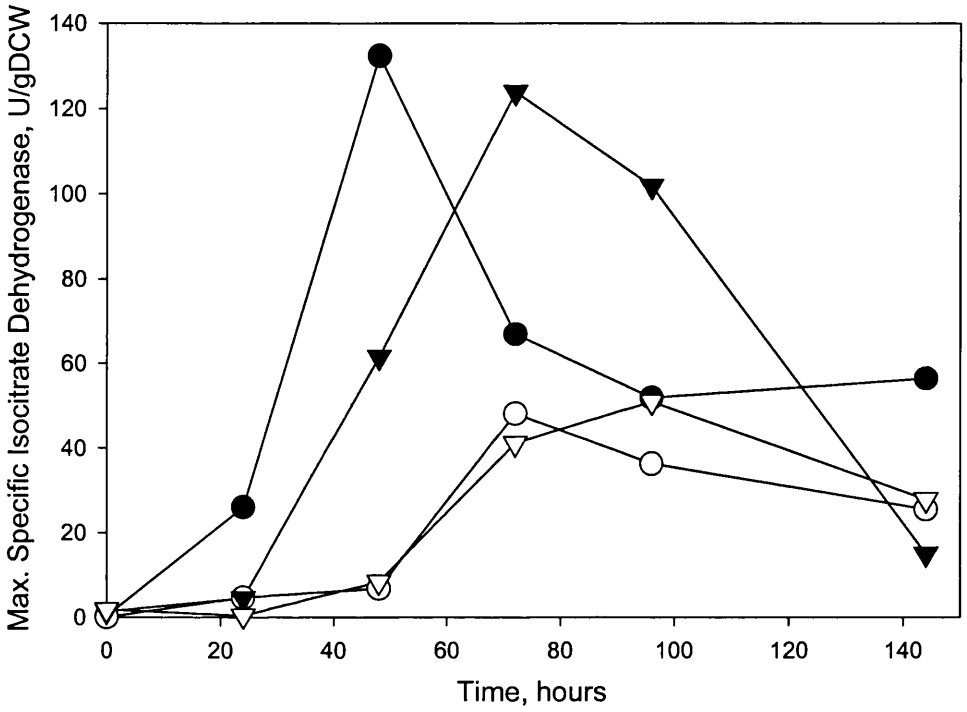


Figure 43: Maximum specific isocitrate dehydrogenase activity for *S. erythraea* grown on glucose or glycerol at C/N of 45 or 15.

(●) C/N = 45 and carbon source is glucose, (○) C/N = 45 and carbon source is glycerol, (▼) C/N = 15 and carbon source is glucose, (▽) C/N = 15 and carbon source is glycerol.

Isocitrate dehydrogenase activity peaked as α-ketoglutarate excretion increased. Once nitrate limitation occurs, it appears that the demand for α-ketoglutarate decreased. Nitrate-depletion correlated with increasing levels of α-ketoglutarate excretion, which may have been caused by a reduced need for α-ketoglutarate to the nitrogen assimilation pathways (data not shown).

4.6 Modified α-Ketoglutarate Dehydrogenase Activity Assay

The results from the experiments described above did not yield measurable α-ketoglutarate dehydrogenase activity. This could have been because the lower limit of detection of the assay was not sensitive enough to the levels of activity in the culture, or there may not have been activity to measure. To determine if the assay was indeed not sensitive enough to detect the levels of activity found in *S. erythraea* grown under nitrate-limiting conditions, several changes were made to the procedure for α-ketoglutarate dehydrogenase activity measurement as outlined in section 4.3.

The most significant change made to the existing procedure was that a 300 mL culture was grown and the cell concentrated to provide a larger signal if in fact there was low levels of activity present. A second precaution was to harvest, prepare, and assay the cells in the same day as culture harvest to prevent loss of activity during freeze thaw process if in fact this was occurring.

This approach was indeed successful and α-ketoglutarate dehydrogenase activity was measured. The α-ketoglutarate dehydrogenase activity of *S. erythraea* grown under nitrate-limited condition at 48 hours was measured to be 7.4 +/- 0.065 nmol/min/mg protein. The specific activity values of other organisms are listed in Table 30. Even though these organisms are different from *Saccharopolyspora spp.*, the values are within the range of reported activities which provides a level of confidence that the measurements of *S. erythraea* are reliable. It is also reassuring that all of the specific activities documented are in the nmol/minute/mg protein range. The specific activities of other enzymes in the same pathways as α-ketoglutarate dehydrogenase, such as isocitrate dehydrogenase, which is the enzyme that produces α-ketoglutarate, is generally in the μmol/minute/mg protein range.

Table 30: Specific α-ketoglutarate dehydrogenase activity for a range of organisms.

Organism	**Specific α-KGDH activity, nmol/min/mg prot**	**Reference**
Saccharopolyspora erythraea wt RV	7.4 +/- 0.065	this work
Rhizobium leguminosarum	109 +/-10	(Walshaw *et al.*, 1997)
Pseudomonas fluorescens	34	(Hamel and Appanna, 2001)
Bradyrhizobium japonicum	46 +/- 7.6	(Green *et al.*, 2000)
Bradyrhizobium japonicum	69 +/- 12.2	(Green and Emerich, 1997)
Mesorhizobium loti R7A	22.7	(Green *et al.*, 2000)

The modified assay procedure was used for subsequent experiments as presented in section 7.3.3.

4.7 Discussion and Conclusions

Organic acid excretion appeared to be controlled, at least to some extent, by the activity of enzyme consuming or producing the metabolite of interest. As pyruvate was excreted and re-consumed, pyruvate dehydrogenase activity appeared to be increasing and decreasing in a manner correlating with pyruvate excretion. Similarly, as isocitrate dehydrogenase activity increased, α-ketoglutarate excretion began to increase and then remained outside the cell and isocitrate dehydrogenase activity continued to decrease for the remainder of the growth cycle. α-Ketoglutarate dehydrogenase activity was orders of magnitude lower than that of isocitrate dehydrogenase which may explain why α-ketoglutarate was excreted to higher levels than other organic acids, and was not re-consumed. Non-detectable levels of α-ketoglutarate dehydrogenase activity has also been found with *S. venezuelae*, and α-ketoglutarate excretion was occurred with the loss of activity (Ahmed *et al.*, 1984).

When the pyruvate dehydrogenase, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase activities were compared on a per volume (or per g DCW) basis, it was found that α-ketoglutarate dehydrogenase activity was three orders of magnitude less than isocitrate dehydrogenase activity and two orders of magnitude less than pyruvate dehydrogenase activity as shown in Table 31.

Table 31: Comparison of enzyme activities for pyruvate dehydrogenase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase of *S. erythraea* at 48 hours grown under nitrate-limited conditions.

Enzyme	U/mL (μmol/min/mL)	Normalized Activity
Pyruvate dehydrogenase	0.04	67
Isocitrate dehydrogenase	0.6	1000
α-Ketoglutarate dehydrogenase	0.0006	1

The results shown in Table 31 allow a model to be proposed of α-ketoglutarate excretion based on enzyme activity results on α-ketoglutarate excretion physiology data. These values are illustrated in Figure 44 to provide a visual interpretation of why α-ketoglutarate was perhaps excreted. Isocitrate dehydrogenase activity was high and produces high levels of α-ketoglutarate. The low activity of α-ketoglutarate dehydrogenase caused an over flow of α-ketoglutarate which was then excreted outside the cell as waste. Pyruvate excretion was not included because pyruvate was re-consumed by the cell and the focus here was to understand why carbon was lost outside the cell for the potential of re-channeling the carbon to product formation.

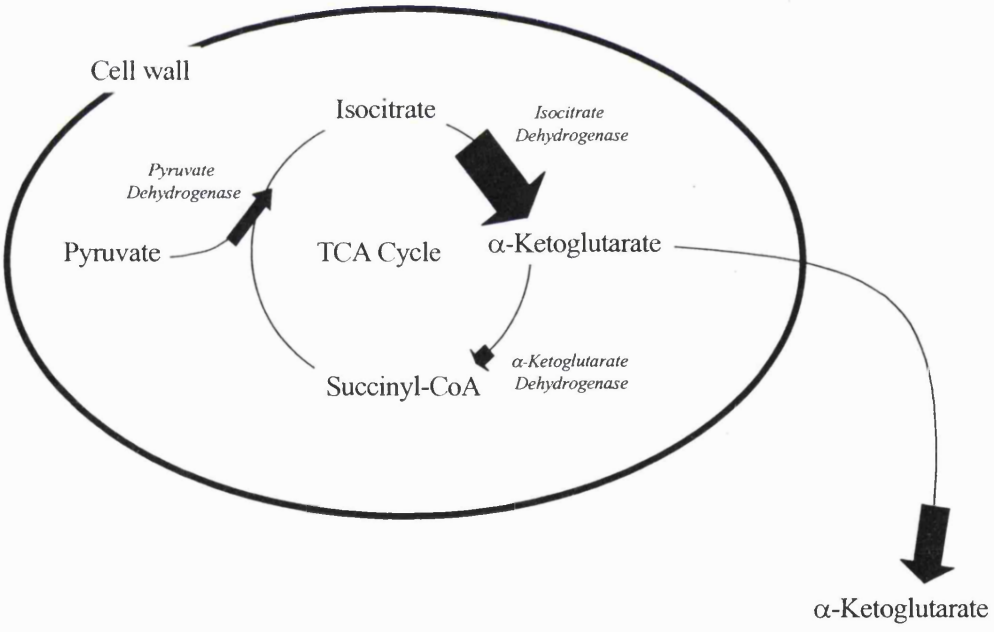


Figure 44: Illustration of α-ketoglutarate excretion model.

It is important to note that α-ketoglutarate excretion occurs as a result of nitrate-depletion and hence the end of the growth phase. This prevents the need for α-ketoglutarate to be used for assimilation of ammonia and production of glutamine and glutamate which supports why α-ketoglutarate excretion does occur until growth ceases.

There are two main approaches that could be utilized to try to reduce α-ketoglutarate excretion. The first is to modify the medium so that nitrate is not limiting and, therefore, excretion is prevented. The second approach is to try to increase the enzyme activity of α-ketoglutarate dehydrogenase. Physiological experiments have been performed to address the approach of media modification as described previously in sections 3.5 and 3.7.2. The results showed that the increased nitrate or non-nitrate-limited conditions prevented high levels of α-ketoglutarate excretion and that the carbon either went to biomass or red pigment production. A fed-batch strategy where low levels of nitrate are fed may also serve to prevent organic acid excretion. It may also be valuable to modify the enzyme activity so that the conditions could be kept such that the carbon is not excreted by the cell and, therefore, available for product production. This approach is described further in Chapters 6 and 7.

This chapter described results of enzyme activities that are critical for the better understanding pyruvate and α-ketoglutarate excretion. The data presented here together with the physiological data from Chapter 3 and metabolic modelling predictions presented in Chapter 5 provide the basis for the formation and testing of a hypothesis that connects organic acid excretion with secondary metabolite production.

5 Metabolic Flux Analysis of *Saccharopolyspora erythraea*

5.1 Summary

There have been several approaches implemented to model biochemical behavior. These approaches have evolved from modelling simple growth kinetics to complex metabolic networks. This chapter describes the metabolic modelling of *Saccharopolyspora erythraea*. Metabolic flux analysis was the approach chosen for the purpose of this thesis because of the large network involved and the lack of kinetic data for *S. erythraea*. There have been five examples in the literature that model Streptomycetes metabolism by metabolic flux analysis, however, this is the first time, to the best of our knowledge, with *S. erythraea*.

A reaction network was constructed that comprises the pathways of primary metabolism, erythromycin biosynthesis, red pigment biosynthesis, biomass formation, and maintenance energy. Sensitivity analysis was performed and revealed that the network was more sensitive to changes to glucose uptake, nitrate uptake, specific growth rate and oxygen consumption compared to changes in α-ketoglutarate, pyruvate, and carbon dioxide production. It also revealed that erythromycin production was sensitive to changes in flux to the TCA cycle.

Metabolic flux analysis was performed to compare differences between *S. erythraea* wild-type and *S. erythraea* Δ*eryA*. Flux spilt ratios were used to examine how the flux to the pentose phosphate pathway changes from growth to stationary phase. It was also used to examine how excretion of α-ketoglutarate affects the flux split ratio between TCA cycle reactions.

Metabolic flux analysis was also used to determine how changes in flux from α-ketoglutarate to succinyl-CoA affect flux to both erythromycin and red pigment. The results of this analysis identified α-ketoglutarate dehydrogenase as a metabolic engineering target with the intent to re-channel carbon, lost to α-ketoglutarate

excretion, back into primary metabolism and subsequently to secondary metabolite formation.

5.2 Background

Approaches to describe cellular metabolism are broadly divided into the two categories of structured and unstructured models. Unstructured models include kinetic models such as the Monod model which is based on Michaelis-Menton type enzyme kinetics (Monod, 1942). This model has been useful in characterizing growth when one substrate is limiting. Improvements have been made on the Monod model by including terms for maintenance (non-growth associated substrate consumption), growth on multiple substrates, and growth inhibition (Herbert, 1959; Pirt, 1964; Tsao and Hanson, 1975; Han and Levenspiel, 1988). In general, the unstructured models treat all cellular components as biomass which is represented and modelled as one reaction and can be adequate for steady state conditions.

Structured models include qualitative elements of cellular behavior. Generally they provide a good frame work where details can be added to represent as closely as possible biochemical system. Simple structured models include compartments where cell components of similar function are grouped (Nielsen and Villadsen, 1992). Cybernetic models have been used to simulate growth on multiple substrates (Kompala *et al.*, 1986). Comprehensive single cell models have been developed but only for well characterized organism such as *E. coli* (Shuler *et al.*, 1979; Domach *et al.*, 1984). These models have since been simplified and shown not to lose the main features of the comprehensive model (Palsson and Joshi, 1987; Joshi and Palsson, 1988).

There are four main approached to modelling metabolic pathways which are 1) kinetic modelling, 2) metabolic control analysis, 3) biochemical systems theory and, 4) metabolic flux analysis. Kinetic modelling has been shown to be an acceptable approximation of enzyme kinetics. However, the enzyme analysis is usually performed *in vitro* and may not represent what is happening inside the cell. The kinetic equations are not amenable for large number of reactions but can be useful for small systems if the kinetic data is available (Malmberg and Hu, 1991). Metabolic control analysis (MCA) has been used for determining the metabolic regulation at a

steady state (Kacser and Burns, 1973; Heinrich and Rapoport, 1974). This analysis can be useful for small systems but tends not to be predictive for environments that differ largely from the original operating point. For unbranched pathways, metabolic control analysis can provide a good assessment of the control of enzyme reactions (Fell, 1992; Kacser *et al.*, 1995). Biochemical systems theory elucidate which enzymes in the pathway have a significant effect on the pathway flux (Savageau, 1970). It, thereby, highlights the relative importance of different enzymes and regulatory mechanisms. However, it is mathematically more complicated in that it incorporates non-linear elements into the model. The final approach to modelling metabolic pathways is metabolic flux analysis. This is the approach chosen for this thesis and is described in more detail in the sections below.

5.2.1 Metabolic Flux Analysis

Metabolic flux analysis (MFA) is a method used to evaluate the flow of carbon through a metabolic network. The intracellular fluxes are calculated using a stoichiometric model and a set of extracellular measured fluxes. MFA is a linear model and can accommodate large numbers of reactions which kinetic modelling and MCA would have difficulty managing. The stoichiometric model is assembled by applying mass balances around the metabolites. MFA can provide insight into many areas of metabolism including the following: 1) quantification of pathway fluxes, 2) identification of branch point control, 3) identification of alternative pathways, and 4) calculation of maximum theoretical yields (Stephanopoulos *et al.*, 1998).

MFA is based on metabolite mass balances in the form of a stoichiometric model. Each metabolite is designated as a substrate, metabolic product, biomass constituent, or intracellular metabolite. A substrate is defined as a compound found in the medium that can be metabolized by the cell. A metabolic product is defined as a compound produced by the cell that is excreted to the extracellular medium. Biomass constituents are defined as macromolecules that make up the composition of biomass, including proteins, RNA, lipids, carbohydrates, etc. And intracellular metabolites are defined as all other compounds found inside the cell (Stephanopoulos *et al.*, 1998).

One advantage of using MFA is that kinetic information, which is difficult to obtain, is not necessary to calculate the internal fluxes. However, the disadvantages are that

no dynamic and regulatory information is obtained. MFA is a useful tool, and has been applied to a metabolic network of *S. erythraea* to study carbon flow to the precursors of polyketide synthesis, in the work described in this report.

5.2.1.1 Metabolic Flux Analysis of Streptomycetes Organisms

There are five examples of metabolic flux analysis with Streptomycetes organisms in the literature. In chronological order, the first was a theoretical study with *S. lividans* which reported that biomass fluctuations up to 20% did not significantly impact flux distribution, while oxygen utilization had the greatest impact in when grown on glucose and animo acids in batch culture (Daae and Ison, 1999). The second example used physiology data, generated by Melzoch *et. al.* (1997), from *S. coelicolor* grown on various nutrient limitations in chemostat culture to model the flux distribution (Naeimpoor and Mavituna, 2000). The results showed that nitrogen-limitation produced the highest levels of actinorhodin (secondary metabolite product), however, since the flux to actinorhodin was very low compared to primary metabolism fluxes, changes in flux to actinorhodin had no impact on flux distribution. The third example was with *S. noursei*, grown in batch culture, which reported on metabolic flux analysis using data from GC-MS measurements of labeled carbon substrate (Jonsbu *et al.*, 2001). The results showed that changes in primary and secondary metabolism are coordinated in that changes in fluxes to TCA cycle and pentose phosphate pathway increased or decreased according to growth rate and nystatin (secondary metabolite product) production. The fourth example examined *S. lividans* in chemostat culture under different dilution rates and different carbon sources (Rossa *et al.*, 2002). The results showed that increased growth rates increased flux though glycolysis and the pentose phosphate pathway. The results also showed that decreased flux through the pentose phosphate pathway correlated with increased synthesis of secondary the metabolites actinorhodin and undecylprodigiosin. The fifth example is a theoretical analysis of *S. lividans* grown under various carbon sources in batch culture (Bruheim *et al.*, 2002). This study focuses on the availability of NADPH and actinorhodin production. They conclude that NADPH is in excess for the actinorhodin levels observed. In a subsequent study, it was shown that if flux through the pentose phosphate pathways was reduced antibiotic levels increased (Butler *et al.*, 2002). It was suggested that decreased flux through the pentose phosphate pathway increased flux through glycolysis which led to increased product formation. However, if total

flux to the pentose phosphate was eliminated then lower levels of antibiotic were produced.

Evident in these examples of metabolic flux analysis of Streptomycetes organisms is the difficulty of studying the impact of primary metabolism flux distribution on secondary metabolite production. This is caused by the fact that typically the flux to secondary metabolite products are much lower than primary metabolism fluxes. It should also be noted that only, very recently, the last of the studies discussed above tested the model prediction by performing the genetic engineering believed necessary to increase secondary metabolite production (Bruheim *et al.*, 2002; Butler *et al.*, 2002). In the work presented here, we have studied the flux to the precursors of secondary metabolism as markers for the potential increase in secondary metabolite formation. This has enabled us to work with higher flux values as we will have been working with primary metabolism which is likely to confer higher confidence in flux distribution results.

5.3 Materials and Methods

5.3.1 *S. erythraea* Fermentations

5.3.1.1 Media

The fermentation experiments were performed with defined minimal media as described previously (McDermott *et al.*, 1993). Two molar sulfuric acid and two molar sodium hydroxide was used to control pH. The media components and concentrations used for the fermentation experiments were identical to the nitrogen-limited medium described in section 3.3.2.1 with the exception that no MOPS was added to the fermentation medium.

5.3.1.2 Growth Conditions

Each fermentation experiment was prepared in the same manner and run under the same conditions. One 1mL liquid stock was used to inoculate 45 mL of nutrient broth autoclaved in a 500 mL baffled flask. The nutrient broth culture was allowed to incubate at 28°C for 48 hours in a rotary shaker. Forty mL of the nutrient broth was used to inoculate ~360 mL of defined media autoclaved in a 2 L baffled flask. The culture was allowed to incubate at 28°C for 48 hours in a rotary shaker. The entire

contents of the defined medium culture was used to inoculate a 7L fermenter, which had been previously sterilized, to bring the working volume to 5L. The fermentations were run at pH 7, 28°C, 5 L/minute of air (1 vvm), and a 500 rpm agitation rate. Fifteen samples were aseptically removed from the fermenter over the course of the fermentation. The dry cell weight of each sample was determined, then the sample was immediately centrifuged, and the supernatant was removed from the solids and stored at -20°C until further analysis. Oxygen and carbon dioxide were measured on-line by mass spectrometry. Glucose and nitrate uptake, organic acid production, erythromycin production, and red pigment intensity were measured off-line as described in section 3.3.3.

5.3.2 Data Preparation for Metabolic Flux Analysis

After the fermentation data was collected and analyzed it was then necessary to calculate the fluxes. The fluxes determined from the fermentation data were used as the measured fluxes vector ($\mathbf{v}_m$) in the MFA calculations. There were three steps in preparing the measured flux vector. These steps are 1) to convert data to appropriate units, 2) to partition data into phases of linear change, and 3) to calculate the slope or flux for each measured variable for each phase.

The first step was to convert the data in to useful units. The choice of units was dependent on the goal of the analysis, however, typical units are millimole per gram of dry cell weight (mmol/g DCW). Another choice may be on a per carbon basis such as millimole carbon per gram dry cell weight (mmol C/g DCW). These converted data were then plotted versus time.

The next step was to partition the data into phases where all variables are changing linearly. This was critical to the analysis because of the pseudo steady-state assumption which assumes that the metabolite pools are not accumulating. The plotted data could help determine where to partition data. This was necessary because the change in each variable must be linear in each phase. For instance, one phase boundary would be at the change from growth to stationary phase. The slope of the biomass changes at this point and therefore the data cannot be included in the same phase. Figure 46 and Figure 47 in section 5.5.1.1 show examples of how data were partitioned. The phases are indicated in roman numerals above the graph.

Once the data were partitioned in to phases, the flux of each variable for each phase was calculated. This was simply the slope of the line in a particular phase. The resulting flux units used were millimole per g dry cell weight per hour (mmol/g DCW/hour). For example, if there were 4 measured variables and 4 phases then there will be 16 measured fluxes in total. The MFA is performed for each phase where the 4 measured fluxes were used to constrain the matrix. The 4 phases could then be compared to determine how the internal fluxes changed due to changes in growth, substrate uptake, product formation, etc.

5.3.3 Calculations for Metabolic Flux Analysis

Metabolic flux analysis (MFA) is based on metabolite mass balances in the form of a stoichiometric model. To perform MFA, a stoichiometric model must be constructed by arranging the stoichiometric coefficients of each metabolite of each reaction into a matrix. A matrix is constructed for the substrates (S), products (P), biomass constituents (M), and intracellular metabolites (X) the sum of which is equal to 0. Equality to zero is specified based on the assumption that the metabolite pools are not accumulating and are therefore at a pseudo-steady state. In matrix notation the stoichiometry for all cellular reactions is

$$\mathbf{AS} + \mathbf{BP} + \Gamma\mathbf{M} + \mathbf{GX} = 0 \qquad (1)$$

where the matrices **A**, **B**, Γ, and **G** are the stoichiometric matrices containing the coefficients of the J reactions for the substrates, products, biomass constituents, and intracellular metabolites, respectively. In the matrices the rows represent the reaction and the columns represent the metabolites. Therefore, the element is the *j*th row and the *i*th column of **A** specifies the stoichiometric coefficient for the *i*th substrate in the *j*th reaction (Stephanopoulos *et al.*, 1998).

The stoichiometry defined above describes the relative amounts of the metabolites produced or consumed for each reaction. However, it does not provide information about the reaction rates. Reaction rates can be coupled to the stoichiometry to obtain the overall rate of product secretion. The rate of a reaction is defined as v such that a metabolite with stoichiometric coefficient B is formed at the rate of Bv. Each reaction

rate of the J reactions are composed into the rate vector **v**. Therefore, $B_{ij}v_i$ specifies the specific rate of formation r of the *i*th metabolic product in the *j*th reaction. In matrix notation the specific rates of formation can be described as

$$\mathbf{0} = \mathbf{r}_s = -\mathbf{A}^T\mathbf{v} \quad (2)$$

$$\mathbf{0} = \mathbf{r}_p = \mathbf{B}^T\mathbf{v} \quad (3)$$

$$\mathbf{0} = \mathbf{r}_m = \Gamma^T\mathbf{v} \quad (4)$$

$$\mathbf{0} = \mathbf{r}_x = \mathbf{G}^T\mathbf{v} \quad (5).$$

The objective of MFA is to calculate the intracellular rate vector **v** to determine the intracellular pathway fluxes represented by equation (5). If J is the number of reactions and K is the number of metabolites, then the number of degrees of freedom F is J – K. In the case described here, the degrees of freedom is a positive number. Therefore, some elements of **v** must be measured, e.g. glucose uptake rate, product formation rate, etc., in order to solve all internal fluxes for the system. If the number of measurements is equal to F then the system is determined, greater than F the system is overdetermined, and less than F the system is underdetermined.

If the system is determined or overdetermined, **v** can be solved using linear algebra. First, the matrix **G** and the rate vector **v** must be partitioned into calculated and measured portions yielding the equation

$$\mathbf{0} = \mathbf{G}^T\mathbf{v} = \mathbf{G}_m{}^T\mathbf{v}_m + \mathbf{G}_c{}^T\mathbf{v}_c \quad (6).$$

In an overdetermined system, as the one used in this study, $\mathbf{G}_c$ is non-square and the pseudo-inverse ($^{\#}$) must be employed to solve $\mathbf{v}_c$

$$\mathbf{v}_c = -(\mathbf{G}_c{}^T)^{\#}\,\mathbf{G}_m{}^T\mathbf{v}_m \quad (7)$$

where the pseudo inverse of $(\mathbf{G}_c{}^T)^{\#}$ is given as

$$(\mathbf{G}_c{}^T)^{\#} = (\mathbf{G}_c\mathbf{G}_c{}^T)^{-1}\mathbf{G}_c \quad (8).$$

Equation (7) represents the least squares estimate for the non-measured fluxes. It is required that the system of equations are linearly independent in order to solve $\mathbf{v}_c$ for both determined and overdetermined systems. Linear independent means that the matrix $\mathbf{G}_c$ must be of full rank or that the determinant is nonzero.

For metabolic flux analysis in this study, the stoichiometric matrix was assembled in Microsoft Excel and then imported into Mathwork's MATLAB software for the flux calculations.

5.4 *S. erythraea* Metabolic Network

A metabolic network for *S. erythraea* was assembled for primary metabolism and erythromycin biosynthesis pathways. The primary metabolism network was based on the network for *Streptomyces lividans* (Daae and Ison, 1999). Figure 45 shows a schematic of the *S. erythraea* metabolic network. The numbers in the boxes indicate the reaction number as described in Appendix 1.

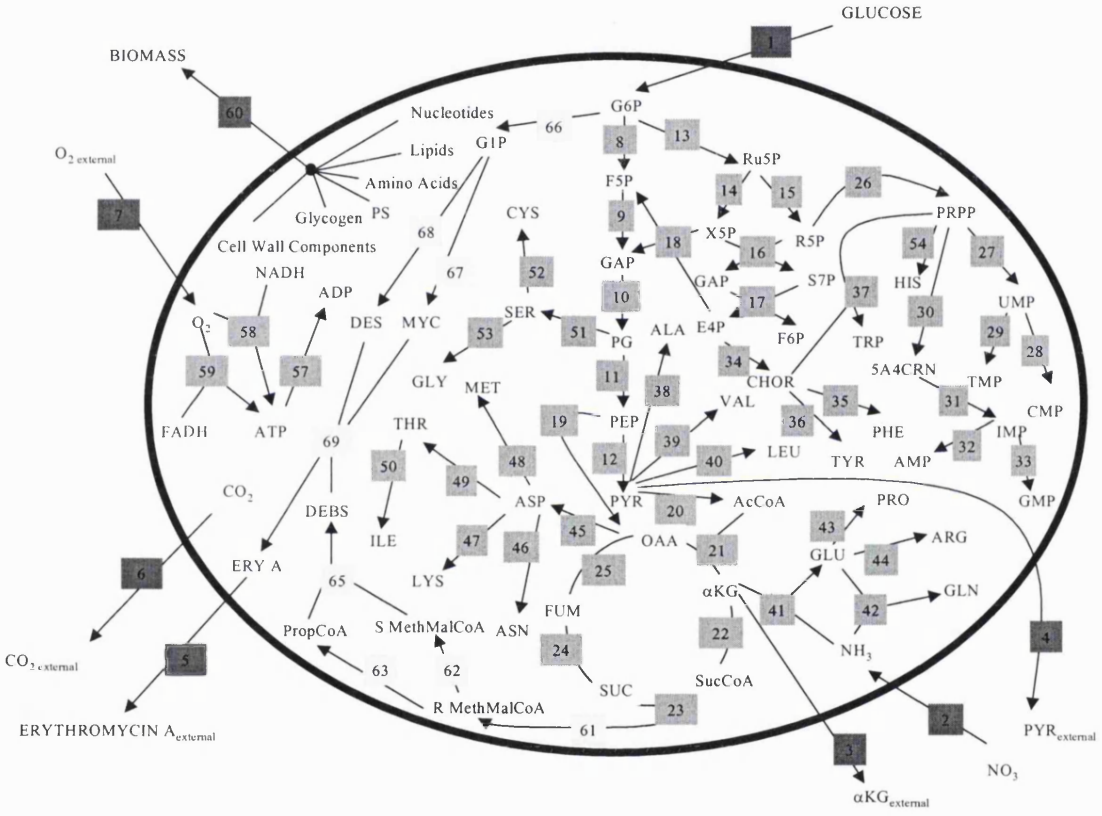


Figure 45: *S. erythraea* metabolic reaction network.

5.4.1 Metabolic Reactions

The reactions used in the metabolic network are listed in Appendix 1. The list is divided into groups based on the function of the reaction in metabolism. The full name of each metabolite is listed in Appendix 2.

5.4.2 Metabolites

The abbreviations and full names of each metabolite used in the metabolic network are shown in Appendix 2.

5.4.3 Sensitivity Analysis of *S. erythraea* Metabolic Network

As described in section 5.2.1, MFA can be performed and internal fluxes calculated. However, the results are only as accurate as the accuracy of the network and measured fluxes. It is important to evaluate that the model is providing information that is representative of the biological system. There are several methods of evaluating the mathematical system of equation used for the metabolic network. These methods include calculation of the condition number, and sensitivity analysis of the calculated values to changes of the measured values. The measured rates used for the various evaluations of the *S. erythraea* network were 1) glucose uptake, 2) nitrate uptake, 3) α-ketoglutarate production, 4) pyruvate production, 5) specific growth rate, 6) carbon dioxide production, and 7) oxygen consumption.

The network described here for *S. erythraea* incorporates 67 reactions and 62 metabolites (which are listed in Appendix 1 and Appendix 2). The condition number for this system was 140 which indicated a well-conditioned system for a network of this size (Savinell and Palsson, 1992). This meant that it was reasonable to use data generated by typical biological assays for network analysis.

Sensitivity analysis evaluates how sensitive the calculated values ($\mathbf{v}_c$) are to changes in the measured values ($\mathbf{v}_m$). The sensitivities are calculated by the equation

$$\delta\mathbf{v}_c/\delta\mathbf{v}_m = -(\mathbf{G}_c^T)^{-1}\mathbf{G}_m^T \qquad \textbf{(9)}.$$

For the *S. erythraea* network sensitivity analysis has been conducted for measured fluxes of glucose uptake, nitrate uptake, α-ketoglutarate production, pyruvate production, carbon dioxide production, oxygen consumption, and specific growth rate. The accumulated sensitivities are shown in Table 32. The accumulated values indicate the overall sensitivity of the system to variations in one measured rate relative to the other measured rates.

Table 32: Accumulated sensitivities of measured fluxes.

Metabolite Fluxes Measured	Accumulated Sensitivities
Glucose Uptake	13.68
Nitrate Uptake	16.92
α-Ketoglutarate Production	8.84
Pyruvate Production	7.52
Specific Growth Rate	14.40
Carbon Dioxide Production	7.43
Oxygen Consumption	12.67

The accumulated sensitivities showed that changes in glucose uptake, nitrate uptake, specific growth rate and oxygen consumption had more of an impact on the system than changes in α-ketoglutarate, pyruvate, and carbon dioxide production. Even though changes in nitrate uptake had a large effect on the system compared to the other measured fluxes, the average sensitivity per reaction is 0.282, which may be considered low. The values of the accumulated sensitivities were similar to an analysis performed for *S. lividans* (Daae and Ison, 1999).

The specific reactions with high sensitivities could also be analyzed to gain insight into which regions of the metabolism were most sensitive to changes in specific measured fluxes. Appendix 3 lists the ten reactions with the highest sensitivities to changes in each of the seven measured fluxes. The reactions were categorized by metabolic function, designated as glycolysis (GLY), pentose phosphate pathway (PPP), TCA cycle (TCA), amino acid synthesis (AA), nucleotide synthesis (NS), erythromycin production (ERY), and cell maintenance (CM). Table 33 shows the value and the metabolic function of the five reactions that had the highest sensitives to changes in each measured rate.

Table 33: Metabolic function of the five most sensitive reactions to changes in measured fluxes.

Glucose	Nitrate	α-Keto	Pyruvate	Growth	CO_2	O_2
1.29	-2.33	-1.13	0.83	-2.61	-0.52	-4.49
GLY	CM	ERY	GLY	CM	ERY	CM
1.25	1.54	-0.96	-0.64	0.89	-0.50	-1.96
GLY	PPP	ERY	ERY	TCA	TCA	CM
0.92	-1.31	-0.91	-0.63	0.75	0.47	-0.44
TCA	GLY	TCA	TCA	GLY	CM	TCA
0.91	0.94	0.53	-0.55	0.69	-0.44	-0.43
ERY	PPP	CM	ERY	GLY	ERY	TCA
0.79	0.73	0.37	0.43	0.69	-0.42	-0.37
ERY	AA	GLY	GLY	PPP	CM	ERY

If the reactions with sensitivities values greater than 0.5 or less than –0.5 were considered to have an impact on the outcome of MFA on a given region of metabolism, then the results indicated that the glycolysis pathway was most sensitive to the glucose uptake, nitrate uptake and specific growth rate measurements. This would be expected because glucose and nitrate are highly important nutrients contributing to biomass generation. The pentose phosphate pathway was most sensitive to the nitrate uptake and specific growth rate measurements. Since, nitrate assimilation and biomass generation requires NADPH, it was not surprising that the pentose phosphate pathway, which generates NADPH, was affected by these two measured factors. The TCA cycle was sensitive to all of the measured rates except nitrate uptake, and carbon dioxide production. The TCA cycle is critical to the growth and respiration of the cell and it would be expected that many of the reactions in the TCA cycle were sensitive to the measured rates. Similarly, ATP maintenance was affected by most of the measured rates except glucose uptake and carbon dioxide production. Oxidative phosphorylation was affected most by carbon dioxide production and oxygen consumption, which would be expected because these rates are critical for respiration. Amino acid production was only affected by nitrate uptake which made sense since amino acids require nitrogen. Erythromycin production was affected by all measured rates except nitrate uptake and specific growth rate.

Erythromycin was most sensitive to α-ketoglutarate, pyruvate, and carbon dioxide production, all of which are integral to the TCA cycle. It appeared that erythromycin production was greatly affected by the TCA cycle which made sense since the TCA cycle is a main source of precursors for erythromycin production. The sensitivity analysis supported further investigation of the TCA cycle in examining erythromycin production.

From the sensitivity analysis, it is concluded that the mathematical system representing the metabolism of *S. erythraea* is well-conditioned and should be suitably robust to handle measured data for MFA without generating unreliable calculated fluxes.

5.5 *S. erythraea* Metabolic Flux Analysis

In this section on metabolic flux analysis, two applications of MFA to identify targets for metabolic engineering, and further understanding of the system of interest, are described. The first application examined the differences in the flux spit ratio between two strains under the same condition, or the same strain under different conditions. This analysis was used to identify what reactions may have a greater influence on the whole system. The second analysis involved increasing or decreasing a given flux to observe how the remainder of the system then changes. The choice of which flux to vary was determined from experimental data and previous metabolic model analysis.

5.5.1 Flux Analysis Comparison of *S. erythraea* wild-type and *S. erythraea* Δ*eryA*

5.5.1.1 Fermentation Results

5.5.1.1.1 *S. erythraea* Red-Variant Wild-Type

Two batch fermentations were performed with the *S. erythraea* wild-type under nitrogen-limited growth conditions. Figure 46 shows the batch fermentation data for the *S. erythraea* wild-type. The different phases of the fermentation are shown in roman numerals and are described further in the following section on metabolic flux analysis.

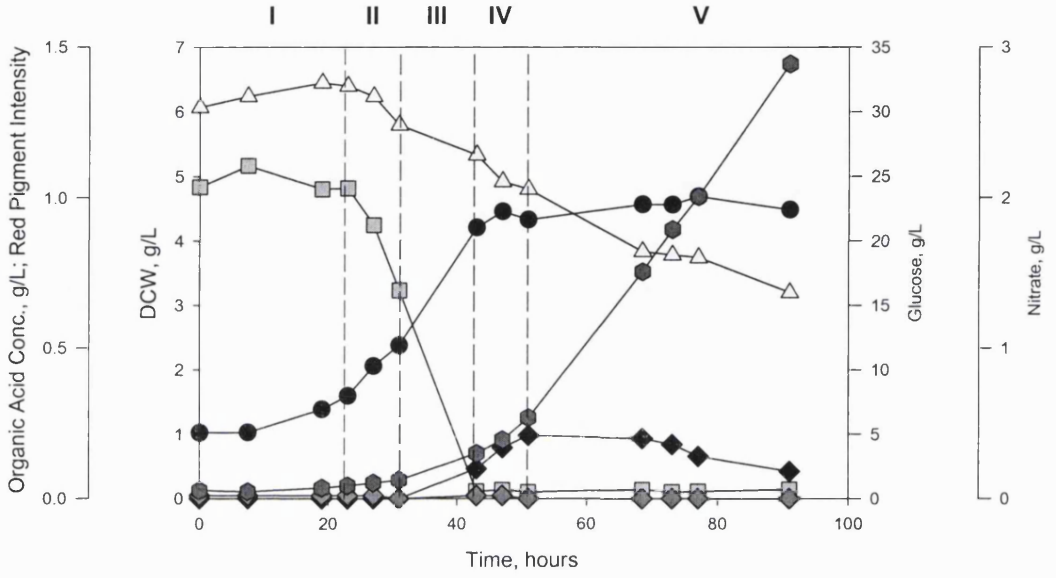


Figure 46: Growth and product formation of *S. erythraea* wild-type grown in 7 L batch bioreactor under nitrate-limitation.

(●) Dry cell weight, (▲) Glucose, (■) Nitrate, (◆) α-Ketoglutarate, (◆) Pyruvate, and (●) Red pigment.

Figure 46 shows the concentrations of residual substrate and product formation. A comparison between the *S. erythraea* wt RV batch fermentation and shake-flask results (see section 3.5) showed that growth rate, substrate uptake, and red pigment production were similar. Also, no erythromycin could be detected in either the shake-flask or fermenter cultures. This could be due to a repressive effects of high residual levels of glucose on erythromycin production (Escalante *et al.*, 1982). The results differed in the levels of organic acid produced. In the fermentation experiments, approximately one-half as much α-ketoglutarate was produced, and essentially no pyruvate was produced compared to the shake-flasks. It is believed that the shake-

flask cultures were not oxygen limited since the biomass and substrate uptake rates were similar between the two systems. Also, the biomass levels were low and, therefore, would not require large amounts of oxygen.

5.5.1.1.2 *S. erythraea ΔeryA*

One batch fermentation was performed with the *S. erythraea ΔeryA* mutant under nitrogen-limited growth conditions. Figure 47 shows the batch fermentation data for *S. erythraea ΔeryA* mutant. The different phases of the fermentation are shown in roman numerals and are described further in the following section on metabolic flux analysis as in wild-type results.

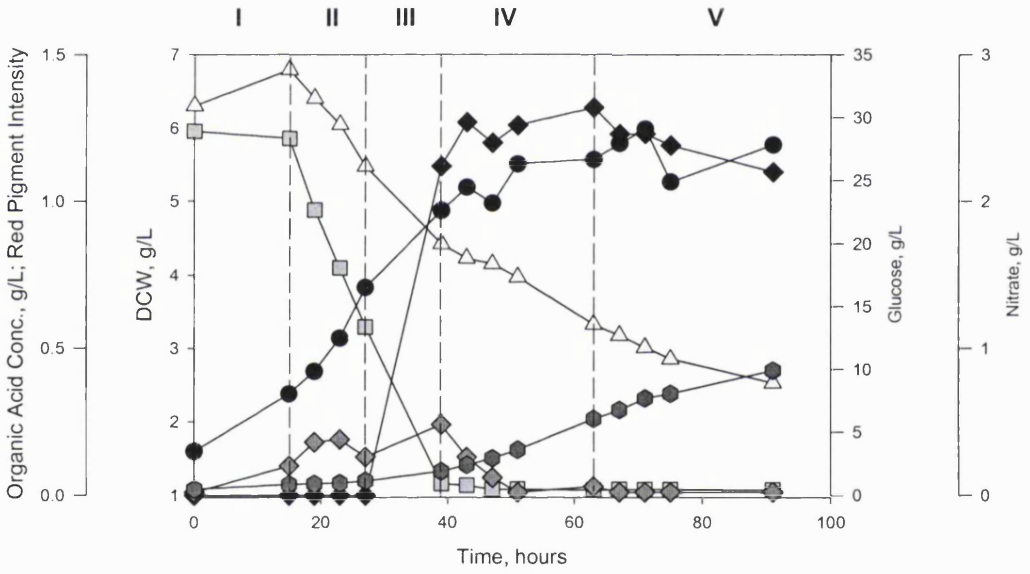


Figure 47: Growth and product formation *S. erythraea ΔeryA* mutant grown in 7 L batch bioreactor under nitrate-limitation.

(●) Dry cell weight, (▲) Glucose, (■) Nitrate, (◆) α-Ketoglutarate, (◆) Pyruvate, and (●) Red pigment.

A comparison between the *S. erythraea ΔeryA* batch fermentation and shake-flask results (see section 3.5) showed that growth rate and substrate uptake, were slightly slower in the fermentation. The results also differed in the levels of organic acid produced. In the fermentation experiments, approximately twice as much α-ketoglutarate was produced, and one-quarter of pyruvate was produced compared to the shake-flasks. The red pigment was produced to the same levels in both systems. It is unclear at this time why these differences were observed in the two systems.

The bioreactor fermentation results of *S. erythraea ΔeryA* compared to the *S. erythraea* wt showed similar trends as observed in the shake-flask experiments. Greater levels of organic acids were produced in the *S. erythraea ΔeryA* fermentation, and greater levels of red pigments were found in the wt fermentation. In the shake-flask experiments, there was a larger difference in growth rate between the two strains, however, the growth rates were similar in the fermentations, in that the *S. erythraea ΔeryA* strain grew slower that in the shake-flasks. Furthermore, in agreement with the shake-flask experiments, no erythromycin was detected.

5.5.1.2 Metabolic Flux Analysis Results

Metabolic flux analysis was performed with the *S. erythraea* wild type and *S. erythraea* Δ*eryA* mutant. The measured fluxes were calculated from the batch fermentation data described in section 5.3.3. To perform metabolic flux analysis with batch fermentation data, the data were divided into phases. The phases represented linear trends in the data so that rates could be calculated from the slopes generated for each metabolite. The fermentation data were divided into five phases as described in Table 34.

Table 34: Fermentation phases for metabolic flux analysis.

Phase	Description
I	Growth Phase with Sufficient Glucose and Nitrate
II	Growth Phase as Nitrate is Depleted
III	Growth Phase with Increasing Organic Acid Production
IV	Stationary Phase with Increasing Organic Acid Production
V	Stationary Phase with Decreasing Organic Acid Production

Flux analysis was performed for phase III and phase IV for the *S. erythraea* wild-type and Δ*eryA* mutant. These analyses showed the type of information one can obtain from MFA. It is important to consider the accuracy of the measured data when interpreting the results of the flux analysis.

As shown in Figure 48, the model predicted that as the transition was made from the growth phase to the stationary phase, the flux to the pentose phosphate pathway was reduced from about 60 % of carbon flux to less than 5 % for both the wild-type and *eryA* mutant. Reduction of flux to the pentose phosphate pathway, from growth to stationary phase, had been previously observed (Jonsbu *et al.*, 2001). However the reduction in flux was about 50 % for *S. noursei* compare to 98 % in *S. erythraea*. The difference could be due to the change in growth rate between the two phases was 58 % for *S. noursei* and 92 % form *S. erythraea*.

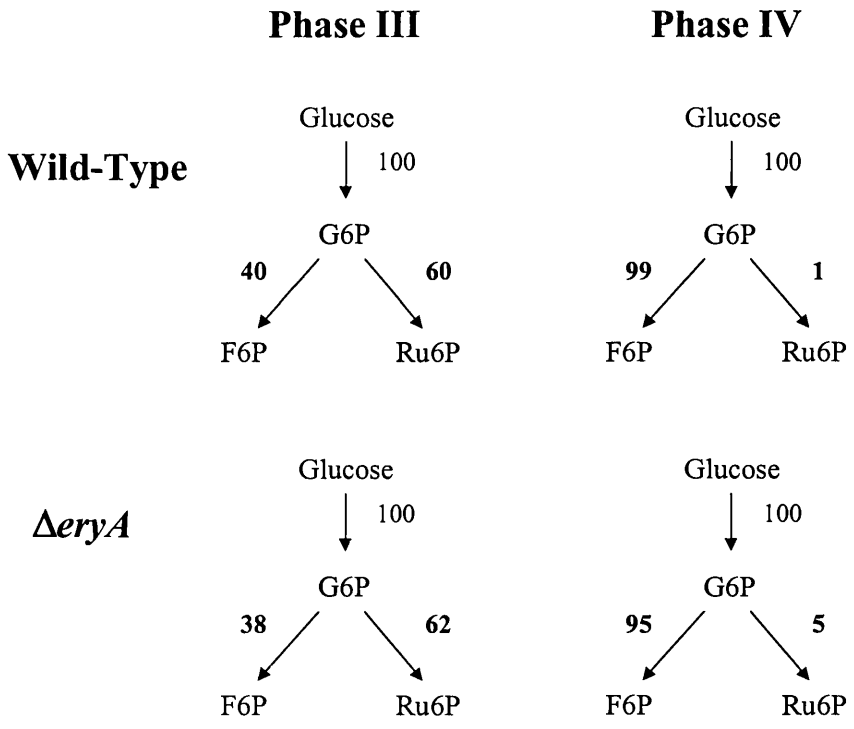


Figure 48: Glycolysis and pentose phosphate pathway flux split ratio for Phases III an IV for *S. erythraea* wt RV and *S. erythraea* Δ*eryA*.

The model also predicted the differences between the strains in phase IV, where α-ketoglutarate was excreted as shown in Figure 49. The flux analysis showed that the wild-type had a greater flux going from α-ketoglutarate to succinyl-CoA than the *S. erythraea ΔeryA* mutant because of the higher flux to extracellular α-ketoglutarate in the *S. erythraea ΔeryA* mutant. Also, the flux through PEP carboxylase to the TCA cycle was greater for the *S. erythraea ΔeryA* mutant, which was assumed to compensate for the carbon loss to extracellular α-ketoglutarate.

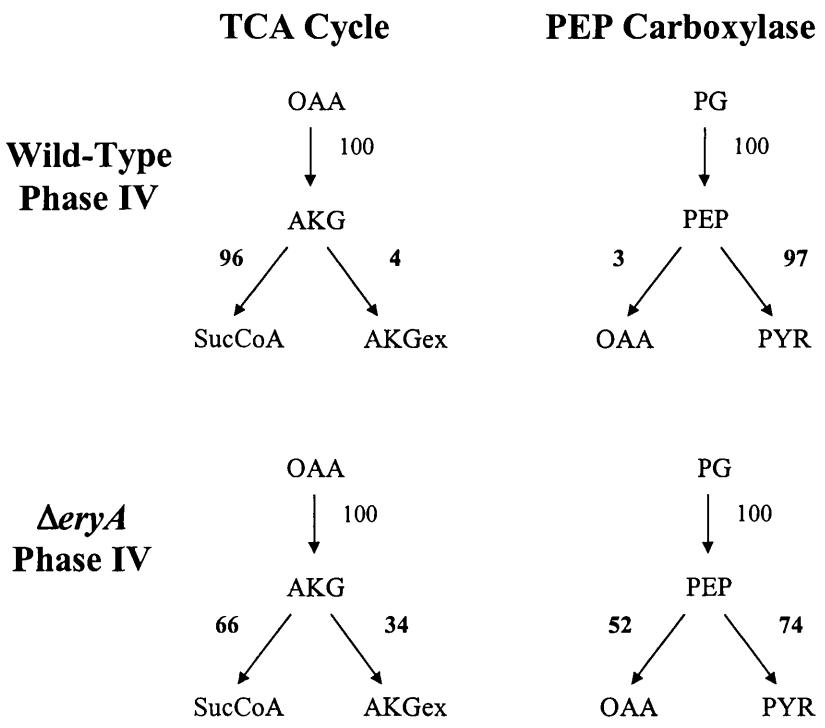


Figure 49: Flux split ratio for the TCA cycle and PEP carboxylase in Phase IV for *S. erythraea* wt RV and *S. erythraea ΔeryA*.

5.5.2 Flux Analysis of Re-channeling Organic Acids to Polyketide Biosynthesis

Another application of MFA is to determine how fluxes change due to increase or decrease of a given flux. Where flux split ratio analysis compares two strains or two phases, varying a given flux can provide information on what may happen to the flux distribution if a gene for a given enzyme is over-expressed or deleted. This section describes the type of information obtained when increased flux of reactions influencing organic acid excretion was simulated.

Organic acids in the form of pyruvate and α-ketoglutarate were excreted outside the cell, as described in Chapter 3. It is interesting to consider if the carbon lost to these excreted acids could be re-channeled to increase polyketide precursors and hence increase polyketide products. To this end, MFA was performed with *S. erythraea* wild-type during the phase in which organic acids were accumulating outside the cell. Two situations analyzing the influence of re-channeling organic acids to polyketide precursors are discussed below. Figure 50 shows a simplified metabolic reaction network that highlights the relationship between pyruvate, acetyl-CoA, malonyl-CoA and red pigment, and α-ketoglutarate, succinyl-CoA, methylmalonyl-CoA, and 6-deoxyerythronolide.

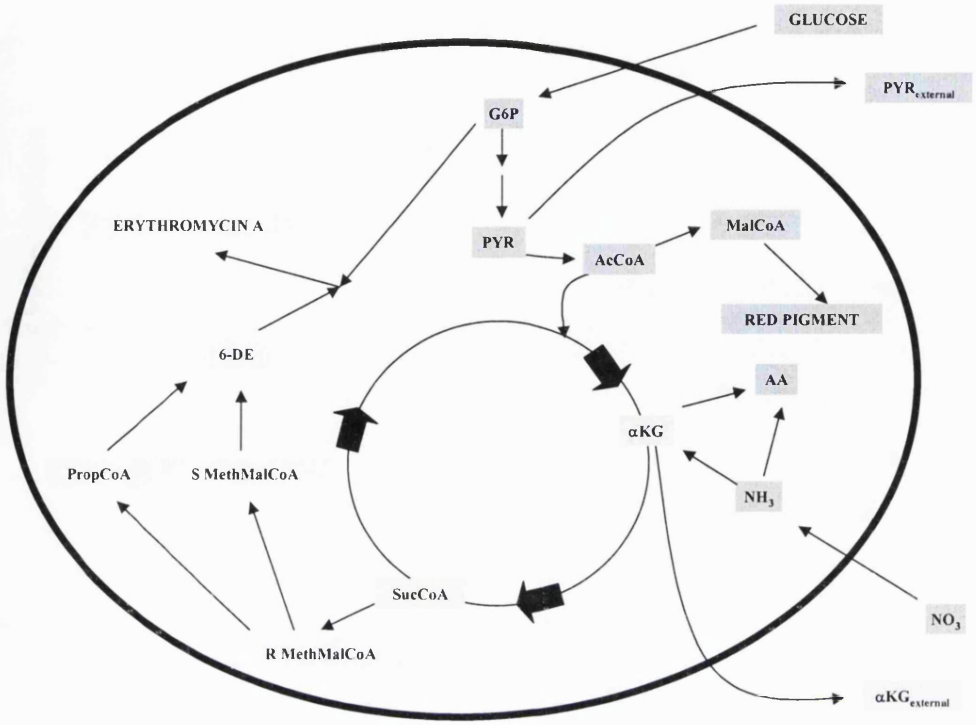


Figure 50: Simplified *S. erythraea* metabolic reaction network of red pigment and 6-deoxyerythronolide biosynthesis.

5.5.2.1 Increased Flux from Pyruvate to Acetyl-CoA

Pyruvate can be converted to acetyl-CoA by pyruvate dehydrogenase. Acetyl-CoA is a critical precursor for many reactions involved primary metabolism, e.g. TCA cycle. It can also be converted to malonyl-CoA which may be a building block for red pigment biosynthesis by *S. erythraea* (Cortes *et al.*, 2002). MFA was performed to determine if increasing the flux from pyruvate to acetyl-CoA increases red pigment production. The question to be addressed was "can carbon lost by pyruvate excretion be re-channeled to increase red pigment production?" The influence on 6-deoxyerythronolide (polyketide moiety of erythromycin) production was also analyzed in the same manner.

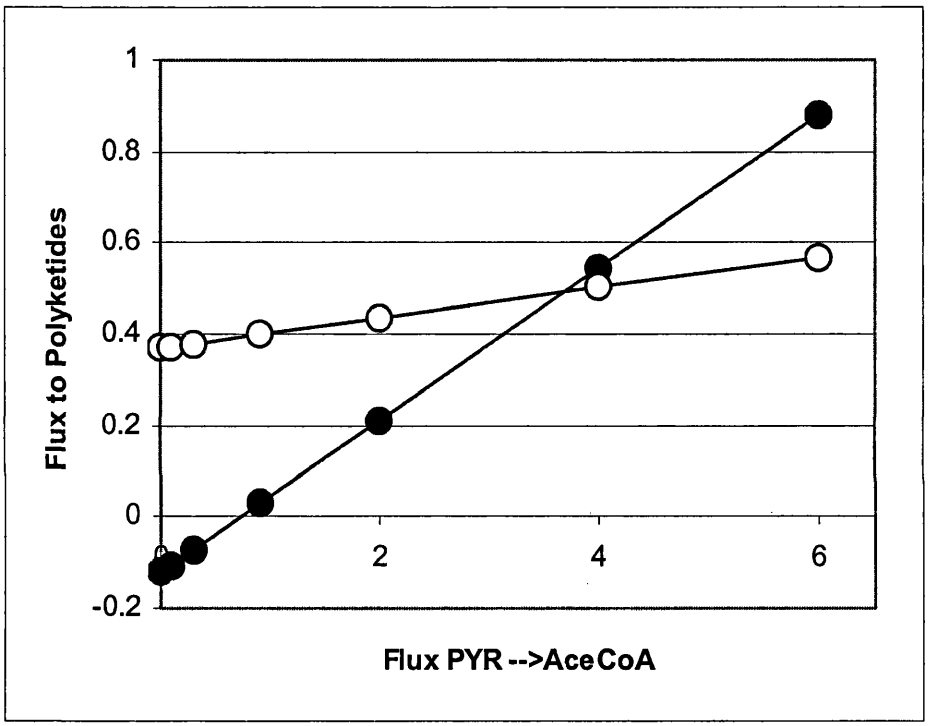


Figure 51: Model prediction of the influence of flux to acetyl-CoA on flux to red pigment and 6-deoxyerythronolide.

(●) Red Pigment and (○) 6-Deoxyerythronolide.

Figure 51 shows the model prediction of how increased flux to acetyl-CoA would influence flux to red pigment and 6-deoxyerythronolide. Both red pigment and 6-deoxyerythronolide flux would increase if flux to acetyl-CoA increases. However, flux to red pigment increases more than flux to 6-deoxyerythronolide. This is reasonable if it is assumed that as the carbon is channeled to acetyl-CoA, the precursor to red pigment is in closer proximity to the acetyl-CoA pool compared to 6-deoxyerythronolide and therefore may be influenced by increased levels of acetyl-CoA. Flux to 6-deoxyerythronolide would also be increased, probably due to higher levels of carbon in the system, but to a lesser extent than red pigment. This may be because the carbon could be re-directed to other pathways before reaching the 6-deoxyerythronolide pool.

5.5.2.2 Increased Flux from α-Ketoglutarate to Succinyl-CoA

α-Ketoglutarate can be converted to succinyl-CoA by α-ketoglutarate dehydrogenase. Succinyl-CoA is a component of the TCA cycle, and it can also be converted to methylmalonyl-CoA, which is the building block for 6-deoxyerythronolide (the polyketide moiety of erythromycin) biosynthesis by *S. erythraea*. MFA was performed to determine if increasing the flux from α-ketoglutarate to succinyl-CoA increases 6-deoxyerythronolide production and hence erythromycin production. The question to be answered was "could carbon lost by α-ketoglutarate excretion be re-channeled to increase erythromycin production?" The influence on red production was also analyzed.

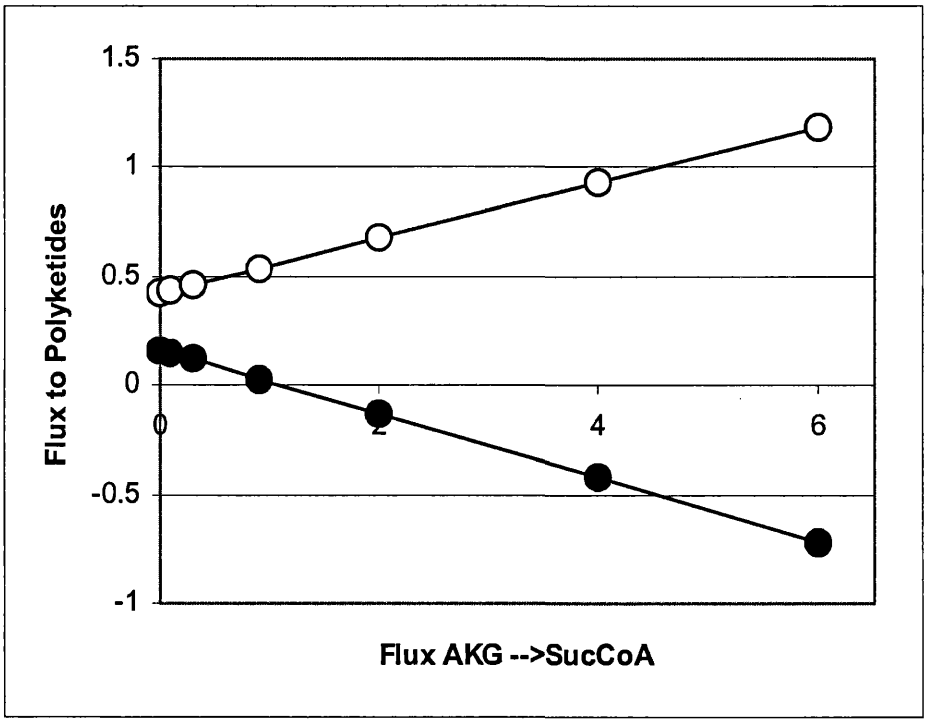


Figure 52: Model prediction of the influence of flux to succinyl-CoA on flux to red pigment and 6-deoxyerythronolide.

(●) Red Pigment and (○) 6-Deoxyerythronolide.

Figure 52 shows the influence of increasing flux from α-ketoglutarate to succinyl-CoA on flux to red pigment and 6-deoxyerythronolide. The model predicted that the

flux to red pigment production would decrease and that the flux to 6-deoxyerythronolide would increase. This may be reasonable because if the flux to succinyl-CoA is increasing then the carbon could be "pulled" away from upstream reactions such as those going to red pigment production. More and more carbon would be drawn away from upstream reactions as the flux to succinyl-CoA increased. Conversely, the flux to 6-deoxyerythronolide would increase because some of the increased flux from succinyl-CoA would go to methylmalonyl-CoA and then to 6-deoxyerythronolide.

5.6 Discussion and Conclusions

This chapter discussed the processes involved in constructing a metabolic model, performing a sensitivity analysis on the model network, generating the experimental data for model analysis, and executing metabolic flux analysis. The utility of MFA was shown in two ways. The first was in comparison of flux split ratios by determining the differences between two strains under the same condition, or the same strain under different conditions. This analysis is useful to determine where the critical reactions are which may be useful targets for metabolic engineering. A second analysis was shown in which a reaction flux was deliberately increased or decreased to determine the influence of that flux on the whole system. This approach can also be used to determine which reaction have greater influence on the whole system.

The second analysis was demonstrated by simulating the outcome of increasing the flux from pyruvate to acetyl-CoA which could be experimentally tested by increasing expression of the gene coding for pyruvate dehydrogenase. Or alternatively, increasing flux from α-ketoglutarate to succinyl-CoA which could be experimentally tested by increasing expression of the gene coding for α-ketoglutarate dehydrogenase. Executing the genetic engineering is a time consuming challenge and if the model is predictive then large amounts of time could be saved by implementing metabolic flux analysis. The assumption, however, is that the model is predictive. Before confidence can be held in the predictions, thorough validation of the model must be performed. This could take the form of executing the genetic engineering and testing if the model prediction was correct.

5.6.1 Hypothesis to Re-channel Carbon in to Primary Metabolism and Improve Flux to Erythromycin

Based on the predictions of the metabolic flux analysis it was hypothesized that if the flux from α-ketoglutarate to succinyl-CoA is increased then 1) the flux to excreted α-ketoglutarate would be decreased and 2) the erythromycin flux would be increased. This hypothesis was supported with data previously presented in this thesis. The first supporting data are the physiology studies described in Chapter 3 where it was shown that carbon was lost to α-ketoglutarate excretion, which provides a source of carbon to be re-channeled. The second came from the enzyme activity analysis described in Chapter 4 where it was shown that the activity of α-ketoglutarate dehydrogenase was very low compared to the other TCA cycle enzymes measured indicating that the activity of this enzyme could possibly be increased to produced more succinyl-CoA.

To test this hypothesis, it was proposed to over-express the α-ketoglutarate dehydrogenase gene in *S. erythraea* wild-type RV. The molecular biology to construct this new strain is describe in next chapter (Chapter 6) and the physiology results comparing the over-expression strain to the wild-type are presented in Chapter 7.

6 Genetic Engineering of *Saccharopolyspora erythraea*

6.1 Summary

The relationship between primary and secondary metabolism is of interest when considering the production of polyketides. Many polyketides are produced with similar building block units from primary metabolism, in particular malonyl-CoA and methylmalonyl-CoA. By better understanding how the precursor units are supplied though the complicated network of bacterial metabolism, it may be possible to construct organisms that are optimized for polyketide production. *S. erythraea* produces erythromycin using a type I polyketide synthase, and produces red pigments possibly by a type III polyketide synthase (Cortes *et al.*, 2002). The physiology of *S. erythraea* was studied under nitrogen-limited conditions which showed that as the nitrate in the medium decreased, the levels of red pigment and organic acid excretion increased. Enzyme activity analysis showed that the levels of activity correlated with the changes in pyruvate and α-ketoglutarate excretion over time. Isocitrate dehydrogenase exhibited much greater activity than α-ketoglutarate dehydrogenase which may explain the high levels of α-ketoglutarate excretion. Metabolic flux analysis was also performed which predicted that if the flux from α-ketoglutarate to succinyl-CoA increases, then the flux to erythromycin A increases. It was proposed to construct a *S. erythraea* strain that overproduces α-ketoglutarate dehydrogenase and a red pigment knockout strain to determine if the carbon lost to α-ketoglutarate or red pigment production could be re-channeled to primary metabolism and potentially increase erythromycin production compared to the wild-type. This hypothesis was based on previous work presented in this thesis: physiology data (Chapter 3), enzyme activity analysis (Chapter 4), and metabolic flux analysis (Chapter 5).

Three sets of *S. erythraea* strains were constructed in this work. Each set name refers to the plasmid DNA integrated into the following host *S. erythraea* strains: 1) *S. erythraea* wild-type red-variant (wt RV), 2) *S. erythraea* JC2 (Δ*eryA*), and in some cases 3) *S. erythraea* JC103 (P_{actI} *eryA*). The first set (pMU5) had the α-ketoglutarate dehydrogenase from *S. coelicolor* over-expressed. The second set (pMU4) had the

rppA gene (red pigment synthesis) knocked out. The third set (pMU4 + pMU5) had both the α-ketoglutarate dehydrogenase from *S. coelicolor* over-expressed and the *rppA* gene knocked out. Construction was completed or initiated for each of these strains. Several physiological characteristics of these new stains were studied, including the α-ketoglutarate excretion levels, α-ketoglutarate dehydrogenase activity, red pigment production, and erythromycin production. This chapter contains the materials and methods used to construct novel *S. erythraea* strains that were then used to help further study the interaction between primary and secondary metabolism. As several Actinomycetes excrete organic acids, the results may provide a general tool to manipulate primary metabolism to enhance secondary metabolite production.

6.2 Background

This chapter discusses the genetic engineering approach to increase precursor availability for polyketide biosynthesis with the aim of increasing levels of polyketide production. The approach focuses on the red pigment synthase genes and the α-ketoglutarate dehydrogenase gene to attempt to supply precursors to erythromycin biosynthesis. These genes are discussed below, as well as the VWB integrase system, which was used for the first time in this work for plasmid integration into the *Saccharopolyspora erythraea* genome.

6.2.1 RppA

RppA is a type III (or chalcone-like) polyketide synthase (PKS) that produces 1, 3, 6, 8-tetrahydroxynapthlene (THN) which is oxidized to produce various red pigments by *S. erythraea* wt red-variant (Cortes *et al.*, 2002). THN is synthesized using five malonyl-CoA units which can be derived from acetyl-CoA (Funa *et al.*, 1999). Pigment production and organic acid excretion have been measured in three *S. erythraea* strains: 1) *S. erythraea* wt RV, 2) *S. erythraea* JC2 (erythromycin PKS (*eryA*) genes knocked-out), 3) *S. erythraea* JC103 (erythromycin PKS genes under the control of the P_{actI} promoter system). It has been found that under nitrate-limited conditions, *S. erythraea* wt RV produces up to 2 g/L of pigmented compounds (see section 3.4.3.2). The results implied that the pathways of pigment and erythromycin biosynthesis may be related at points where they branch from primary metabolic pathways because it appeared that deletion of the erythromycin PKS genes affects the levels of pigment produced (see section 3.5). The level of α-ketoglutarate excretion

was also influenced by changes to the erythromycin PKS genes. When the erythromycin PKS genes are deleted, higher levels of α-ketoglutarate excretion have been found and conversely, when the erythromycin PKS genes are under an enhanced promoter system, lower levels of α-ketoglutarate excretion were observed. The levels of α-ketoglutarate appear to reflect the carbon flux to erythromycin in that there is excess carbon (in the form of α-ketoglutarate) when flux to erythromycin is decreased (JC2), and there is less excess carbon when flux to erythromycin is increased (JC103).

To explore further the relationship between pigment, α-ketoglutarate, and erythromycin production, construction of *S. erythraea* strains with the *rppA* gene deleted was initiated. If the red pigment could not be produced then it may be possible that the levels of α-ketoglutarate would increase due to increased carbon flow through the TCA cycle, and an apparent TCA cycle regulation point at α-ketoglutarate dehydrogenase (Chapter 4).

6.2.2 α-Ketoglutarate Dehydrogenase

α-Ketoglutarate dehydrogenase is a multi-subunit enzyme complex that converts α-ketoglutarate to succinyl-CoA. The α-ketoglutarate dehydrogenase complex is very similar to the pyruvate dehydrogenase enzyme complex and consists of three subunits, α-ketoglutarate dehydrogenase (E_1), dihydrolipoamide S-succinyltransferase (E_2), and dihydrolipoamide dehydrogenase (E_3). The E_1 and E_2 subunits are unique to the α-ketoglutarate dehydrogenase complex where the E_3 subunit is identical to the E_3 subunit of the pyruvate dehydrogenase complex in organisms such as *E. coli* where detailed studies have been performed (Gottschalk, 1986). The product of the α-ketoglutarate dehydrogenase complex, succinyl-CoA, can be converted into propionyl-CoA and methylmalonyl-CoA which are both precursors to erythromycin (Kaneda *et al.*, 1962; Marsden *et al.*, 1994).

Physiological studies of *S. erythraea* wt RV grown under nitrate-limited conditions showed that the organism excreted up to 3 g/L of α-ketoglutarate (see Chapter 3) which could be considered lost carbon that could be used to make other products, including erythromycin. Under the above conditions, α-ketoglutarate dehydrogenase also had little to no activity. Metabolic flux analysis, which calculates the flux of

carbon through each reaction in a metabolic pathway network, predicted that if the flux from α-ketoglutarate to succinyl-CoA was increased then the flux from succinyl-CoA to erythromycin should increase thereby increasing the amount of erythromycin produced (see Chapter 5). It was, therefore, hypothesized that if α-ketoglutarate dehydrogenase could be over-expressed, its activity would be increased and convert the α-ketoglutarate, that would otherwise be wasted, to succinyl-CoA. It was also hypothesized that the increased amounts of succinyl-CoA could then be channeled to erythromycin biosynthesis.

To test this hypothesis, *S. erythraea* strains with an over-expressed α-ketoglutarate dehydrogenase gene were constructed. To attempt to circumvent the existing enzyme regulation in *S. erythraea* a heterologous gene sequence was used for over-expression. To this end, sequence analysis was performed comparing the *S. erythraea* α-ketoglutarate dehydrogenase gene sequence to that of *S. coelicolor*, *M. leprae*, and *M. tuberculosis* (M. Gregory, personal communication). Due to sequence similarity, it was presumed that the E_1 and E_2 subunits of *S. coelicolor* α-ketoglutarate dehydrogenase would be compatible with *S. erythraea* and were, therefore, chosen for over-expression in *S. erythraea*.

Several physiological characteristics were studied including the α-ketoglutarate excretion levels, α-ketoglutarate dehydrogenase activity, red pigment production, and erythromycin production (see Chapter 7). We expected to observe reduced levels of α-ketoglutarate excretion and red pigment production, and increased levels of erythromycin production. As several Actinomycetes excrete organic acids, the results may provide a general tool to manipulate primary metabolism to enhance secondary metabolite production in other Actinomycetes.

6.2.3 VWB-Based Integrative Vector

To integrate plasmid DNA into the chromosomal DNA of *S. erythraea*, a suitable integration system had to be established. The ϕC31 integration system is readily used with *S. coelicolor* and other *Streptomyces* (van Wezel *et al.*, 2000; Thorpe *et al.*, 2000; Combes *et al.*, 2002). However, this system is not compatible with *S. erythraea* because it does not contain the integration site where the plasmid DNA combines with

the chromosomal DNA. Site specific integration of the VWB integrase had been shown for *S. venezuelae* and *S. lividans* which initially suggested that this technique may be easily transferable to *S. erythraea* (Van Mellaert *et al.*, 1998). After searching the *S. erythraea* genome database, the attachment site was found which gave confidence that this integration system may work with *S. erythraea.*

The temperate bacteriophage VWB integrates, site-specifically, into the chromosome of *S. venezuelae* ETH 14630 (Anne *et al.*, 1990). Van Mellaert, *et al.* (1998) identified the integrase gene required and the sequence sites involved for integration. The specific integration site is at the 3' end of a putative $tRNA^{Arg}$ (AGG) gene. Homologous recombination of the VWB *attP* region and the chromosomal *attB* region combine to form the *attL* and *attR* host-phage junction sites. The *attL* junction site restores the original $tRNA^{Arg}$ (AGG) gene sequence. Subsequent to the identification of the integrase gene and sites, a integrative vector (pKT012) was constructed using the VWB *int* gene and *attP* site sequences. This vector was also successfully integrated into the chromosome of *S. lividans* TK24 (Van Mellaert *et al.*, 1998).

The work presented here utilizes the VWB integration system. It was determined that *S. erythraea* contains the $tRNA^{Arg}$ (AGG) gene sequence, and therefore, the *attB* integration site (M. Gregory, personal communication). The integrative vector pMU3 was constructed in the thesis and contains the VWB *int* gene and *attP* site for integration, an apramycin resistance gene for selection, the P_{actI} promoter system for gene expression, convenient restriction sites for gene cloning, an origin for replication in *E. coli*, and an origin of transfer for conjugation (see Figure 54). This vector was successfully integrated into *S. erythraea.* An expression plasmid, pMU5, was constructed by inserting the α-ketoglutarate dehydrogenase gene from *S. coelicolor* into the pMU3 vector. pMU5 was subsequently used to over-express a heterologous α-ketoglutarate dehydrogenase gene in *S. erythraea.*

6.3 Materials and Methods

6.3.1 Materials

6.3.1.1 Chemicals

Water was purified using the Millipore Milli-Q water purification system. Chemicals were analytical grade or best commercially available. Agarose was electrophoresis grade. Antibiotics were obtained from Sigma and deoxyribonucleotides were from Pharmacia.

6.3.1.2 Buffers and Solutions

The buffers and solutions used in this work are found in Table 35 with their components and component concentrations.

Table 35: Buffers and solutions.

Buffer or Solution Name	Component Name	Concentration, mM
TE, pH 8.0	Tris	10
	EDTA	1
TAE, pH 8.0	Tris-HCl	40
	EDTA	2
	CH_3COOH	24

6.3.1.3 Culture Media

The culture media used in this work are found in Table 36 with their components and component concentrations.

Table 36: Culture media.

Culture Media Name	Component Name	Concentration, g/L
2TY	Tryptone	10
	Yeast Extract	10
	NaCl	5
	Agar (for solid media)	15
TAXI	2TY (see above)	
	Ampicillin	0.1
	X-gal	0.25
	IPTG	0.25
TSB	Tryptone Soy Broth (Difco)	30
MAM	Wheat Starch (Sigma)	10
	Corn Steep Powder (Sigma)	2.5
	Yeast Extract (Difco)	3
	Calcium Carbonate	3
	Iron Sulfate · 7 H_2O	0.1
	Agar (Difco)	20

6.3.1.4 Antibiotics

Antibiotics, which are listed in Table 37, were prepared and used for selection of the appropriate strains of *E. coli* and *Streptomyces spp.* according to the procedures described previously (Sambrook *et al.*, 1989; Kieser *et al.*, 2000).

Table 37: Antibiotics used for *E. coli* and *S. erythraea* selection.

Antibiotic	Stock Conc., mg/mL	Final Conc., μg/mL	Volume added, mL/L
Ampicillin	100	100	1.0
Apramycin	100	50	0.5
Chloramphenicol	25	25	1.0
Kanamycin	50	50	1.0
Nalidixic Acid	50	25	0.5
Thiostrepton	25	8	0.16

6.3.1.5 Enzymes

The enzymes used in these studies and their manufacturers are listed in Table 38.

Table 38: Enzymes used in molecular cloning.

Enzyme Name	Manufacturer Name	Manufacturer Location
Restriction Endonuclease	New England Biolabs	Hitchin, UK
T4 DNA Ligase	New England Biolabs	Hitchin, UK
T4 Polynucleotide Kinase	New England Biolabs	Hitchin, UK
Taq I DNA Polymerase	Promega	Wisconsin, USA
Pfu DNA Polymerase	Strategene	La Jolla, USA
Shrimp Alkaline Phosphatase	Amersham Life Science	Amersham, UK

6.3.1.6 Oligonucleotides

The oligonucleotides used in this work are described in Table 39 and were purchased from Oligo Europe (Germany). MUSCLF and MUSCLR were the primers used to amplify the α-ketoglutarate dehydrogenase gene from *S. coelicolor*. MUSERPF and MUSERPR were the primers used to amplify an internal fragment of the *rppA* gene from *S. erythraea*.

Table 39: List of oligonucleotides used for PCR.

Primer Name	Primer Sequence
MUSCLF	TCCATATGTCGCCACAGTCCCCCAGTAACTCGAGC
MUSCLR	TCTCTAGATCGCGCCTCGAACACCTCACGCACCAGCT
MUSERPF	GGTCTAGAAGCCGCCCCTCCTCGATGATGCGCC
MUSERPR	CATCTAGAGCGTGCGCGAGCGCCATCTGA

6.3.1.7 Bacterial Strains

The bacterial strains used in this work are described in Table 40. All strains were available in the P.F. Leadlay laboratory or obtained from the NRRL culture Collection (Northern Regional Research Laboratory) which is also known as the ARS Culture Collection (Agriculture Research Service of the US Agriculture Department). The *E. coli* DH10B strain was used for routine cloning, and the *E. coli* ET12567 containing plasmid pUZ8002 was used for conjugation with *S. erythraea*. The *E. coli* ET12567 prevent methylation of DNA which is needed for conjugation with *S. erythraea* requires unmethylated DNA. The plasmid pUZ8002 contains the machinery need for conjugating *E. coli* to *S. erythraea* minus the *oriT*, which is on the integrating plasmid. The three *S. erythraea* strains were used as gene expression vectors.

Table 40: Bacterial strains and description.

Strain	Description	Reference/ Source
E. coli DH10B	F⁻*mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), ϕ80d*lac*ZΔM15, Δ*lac*X74, *deo*R, *rec*A1, *end*A1, *ara*Δ139, Δ(*ara*, *leu*)7697, *gal*U, *gal*K, λ⁻, *rps*L, *nup*G.	Gibco BRL, Inc., USA
E. coli ET12567 pUZ 8002	F⁻*dam*-13::Tn9, *dcm*-6, *hsd*M, *hsd*R, *zjj*-202::Tn10, *rec*F143, *gal*K2, *gal*T22, *ara*-14 *lac*Y1, *xyl*-5, *leu*B6, *thi*-1, *ton*A31, *rps*L136, *his*GF4, *tsx*-78, *mtl*-1, *gln*V44	(MacNeil *et al.*, 1992; Kieser *et al.*, 2000)
S. erythraea wt Red-Variant	NRRL 2338	NRRL, USA
S. erythraea JC2	NRRL 2338 Δ*ery*A	(Rowe *et al.*, 1998)
S. erythraea JC103	NRRL 2338 *act*II-ORF4 *eryA*	J. Cortes, unpublished work

6.3.1.8 Plasmids

The plasmids used in this work were are listed in Table 41 and were either available in the P.F. Leadlay laboratory or constructed as part of this work.

Table 41: Plasmids used or constructed.

Name	Reporter Gene(s)	Size, kb	Reference/Source
pMG55	*apr*R	3.8	M. Gregory, unpublished work
pKT02	*ampR*	6.1	(Van Mellaert *et al.*, 1998)
pCJW90	*ampR* / *tsrR*	4.7	(Wilkinson, 2001)
pCJW192	*ampR* / *tsrR*	17.6	(Wilkinson, 2001)
pUC18	*ampR*, *lacZ*a	2.7	(Yanisch-Perron *et al.*, 1985)
pMU1	*apr*R	6.1	this work
pMU2	*ampR*	6.4	this work
pMU3	*apr*R	6.9	this work
pMU4	*ampR* / *tsrR*	5.0	this work
pMU5	*apr*R	10.6	this work

6.3.2 Bacterial Methods

Standard sterile techniques for microbiological work were maintained throughout. All bacteriological procedures were performed either on ethanol-swabbed work surface with attendant flame, or in a TC48 laminar flow hood (Gelaire Laboratories). Sterilization, by autoclaving, was achieved at 121 °C for 20 minutes or by passage of heat-liable liquids thought a sterile 0.22 μm millex-GS filter (Millipore, France).

6.3.2.1 Growth and Maintenance of Bacterial Strains

Long term storage of *S. erythraea* and *E. coli* strains were prepared by combining 500 μL of culture with an equal volume of sterile 20 % glycerol and stored at –80 °C. *S. erythraea* viable cells were recovered by adding one 1 mL frozen stock culture to 10 mL TSB and incubating in 30 °C shaker for ~48 hours. *E. coli* strains were maintained on liquid or solid 2TY medium supplemented with appropriate antibiotic selection and incubated at 37 °C.

6.3.2.2 Transformation of *E. coli*

Electrocompetent cells (*E. coli* DH10B) were produced by the method described previously (Dower *et al.*, 1988). One μL of plasmid DNA or ligation mixture was mixed with 30 μL suspension of electrocompetent cells on ice and pipetted into 1 mm electroporation cuvette (BioRad, UK). The cells were transformed using a BioRad gene pulser at 1.7 kV/mm, 25 mF, and 200 Ω. After electroporation, 500 μL of 2TY medium was added to the cells and incubated in 37 °C water bath for 1 hour. The cells were then plated on to 2TY medium plates with appropriate antibiotic selection and incubated in 37 °C static incubator overnight. Single colonies were picked and grown overnight in 3 mL 2TY with antibiotic selection and DNA was extracted from the resulting cultures. For conjugation experiments, plasmids to be conjugated were first electroporated into *E. coli* ET12567 with a voltage of 1.25 kV.

6.3.2.3 Conjugation of *S. erythraea* and *E. coli*

Conjugation and integration techniques, modified by M. Gregory (personal communication), were used to transfer DNA as a plasmid from *E. coli* to the genomic DNA of *S. erythraea* (Kieser *et al.*, 2000). The conjugation procedure requires four days plus growth of conjugated colonies after selection and patching. On day one, the plasmid DNA of interest was transformed with *E. coli* ET12567 pUZ8002, incubated for 1 hour at 37 °C, plated on appropriate antibiotic selection plates, and incubated overnight at 37 °C. On day two, the *S. erythraea* strain was inoculated (at about mid-day) into 10 mL TSB medium and grown at 30 °C, and single colonies of *E. coli* transformation from day one were inoculated (in late afternoon) into 2TY with appropriate antibiotics. On day three, 0.5 - 1 mL of *E. coli* culture was inoculated into 25 mL 2TY with appropriate antibiotics and grown until OD (@ 600nm) was between 0.3 and 0.8 (cells need to be in log phase). The cells were then harvested by centrifugation at 2500 at 20 °C for 12 minutes in 50 mL tubes. The cells were washed twice with 2TY and then re-suspended in 2.5 mL 2TY. Meanwhile, 1 mL of *S. erythraea* culture was centrifuged at full speed in microcentrifuge and washed once with 500 μL 2TY and re-suspended in 500 μL 2TY. Once both the *E. coli* and *S. erythraea* cells were prepared, 500 μL of *E. coli* was added to the 500 μL of *S. erythraea* and plated on to MAM agar with no antibiotic. The plates were dried and then incubated at 30 °C overnight. On day four, the plates were overlaid with

nalidixic acid to kill *E. coli* and appropriate antibiotic to kill un-conjugated *S. erythraea*. The plates were then incubated at 30 °C for about 7 days and then single colonies were patched on the MAM agar with appropriate antibiotics to confirm the conjugated strain.

6.3.3 DNA Methods

All pipette tips, tubes, and water were sterilized prior to use. DNA preparation were stored in sterile Tris-EDTA buffer (TE) at -20 °C. Protein and other contaminants were removed from DNA preparations with ethanol extraction by adding 1 μL glycogen (5mg/mL), 10 μL sodium acetate (3 M), and 250 μL ethanol (100%) to 100 μL DNA. The mixture was then incubated at –20 °C for 1 hour and then centrifuged at high speed for 15 minutes, washed with ethanol (70%), air dried, and re-suspended in appropriate volume of TE.

6.3.3.1 Preparation of DNA

Small scale preparation of DNA was performed using the Promega Wizard Plus (Promega Corp., Madison, WI, USA) miniprep kits. Plasmid DNA was extracted from 3 mL cultures were grown overnight in 2TY containing appropriate antibiotic and purified according to Promega Wizard Plus instructions.

6.3.3.2 Agarose Gel Electrophoresis

Agarose gels (0.7 – 1.5 % w/v in TAE buffer) were cast as horizontal slab gels and run submerged in TAE buffer at 100 V for 1-4 hours. Prior to gel casting, ethidium bromide was added at 0.1 μg/mL. Samples (typically 10 μL) were mixed with loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) and run next to 1kb molecular weight markers (Gibco). The DNA was visualized at 302 nm using a UV transilluminator and photographed using a UVP gel documentation system (UVP Inc., CA, USA). DNA fragment bands of interest were excised from gel and then extracted using QiaexII resin according to manufacturers' instruction (Qiagen, Crawley, UK).

6.3.3.3 Polymerase Chain Reaction

PCR reactions were performed using a Robocycler Gradient 40 heating block (Stratagene, La Jolla, CA, USA). Genomic DNA from *S. erythraea* or *S. coelicolor* (previously isolated in P.F. Leadlay laboratory by S. Boakes) was used as the DNA

template and mixed with 20-50 pmol of each oligonucleotide primer, dNTPs (0.25 mM each), *Pfu* polymerase buffer (10% total volume), DMSO (10% total volume), and water (qs. to 50 μL). The reaction mixture was overlaid with 20 μL mineral oil to prevent evaporation. After the template DNA was denatured at 95 °C for 5 minutes, 1 μL of *Pfu* DNA polymerase was added to each reaction. The reaction was then allowed typically to run at 45 seconds at 95 °C, 1 minute at 68 °C, and 7 minutes at 72 °C for 30 cycles and finished with 10 minutes at 72 °C and was held at 4 °C until further analysis.

6.3.3.4 Molecular Cloning Techniques

6.3.3.4.1 Restriction Endonuclease Digestion

In general, 1-5 μg (5 μL of miniprep DNA) was digested with 5-20 units of enzyme at 37 °C for 1-3 hours with the relevant buffers supplied by the manufacturer. The volume of enzyme was not to exceed 10 % (v/v) to avoid reducing enzyme specificity due to high level of enzyme or glycerol content. After digestion, the DNA plus loading buffer was loaded directly onto agarose gel for electrophoresis analysis.

6.3.3.4.2 Dephosphorylation of Linearized DNA

To prevent re-ligation of vector DNA that had been digested to give complementary ends, shrimp alkaline phosphatase (SAP) was routinely used to remove 5'-phosphate groups, as per the manufacturer instructions. The mixture was incubated at 37 °C for 15 minutes and then the enzyme was inactivated at 65 °C for 10 minutes.

6.3.3.4.3 Addition of Phosphate Groups to Linearized DNA

To clone PCR products into *Sma* I-cut pUC18 vector, T4 polynucleotide kinase with appropriate buffer and ATP (20 mM) was used to add phosphate groups to the 5' end of each PCR product. The mixture was incubated at 37 °C for 15 minutes and then the enzyme was inactivated at 65 °C for 10 minutes.

6.3.3.4.4 Ligation of DNA

Ligations were performed as described by Sambrook (1989). Typically, a 3:1 molar ratio of insert to vector was mixed with 1 μL of T4 DNA ligase with appropriate buffers and incubated in 16 °C waterbath overnight.

6.3.3.4.5 DNA Sequencing

Samples were supplied at a concentration of 100 ng/mL for automated DNA sequencing performed by the DNA Sequencing Facility, Department of Biochemistry, University of Cambridge. An Applied Biosystems 800 molecular biology CATALYST robot was used to perform *Taq*I dideoxy terminator sequencing reaction, which were then applied to an ABI 373A sequencer according to manufacturer's instructions (Applied Biosystems Inc., USA).

6.4 Plasmid Construction

Five plasmids were constructed in this work to produce an α-ketoglutarate dehydrogenase over-expression vector, and a red pigment knock-out vector. The various components (e.g. genes, sites) assembled in the final plasmids are listed and described Table 42.

Table 42: Plasmid component definitions.

Plasmid Component	Description
*act*II-ORF4	Gene that encodes protein that activates P_{actI} and P_{actIII}.
P_{actI}	Site that protein, resulting from *act*II-ORF4 gene, binds to for initiating replication of gene of interest.
P_{actIII}	Second site that transcribes in opposite direction of P_{actI} on dsDNA .
oriC	Origin of replication for *E. coli*. Includes all genes and sites necessary for a plasmid to replicate in *E. coli*.
oriT	Origin of transfer from *E. coli* to another bacteria cell. Needed for conjugation. All other required machinery (except *oriT*) for conjugation are on pUZ8002 in *E. coli* strain ET12567. This is to reduce the number of genes needed on plasmid of interest but still allow conjugation. pUZ8002 will not be transferred from *E. coli*.
int (VWB)	Gene that codes for integrase protein which allows specific integration of plasmid DNA into genomic DNA. Integration is important to ensure that DNA of interest is replicated as single copy.
attP (VWB)	Site on plasmid that integrase binds to for integration. There is an *attB* VWB site in *S. erythraea* genome that the same protein binds to for specific site directed integration. *P* = plasmid and *B* = bacteria.
aprR	Apramycin resistance gene.
tsrR	Thiostrepton resistance gene.
ampR (*bla*)	Ampicillin resistance gene.
rppA	fragment of RppA red pigment synthesis enzyme.
α-ketoglutarate dehydrogenase gene (*S. coelicolor*)	E1 and E2 subunits of α-ketoglutarate dehydrogenase gene from *S. coelicolor*.

6.4.1 α-Ketoglutarate Dehydrogenase Over-expression in *S. erythraea*

6.4.1.1 pMU1

pMU1, shown in Figure 53, is an integrative vector containing VWBint, *att*B VWB, *oriC*, *oriT*, and *aprR*. pMU1 was constructed by digesting pMG55 with *Nhe* I and *Apa* I and pKT02 with *Xba* I and *Apa* I. The 3.8 kb fragment of pMG55, containing *oriC*, *oriT*, and *aprR*, was SAP treated and ligated to the 2.2kb fragment of pKT02, containing VWBint and *attP* VWB. pMU1 is apramycin resistant in both *E. coli* and *S. erythraea*.

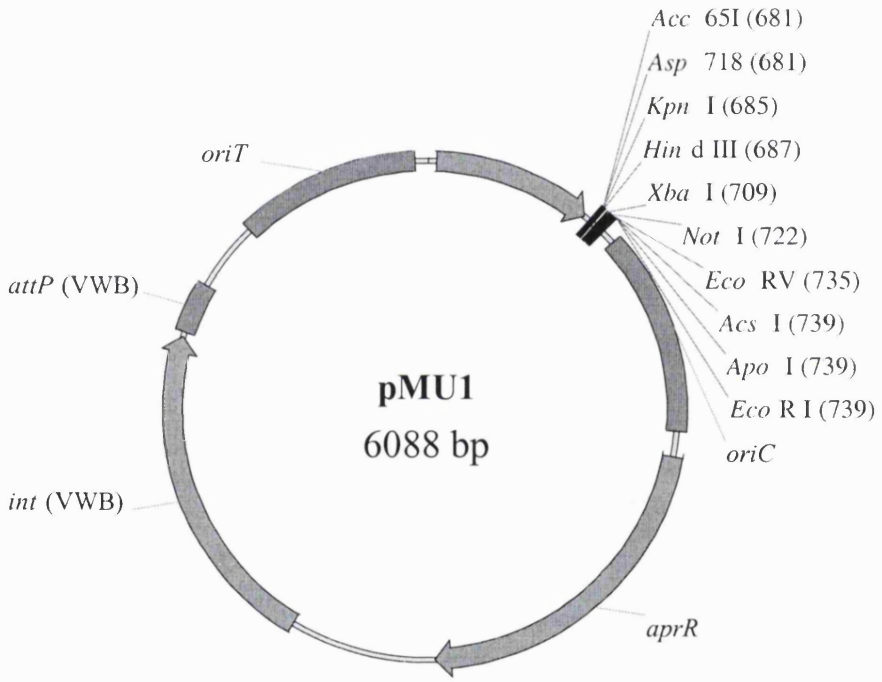


Figure 53: Diagram of pMU1.

6.4.1.2 pMU2

pMU2 is an α-ketoglutarate dehydrogenase coding sequence plasmid containing the PCR gene product of the *S. coelicolor* α-ketoglutarate dehydrogenase which has a methionine start codon engineered at the beginning of the sequence using an *Nde* I site, and an *Xba* I site at the end for excision from the intermediate vector pUC18. The *S. coelicolor* α-ketoglutarate dehydrogenase sequence was amplified by PCR using *S. coelicolor* genomic DNA as the template and oligonucleotides MUSCLF and MUSCLR as primers which introduce the *Nde* I and *Xba* I restriction sites. The 3.8 kb PCR fragment was isolated and 5'-phosphorylated with T4 polynucleotide kinase. It was then ligated to *Sma* I digested and SAP treated pUC18 and confirmed by sequencing. pMU2 is ampicillin resistant in *E. coli* and contains *lacZ*α for blue-white selection in the presence of IPTG and X-gal.

6.4.1.3 pMU3

pMU3, shown in Figure 54, is an expression and integrative vector containing the components of pMU1 plus the promoter system *act*II-ORF4, P_{actI}, and P_{actII}. pMU3 was constructed by digesting pMU1 with *Sph* I and *Xba* I and pCJW90 with *Sph* I and *Spe* I. The 5.4 kb fragment from pMU1 was then ligated to the 1.5 kb fragment from pCJW90. pMU3 is apramycin resistant in *E. coli* and *S. erythraea*. pMU3 contains all the necessary components to over-express a gene using the P_{actI} promoter system and to specifically integrate the plasmid DNA using the VWB integrase system (so long as the bacterial chromosome contains an *attB* binding site).

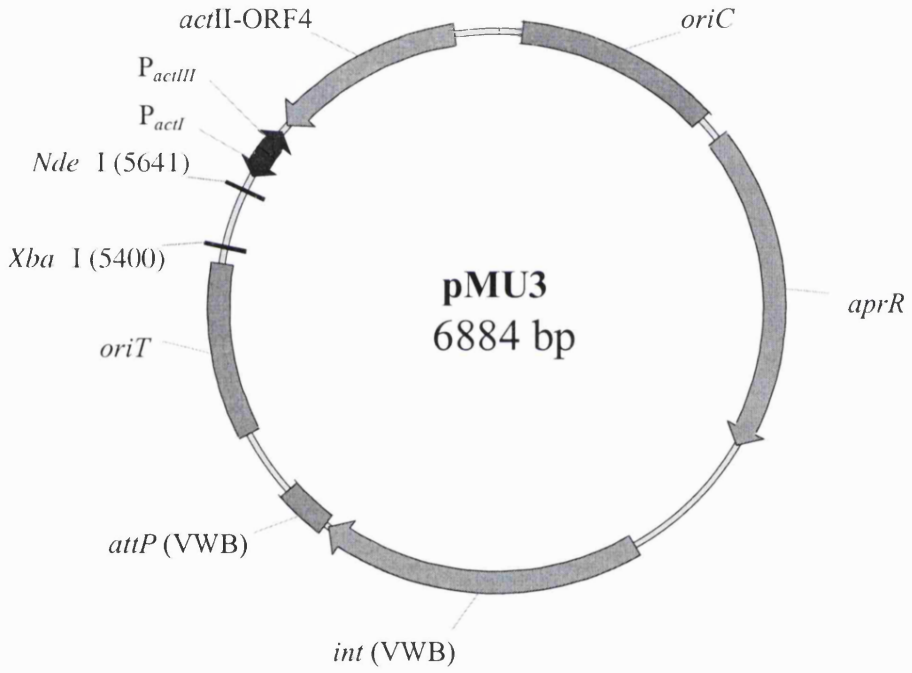


Figure 54: Diagram of pMU3.

6.4.1.4 pMU5

pMU5, shown in Figure 55, is an *S. coelicolor* α-ketoglutarate dehydrogenase expression and integrative vector containing the components of pMU3 plus the *S. coelicolor* α-ketoglutarate dehydrogenase gene sequence over-expressed using the P_{actI} promoter system. pMU5 was constructed by digesting both pMU2 and pMU3 with *Nde* I and *Xba* I. The 6.8 kb fragment from pMU3 was SAP treated and ligated to the 3.8 kb fragment of pMU2. pMU5 is apramycin resistant in *E. coli* and *S. erythraea*.

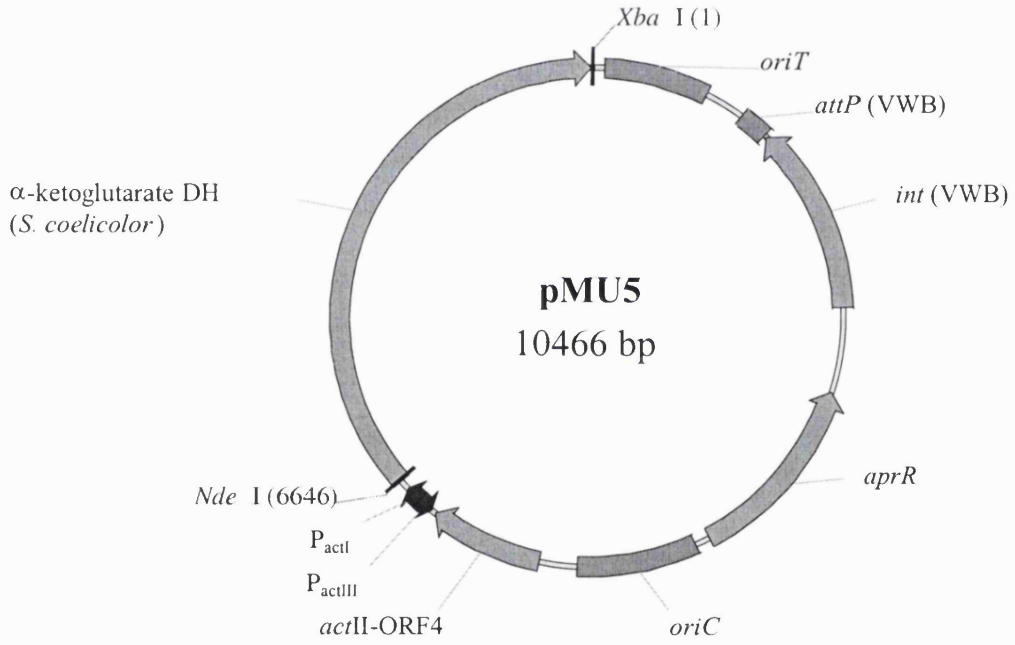


Figure 55: Diagram of pMU5.

6.4.2 Red Pigment Synthase Gene Knock-Out in *S. erythraea*

6.4.2.1 pMU4

pMU4, shown in Figure 56, is a recombination vector containing *oriC*, *oriT*, *ampR*, *tsrR*, and 1 kb of sequence homology to *rpp*A of *S. erythraea*. The 1 kb *rpp*A fragment was amplified by PCR using *S. erythraea* genomic DNA as the template, and MUSERPF and MUSERPR as the primers which also engineered *Xba* I restriction sites at each end. pMU4 was constructed by digesting pCJW192 with *Xba* I and *Spe* I, and digesting the isolated PCR fragment with *Xba* I. *Xba* I and *Spe* I have compatible sticky ends that can be ligated but then do not reform either site. The 4.1 kb pCJW192 fragment was SAP treated and ligated to the 1 kb PCR fragment. pMU4 is ampicillin resistant in *E. coli* and thiostrepton resistant in *S. erythraea*.

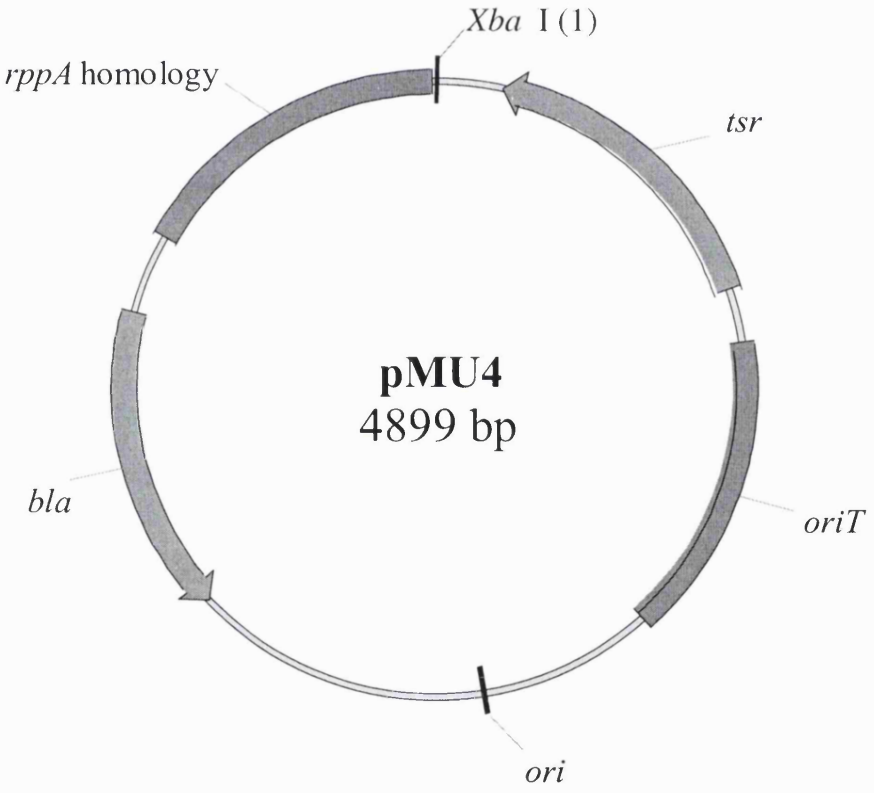


Figure 56: Diagram of pMU4.

6.5 Conjugated *S. erythraea* Strains

Table 43 describes the three sets of strains constructed or initiated in this work. The first three strains (pMU5 set), had the α-ketoglutarate dehydrogenase from *S. coelicolor* over-expressed. The second three strains (pMU4 set) had the *rppA* gene (red pigment synthesis) knocked out. The third three strains (pMU4 + pMU5 set) had both the α-ketoglutarate dehydrogenase from *S. coelicolor* over-expressed and the *rppA* gene knocked out.

Table 43: Conjugated *S. erythraea* strains.

Strain Name	Host Strain	Plasmid	Description
S. erythraea MU1	*S. erythraea* wt RV	pMU5	*S. erythraea spp.* with integrative expression vector that over-expresses α-ketoglutarate dehydrogenase.
S. erythraea MU2	*S. erythraea* JC2	pMU5	
S. erythraea MU3	*S. erythraea* JC103	pMU5	
S. erythraea MU4	*S. erythraea* wt RV	pMU4	*S. erythraea spp.* with Δ*rppA* by homologous recombination.
S. erythraea MU5	*S. erythraea* JC2	pMU4	
S. erythraea MU6	*S. erythraea* wt RV	pMU4, pMU5	*S. erythraea spp.* with integrative expression vector that over-expresses α-ketoglutarate dehydrogenase and with Δ*rppA* by homologous recombination.
S. erythraea MU7	*S. erythraea* JC2	pMU4, pMU5	

6.6 Discussion and Conclusions

Five new plasmids and seven novel *S. erythraea* strains were produced to further the understanding of the interaction of primary and secondary metabolism for the production of polyketides. The primary metabolic enzyme, α-ketoglutarate dehydrogenase, was over-expressed to determine if carbon lost to excess α-ketoglutarate could be re-channeled back into primary metabolism. The *rppA* gene, which produces THN (the core of the red pigments), was knocked-out to determine if

the carbon used for red pigment synthesis could also be re-channeled back into primary metabolism. The α-ketoglutarate dehydrogenase over-expression and *rppA* knock-out were also produced in combination with erythromycin knock-out (JC2) and/or deregulated erythromycin expression (JC103) *S. erythraea* strains to extend the studies of re-channeling carbon to erythromycin. Experiments were performed to determine the changes in physiology of the new strain produced in this work compared to the *S. erythraea* wild-type red-variant and these experiments are described in Chapter 7.

In addition to the construction of the novel *S. erythraea* strains, an integrative vector (pMU3) was constructed that should have general use for site-specific integration of genes cloned in pMU3 into *S. erythraea* chromosomal DNA. The pMU3 vector has all of the necessary components for conjugation and integration between *E. coli* and *S. erythraea* including the VWB integration machinery, the P_{actI} promoter system, an apramycin resistance selection marker, a restriction enzyme cloning site, an origin of replication in *E. coli*, and an origin of transfer from *E. coli*. This was the first report of using the VWB integration system in *S. erythraea* to the best of our knowledge. The pMU3 and pMU5 plasmids were both successfully conjugated and integrated into *S. erythraea* wt RV, *S. erythraea* JC2, and *S. erythraea* JC103 strains. Southern blot analysis and PCR verification of plasmid integration was not performed at this stage of the research. However, it was believed that successful integration was achieved for several reasons. The first reason was that the apramycin resistance gene was used as selection marker and used throughout the construction process. Also, several apramycin resistant clones could readily be obtained (more than 1000 per plate) suggesting that the plasmid was easily incorporated into the chromosome implying site specific integration (Van Mellaert *et al.*, 1998). Second, was that the expected physiology was observed in that α-ketoglutarate excretion was decreased and α-ketoglutarate dehydrogenase activity level was increased (see Chapter 7). And third, site specific integration of the VWB integrase has been shown for *S. venezuelae* and *S. lividans* which initially suggested that this technique may be easily transferable to *S. erythraea* (Van Mellaert *et al.*, 1998).

While this chapter describes the molecular biology techniques used to construct the new *S. erythraea* strains, the following chapter (Chapter 7) described the details of the experiments performed to determine the changes in physiology of the new strains compared to the *S. erythraea* wild-type red-variant.

7 Effect of Over-expressed α-Ketoglutarate Dehydrogenase on Organic Acid Excretion and Polyketide Synthesis by *Saccharopolyspora erythraea*

7.1 Summary

The objective of this study was to determine the effect of an over-expressed heterologous α-ketoglutarate dehydrogenase on organic acid excretion and polyketide synthesis by *S. erythraea*. The α-ketoglutarate dehydrogenase gene from *S. coelicolor* was integrated into the genomic DNA of *S. erythraea* wt RV as described in the previous Chapter 6. This chapter describes the physiological effect the α-ketoglutarate dehydrogenase over-expression.

It was hypothesized that over-expression of α-ketoglutarate dehydrogenase would decrease excretion of α-ketoglutarate outside the cell, decrease red pigment production, and increase erythromycin. This hypothesis was based on results presented in previous chapters of this thesis. The physiology of *S. erythraea*, presented in Chapter 3, was studied under nitrogen-limited conditions which showed that as the nitrate in the medium decreased, the levels of red pigment and organic acid excretion increased. Enzyme activity analysis, presented in Chapter 4, showed that the levels of activity correlated with the changes in pyruvate and α-ketoglutarate excretion over time. Isocitrate dehydrogenase exhibited much greater activity than α-ketoglutarate dehydrogenase which may have caused the high levels of α-ketoglutarate excretion (see Figure 39). As presented in Chapter 5, metabolic flux analysis was performed which predicted that if the flux from α-ketoglutarate to succinyl-CoA increases, then the flux to erythromycin A should increase and the flux to should red pigment decrease. With the support of the physiology, enzyme activity, and metabolic flux analyses, it was proposed to construct a *S. erythraea* strain that overproduces α-ketoglutarate dehydrogenase to determine if the carbon lost to α-

ketoglutarate excretion could be re-channeled back in to primary metabolism and potentially increase erythromycin production compared to the wild-type.

The *S. erythraea* MU1 strain, which contains an over-expressed α-ketoglutarate dehydrogenase gene, was grown in nitrate-limited medium and the growth, substrate uptake, organic acid excretion, red pigment production, and erythromycin production were measured. The results showed that *S. erythraea* MU1 and *S. erythraea* wt RV were equivalent with respect to growth rate and maximum biomass accumulation. *S. erythraea* MU1 showed about a 50 % reduction of α-ketoglutarate excretion, 30 % reduction in erythromycin production, and 10% reduction in red pigment production compared to the *S. erythraea* wt RV when grown under nitrate-limited conditions. The specific activity of α-ketoglutarate dehydrogenase was approximately 10-fold greater for *S. erythraea* MU1 compared to *S. erythraea* wt RV when grown on nitrate-limited medium. When the two strains were grown on three different types of rich media, the composition did not affect the levels of erythromycin between the strains. These results indicated that the carbon lost to α-ketoglutarate excretion could be re-channeled back into the cell. However, there are further strain improvements that need to be made to direct the carbon to polyketide production.

7.2 Materials and Methods

In general, the materials and methods used for the experiments in this chapter are found in section 3.3, unless otherwise stated.

7.2.1 Strains

Two strains were used in this study. This first was the genetically engineered strain *S. erythraea* MU1, which has an over-expressed heterologous α-ketoglutarate dehydrogenase gene. The second strain was the control, *S. erythraea* wild-type red-variant.

7.2.2 Media and Analytical Methods

Nitrate-limited medium as described in section 3.3.2.1 was used for all experiments in this chapter. The liquid-stocks were prepared in 30 g/L tryptone soy broth instead of nutrient broth as described in section 3.3.1.

7.2.3 Experimental Conditions

The experimental condition for the studies presented in this chapter are described in Table 44.

Table 44: Experimental conditions for testing the physiological effect of an over-expressed heterologous α-ketoglutarate dehydrogenase gene in *S. erythraea* grown under nitrate-limitation.

Strains	*S. erythraea* MU1 (see section 3.3.1) *S. erythraea* wt RV (see section 3.3.1)
Media	Nitrate-limited medium. (see section 3.3.2.1) Rich media (see Table 45).
Growth Mode	Batch 2L Shake-Flask
Temperature	28° C
Shaker RPM	200
Sampling Times, hours	0, 10, 24, 34, 43, 48, 53, 58, 67, 72, 77, 96, 120, and 144.
Experimental Duplication	Two isolates of each strain were grown in nitrate-limited medium independently two times. All analytical analyses were performed twice for each sample. The error bars represent the 95% confidence interval.

Table 45: Components of rich media.

Media	Component	Concentration, g/L
Tryptone Soy Broth/Glucose Media, pH 6.0		
	Tryptone Soy Broth	30
	Glucose	10
	2-Morpholinoethanesulfonic acid	21.3
Oil-Base Media, pH 6.8		
	Soya Flour	30
	Dextrin	10
	Rapeseed Oil	23
	KH_2PO_4	1.2
Sucrose/Succinate Defined Media, pH 6.5		
	Sucrose	68.5
	Succinic Acid	2.4
	K_2HPO_4	3.5
	$MgSO_4$	0.6
	KNO_3	10.1
	Trace Elements (see Table 2)	

7.3 Results and Discussion

7.3.1 Growth and Substrate Uptake

The maximum specific growth rate, glucose uptake, and nitrate uptake are shown in Table 46 for *S. erythraea* wt RV and *S. erythraea* MU1. For these three parameters, *S. erythraea* MU1 has slightly greater values, however, the differences are 10 % or less compared to the wild-type. The data suggested that the cultures were nitrate-limited and glucose was not depleted throughout the 144 hours of the experiment. Nitrate depletion coincided with the beginning of stationary phase. Biomass, glucose in the supernatant, and nitrate in the supernatant are shown in Figure 57, Figure 58, and Figure 59, respectively.

Table 46: Growth rates and substrate uptake rates for *S. erythraea* wt RV and *S. erythraea* MU1 grown under nitrate-limiting conditions.

Parameter	*S. erythraea* wt RV	*S. erythraea* MU1
Maximum Specific Growth Rate, h^{-1}	0.041	0.043
Maximum Glucose Uptake Rate, g /h	0.293	0.323
Maximum Nitrate Uptake rate, g/h	0.092	0.100

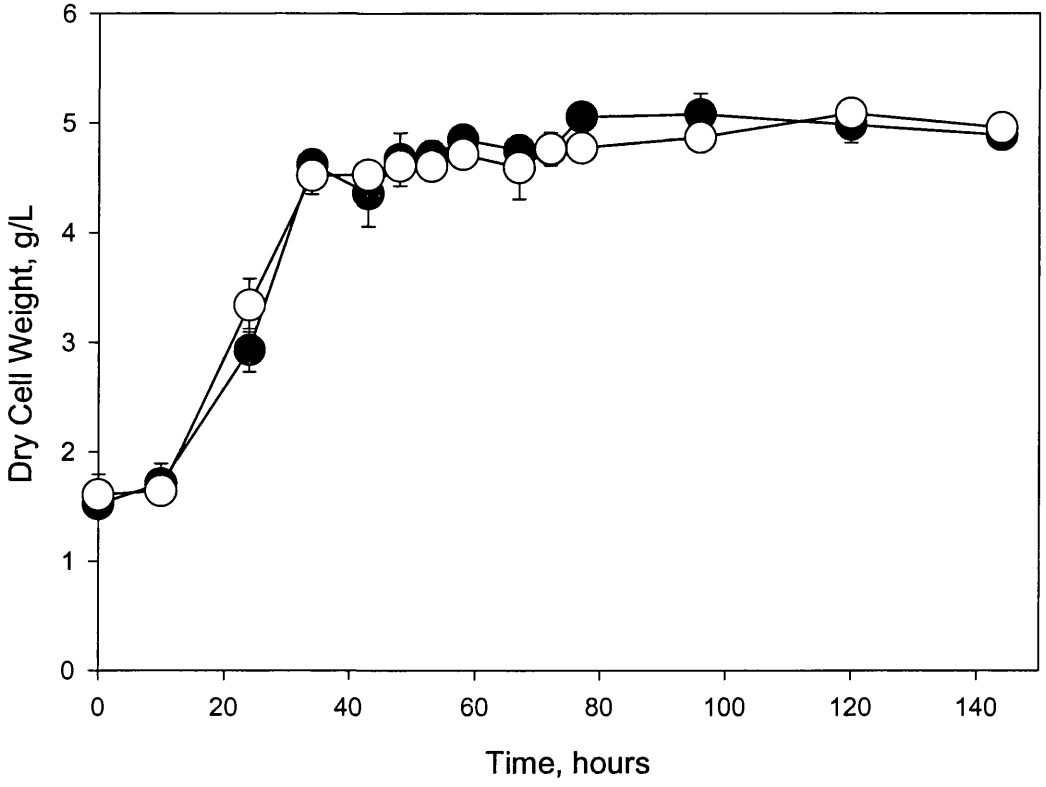


Figure 57: Biomass formation over time for *S. erythraea* wt RV and *S. erythraea* MU1.

(●) *S. erythraea* wt RV and (○) *S. erythraea* MU1.

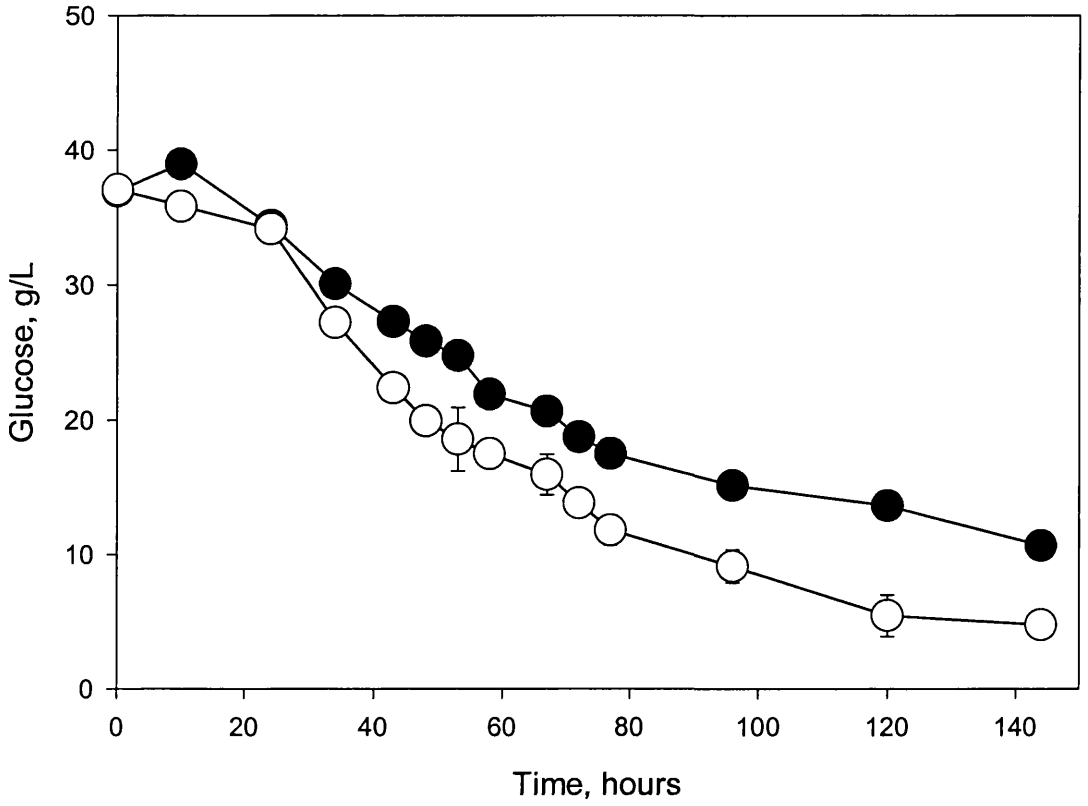


Figure 58: Glucose uptake over time for *S. erythraea* wt RV and *S. erythraea* MU1.

(●) *S. erythraea* wt RV and (○) *S. erythraea* MU1.

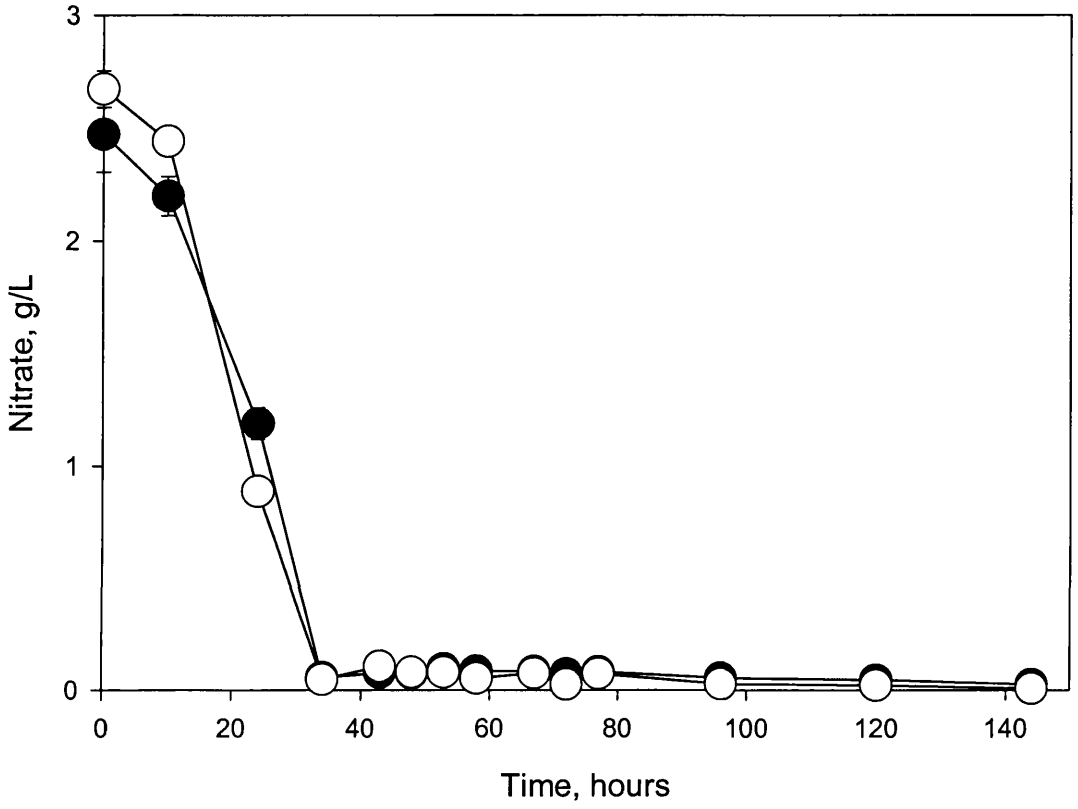


Figure 59: Nitrate uptake over time for *S. erythraea* wt RV and *S. erythraea* MU1.

(●) *S. erythraea* wt RV and (○) *S. erythraea* MU1.

The results indicated that *S. erythraea* MU1 grew slightly faster and had slightly greater substrate uptake rates compared to the wild-type. This may be caused by increased activity of α-ketoglutarate dehydrogenase increasing flux though the TCA cycle due to re-channeling of the carbon formerly excreted to α-ketoglutarate. Increased flux through the TCA cycle may have caused more carbon to be going through primary metabolism and, hence, increased glycolytic flux. The increase in glycolytic flux could be caused by the TCA cycle of the recombinant strain "pulling" carbon through glycolysis faster compared to the wild-type. The extra carbon, presumably, in the MU1 strain compared to the wild-type may be being "burned up" by the TCA cycle as carbon dioxide.

7.3.2 α-Ketoglutarate Excretion

The α-ketoglutarate excretion is shown in Figure 60. The amount α-ketoglutarate excreted outside the cell by *S. erythraea* MU1 is about 50 % less compared to *S. erythraea* wt RV. These results imply that the over-expression of α-ketoglutarate dehydrogenase is preventing excretion, and is keeping the carbon, formerly wasted, within the cell.

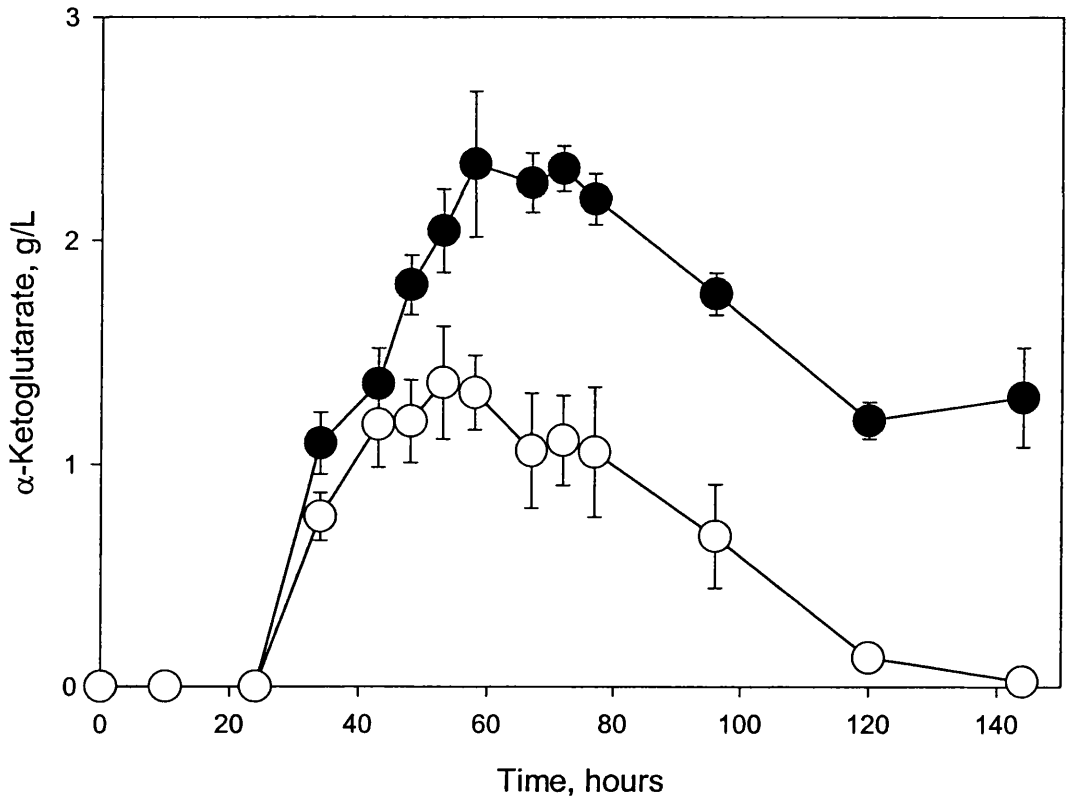


Figure 60: α-Ketoglutarate excretion over time by *S. erythraea* wt RV and *S. erythraea* MU1.

(●) *S. erythraea* wt RV and (○) *S. erythraea* MU1.

Decreased levels of α-ketoglutarate excretion were predicted by the hypothesis stated in Section 7.1. The results suggested that carbon lost to organic acid excretion may be re-channeled to primary metabolism. This is encouraging with respect of being able to direct carbon to defined reactions within the pathways of the cell. However, it should be noted that not all excretion was prevented. This may be due to the complex regulation placed specifically on α-ketoglutarate dehydrogenase which could not be

overcome with simply supplying more enzyme. α-Ketoglutarate is inhibited by NADH and the reaction product, succinyl-CoA. Therefore, even if high levels of enzyme are produced, the enzyme activity may not be proportionally increased.

7.3.3 α-Ketoglutarate Dehydrogenase Activity

The activity of α-ketoglutarate dehydrogenase was measured to determine if over-expression of the gene was leading to increased activity of the expressed enzyme. Presumably, if greater levels of enzyme were produced as a result of the over-expression, then greater levels of specific activity should be measured compared to the wild-type. The activities, depicted in Figure 61, show that the activity was indeed increased, and approximately 10-fold greater for the *S. erythraea* MU1 strain compared to the wild-type. These results suggested that the differences observed between the *S. erythraea* MU1 and wild-type are due to the increased gene expression and hence activity of α-ketoglutarate dehydrogenase, rather than non-specific changes in physiology derived from genetic manipulation.

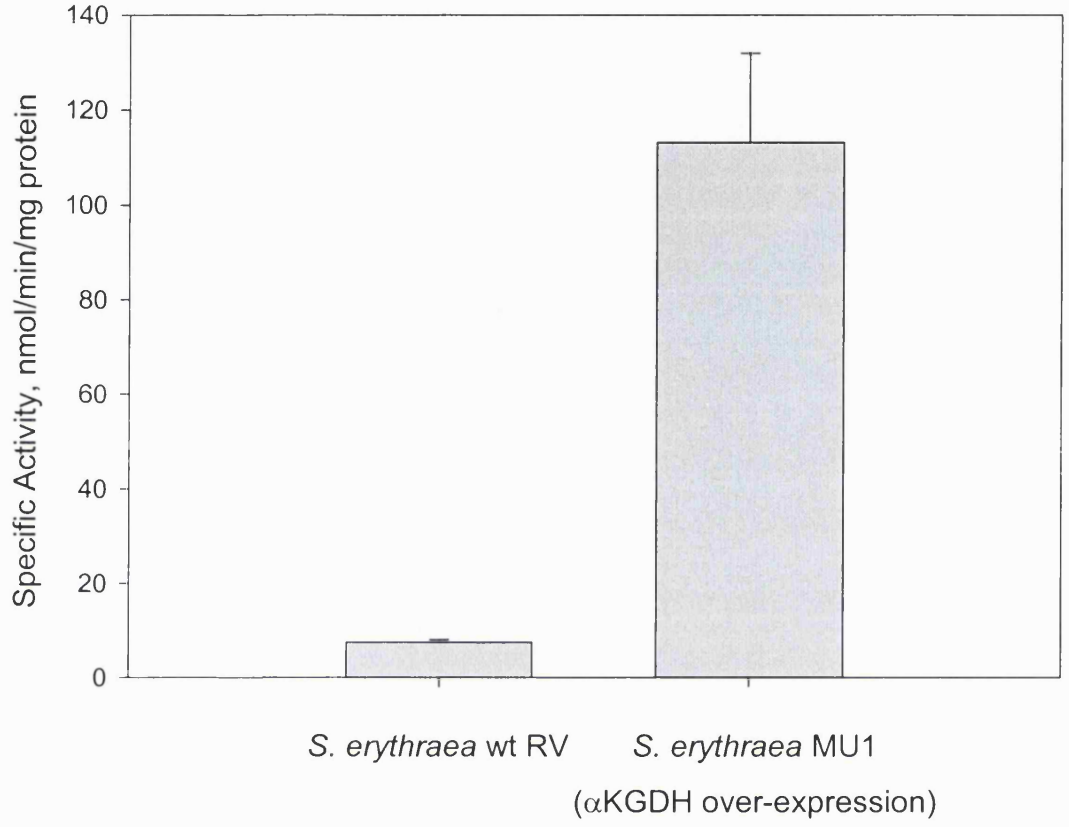


Figure 61: α-Ketoglutarate dehydrogenase activity for *S. erythraea* wt RV and *S. erythraea* MU1.

7.3.4 Red Pigment Production

Red pigment production is shown in Figure 62. Red pigment production was slightly decreased by *S. erythraea* MU1 compared to the wild-type by about 10 %.

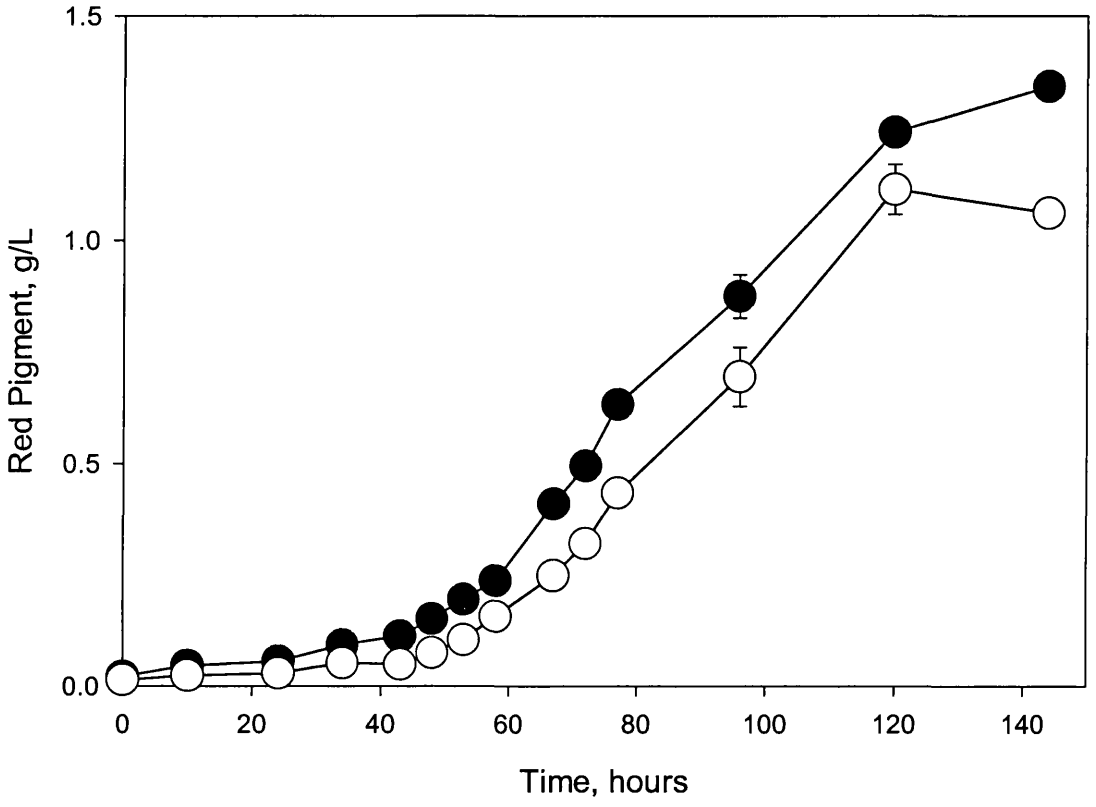


Figure 62: Red pigment production over time by *S. erythraea* wt RV and *S. erythraea* MU1.

(●) *S. erythraea* wt RV and (○) *S. erythraea* MU1.

It was predicted that flux to red pigment would decrease due to increased activity of α-ketoglutarate dehydrogenase because carbon would be "pulled" from glycolysis to the TCA cycle. Even though the results show a decreased production of pigment by *S. erythraea* MU1, the differences are small. This could be due to the fact that the increased demand for carbon due to higher levels of α-ketoglutarate dehydrogenase

are satisfied by the re-channeled carbon formerly excreted outside the cell. If the demand was satisfied then the carbon going to red pigment production, which is presumably upstream (acetyl-CoA branch point) of the TCA cycle, would not be expected to change to a great extent.

7.3.5 Erythromycin Production

Erythromycin production is shown in Figure 63. Erythromycin production *by S. erythraea* MU1 was decreased about 30 % compared to the wild-type.

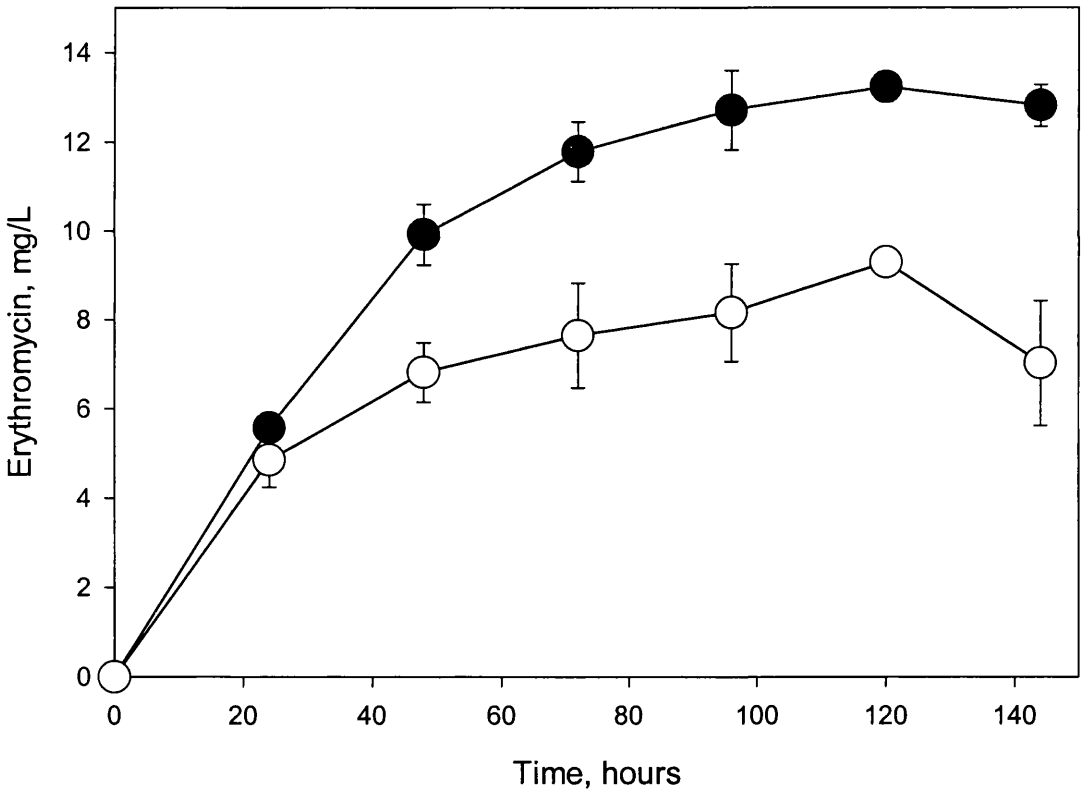


Figure 63: Erythromycin production over time by *S. erythraea* wt RV and *S. erythraea* MU1 grown in nitrate-limited medium.

(●) *S. erythraea* wt RV and (○) *S. erythraea* MU1.

Even though, α-ketoglutarate excretion was decreased by over-expressing the α-ketoglutarate dehydrogenase, erythromycin levels decreased when grown under nitrate-limited conditions. It is not clear why erythromycin synthesis is reduced under these conditions and it would be of interest to compare erythromycin production between *S. erythraea* MU1 and the wild-type when grown on a richer media. Perhaps a complex medium used for erythromycin production (oil-based medium), or even simply increasing the carbon and nitrogen levels in defined media may increase erythromycin levels. Modification of the defined media may only lead to increased levels of biomass, however, the nitrate-limited medium used in these studies may also limit the levels of secondary metabolites. To test if the media has an effect on the level of erythromycin produced by *S. erythraea* MU1 compared to the wild-type, three different media were tested.

The first medium was a sucrose/succinate defined medium used previously to test for erythromycin and erythromycin intermediates (Caffrey *et al.*, 1992; Gaisser *et al.*, 1997; Gaisser *et al.*, 2000). The second was a tryptone soy broth based soluble complex medium. The third was an oil based medium with soya flour. The results showed that the levels of biomass and erythromycin were similar for both strains for each of the media tested. However, the different media produced different levels of erythromycin as shown in Figure 64.

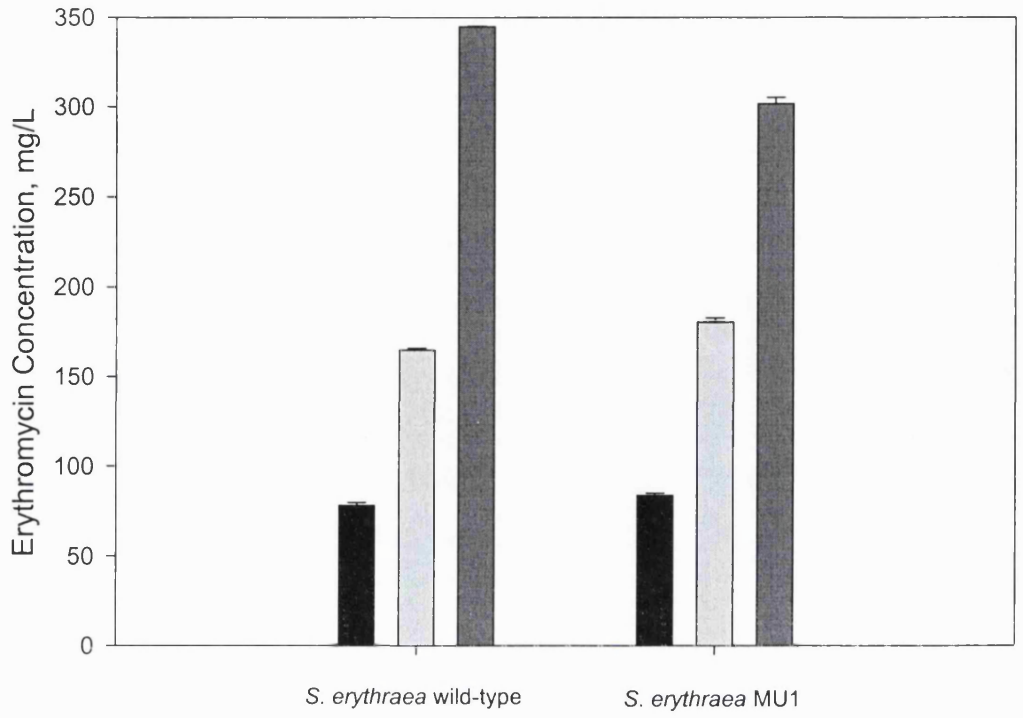


Figure 64: Erythromycin Concentration for S. erythraea wild-type and MU1 Grown on Various Rich Media.

(■) Sucrose/Succinate Defined Medium, (■) Tryptone Soy Broth Based Soluble Complex Medium, and (■) Oil-Based Medium.

All three media produced more erythromycin compared to the nitrate-limited medium as shown in Figure 63. The sucrose/succinate defined medium produced approximately 80 mg/L of erythromycin, and the tryptone soy broth medium produced approximately 170 mg/L erythromycin for both strains. The oil-based medium produced approximately 345 mg/L for the wild-type and 300 mg/L for the MU1 strain. Overall, the richer media did not lead to higher levels of erythromycin by the *S. erythraea* strain with an overexpressed α-ketoglutarate dehydrogenase (*S. erythraea* MU1) compared to the wild-type.

7.4 Conclusions

A hypothesis based on previously described physiology and metabolic modelling results stated that increased flux from α-ketoglutarate to succinyl-CoA would decrease flux to α-ketoglutarate excretion, increase flux to erythromycin, and decrease flux to red pigment production. This hypothesis attempted to link the precursor availability for polyketides synthesis with secondary metabolism. This hypothesis was tested by genetically engineering a *S. erythraea* strain to over-express a heterologous α-ketoglutarate dehydrogenase gene from *S. coelicolor* (see Chapter 6 for details). Even though a *S. coelicolor* enzyme might be under similar physiological control as the *S. erythraea* α-ketoglutarate dehydrogenase gene, it was thought that a heterologous gene could attempt to avoid the *S. erythraea* specific metabolic regulation.

The *S. erythraea* strain with over-expressed α-ketoglutarate dehydrogenase (*S. erythraea* MU1) was grown under nitrate-limited conditions and was compared to the wild-type. The results of this comparison with regard to growth, substrate uptake, α-ketoglutarate excretion, red pigment production, and erythromycin production were presented in this chapter. The results led to the conclusions that carbon lost to excreted organic acids could presumably be re-channeled into primary metabolism for the potential re-direction to product formation. Re-channeling the carbon, however, did not increase flux to red pigment or erythromycin production. This is most likely due to other regulatory features present in the cell.

The levels of erythromycin produced were not increased by decreasing the flux to excreted α-ketoglutarate even though succinyl-CoA levels may increase as a results of increased activity of α-ketoglutarate dehydrogenase. Succinyl-CoA is one of the important intermediates to produce precursors needed for erythromycin biosynthesis. As erythromycin production was not increased, the re-channeled carbon might instead be directed to several other areas of metabolism. It would be possible to measure intracellular levels of succinyl-CoA levels and other TCA metabolites to determine the flow of carbon. Another reason could be that α-ketoglutarate is converted into glutamate or glutamine which could then lead to increased levels of proline or arginine. It would be possible to measure the intracellular amino acid levels to

determine if increased levels of glutamate, glutamine, proline, or arginine occur, particularly glutamate as it is the first product in this pathway. It may also be that the re-channeled carbon is being cycled through the TCA cycle and producing carbon dioxide. If the cell cannot immediately utilize the α-ketoglutarate for metabolite production then it may simply be "burning off" the carbon as carbon dioxide through TCA cycle activity. The carbon evolution rate determined by mass spectrometry off-gas analysis during fermentation could establish if increased levels of carbon dioxide were produced by *S. erythraea* MU1 compared to the wild-type. It would also be important to measure the levels of succinyl-CoA to establish if increased activity of a-ketoglutarate dehydrogenase did in fact lead to increased levels of succinyl-CoA.

α-Ketoglutarate excretion was decreased by ~50 % by over-expressing the α-ketoglutarate dehydrogenase. However, erythromycin levels were not increased when grown under nitrate-limited conditions. Comparison of erythromycin production between *S. erythraea* MU1 and the wild-type when grown on a richer media did not show differences. The results indicated that control of secondary metabolite production is more complicated than simply providing more carbon in the system for potential synthesis of metabolites. Even though the *S. erythraea* MU1 may re-channel carbon into primary metabolism, activity of other erythromycin pathway enzymes may need to be increased to take advantage of the increased carbon in the system. For instance, succinyl-CoA can be converted to methylmalonyl-CoA by methylmalonyl-CoA mutase to then used as a direct precursor for erythromycin biosynthesis (see section 2.2.1.1). This enzyme may be a good next target for metabolic engineering to add to the existing improvements of *S. erythraea* MU1.

8 Red Pigment Characterization

8.1 Summary

S. erythraea produces one or more red pigments. The red-variant wild-type of *S. erythraea* produces more pigment and less erythromycin compared to the white-variant wild-type of *S. erythraea*. Characterization of the pigment was performed, and the isolation and antimicrobial activity were examined.

It was found that four soluble pigments were produced. These pigments were separated by thin layer chromatography. Two red pigments and two yellow pigments were observed and the two yellow bands had fluorescent properties. This mixture of pigment was found to have antimicrobial activity against *Staphylococcus aureus*, *Bacillus subtilis*, and *Arthrobacter citreus*. No antimicrobial activity was found against *Pseudomonas aeruginosa* Pao2 F or *Escherichia coli* DH5α.

8.2 Background

Sections 8.2.1 and 8.2.2 are reproduced from Chapter 3 sections 3.2.1.4 and 3.2.1.5.3. It seemed prudent to include these background sections again here since the information is important to introduce the characterization of the red pigment as well as the physiological control of the red pigment (Chapter 3).

8.2.1 Pigments Produced by Streptomycetes

It has been well documented that *Streptomyces* spp. produce various pigmented secondary metabolites, such as rubrolone produced by *S. echinoruber* sp. nov. (Palleroni *et al.*, 1978), metacycloprodigiosin produced by *S. longisporus ruber* (Wasserman *et al.*, 1969), roseoflavin produced by *Streptomyces* strain No. 768 (Otani *et al.*, 1974), undecylprodigiosin and actinorhodin produced by *S. coelicolor* A3(2) (Rudd and Hopwood, 1980; Bermudez *et al.*, 1998), a melanin-like pigment produced by *S. aureofaciens* (Nakano *et al.*, 2000), and unidentified sporulation pigment by *S. venezuelae* S13 (Scribner *et al.*, 1973). These pigments are considered secondary metabolites and, in some cases, exhibit antimicrobial activity (Otani *et al.*, 1974; Gerber and Lechevalier, 1976; Rudd and Hopwood, 1980). In some *Streptomyces* strains, a mixture of pigments is produced in conjunction with the major

pigment product, as determined by thin layer chromatography (Wasserman *et al.*, 1969; Palleroni *et al.*, 1978; Tsao *et al.*, 1985).

There have been a few reports on the physiological factors influencing pigment production in Streptomycetes. The nutritional requirement for pigment production was studied in *S. venezuelae* (Scribner *et al.*, 1973), for which higher amino acid concentrations and added $FeCl_3$ promoted pigment production. In *S. coelicolor* A3(2), the influence of phosphate and ammonium on the production of undecylprodigiosin and actinorhodin was examined (Hobbs *et al.*, 1990). Although undecylprodigiosin was not sensitive to either, actinorhodin was inhibited by ammonium and phosphate to varying degrees depending on the ammonium to phosphate ratio.

8.2.2 Red Pigments Produced by *Saccharopolyspora erythraea*

Saccharopolyspora erythraea produces the commercially important macrolide antibiotic, erythromycin A, in addition to an unidentified red pigment from which the organism obtained it name (Labeda, 1987). Hsieh and Kolattukudy (1994) investigated the effect of malonyl-CoA decarboxylase disruption on erythromycin production. They observed that the mutant strain lost the capacity to produce erythromycin and a red pigment, while the *S. erythraea* CA 340 parent strain produced erythromycin and 'became red as the culture grew dense'. They also reported that upon addition of exogenous propionate to the growth media, the mutant was able to re-establish erythromycin production but was not able to restore red pigment production. Clark *et. al.* (1995) examined the effect of controlled oxygen limitation on secondary metabolite formation and observed that *S. erythraea* wt NRRL 2338 produced an 'unidentified soluble red pigment' under oxygen-limited conditions.

Recently, the genes involved in the production of a red pigment produced by *S. erythraea* wt RV have been identified and characterized (Cortes *et al.*, 2002). Their work also shows that a variety of pigments derived from tetrahydroxynaphthalene are produced. Consistent with these results, Udea *et. al.* (1995) identified a water-soluble red-pigment produced by *S. griseus* and determined that the 2 genes *rppA* and *rppB* were necessary for pigment production (Ueda *et al.*, 1995). Funa *et. al.* (1999)

identified the red-pigment product of *rppA* produced by *S. griseus* as 1, 3, 6, 8-tetrahydroxynaphthalene (THN). They proposed that a chalcone synthase-like enzyme incorporates five malonyl-CoA units into a pentaketide before cyclizing to THN, which can be oxidized to form flaviolin. Flavolin can form various pigmented compounds which was evident when *rppA* was subcloned into *E. coli* a mixture of pigmented compounds was produced (Funa *et al.*, 1999). Even though we have not yet structurally characterized the pigments discussed in this report, it is possible that they are similar or identical to the compounds reported above derived from THN. However, very recently in a poster presentation, Weber and colleagues (2002) found that one of the red pigments produced by S. *erythraea* was pyomelanin, a shunt product of tyrosine metabolism. This result presents a new possible metabolic route to red pigment biosynthesis to compare to the THN hypothesis.

8.3 Materials and Methods

8.3.1 Pigment Isolation

8.3.1.1 Solid Phase Extraction

The red pigment was isolated from filtered culture supernatant using a Phenomenex C18 bond elute solid phase extraction cartridge. The procedure is identical to the erythromycin extraction method described in section 3.3.3.6.1 with the exception that the methanol elution was performed in 10 fractions. The red pigment was eluted in the second fraction as indicated by the visually intense red color compared to all other fractions. The procedure was repeated several times to obtain a sufficient amount of pigment. To verify purity of the red pigment and the absence of any erythromycin, the pooled second fractions were run on the erythromycin HPLC assay described in section 3.3.3.6.2. Experiments were previously performed to make certain the red pigment and erythromycin are clearly separated peaks by the extraction and HPLC method as described in section 3.3.3.6.3.

8.3.1.2 Thin Layer Chromatography

Thin layer chromatography was performed by spotting 3μL of sample onto Whatman silica gel 60A fluorescent plates. Samples were run for approximately 3 hours with a mobile phase of 50 % methanol in dH_2O. The plates were analyzed under visible light and UV light (254 nm).

8.3.2 Antimicrobial Activity Assay

The red pigment was isolated as described above and samples were prepared by diluting the isolated pigment to concentrations of 5.00 g/L, 2.50 g/L, 1.25 g/L, 0.50 g/L and 0 g/L in 1 mL methanol. The samples were then dried down to a powder using rotary vacuum and re-suspended in 1 mL dH_2O. Liquid cultures of *Arthrobacter citreus*, *Bacillus subtilis*, and *Staphylococcus aureus* F were grown for 20 hours at 28 °C for *A. citreus* and 37 °C for *B. subtilis* and *S. aureus* before being plated onto nutrient agar. Wells (3/4 inch) were then bored into the agar (prepared in 10 cm round petri dishes) and 100 μL of each sample was dispensed into each well. The plates were incubated for 24 hours at which time the diameters of the zones of inhibition were measured. To compare the antimicrobial activity of the pigments to erythromycin, the above procedure was performed as described with the exception that the erythromycin concentrations tested were 0.200 g/L, 0.100 g/L, 0.050 g/L, 0.010 g/L, 0.005 g/L and 0 g/L.

The *Bacillus subtilis*, *Staphylococcus aureus* F, *Escherichia coli* DH5α, and *Pseudomonas aeruginosa* Pao2 strains used in the antimicrobial activity assay were from the culture collection of Dr. J. Ward. The *Arthrobacter citreus* (# NRRL B-1258) was obtained from the NRRL culture collection.

8.4 Results

8.4.1 Pigment General Characteristics and Effect of pH

The red pigment was found to be a water-soluble compound in liquid culture, and freely-diffusible from the cell. On solid medium, as the pigment permeates into the solid agar substrate, the mycelium first became red and then the solid medium substrate turns red. Upon addition of acid (HCl, to pH = 0.1) the red pigment solution turned a bright yellow color, and upon addition of alkali (NaOH, to pH = 13.5), the red pigment solution turned a blackish purple color.

8.4.2 Thin Layer Chromatography

Thin layer chromatography (TLC) separation of the isolated fraction of the red pigment revealed four visible bands at Rf values of 0.92 (pigment A), 0.88 (pigment B), 0.79 (pigment C), and 0.70 (pigment D) as shown in Figure 65. Pigment B yielded the largest band and was a dark red color. The band of pigment A was approximately 50% of the area covered on TLC plate by pigment B and was a red-orange color. The bands of pigments C and D were approximately 20% of the area covered on the TLC plate by pigment B and were yellow. The same four bands only were present under UV light as shown in Figure 65. Under the UV light (254 nm), pigments A and B were dark bands where pigments C and D were bright bands, establishing that they were fluorescent compounds.

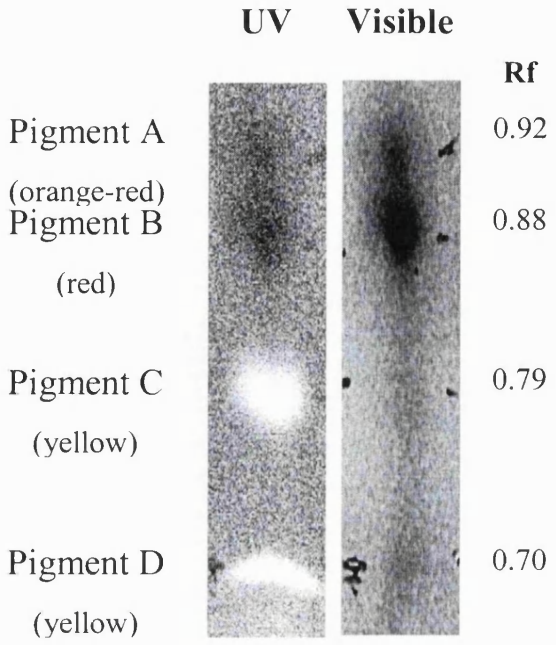


Figure 65: Thin layer chromatography results of pigments produced by *S. erythraea* wild-type red-variant under UV and visible light.

8.4.3 Antimicrobial Activity

Samples of 5.00 g/L, 2.50 g/L, 1.25 g/L, 0.50 g/L and 0 g/L pigment (same pigment mixture used for TLC in section 8.4.2) in dH_2O were tested against *Bacillus subtilis*, *Arthrobacter citreus*, and *Staphylococcus aureus* for antimicrobial activity. The pigment had antimicrobial activity against all three strains at the lowest concentration tested (0.50 g/L) with *A. citreus* being the most sensitive with a 2.5 cm zone of inhibition, followed by *B. subtilis* (1.6 cm at 0.50 g/L) and *S. aureus* (1.0 cm at 0.50 g/L). The pigment was not active against *Escherichia coli* or *Pseudomonas aeruginosa.* Figure 66 shows the 5 g/L and 0 g/L results for each strain tested. Erythromycin also exhibited antimicrobial activity to the three organisms tested. For comparison, 0.005 g/L of erythromycin produced zones of inhibition of at least 2.5 cm for each strain tested, suggesting that the pigment is far less active against these organisms than erythromycin.

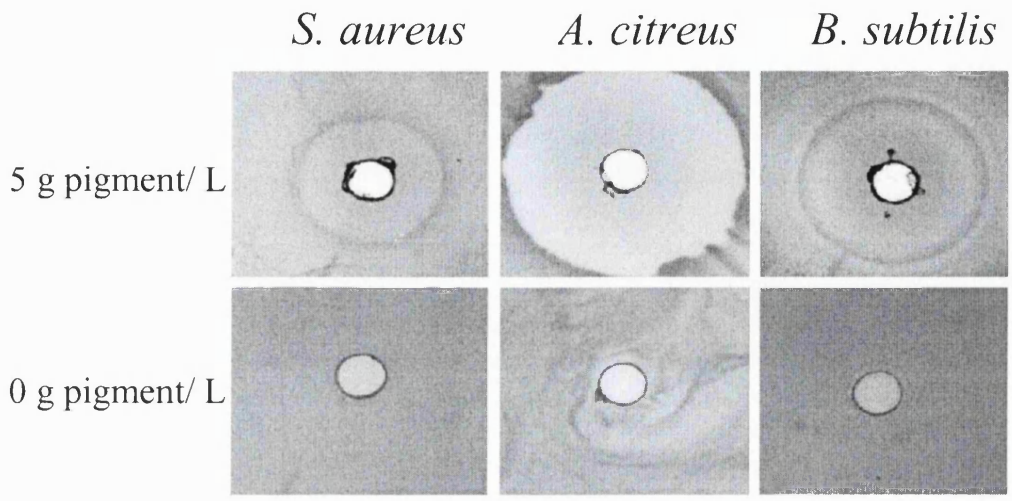


Figure 66: Antimicrobial assay results of 5 g pigment/L and 0 g pigment/L for test organisms of *S. aureus*, *A. citreus*, and *B. subtilis*.

8.5 Discussion and Conclusions

Challenges for future work include elucidation of the pigment structures which would be useful in identifying similarities between the pigments and erythromycin. An understanding of its structure may suggest if its biosynthetic precursors or pathways are related to those of erythromycin, and thereby extend our understanding of secondary metabolite production in this important Actinomycete. Efforts to elucidate the structure of the major red pigment (pigment B in Figure 65) had been ongoing but had not yet produced a definitive structure.

Recently, in *S. erythraea* wt RV, the genes for a type III polyketide synthase have been identified and cloned, the disruption of which prevented the synthesis of red pigments identified as oxidized and polymerized products of 1, 3, 6, 8-tetrahydroxynaphthalene (THN) (Cortes *et al.*, 2002). However, even though red pigment was not produced in the disruptant, erythromycin levels were not increased. The genes from *S. erythraea* are very similar to those necessary for THN synthesis in *S. griseus* (Cavalier-Smith, 1992; Funa *et al.*, 1999). Even though we have not yet structurally characterized the pigments discussed in this report, it is possible that they are THN derivatives (Cortes *et al.*, 2002). If so, this is the first documentation of their antimicrobial activity. Furthermore, elucidating the differences between the *S. erythraea* wild-type, the red-variant wild-type, and the *S. erythraea* Δ*eryA* mutant at the genetic level may determine which genes influence the production of pigments compared to production of erythromycin.

9 Discussion and Conclusions

This thesis described the metabolic engineering approach used to optimize *S. erythraea* for production of two polyketides, which were erythromycin and a red pigment. The majority of the research focused on how carbon directed to organic acid excretion could be re-channeled to erythromycin precursors to produce more erythromycin. The research also investigated the production of red pigment by *S. erythraea* which was a rarely studied compound at the beginning of this thesis research.

9.1 Metabolic Engineering of α-Ketoglutarate Dehydrogenase for Re-Channeling of Carbon from Organic Acid Excretion to Erythromycin Biosynthesis

The investigation of α-ketoglutarate dehydrogenase as a target for metabolic engineering to re-directed carbon from organic acid excretion to erythromycin precursors had several components, each representing a phase of the metabolic engineering cycle. The metabolic engineering cycle involves analysis, design and synthesis of the organism. The analysis involves characterization of the organism, which may include physiological studies and flux analysis. The design involves interpretation of the analysis to propose genetic modifications for strain improvement. The synthesis entails construction of a new strain using recombinant DNA technology. The cycle continues with another round of analysis of the new strain, and so on (Nielsen, 2001). This research was distinctive in that it completes a full sequence of the metabolic engineering cycle. This section aims to summarize and discuss the components, in one section, of the investigation involving over-expression of α-ketoglutarate dehydrogenase in *S. erythraea*, which have been previously discussed in various sections throughout different chapters.

The components of the analysis were described in Chapters 3, 4, and 5, where physiological characterization, enzyme analysis, and metabolic flux analysis were presented. The physiological characterization of *S. erythraea* (Chapter 3) showed that organic acids, in the form of pyruvate and α-ketoglutarate, were excreted from the cell at levels of up to 20% of the carbon consumed. It was thought that this carbon

lost from the cell could be re-channeled (or prevented from excretion) into the cell and used for product (erythromycin) biosynthesis. It was also found that pyruvate was excreted and then re-consumed such that there was no pyruvate outside the cell at the end of the stationary phase. However, α-ketoglutarate was excreted and little to no re-consumption occurred. Also, organic acid excretion did not occur until nitrate was depleted which corresponded with the end of the growth phase and the onset of the stationary phase. This same trend has been observed in several Streptomycetes (Ahmed *et al.*, 1984).

To determine if enzyme activity levels may be influencing organic acid excretion pyruvate dehydrogenase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase activity were measured (Chapter 4). It was found that pyruvate dehydrogenase activity varied over the course of the growth and stationary phases, which corresponded to the excretion and consumption profile. This indicated that pyruvate dehydrogenase may be participating the control of pyruvate availability for the cell. Isocitrate dehydrogenase activity was 1000-fold higher that α-ketoglutarate dehydrogenase activity which indicated that the cell appears to have a large capacity for α-ketoglutarate synthesis. However, the observation that α-ketoglutarate dehydrogenase activity was so much lower may explain why high levels of α-ketoglutarate excretion were observed. At this point, α-ketoglutarate dehydrogenase was considered as a potential metabolic engineering target. If the activity of α-ketoglutarate dehydrogenase could be increased then perhaps α-ketoglutarate excretion would be prevented and more succinyl-CoA would be produced which could be converted to make all of the precursors needed for erythromycin biosynthesis. Successful attempts to increase TCA cycle metabolites have been reported. For example, fumarate reductase was over-expressed in *E. coli* and, when the recombinant strain was grown on glucose, conversion of fumarate to succinate had higher rates and yields compared to wild-type (Goldberg *et al.*, 1983). Other attempts, including over-expression of malic enzyme has also been successful in producing higher levels of succinic acid in *E. coli* (Millard *et al.*, 1996; Stols and Donnelly, 1997; Wang *et al.*, 1998; Hong and Lee, 2001). Succinic acid was produced with high yield and productivity when *A. succiniciproducens* was grown on glucose (20 g/L) and with a specific ratio of external hydrogen to carbon dioxide of 5:95%,

indicating that high levels of succinic acid could be produced by process optimization (Lee *et al.*, 1999). However, in a report on an α-ketoglutarate dehydrogenase knock-out in *B. japonicum*, it had been suggested that α-ketoglutarate could be metabolized by α-ketoglutarate decarboxylase and succinate semialdehyde dehydrogenase which would provide an alternative pathway for α-ketoglutarate catabolism (Green *et al.*, 2000). This study indicated that even though α-ketoglutarate dehydrogenase had low activity, α-ketoglutarate may be consumed by other pathways and would not be available for conversion to succinyl-CoA even if α-ketoglutarate dehydrogenase activity was increased. Other data with *B. japonicum* indicated that the organism could compensate for inhibition of α-ketoglutarate dehydrogenase during nitrogen fixation (Green and Emerich, 1997). It would be interesting to search for the genes of α-ketoglutarate decarboxylase and succinate semialdehyde dehydrogenase in the *S. erythraea* genome once it becomes available.

Metabolic flux analysis was performed (Chapter 5) to see if the metabolic model also suggested that α-ketoglutarate dehydrogenase might be a good target for metabolic engineering. The analysis showed that if flux from α-ketoglutarate to succinyl-CoA could be increased then erythromycin levels should increase. This prediction was presumably obtained because if increased levels of carbon were in the system then some of it will be channeled to the various pathways, including erythromycin biosynthesis. One of the limitations of metabolic flux analysis is that it is not possible to incorporate enzyme regulation into the model.

Once the analysis was completed, design of an improved *S. erythraea* strain was suggested. It was proposed that α-ketoglutarate dehydrogenase should be over-expressed in *S. erythraea* to attempt to channel carbon, that would previously go to α-ketoglutarate excretion, to succinyl-CoA and further on to erythromycin production. The expected results were that α-ketoglutarate excretion would be decreased and erythromycin production would be increased.

The synthesis of the new strains were described in Chapter 6. A heterologous α-ketoglutarate dehydrogenase gene from *S. coelicolor* was over-expressed in *S. erythraea* wild-type red-variant. The plasmid containing the E_1 and E_2 subunits of the

α-ketoglutarate dehydrogenase gene was site specifically integrated into the *S. erythraea* chromosomal DNA using the VWB integrase system which was used for the first time with *S. erythraea* in this work. Other novel *S. erythraea* strains were constructed at the same time but physiological studied were focused on the comparison between the α-ketoglutarate dehydrogenase over-expression strain and the wild-type due to time constraints. The E_3 subunit of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase are assumed to be identical in S. *erythraea*. It is possible that both enzyme complexes compete for the E_3 subunit, which would make it limiting for increased enzyme activity.

Physiological characterization of the α-ketoglutarate dehydrogenase over-expression strain and the wild-type was performed under nitrate-limited conditions as described in Chapter 7. This represented the analysis stage of the second cycle of metabolic engineering. The physiological comparison showed that α-ketoglutarate excretion was reduced by ~50% and the α-ketoglutarate dehydrogenase activity increased 10-fold compared to the wild-type. However, erythromycin production did not increase compared to the wild-type. Both strains were grown in three different media that had been used for erythromycin production. No significant differences in erythromycin production were observed between the two strains. It is, however, encouraging that α-ketoglutarate excretion was reduced, which indicated that carbon could be re-channeled. There have been other examples of TCA enzymes over-expressed or knocked-out in different organisms, and specifically knock-outs of α-ketoglutarate dehydrogenase in *E. coli* which showed that the organisms could not grow as well as the wild-type (Subik *et al.*, 1972; Walshaw *et al.*, 1997). However, to the best of our knowledge, this was the first attempt to over-express α-ketoglutarate dehydrogenase in any organism.

It is clear that further work is required to determine how to direct the carbon to increase erythromycin biosynthesis. There are several studies that could be performed to help determine where the next targets for metabolic engineering may be. Analysis of intracellular TCA metabolites, especially succinyl-CoA, could be performed. This could show if the carbon from α-ketoglutarate was, in fact, directed to succinyl-CoA. If the carbon is going to succinyl-CoA then the pathways to methylmalonyl-CoA and

propionyl-CoA could be studied. If the carbon was not going to succinyl-CoA then other pathways utilizing α-ketoglutarate could be studied. The reaction involving glutamate and glutamine may be good targets. Radioactively labeled substrate studies may be very helpful in determine where the carbon is directed throughout the metabolism, as has been showed by others (Inbar and Lapidot, 1991; Schmidt *et al.*, 1998; Christensen and Nielsen, 2000). This type of data would also increase the amount of information that could be obtained from metabolic flux analysis. There have been other reports that suggest control of secondary metabolite synthesis can involve co-factor levels in the cell (Kwon and Kim, 1998, van Gulik *et al.*, 2000). Co-factors and/or ratios of co-factors, e.g. CoA, ADP, ATP, NAD, NADH, NADP, NADPH, FAD, and FADH, could be measured to determine if erythromycin synthesis is limited/controlled by co-factor regulation. In a recent study, it was shown that glycolytic flux was controlled by the demand for ATP in *E. coli* (Koebmann *et al.*, 2002). This report demonstrated why little success, in controlling glycolysis, had been made with manipulating individual reaction of glycolysis, and supports investigating global regulation of the cell. Overall, the results of over-expressing α-ketoglutarate dehydrogenase in *S. erythraea* provide insight in to the relationship between primary metabolism and secondary metabolism with respect to polyketide biosynthesis.

9.2 Red Pigment Characterization

This thesis also studied the factors influencing red pigment production and attempted to characterize the pigments. The story is not complete and this section attempts to describe what is known from the thesis research described in Chapters 3 and 8, and from other researchers. When this thesis research was initiated, there was little information regarding a red pigment produced by *S. erythraea*. Two papers in the literature mentioned that a red pigment had been observed when reporting on other studies using *S. erythraea* (Hsieh and Kolattukudy, 1994; Clark *et al.*, 1995). However, other groups had determined that 2 genes *rppA* and *rppB* were necessary for red-pigment production by *S. griseus*, and determined that the product was derived from 1, 3, 6, 8-tetrahydroxynaphthalene (THN) (Ueda *et al.*, 1995; Funa *et al.*, 1999). They proposed that a chalcone synthase-like enzyme incorporates five malonyl-CoA units into a pentaketide before cyclizing to THN which could then be oxidized to form a variety of pigments.

In the research presented in Chapter 3, it had been observed that high levels of pigment were produced (1.5 g/L) when the *S. erythraea* wild-type red-variant was gown on nitrate-limited medium. In the same experiments it was observed that no erythromycin was produced (see section 3.4). It was also found that when an erythromycin polyketide synthase *S. erythraea* knock-out was grown on nitrate-limited medium a four-fold reduction in red pigment compared to the wild-type occurred (see section 3.5). It was decided that characterization of the red pigment should be performed in order to determine if there was any similarity with erythromycin or with THN. If the pigment was similar to THN, it could be possible that similar precursor pools were being used for both erythromycin and red pigment synthesis. Modulation between the two products could, therefore, be a possible area for metabolic engineering.

Pigment isolation and antimicrobial activity studies were performed as described in Chapter 8. It was found that a family of four pigments were produced *when S. erythraea* was grown on nitrate-limited medium. Two red and two yellow bands were separated by thin layer chromatography. This mixture of pigments exhibited anti-microbial activity for three gram-positive bacteria, and no anti-microbial activity for

two gram-negative bacteria (see section 8.4). It is possible that other pigments or combination of pigments are produced when *S. erythraea* is grown on other media. It has been observed that different media produce different colors of supernatant which can range from dark brown to light pink (data not presented).

In early 2002, Cortes *et al.* reported that the genes involved in the production of a red pigment produced by *S. erythraea* wt RV were the *rppA* and *rppB*, and that the pigment was derived from THN similar to that of *S. griseus* (Cortes *et al.*, 2002). Their work also showed that a variety of pigments derived from tetrahydroxynaphthalene were produced. This new information means that the pathways to red pigment biosynthesis was known and could be incorporated in to the metabolic model. However, very recently (late 2002) in a poster presentation, Weber and colleagues found that one of the red pigments produced by S. *erythraea* was pyomelanin, a shunt product of tyrosine metabolism. This result presents a new possible metabolic route to red pigment biosynthesis compared with the THN hypothesis.

In this thesis, it was shown red pigment production can be controlled by the level of nitrate in the medium. It was also shown that *S. erythraea* produced several pigments and one or more of them have anti-microbial activity against the gram-positive bacteria tested. There is a great deal of work that can be done to research further the metabolic origins of the red pigments and how, or if, they influence erythromycin production. Firstly, the structure of the red pigment produced under nitrate-limited conditions by the *S. erythraea* strain studied here should be elucidated so that the metabolic pathway can be derived. Red pigment could also be perceived as wasted carbon and efforts to re-channel the carbon to central metabolism, as in the α-ketoglutarate excretion study (see section 9.1), could be performed. Construction of a red pigment knock-out strain was initiated (Chapter 6) and could be useful to investigate carbon flow from red pigment synthesis. Radioactively labeled substrate studies would also be useful here to determine what components are incorporated into the red pigment. It would also be interesting to determine if the pyruvate that is re-consumed during stationary phase is used to produce red pigment. Overall,

characterization of the red pigment is proving to be in interesting challenge but is worth pursuing because of its potential influence on erythromycin production.

9.3 Organic Acid Excretion Model

The results gathered from different studies in this thesis were used to form a model of a mechanism by which organic acids are excreted from the cell. The model attempted to incorporate physiological data and enzyme activity data into scenarios of what might have happened at the transition from growth phase to early stationary phase to cause organic acid excretion, and also from early stationary phase to late stationary phase.

The parameters that were considered were the fluxes through 1) glycolysis (which included glucose uptake, 2) the TCA cycle, 3) nitrate uptake, 4) pyruvate excretion, 5) α-ketoglutarate excretion, 6) glutamate/glutamine cycle, and the enzyme activities of 1) pyruvate dehydrogenase, 2) isocitrate dehydrogenase, and 3) α-ketoglutarate dehydrogenase. Each parameter was assumed to have a "high" and "low" setting for simplification. At the transition between growth phase and stationary phase, the parameters either decrease from "high" to "low", increase to "low" to "high", or remain unchanged. Figure 67 shows the biomass, substrate uptake, organic acid excretion levels, as found in Figure 37 of Chapter 4, which was used to direct and verify some of the model assumptions. This graph was divided into the three phases (exponential growth, early stationary, and late stationary phase) used to illustrate the organic acid excretion model. Figure 38 and Figure 39, in Chapter 4, could also be referred to for enzyme activity data for the same culture.

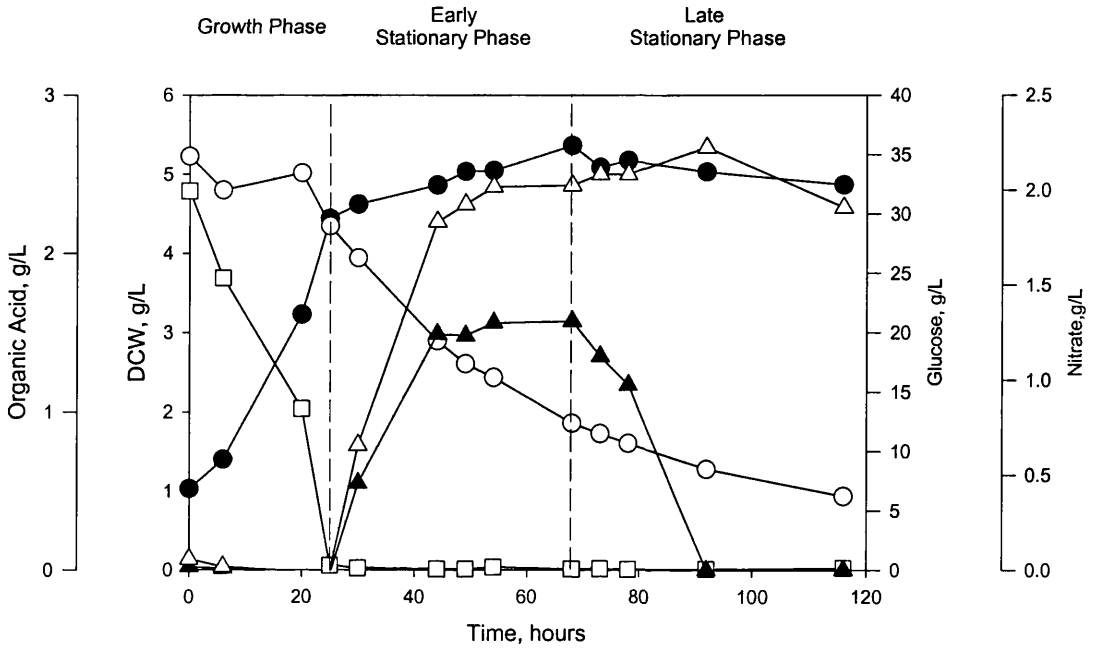


Figure 67: Batch fermentation kinetics for *S. erythraea* wt RV grown under nitrate-limited conditions.

(●) Dry cell weight concentration, (○) Glucose in supernatant, (□) Nitrate in supernatant, (▲) Pyruvate in supernatant, (△) α-Ketoglutarate in supernatant.

9.3.1 Exponential Growth Phase

During the growth phase it was assumed that fluxes through glycolysis, the TCA cycle, nitrate uptake, and the glutamate/glutamine cycle are "high" because the cell needs energy and biomass components for growth. The fluxes to pyruvate and α-ketoglutarate excretion were considered "low" because no excretion was observed during growth phase. The enzymes activities of pyruvate dehydrogenase and isocitrate dehydrogenase were "high" and that of α-ketoglutarate dehydrogenase was "low" based on activity data (see Chapter 4). It should be noted that the relative activities of the three enzymes are shown by the size of the arrows in the illustrations. Even though both pyruvate dehydrogenase and isocitrate dehydrogenase were considered "high" the relative activity of isocitrate dehydrogenase was much greater compared to pyruvate dehydrogenase, and both were greater compared to α-ketoglutarate dehydrogenase. These parameters were depicted in Figure 68. The heavy lines denoted "high" flux and the dotted lines denoted "low" flux.

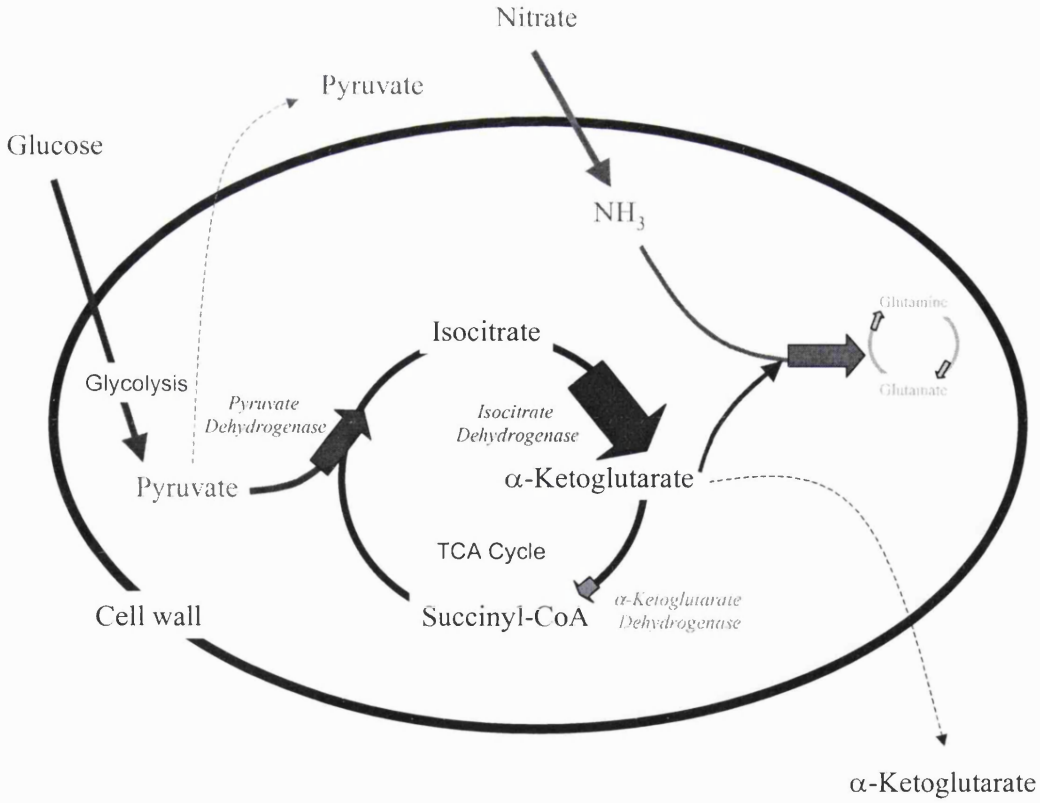


Figure 68: Illustration of the organic acid excretion model during growth phase.

9.3.2 Early Stationary Phase

Shortly after nitrate was depleted, or early stationary phase, growth stopped and organic acid excretion began. It was assumed that growth stopped because of nitrogen source depletion. This led to decreased nitrate uptake, and flux through the glutamate/glutamine cycle. However, there were still high levels of glucose available which may have caused glycolysis and the TCA cycle to continue at relatively "high" rates for some given time (until late stationary phase). The activity of pyruvate dehydrogenase decreased but that of isocitrate dehydrogenase (high) and α-ketoglutarate dehydrogenase (low) remained the same. This model predicted that pyruvate excretion was caused by decreased activity of pyruvate dehydrogenase with glycolysis flux still relatively high. This would have caused an excess of pyruvate in the cell which would then be excreted. α-Ketoglutarate excretion would occur because the glutamate/glutamine cycle no longer required α-ketoglutarate but it was still produced by isocitrate dehydrogenase at high levels. Therefore, accumulation of α-ketoglutarate occurred in the cell which was then excreted.

Other groups have attempted to understand the connection between primary and secondary metabolism. Bialaphos, a secondary metabolite produced by *S. hygroscopicus*, has been studied. When bialaphos was produced by a highly productive strains, the TCA cycle enzyme activities were lower, and the glyoxylic acid cycle enzyme activities were higher, than those with a lower productivity strain. The results suggested that the highly productive strain suppressed flux from pyruvate and acetyl-CoA to the TCA cycle and activated the glyoxylic acid cycle. They suggested that carbon flux from pyruvate and acetyl-CoA was instead directed to secondary metabolism (Takebe *et al.*, 1991). Organic acid excretion data was not presented, however, it would be interesting to see if *S. hygroscopicus* excreted pyruvate under these conditions. It may be possible that pyruvate excretion may occur as a result of the lower flux through the TCA cycle, compared to glycolysis, and not because of reduced pyruvate dehydrogenase activity. Figure 69 shows the changes in physiology from the growth phase to the early stationary phase.

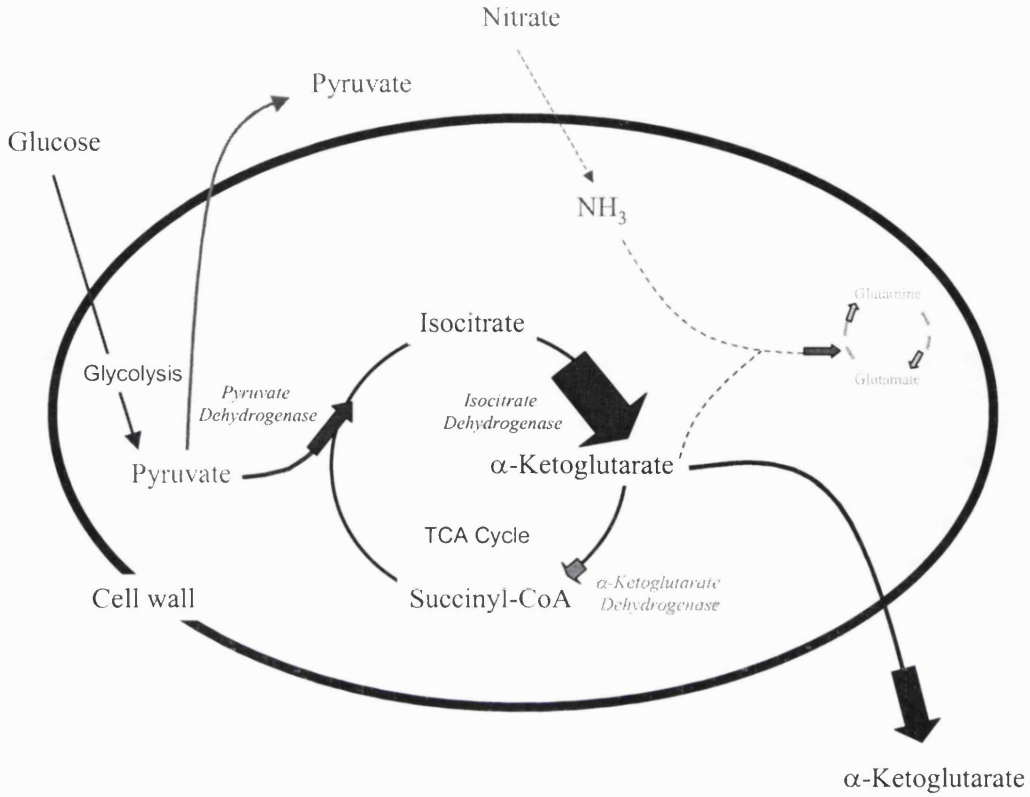


Figure 69: Illustration of the organic acid excretion model during early stationary phase.

9.3.3 Late Stationary Phase

Once the majority of glucose was consumed and pyruvate was beginning to be re-consumed, the culture was assumed to be in late stationary phase. In this phase, the fluxes through glycolysis, the TCA cycle, and α-ketoglutarate excretion were decreased. The enzyme activity of pyruvate dehydrogenase was increased and that of isocitrate dehydrogenase was decreased. All other parameters remained the same as early stationary phase. In this phase, pyruvate was re-consumed because of increased activity of pyruvate dehydrogenase, presumably for cell maintenance, and because there was low carbon flux through central metabolism. α-Ketoglutarate excretion had stopped but there were still high levels accumulated outside the cell. Figure 70 illustrates the cellular fluxes during late stationary phase.

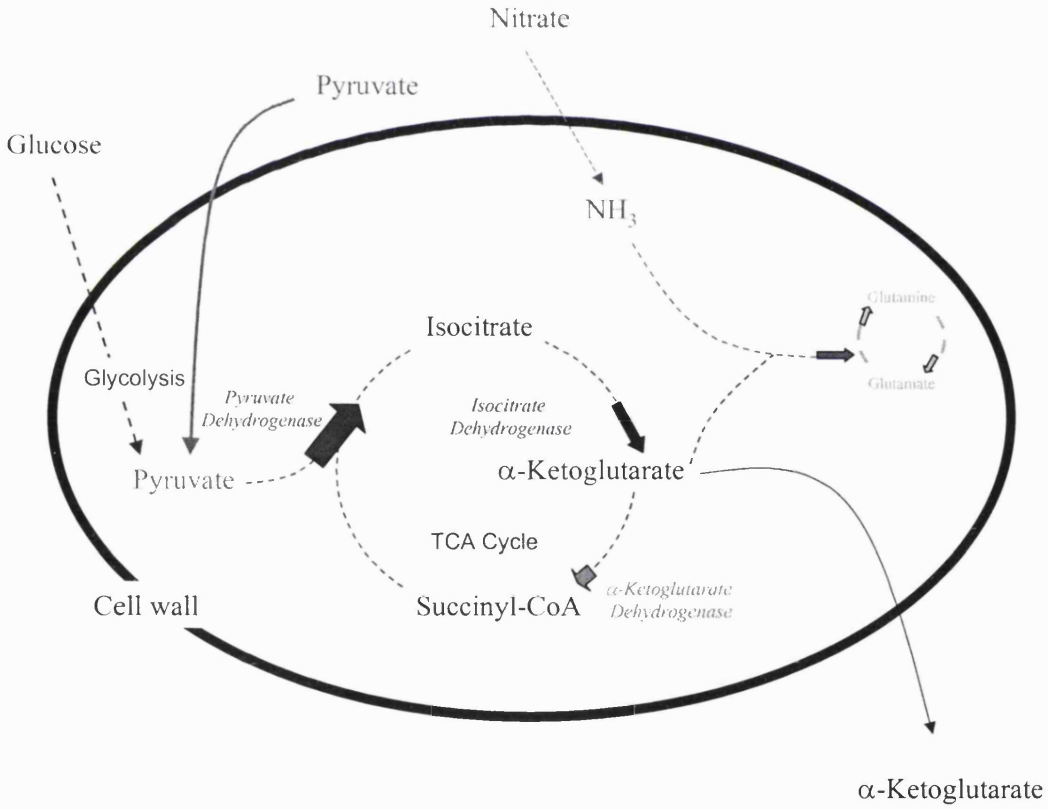


Figure 70: Illustration of the organic acid excretion model during late stationary phase.

These descriptions of organic acid excretion were in agreement with physiology data presented in Chapter 4. However, this is a relatively simple model and several studies would need to be performed to verify this theory. As suggested before, radio labeled substrate studies would be useful to determine if α-ketoglutarate was directed to other parts of metabolism after growth has stopped. It would also be possible to measure intracellular metabolite levels of glutamate and glutamine to determine if they were produced during stationary phase. Enzyme activity of other TCA cycle enzymes would help to verify if the whole cycle was active or inactive, or if parts of the cycle acted independently. In *E. coli*, it appears that oxygen and carbon control of α-ketoglutarate dehydrogenase and coenzyme A synthase occurs by transcriptional regulation an the upstream promoter *sdhC* (Chao *et al.*, 1997). These findings suggested that eight genes for three TCA cycle enzymes, including α-ketoglutarate dehydrogenase, were controlled by one promoter indicating at least part of the cycle may act at one unit. However, studies with *R. capsulatus* suggested that control of the activity of the α-ketoglutarate dehydrogenase subunits is a posttranslational process implying that even though several genes may be expressed at the same time it does not mean that all enzymes are active simultaneously (Dastoor *et al.*, 1997). Overall, the model agrees with the experimental data and if validated further could facilitate reducing carbon wasted to organic acid excretion by Streptomycetes.

9.4 Closing

In closing, the research presented in this thesis represents a metabolic engineering approach to examine polyketide production by *Saccharopolyspora erythraea*. The investigation attempted to combine physiological characterization with metabolic modelling to determine targets for genetic manipulation that would increase the production of erythromycin. To this end, a hypothesis was put forward that identified α-ketoglutarate dehydrogenase as a potential bottle-neck of flux to erythromycin biosynthesis. A new S. *erythraea* strain with an over-expressed α-ketoglutarate dehydrogenase gene was constructed and tested. The new strain was able to reduce the levels of α-ketoglutarate excretion, however, it did not produce greater amounts of erythromycin. Even though erythromycin levels were not increased this research could be considered as a first step to rational strain development for erythromycin production. It could also be regarded, in a greater sense, as proof of concept for use of the *S. erythraea* sequence data to identify genes of interested, and the ability to genetically manipulate the central metabolism of *S. erythraea*.

The research also investigated the physiological control and anti-microbial activity of the red pigment(s) produced by *S. erythraea*. The physiological studies showed that red pigment is produced at high levels when *S. erythraea* is grown under nitrate-limited conditions and that several pigments are formed. It was also shown that the red pigment(s) have some anti-microbial activity against gram-positive bacteria. As new studies regarding the red pigments are reported, it will be interesting to determine which of the proposed synthesis pathways are accurate.

This work could be the starting point for further research in three areas, which have been discussed in sections 9.1, 9.2, and 9.3. The first is strain improvement for erythromycin over-production. This is important because antibiotics are naturally produced at low levels and progress toward a rational approach to increase yield will be needed for all new and existing products. The second area is understanding of how to control organic acid excretion. This is important because several antibiotic producing strains excrete organic acids. It would be very useful if there was a method to re-direct the carbon to product formation. The third area is red pigment

characterization. This is important because it is a new compound and little is known about it, and it would be useful to re-direct the carbon used to make red pigment to erythromycin production.

Metabolic engineering is a powerful tool that enables the rational design and optimization of organisms that produce important products such as polyketides. With each new piece of research comes information that will contribute to and advance the field of metabolic engineering. With these efforts, the tools to rapidly and successfully generate efficient and economical biological processes will increasingly become available.

10 References

Aharonowitz, Y. (1980). Nitrogen metabolite regulation of antibiotic biosynthesis. Annual Reviews in Microbiology *34*, 209-233.

Ahmed, Z. U., Shapiro, S., Vining, L. C. (1984). Excretion of alpha-keto acids by strains of *Streptomyces venezuelae*. Can.J Microbiol *30*, 1014-1021.

Anne, J., Van Mellaert, L., Decock, B., Van Damme, J., Van Aerschot, A., Herdewijn, P., Eyssen, H. (1990). Further biological and molecular characterization of actinophage VWB. Journal of General Microbiology *136 (Pt 7)*, 1365-1372.

Bailey, J. E. (1991). Toward a science of metabolic engineering. Science *252*, 1668-1675.

Bermudez, O., Paddilla, P., Huitron, C., Flores, M. E. (1998). Influence of carbon and nitrogen source on synthesis of NADP+ -isocitrate dehydrogenase, methylmalonyl-coenzyme A mutase, and methylmalonyl-coenzyme A decarboxylase in *Saccharopolyspora erythraea* CA 340. FEMS Letters *164*, 77-82.

Bormann, E. J., Herrmann, R. (1968). [On the secretion of pyruvate and ketoglutarate by Streptomyces rimosus]. Arch.Mikrobiol. *63*, 41-52.

Bruheim, P., Butler, M., Ellingsen, T. E. (2002). A theoretical analysis of the biosynthesis of actinorhodin in a hyper-producing *Streptomyces lividans* strain cultivated on various carbon sources. Appl.Microbiol Biotechnol *58*, 735-742.

Brunker, P., Minas, W., Kallio, P. T., Bailey, J. E. (1998). Genetic engineering of an industrial strain of *Saccharopolyspora erythraea* for stable expression of the *Vitreoscilla* haemoglobin gene (*vhb*). Microbiology *144 (Pt 9)*, 2441-2448.

Bushell, M. E. (1988). Growth, Product Formation and Fermentation Technology. In: Actinomycetes in Biotechnology, ed. M. Goodfellow, S. T. Williams, Mordarski.M, London: Academic Press, 185-217.

Bushell, M. E., Dunstan, G. L., Wilson, G. C. (1997b). Effect of small scale culture vessel type on hyphal fragment size and erythromycin production in *Saccharopolyspora erythraea*. Biotechnology Letters *19*, 849-852.

Bushell, M. E., Smith, J., Lynch, H. C. (1997a). A Physiological Model for the Control of Erythromycin Production in Batch and Cyclic Fed Batch Culture. Microbiology *143*, 475-480.

Butler, M., Bruheim, P., Jovetic, S., Marinelli, F., Postma, P. W., Bibb, M. (2002). Engineering of Primary Carbon Metabolism for Improved Antibiotic Production in *Streptomyces lividans*. Applied and Environmental Microbiology *68*, 4731-4739.

Caffrey, P., Bevitt, D. J., Staunton, J., Leadlay, P. F. (1992). Identification of DEBS 1, DEBS 2 and DEBS 3, the multienzyme polypeptides of the erythromycin-producing polyketide synthase from *Saccharopolyspora erythraea*. FEBS Lett *304*, 225-228.

Cane, D., Hasler, H., Liang, T. (1981). Macrolide Biosynthesis. Orgin of the Oxygen Atoms in the Erythromycins. Journal of the American Chemical Society *109*, 5960-5962.

Cane, D. E. (1994). Polyketide biosynthesis: molecular recognition or genetic programming? Science *263*, 338-340.

Cane, D. E. (1997). Polyketide and Nonribosomal Polypeptide Biosynthesis. From Collie to *Coli*. Chemical Reviews *97*, 2463-2464.

Cataldo, D. A. (1975). Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. Commun Soli Sci Plan *6*, 71-80.

Cavalier-Smith, T. (1992). Origins of secondary metabolism. Ciba Found.Symp. *171*, 64-80.

Chao, G., Shen, J., Tseng, C. P., Park, S. J., Gunsalus, R. P. (1997). Aerobic regulation of isocitrate dehydrogenase gene (icd) expression in Escherichia coli by the arcA and fnr gene products. J Bacteriol *179*, 4299-4304.

Chen, H. C., Wilde, F. (1991). The Effect of Dissolved Oxygen and Aeration Rate on Antibiotic Production of *Streptomyces fradiae*. Biotechnology and Bioengineering *37*, 591-595.

Cheng, Y. R., Hauck, L., Demain, A. L. (1995). Phosphate, ammonium, magnesium and iron nutrition of *Streptomyces hygroscopicus* with respect to rapamycin biosynthesis. J Ind.Microbiol *14*, 424-427.

Christensen, B., Nielsen, J. (2000). Metabolic network analysis of Penicillium chrysogenum using (13)C-labeled glucose. Biotechnology and Bioengineering *68*, 652-659.

Clark, G. J., Langley, D., Bushell, M. E. (1995). Oxygen limitation can induce microbial secondary metabolite formation: investigations with miniature electrodes in shaker and bioreactor culture. Microbiology *141*, 663-669.

Combes, P., Till, R., Bee, S., Smith, M. C. (2002). The streptomyces genome contains multiple pseudo-attB sites for the (phi)C31-encoded site-specific recombination system. Journal of Bacteriology *184*, 5746-5752.

Corcoran, J. W., Vygantas, A. M. (1977). Hydroxylation Steps in Erythromycin Biogenesis. Federation Proceedings *36*, 663.

Corcoran, J. W., Vygantas, A. M. (1982). Accumulation of 6-Deoxyerythronolide B in a Normal Strain of *Streptomyces erythreus* and Hydroxylation at Carbon 6 of the Erythranolide Ring System by a Soluble Noninduced Cell-free Enzyme System. Biochemistry *21*, 263-269.

Cortes, J., Haydock, S. F., Roberts, G. A., Bevitt, D. J., Leadlay, P. F. (1990). An unusually large multifunctional polypeptide in the erythromycin-producing polyketide synthase of *Saccharopolyspora erythraea*. Nature *348*, 176-178.

Cortes, J., Velasco, J., Foster, G., Blackaby, A. P., Rudd, B. A., Wilkinson, B. (2002). Identification and cloning of a type III polyketide synthase required for diffusible pigment biosynthesis in *Saccharopolyspora erythraea*. Mol Microbiol *44*, 1213-1224.

Daae, E. B., Ison, A. P. (1999). Classification and sensitivity analysis of a proposed primary metabolic reaction network for *Streptomyces lividans*. Metab Eng *1*, 153-165.

Dastoor, F. P., Forrest, M. E., Beatty, J. T. (1997). Cloning, sequencing, and oxygen regulation of the Rhodobacter capsulatus alpha-ketoglutarate dehydrogenase operon. J Bacteriol *179*, 4559-4566.

Davies, J. L., Baganz, F., Ison, A. P., Lye, G. J. (2000). Studies on the interaction of fermentation and microfiltration operations: erythromycin recovery from *Saccharopolyspora erythraea* fermentation broths. Biotechnol.Bioeng. *69*, 429-439.

Dayem, L. C., Carney, J. R., Pfeifer, B. A., Khosla, C., Kealey, J. T. (2002). Metabolic Engineering of a Methylmalonyl-CoA Mutase-Epimerase Pathway for Complex Polyketide Biosynthesis in *Escherichia coli*. Biochemistry *41*, 5193-5201.

Demain, A. L. (1992). Microbial secondary metabolism: a new theoretical frontier for academia, a new opportunity for industry. Ciba Found.Symp. *171*, 3-16.

Domach, M. M., Leung, S. K., Chan, R. E., Cocks, G. G., Shuler, M. L. (1984). Computer model for glucose-limited growth of a single cell of *Escherichia coli* B/r-A. Biotechnology and Bioengineering *26*, 203-216.

Donadio, S., Staver, M. J., Katz, L. (1996). Erythromycin production in *Saccharopolyspora erythraea* does not require a functional propionyl-CoA carboxylase. Mol Microbiol *19*, 977-984.

Donadio, S., Staver, M. J., McAlpine, J. B., Swanson, S. J., Katz, L. (1991). Modular organization of genes required for complex polyketide biosynthesis. Science *252*, 675-679.

Doull, J. L., Vining, L. C. (1990). Global Physiological Controls. In: Genetics and Biochemistry of Antibiotic Production, ed. L. C. Vining, C. Stuttard, Boston: Butterworth-Heinemann, 9-63.

Dower, W. J., Miller, J. F., Ragsdale, C. W. (1988). High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res. *16*, 6127-6145.

Drew, S. W., Demain, A. L. (1977). Effect of primary metabolites on secondary metabolism. Annu Rev Microbiol *31*, 343-356.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., Smith, F. (1956). Colorimetric Method for Determination of Sugars and Related Substances. Analytical Chemistry *28*, 350-356.

Dulaney, E. L. (1948). Observations on *Streptomyces griseus*. II. Nitrogen sources for growth and streptomycin production. J Bacteriol *56*, 305-313.

el Mansi, E. M., Holms, W. H. (1989). Control of carbon flux to acetate excretion during growth of *Escherichia coli* in batch and continuous cultures. Journal of General Microbiology *135 (Pt 11)*, 2875-2883.

Escalante, L., Lopez, H., del Carmen, M. R., Lara, F., Sanchez, S. (1982). Transient repression of erythromycin formation in *Streptomyces erythraeus*. Journal of General Microbiology *128 (Pt 9)*, 2011-2015.

Fang, A., Demain, A. L. (1995). Dependence of nitrogen- and phosphorus-regulation of β-lactam antibiotic production by *Streptomyces clavuligerus* on aeration level. J Ind.Microbiol *15*, 407-410.

Fell, D. A. (1992). Metabolic control analysis: a survey of its theoretical and experimental development. Biochem.J *286 (Pt 2)* , 313-330.

Fink, D., Falke, D., Wohlleben, W., Engels, A. (1999). Nitrogen metabolism in *Streptomyces coelicolor* A3(2): modification of glutamine synthetase I by an adenylyltransferase. Microbiology *145 (Pt 9)*, 2313-2322.

Flores, M. E., Sanchez, S. (1985). Nitrogen Regulation of Erythromycin Formation in *Streptomyces erythreus*. FEMS Microbiol Lett *26*, 191-194.

Funa, N., Ohnishi, Y., Fujii, I., Shibuya, M., Ebizuka, Y., Horinouchi, S. (1999). A new pathway for polyketide synthesis in microorganisms. Nature *400*, 897-899.

Gaisser, S., Bohm, G. A., Cortes, J., Leadlay, P. F. (1997). Analysis of seven genes from the eryAI-eryK region of the erythromycin biosynthetic gene cluster in *Saccharopolyspora erythraea*. Mol Gen Genet *256*, 239-251.

Gaisser, S., Bohm, G. A., Doumith, M., Raynal, M. C., Dhillon, N., Cortes, J., Leadlay, P. F. (1998). Analysis of *eryBI*, *eryBIII* and *eryBVII* from the erythromycin biosynthetic gene cluster in *Saccharopolyspora erythraea*. Mol Gen Genet *258*, 78-88.

Gaisser, S., Reather, J., Wirtz, G., Kellenberger, L., Staunton, J., Leadlay, P. F. (2000). A defined system for hybrid macrolide biosynthesis in Saccharopolyspora erythraea. Mol Microbiol *36*, 391-401.

Gerber, N. N., Lechevalier, M. P. (1976). Prodiginine (prodigiosin-like) pigments from *Streptomyces* and other aerobic Actinomycetes. Can.J Microbiol *22*, 658-667.

Goldberg, I., Lonberg-Holm, K., Bagley, E. A., Stieglitz, B. (1983). Improved conversion of fumarate to succinate by *Escherichia coli* strains amplified for fumarate reductase. Appl.Environ.Microbiol *45*, 1838-1847.

Gottschalk, G. (1986). Bacterial Metabolism: 2nd Edition. New York: Springer-Verlag.

Grafe, U., Bocker, H., Reinhardt, G., Thrum, H. (1975). [Inducible accumulation of alpha-ketoglutaric acid in cultures of *Streptomyces hygroscopicus* JA 6599 producing a macrolide antibiotic]. Z.Allg.Mikrobiol. *15*, 575-583.

Green, L. S., Emerich, D. W. (1997). *Bradyrhizobium japonicum* does not require alpha-ketoglutarate dehydrogenase for growth on succinate or malate. J Bacteriol *179*, 194-201.

Green, L. S., Li, Y., Emerich, D. W., Bergersen, F. J., Day, D. A. (2000). Catabolism of alpha-ketoglutarate by a sucA mutant of *Bradyrhizobium japonicum*: evidence for an alternative tricarboxylic acid cycle. J Bacteriol *182*, 2838-2844.

Hamel, R. D., Appanna, V. D. (2001). Modulation of TCA cycle enzymes and aluminum stress in *Pseudomonas fluorescens*. J Inorg.Biochem. *87*, 1-8.

Han, K., Levenspiel, O. (1988). Extended Monod kinetics for substrate, product, and cell inhibition. Biotechnology and Bioengineering *32*, 430-437.

Heinrich, R., Rapoport, T. A. A. (1974). A linear steady-state treatment of enzymatic chains. General properties control and effector strength. European Journal of Biochemistry *42*, 89-95.

Herbert, D. (1959). Some principles of continuous culture. Recent Progress in Microbiology *7*, 381-396.

Heydarian, S. M., Ison, A. P., Mirjalili, N. (1998). A rapid and simplified extraction method of erythromycin from fermentation broth with bond elut C18 cartridge for analysis by HPLC. Biotechnol Tech *12*, 155-158.

Heydarian, S. M., Lilly, M. D., Ison, A. P. (1996). The effect if culture conditions on the production of erythromycin by *Saccharopolyspora erythraea* in batch culture. Biotechnology Letters *18*, 1181-1186.

Ho, N. W. Y., Chen, Z., Brainard, A. P. (1998). Genetically Engineered Saccharomyces Yeast Capable of Effective Cofermentation of Glucose and Xylose. Applied and Environmental Microbiology *64*, 1852-1859.

Hobbs, G., Frazer, C. M., Gardner, D. C., Flett, F., Oliver, S. G. (1990). Pigmented Antibiotic Production by *Streptomyces coelicolor* A3(2): kinetics and the influence of nutrients. Journal of General Microbiology *136*, 2291-2296.

Hobbs, G., Obanye, A. I., Petty, J., Mason, J. C., Barratt, E., Gardner, D. C., Flett, F., Smith, C. P., Broda, P., Oliver, S. G. (1992). An integrated approach to studying regulation of production of the antibiotic methylenomycin by *Streptomyces coelicolor* A3(2). J Bacteriol *174*, 1487-1494.

Holms, W. H. (1986). The central metabolic pathways of *Escherichia coli*: relationship between flux and control at a branch point, efficiency of conversion to biomass, and excretion of acetate. Curr.Top.Cell Regul. *28*, 69-105.

Hong, S. H., Lee, S. Y. (2001). Metabolic Flux Analysis for Succinic Acid Production by Recombinant *Escherichia coli* with Amplified Malic Enzyme Activity. Biotechnology and Bioengineering *74*, 89-95.

Hopwood, D. A. (1997). Genetic Contributions to Understanding Polyketide Synthases. Chemical Reviews *97*, 2465-2497.

Hsieh, Y. J., Kolattukudy, P. E. (1994). Inhibition of erythromycin synthesis by disruption of malonyl-coenzyme A decarboxylase gene *eryM* in *Saccharopolyspora erythraea*. J Bacteriol *176*, 714-724.

Hunaiti, A. A., Kolattukudy, P. E. (1984a). Source of methylmalonyl-coenzyme A for erythromycin synthesis: methylmalonyl-coenzyme A mutase from *Streptomyces erythreus*. Antimicrob.Agents Chemother. *25*, 173-178.

Hunaiti, A. R., Kolattukudy, P. E. (1982). Isolation and characterization of an acyl-coenzyme A carboxylase from an erythromycin-producing *Streptomyces erythreus*. Arch.Biochem.Biophys. *216*, 362-371.

Hunaiti, A. R., Kolattukudy, P. E. (1984b). Malonyl-CoA decarboxylase from *Streptomyces erythreus*: purification, properties, and possible role in the production of erythromycin. Arch.Biochem.Biophys. *229*, 426-439.

Hung, P. P., Marks, C. L., Tardrew, P. L. (1965). The Biosynthesis and Metabolism of Erythromycin by *Streptomyces erythreus*. J Biol.Chem. *240*, 1322-1326.

Inbar, L., Lapidot, A. (1991). 13C nuclear magnetic resonance and gas chromatography-mass spectrometry studies of carbon metabolism in the actinomycin D producer Streptomyces parvulus by use of 13C-labeled precursors. J Bacteriol *173*, 7790-7801.

Ives, P. R., Bushell, M. E. (1997). Manipulation of the physiology of clavulanic acid production in *Streptomyces clavuligerus*. Microbiology *143 (Pt 11)*, 3573-3579.

Jonsbu, E., Christensen, B., Nielsen, J. (2001). Changes of in vivo fluxes through central metabolic pathways during the production of nystatin by *Streptomyces noursei* in batch culture. Applied Microbiology and Biotechnology *56*, 93-100.

Joshi, A., Palsson, B. O. (1988). *Escherichia coli* growth dynamics: A three pool biochemically based description. Biotechnology and Bioengineering *31*, 103-116.

Kacser, H., Burns, J. A. (1973). The Control of Flux. In: Rate control of biological processes, ed. D. D. Davies, Cambridge: Cambridge University Press, 65-104.

Kacser, H., Burns, J. A., Fell, D. A. (1995). The control of flux. Biochem.Soc.Trans. *23*, 341-366.

Kaneda, T., Butte, J. C., Taubman, S. B., Corcoran, J. W. (1962). Actinomycete Antibiotics. J Biol.Chem. *237*, 322-328.

Katz, L. (1997). Manipulation of Modular Polyketide Synthases. Chemical Reviews *97*, 2557-2575.

Khetan, A., Hu, W. S. (1999). Metabolic Engineering of Antibiotic Biosynthesis for Process Improvement. In: Metabolic Engineering, ed. S. Y. Lee, E. T. Papoutsakis, New York: Marcel Dekker, Inc., 177-202.

Khetan, A., Malmberg, L. H., Sherman, D. H., Hu, W. S. (1996). Metabolic engineering of cephalosporin biosynthesis in *Streptomyces clavuligerus*. Ann.N.Y.Acad.Sci. *782*, 17-24.

Khosla, C., Bailey, J. E. (1988). Heterologous expression of a bacterial hemoglobin improves the growth properties of recombinant *Escherichia coli*. Nature *331*, 633-635.

Khosla, C., Bailey, J. E. (1989). Characterization of the oxygen-dependent promoter of the Vitreoscilla hemoglobin gene in *Escherichia coli*. Journal of Bacteriology *171*, 5995-6004.

Khosla, C., Curtis, J. E., Deodena, J., Rinas, U., Bailey, J. E. (1990). Expression of intracellular hemoglobin improves protein synthesis in oxygen-limited *Escherichia coli*. Biotechnology *8*, 849-853.

Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F., Hopwood, D. A. (2000). Practical *Streptomyces* Genetics. John Innes Foundation, Norwich , UK.

Koebmann, B. J., Westerhoff, H. V., Snoep, J. L., Nilsson, D., Jensen, P. R. (2002). The Glycolytic Flux in *Escherichia coli* Is Controlled by the Demand for ATP. Journal of Bacteriology *184*, 3909-3916.

Kojima, I., Cheng, Y. R., Mohan, V., Demain, A. L. (1995). Carbon source nutrition of rapamycin biosynthesis in *Streptomyces hygroscopicus*. J Ind.Microbiol *14*, 436-439.

Kompala, D. S., Ramkrishna, D., Jansen, N. B., Tsao, G. T. (1986). Investigation of bacterial growth on mixed substrates: Experimental evaluation of cybernetic models. Biotechnology and Bioengineering *28*, 1044-1055.

Kubicek, C. P., Schreferl-Kunar, G., Wohrer, W., Rohr, M. (1988). Evidence for a cytoplasmic pathways of oxalate biosynthesis in *Aspergillus niger*. Applied and Environmental Microbiology *55*, 633-637.

Labeda, D. P. (1987). Transfer of the Type Strain of *Streptomyces erythraeus* (Waksman 1923) Waksman and Henrici 1948 to the Genus *Saccharopolyspora* Lacey and Goodfellow 1975 as *Saccharopolyspora erythraea* sp. nov., and Designation of a Neotype Strain for *Streptomyces erythraeus*. Int J Syst Bacteriol *37*, 19-22.

Lambalot, R. H., Cane, D. E., Aparicio, J. J., Katz, L. (1995). Overproduction and characterization of the erythromycin C-12 hydroxylase, EryK. Biochemistry *34*, 1858-1866.

Leadlay, P. F., Staunton, J., Aparicio, J. F., Bevitt, D. J., Caffrey, P., Cortes, J., Marsden, A., Roberts, G. A. (1993). The erythromycin-producing polyketide synthase. Biochem.Soc.Trans. *21*, 218-222.

Lee, M. S., Kojima, I., Demain, A. L. (1997). Effect of nitrogen source on biosynthesis of rapamycin by *Streptomyces hygroscopicus*. J Ind.Microbiol Biotechnol. *19*, 83-86.

Lee, P. C., Lee, W. G., Kwon, S., Lee, S. Y., Chang, H. N. (1999). Succinic acid production by *Anaerobiospirillum succiniciproducens*: effects of the H_2 / CO_2 supply and glucose concentration. Enzyme and Microbial Technology *24*, 549-554.

Liras, P., Asturias, J. A., Martin, J. F. (1990). Phosphate control sequences involed in transcriptional regulation of antibiotic biosynthesis. Trends in Biotechnology *8*, 184-189.

Lubbe, C., Wolfe, S., Demain, A. L. (1985). Repression and inhibition of cephalosporin synthetases in *Streptomyces clavuligerus* by inorganic phosphate. Archives of Microbiology *140*, 317-320.

Luli, G. W., Strohl, W. R. (1990). Comparison of growth, acetate production, and acetate inhibition of *Escherichia coli* strains in batch and fed-batch fermentations. Appl.Environ.Microbiol *56*, 1004-1011.

Lynch, H. C., Bushell, M. E. (1995). The physiology of erythromycin biosynthesis in cyclic fed batch culture. Microbiology *141 (Pt 12)*, 3105-3111.

MacNeil, D. J., Gewain, K. M., Ruby, C. L., Dezeny, G., Gibbons, P. H., MacNeil, T. (1992). Analysis of *Streptomyces avermitilis* genes required for avermectin biosynthesis utilizing a novel integration vector. Gene *111*, 61-68.

Madden, T., Ward, J. M., Ison, A. P. (1996). Organic acid excretion by *Streptomyces lividans* TK24 during growth on defined carbon and nitrogen sources. Microbiology *142 (Pt 11)*, 3181-3185.

Majer, J., Martin, J. R., Egan, R. S., Corcoran, J. W. (1977). Antibiotic Glycosides. 8. Erythromycin D, a New Macrolide Antibiotic. Journal of the American Chemical Society *99*, 1620-1622.

Malmberg, L. H., Hu, W. S. (1991). Kinetic analysis of cephalosporin biosynthesis in *Streptomyces clavuligerus*. Biotechnology and Bioengineering *38*, 941-947.

Marsden, A. F., Caffrey, P., Aparicio, J. F., Loughran, M. S., Staunton, J., Leadlay, P. F. (1994). Stereospecific acyl transfers on the erythromycin-producing polyketide synthase. Science *263*, 378-380.

Martin, J. F., Demain, A. L. (1978). Fungal Development and Metabolite Formation. In: The Filamentous Fungi, Volume 3, ed. J. E. Smith, D. R. Berry, London: Edward Arnold, 424-450.

Martin, J. F., Demain, A. L. (1980). Control of antibiotic biosynthesis. Microbiol Rev *44*, 230-251.

Martin, J. R., Perun, T. J., Girolami, R. L. (1966). Studies on the Biosynthesis of the Erythromycins. I. Isolation and Structure of an Intermediate Glycoside, 3-α-L-Mycarosylerythronolide B. Biochemistry *5*, 2852-2856.

Martin, J. R., Rosenbrook, W. (1967). Studies on the Biosynthesis of the Erythromycins. II. Isolation and Structure of a Biosynthetic Intermediate, 6-Deoxyerythronolide. Biochemistry *6*, 435-440.

Martin, S. M., Bushell, M. E. (1996). Effect of hyphal morphology on bioreactor performance of antibiotic-producing *Saccharopolyspora erythraea* cultures. Microbiology *142*, 1783-1788.

McDermott, J. F., Lethbridge, G., Bushell, M. E. (1993). Estimation of the kinetic constants and elucidation of trends in growth and erythromycin production in batch and continuous cultures of *Saccharopolyspora erythraea* using curve-fitting techniques. Enzyme Microb.Technol. *15*, 657-663.

McGuire, J. M., Bunch, R. L., Anderson, R. C., Boaz, H. E., Flynn, E. H., Powell, M., Smith, J. W. (1952). Antibiot.Chemother. *2*, 281.

Melzoch, K., De Mattos, M. J. T., Neijssel, O. M. (1997). Production of Actinorhodin by *Streptomyces coelicolor* A3(2) Grown in Chemostat Culture. Biotechnol.Bioeng. *54*, 577-582.

Millard, C. S., Chao, Y. P., Liao, J. C., Donnelly, M. I. (1996). Enhanced production of succinic acid by overexpression of phosphoenolpyruvate carboxylase in *Escherichia coli*. Appl.Environ.Microbiol *62*, 1808-1810.

Mirjalili, N., Zormpaidis, V., Leadlay, P. F., Ison, A. P. (1999). The Effect of Rapeseed Oil Uptake on the Production of Erythromycin and Triketide Lactone by *Saccharopolyspora erythraea*. Biotechnol.Prog. *15*, 911-918.

Monod, J. (1942). Recherches Sur La Croissance Des Cultures Bacteriennes. Paris: Hermann and Cie.

Naeimpoor, F., Mavituna, F. (2000). Metabolic flux analysis in *Streptomyces coelicolor* under various nutrient limitations. Metab Eng *2*, 140-148.

Nakano, T., Miyake, K., Ikeda, M., Mizukami, T., Katsumata, R. (2000). Mechanism of the incidental production of a melanin-like pigment during 6-demethylchlortetracycline production in *Streptomyces aureofaciens*. Appl.Environ.Microbiol *66*, 1400-1404.

Nielsen, J. (2001). Metabolic Engineering. Applied Microbiology and Biotechnology *55*, 263-283.

Nielsen, J., Villadsen, J. (1992). Modelling of microbial kinetics. Chemical Engineering Science *47*, 4225-4270.

Nisbet, L. J. (1992). Useful functions of microbial metabolites. Ciba Found.Symp. *171*, 215-225.

O'Hagan, D. (1992). Biosynthesis of Polyketide Metabolites. Natural Products Reports *9*, 447-479.

Obregon, A. M., Escalante, L., Gonzalez, R., Rodriguez, R., Sanchez, S. (1994). Physiological studies on gentamicin: phosphate repression of antibiotic formation. Journal of Antibiotics *47*, 1442-1446.

Ostergaard, S., Olsson, L., Nielsen, J. (2000). Metabolic engineering of *Saccharomyces cerevisiae*. Microbiol Mol Biol.Rev *64*, 34-50.

Otani, S., Takatsu, M., Nakano, M., Kasai, S., Miura, R. (1974). Letter: Roseoflavin, a new antimicrobial pigment from *Streptomyces*. J Antibiot.(Tokyo) *27*, 86-87.

Palleroni, N. J., Reichelt, K. E., Mueller, D., Epps, R., Tabenkin, B., Bull, D. N., Schuep, W., Berger, J. (1978). Production of a novel red pigment, rubrolone, by *Streptomyces echinoruber* sp. nov. I. Taxonomy, fermentation and partial purification. J Antibiot.(Tokyo) *31*, 1218-1225.

Palsson, B. O., Joshi, A. (1987). On the dynamic order of structured *Escherichia coli* growth models. Biotechnology and Bioengineering *29*, 789-792.

Pedersen, H., Beyer, M., Nielsen, J. (2000a). Glucoamylase production in batch, chemostat, and fed-barch cultivations by an industrial strain of *Apergillus niger*. Applied Microbiology and Biotechnology *53*, 272-277.

Pedersen, H., Christensen, B., Hjort, C., Nielsen, J. (2000c). Construction and Characterization of an Oxalic Acid Nonproducing Strain of *Aspergillus niger.* Metabolic Engineering *2*, 34-41.

Pedersen, H., Hjort, C., Nielsen, J. (2000b). Cloning and characterization of *oah*, the gene encoding oxaloacetate hydrolase in *Aspergillus niger.* Molecular General Genetics *263*, 281-286.

Pfeifer, B. A., Admiraal, S. J., Gramajo, H., Cane, D. E., Khosla, C. (2001). Biosynthesis of complex polyketides in a metabolically engineered strain of *E. coli.* Science *291*, 1790-1792.

Pirt (1964). The maintenance of energy of bacterial in growing cultures. Proceeding of the Royal Society of London *165*, 224-231.

Potvin, J., Peringer, P. (1994a). Ammonium Regulation in *Saccharopolyspora erythraea.* Part I: Growth and Antibiotic Production. Biotechnol Lett *16*, 63-68.

Potvin, J., Peringer, P. (1994b). Ammonium Regulation in *Saccharopolyspora erythraea.* Part II: Regulatory Effects Under Different Nutritional Conditions. Biotechnol Lett *16*, 69-74.

Prosser, J. I., Tough, A. J. (1991). Growth mechanisms and growth kinetics of filamentous microorganisms. Crit Rev Biotechnol. *10*, 253-274.

Rossa, C. A., White, J., Kuiper, A., Postma, P. W., Bibb, M., Teixeira de Mattos, M. J. (2002). Carbon Flux Distribution in Antibiotic-Producing Chemostat Cultures of *Streptomyces lividans.* Metab Eng *4*, 138-150.

Rowe, C. J., Cortes, J., Gaisser, S., Staunton, J., Leadlay, P. F. (1998). Construction of new vectors for high-level expression in actinomycetes. Gene *216*, 215-223.

Rudd, B. A., Hopwood, D. A. (1980). A pigmented mycelial antibiotic in *Streptomyces coelicolor*: control by a chromosomal gene cluster. Journal of General Microbiology *119*, 333-340.

Salah-Bey, K., Doumith, M., Michel, J. M., Haydock, S., Cortes, J., Leadlay, P. F., Raynal, M. C. (1998). Targeted gene inactivation for the elucidation of deoxysugar

biosynthesis in the erythromycin producer *Saccharopolyspora erythraea*. Mol Gen Genet *257*, 542-553.

Sambrook, J., Fritsch, E. F., Maniatis, T. (1989). Molecular Cloning: a Laboratory Manual. Cold Spring Harbor, New York, USA.

Savageau, M. A. (1970). Biochemical systems analysis: III Dynamic solution using power-law approximation. Journal of Theoretical Biology *26*, 215-226.

Savinell, J. M., Palsson, B. O. (1992). Optimal selection of metabolic fluxes for in vivo measurement. II. Application to *Escherichia coli* and hybridoma cell metabolism. J Theor.Biol. *155*, 215-242.

Schmidt, K., Marx, A., de Graaf, A. A., Wiechert, W., Sahm, H., Nielsen, J., Villadsen, J. (1998). 13C tracer experiments and metabolite balancing for metabolic flux analysis: comparing two approaches. Biotechnol.Bioeng. *58*, 254-257.

Scribner, H. E., Tang, T., Bradley, S. G. (1973). Production of a sporulation pigment by *Streptomyces venezuelae*. Appl.Microbiol *25*, 873-879.

Shapiro, S., Vining, L. C. (1983). Nitrogen metabolism and chloramphenicol production in *Streptomyces venezuelae*. Can.J Microbiol *29*, 1706-1714.

Shuler, M. L., Leung, S. K., Dick, C. C. (1979). A mathematical model for the growth of a single bacterial cell. Ann.N.Y.Acad.Sci. *326*, 35-55.

Smith, C. G., Bungay, H. R., Pittenger, R. C. (1962). Growth-Biosynthesis Relationships in Erythromycin Fermentation. Appl.Microbiol *10*, 293-296.

Stassi, D. L., Donadio, S., Staver, M. J., Katz, L. (1993). Identification of a *Saccharopolyspora erythraea* gene required for the final hydroxylation step in erythromycin biosynthesis. Journal of Bacteriology *175*, 182-189.

Staunton, J., Wilkinson, B. (1997). Biosynthesis of Erythromycin and Rapamycin. Chemical Reviews *97*, 2611-2629.

Stephanopoulos, G., Aristidou, A. A., and Nielsen, J. Metabolic Engineering: Principles and Methodologies. 1998. San Diego, Academic Press.

Stephanopoulos, G., Vallino, J. J. (1991). Network rigidity and metabolic engineering in metabolite overproduction. Science *252* , 1675-1681.

Stocks, S. M., Thomas, C. R. (2001). Strength of mid-logarithmic and stationary phase Saccharopolyspora erythraea hyphae during a batch fermentation in defined nitrate-limited medium. Biotechnol.Bioeng. *73*, 370-378.

Stols, L., Donnelly, M. I. (1997). Production of succinic acid through overexpression of NAD(+)-dependent malic enzyme in an *Escherichia coli* mutant. Appl.Environ.Microbiol *63*, 2695-2701.

Subik, J., Kolarov, J., Lachowicz, T. M. (1972). A mutant of Saccharomyces cerevisiae lacking alpha-ketoglutarate dehydrogenase activity. FEBS Lett *27*, 81-84.

Takebe, H., Matsunaga, M., Hiruta, O., Satoh, A., Tanaka, H. (1991). Relationship between Sugar Consumption and Tricarboxylic Acid Cycle Enzyme Activity in a High Bialaphos-Producing Strain. Journal of Fermentation and Bioengineering *71*, 110-113.

Tang, L., Zhang, Y. X., Hutchinson, C. R. (1994). Amino acid catabolism and antibiotic synthesis: valine is a source of precursors for macrolide biosynthesis in Streptomyces ambofaciens and Streptomyces fradiae. J Bacteriol *176*, 6107-6119.

Thorpe, H. M., Wilson, S. E., Smith, M. C. (2000). Control of directionality in the site-specific recombination system of the Streptomyces phage phiC31. Mol Microbiol *38*, 232-241.

Trilli, A., Crossley, M. V., Kontakou, M. (1987). Relation Between Growth Rate and Erythromycin Production in *Streptomyces erythraeus*. Biotechnology Letters *9*, 765-770.

Tsao, G. T., Hanson, T. P. (1975). Extended Monod equation for batch cultures with multiple exponential phases. Biotechnology and Bioengineering *17*, 1591-1598.

Tsao, S. W., Rudd, B. A., He, X. G., Chang, C. J., Floss, H. G. (1985). Identification of a red pigment from *Streptomyces coelicolor* A3(2) as a mixture of prodigiosin derivatives. J Antibiot.(Tokyo) *38*, 128-131.

Ueda, K., Kim, K. M., Beppu, T., Horinouchi, S. (1995). Overexpression of a gene cluster encoding a chalcone synthase-like protein confers redbrown pigment production in *Streptomyces griseus*. J Antibiot.(Tokyo) *48*, 638-646.

Van Mellaert, L., Mei, L., Lammertyn, E., Schacht, S., Anne, J. (1998). Site-specific integration of bacteriophage VWB genome into *Streptomyces venezuelae* and construction of a VWB-based integrative vector. Microbiology *144 (Pt 12)*, 3351-3358.

van Wezel, G. P., White, J., Hoogvliet, G., Bibb, M. J. (2000). Application of redD, the transcriptional activator gene of the undecylprodigiosin biosynthetic pathway, as a reporter for transcriptional activity in *Streptomyces coelicolor* A3(2) and *Streptomyces lividans*. J Mol Microbiol Biotechnol *2*, 551-556.

Viollier, P. H., Minas, W., Dale, G. E., Folcher, M., Thompson, C. J. (2001b). Role of acid metabolism in *Streptomyces coelicolor* morphological differentiation and antibiotic biosynthesis. J Bacteriol *183*, 3184-3192.

Viollier, P. H., Nguyen, K. T., Minas, W., Folcher, M., Dale, G. E., Thompson, C. J. (2001a). Roles of aconitase in growth, metabolism, and morphological differentiation of *Streptomyces coelicolor*. J Bacteriol *183*, 3193-3203.

Voet, D., Voet, J. G. (1990). Biochemistry. New York: John Wiley & Sons.

Walshaw, D. L., Wilkinson, A., Mundy, M., Smith, M., Poole, P. S. (1997). Regulation of the TCA cycle and the general amino acid permease by overflow metabolism in Rhizobium leguminosarum. Microbiology *143 (Pt 7)*, 2209-2221.

Wang, X., Gong, C. S., Tsao, G. T. (1998). Bioconversion of fumaric acid to succinic acid by recombinant E. coli. Appl.Biochem.Biotechnol *70-72*, 919-928.

Wardell, J. N., Stocks, S. M., Thomas, C. R., Bushell, M. E. (2002). Decreasing the hyphal branching rate of Saccharopolyspora erythraea NRRL 2338 leads to increased resistance to breakage and increased antibiotic production. Biotechnol Bioeng. *78*, 141-146.

Wasserman, H. H., Rodgers, G. C., Keith, D. D. (1969). Metacycloprodigiosin, a tripyrrole pigment from *Streptomyces longisporus* ruber. J Am.Chem.Soc. *91*, 1263-1264.

Weber, J. M., Leung, J. O., Maine, G. T., Potenz, R. H., Paulus, T. J., DeWitt, J. P. (1990). Organization of a cluster of erythromycin genes in *Saccharopolyspora erythraea*. J Bacteriol *172*, 2372-2383.

Weber, J. M., Wierman, C. K., Hutchinson, C. R. (1985). Genetic Analysis of erythromycin Production in *Streptomyces erythreus*. J Bacteriol *164*, 425-433.

Wilkinson, C. J. Chain Initiation on Modular Polyketide Synthases. 2001. University of Cambridge.

Wilson, GC., Bushell, M. E. (1995). The Induction of Antibiotic Synthesis in *Saccharopolyspora erythraea* and *Streptomyces hygroscopicus* by growth rate decrease is accompanied by a down-regulation of protein synthesis rate. FEMS Microbiol Lett *129*, 89-96.

Yanisch-Perron, C., Vieira, J., Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene *33*, 103-119.

11 Appendices

Appendix 1 lists the reaction in the metabolic network discussed in section 5.4.1.

Appendix 1: List of Reactions in Metabolic Network

	Reaction Type	Reaction
1	Transport Reaction	GLC + PEP = G6P + PYR
2	Transport Reaction	NO3 + 4 NADPH = NH4
3	Transport Reaction	AKGin = AGKout
4	Transport Reaction	PYRin = PRYout
5	Transport Reaction	ERYin = ERYout
6	Transport Reaction	CO2in = OC2out
7	Transport Reaction	O2in = O2out
8	EMP Pathway	G6P = F6P
9	EMP Pathway	ATP + F6P = 2 GAP
10	EMP Pathway	GAP = ATP + PG + NADH
11	EMP Pathway	PG = PEP
12	EMP Pathway	PEP = ATP + PYR
13	PPP Pathway	G6P = CO2 + Ru5P + 2 NADPH
14	PPP Pathway	Ru5P = X5P
15	PPP Pathway	Ru5P = R5P
16	PPP Pathway	R5P + X5P = GAP + S7P
17	PPP Pathway	S7P + GAP = F6P + E4P
18	PPP Pathway	E4P + X5P = F5P + GAP
19	PEP Carboxylase	CO2 + PEP = ATP + OAA
20	TCA Cycle	PYR = NADH + CO2 + AcCoA
21	TCA Cycle	OAA + AcCoA = NADH + CO2 +AKG

22	TCA Cycle	AKG = SucCoA + CO2 + NADH
23	TCA Cycle	SucCoA = SUC + ATP
24	TCA Cycle	SUC = FUM + FADH
25	TCA Cycle	FUM = NADH + OAA
26	Purine and Pyrimidine Nucleotide Synthesis	R5P + ATP = PRPP
27	Purine and Pyrimidine Nucleotide Synthesis	GLN + ASP + PRPP + 2 ATP = NADH + GLU + UMP
28	Purine and Pyrimidine Nucleotide Synthesis	UMP + GLN + ATP = GLU + CMP
29	Purine and Pyrimidine Nucleotide Synthesis	MTHF + UMP = TMP
30	Purine and Pyrimidine Nucleotide Synthesis	GLY + FTHF + 2 GLN + PRPP + CO2 + 2 ATP = 2 GLU + 5A4CRN
31	Purine and Pyrimidine Nucleotide Synthesis	5A4CRN + FTHF + ASP + ATP = FUM + IMP
32	Purine and Pyrimidine Nucleotide Synthesis	IMP + ASP + ATP = FUM + AMP
33	Purine and Pyrimidine Nucleotide Synthesis	IMP + GLN + ATP = NADH + GLU + GMP
34	Phenylalanine, Tyrosine, and Tryptophan Synthesis	E4P + NADPH + 2 PEP = CHOR
35	Phenylalanine, Tyrosine, and Tryptophan Synthesis	NH4 + CHOR = CO2 + PHE
36	Phenylalanine, Tyrosine, and Tryptophan Synthesis	NH4 + CHOR = CO2 + TYR
37	Phenylalanine, Tyrosine, and Tryptophan Synthesis	SER + CHOR + GLN + PRPP + ATP = GAP + PEP + CO2 + GLU + TRP

38	Alanine, Valine, and Leucine Synthesis	GLU + PYR = AKG + ALA
39	Alanine, Valine, and Leucine Synthesis	GLU + NADPH + 2 PYR = CO2 + AKG + VAL
40	Alanine, Valine, and Leucine Synthesis	GLU + AcCoA + 2 PYR = 2 CO2 + AKG + LEU
41	Glutamate, Glutamine, Proline, and Arginine Synthesis	NH4 + AKG + NADH + ATP = GLU
42	Glutamate, Glutamine, Proline, and Arginine Synthesis	NH4 + GLU + ATP = GLN
43	Glutamate, Glutamine, Proline, and Arginine Synthesis	GLU + 2 NADPH + ATP = PRO
44	Glutamate, Glutamine, Proline, and Arginine Synthesis	GLU + GLN + ASP + NADPH + CO2 + 4 ATP = AKG + FUM + ARG
45	Aspartate, Asparagine, Lysine, Methionine, Threonine, and Isoleucine Synthesis	GLU + OAA = AKG + ASP
46	Aspartate, Asparagine, Lysine, Methionine, Threonine, and Isoleucine Synthesis	NH4 + ASP + ATP = ASN
47	Aspartate, Asparagine, Lysine, Methionine, Threonine, and Isoleucine Synthesis	GLU + ASP + 2 NADPH + PYR + ATP = CO2 + AKG + LYS
48	Aspartate, Asparagine, Lysine, Methionine, Threonine, and Isoleucine Synthesis	MTHF + ASP + 2 NADPH + 2 ATP = MET
49	Aspartate, Asparagine, Lysine, Methionine, Threonine, and Isoleucine Synthesis	ASP + 2 NADPH + 2 ATP = THR

50	Aspartate, Asparagine, Lysine, Methionine, Threonine, and Isoleucine Synthesis	THR + NADPH + PYR = CO2 + ILE
51	Serine, Cystine, and Glycine Synthesis	GLU + PG = NADH + AKG + SER
52	Serine, Cystine, and Glycine Synthesis	SER = CYS
53	Serine, Cystine, and Glycine Synthesis	SER = MTHF + GLY
54	Histidine Synthesis	GLN + PRPP + ATP = GLU + 5A4CRN + HIS
55	Methylene Tetrahydrofolate Synthesis	NADPH + AcCoA + ATP = NADH + CO2 + FADH + FTHF
56	Methylene Tetrahydrofolate Synthesis	FTHF + NADH = MTHF
57	Excess ATP Turnover	ATP = ADP
58	Oxidative Phosphorylation	0.5 O2 + NADH = 2 ATP
59	Oxidative Phosphorylation	0.5 O2 + FADH = 1.33 ATP
60	Biomass	0.09 HIS + 0.087 CYS + 0.276 ILE + 0.241 THR + 0.146 MET + 0.326 LYS + 0.229 ASN + 0.281 ARG + 0.210 PRO + 0.428 LEU + 0.402 VAL + 0.543 ALA + 0.054 TRP + 0.334 SER + 0.131 TYR + 0.176 PHE + 0.228 GMP + 0.190 AMP + 0.585 GLY + 0.025 TMP + 0.049 MTHF + 0.151 CMP + 0.136 UMP +

		0.548 GLU + 0.250 GLN + 0.229 ASP + 0.028 OAA + 0.024 S7P + 0.024 R5P + 5.188 NADPH + 2.450 AcCoA + 0.028 PYR + 0.051 PEP + 0.024 PG + 0.129 GAP + 4.286 ATP + 0.071 F6P + 0.154 G6P = 0.024 NADH + 0.211 AKG + BIOMASS
61	Erythromycin A Biosynthesis	–SucCoA +RmethMalCoA = 0
62	Erythromycin A Biosynthesis	–RmethMalCoA +SmethMalCoA = 0
63	Erythromycin A Biosynthesis	–RmethMalCoA +PropCoA = 0
64	Erythromycin A Biosynthesis	–PropCoA –ATP –CO2 +SmethMalCoA +ADP = 0
65	Erythromycin A Biosynthesis	–PropCoA –6 SmethMalCoA +DEBS = 0
66	Erythromycin A Biosynthesis	–G6P +G1P = 0
67	Erythromycin A Biosynthesis	–G1P –NAD(P)H +MYC = 0
68	Erythromycin A Biosynthesis	–G1P –NAD(P)H +DES = 0
69	Erythromycin A Biosynthesis	–DEBS –MYC –DES +ERYA = 0

Appendix 2 lists the names and abbreviations of the metabolites in the metabolic network discussed in section 5.4.2.

Appendix 2: List of Metabolites in Metabolic Network

	Metabolite ABBREV.	Metabolite Name
1	5A4CRN	5-Aminoimidazole-4-carboxamide ribotide
2	AcCoA	Acetyl-coenzyme A
3	ADP	Adenosine diphosphate
4	AKG	α-Ketoglutarate
5	ALA	Alanine
6	AMP	Adenosine monophosphate
7	ARG	Arginine
8	ASN	Asparagine
9	ASP	Aspartate
10	ATP	Adenosine triphosphate
11	BIOMASS	Biomass
12	CHOR	Chorismate
13	CIT	Citrate
14	CMP	Cytosine monophosphate
15	CO2	Carbon dioxide
16	CoA	Coenzyme A
17	CYS	Cysteine
18	6-DE	6-Deoxyerythronolide B
19	DES	Desosamine
20	E4P	Erythrose-4-phosphate
21	ERYA	Erythromycin A
22	F6P	Fructrose-6-phosphate
23	FAD	Flavin adenine dinucleotide-O
24	FADH	Flavin adenine dinucleotide-R
25	FTHF	N5-Formyl-tetrahydrofolate
26	FUM	Fumarate
27	G1P	Glucose-1-phosphate

28	G6P	Glucose-6-phosphate
29	GAP	Glyceraldehyde-3-phosphate
30	GLC	Glucose
31	GLN	Glutamine
32	GLU	Glutamate
33	GLY	Glycine
34	GLYC	Glycogen
35	GLYX	Glyoxylate
36	GMP	Guanosine monophosphate
37	H20	Water
38	HIS	Histidine
39	ICIT	Isocitrate
40	ILE	Isoleucine
41	IMP	Inosine monophosphate
42	LEU	Leucine
43	LIP	Lipid
44	LPS	Lipopolysaccharide
45	LYS	Lysine
46	MAL	Malate
47	MDAP	meso-diaminopimelate
48	MET	Methionine
49	MTHF	N5-Methyl-tetrahydrofolate
50	MUR	Murine
51	MYC	Mycarose
52	NAD	Nicotinamide adenine dinucleotide-O
53	NADH	Nicotinamide adenine dinucleotide-R
54	NADP	Nicotinamide adenine dinucleotide phosphate-O
55	NADPH	Nicotinamide adenine dinucleotide phosphate-R
56	NH4	Ammonia
57	NO2	Nitrite
58	NO3	Nitrate
59	O2	Oxygen

60	OAA	Oxaloacetate
61	PCOA	Propionyl-Coenzyme A
62	PEP	Phosphoenolpyruvate
63	PG	3-Phosphoglycerate
64	PHE	Phenylalanine
65	PRO	Proline
66	PROT	Protein
67	PRPP	5-Phosphoribosyl-1-pyrophosphate
68	PYR	Pyruvate
69	R5P	Ribose-5-phosphate
70	RMMCoA	R-Methylmalonyl Coenzyme A
71	RNA	Ribonucleic acid
72	Ru5P	Ribulose-5-phosphate
73	S7P	Sedoheptulose-7-phosphate
74	SER	Serine
75	SMMCoA	S-Methylmalonyl Coenzyme A
76	SUC	Succinate
77	SucCoA	Succinyl Coenzyme A
78	THF	Tetrahydrofolate
79	THR	Threonine
80	TMP	Thymidine monophosphate
81	TRP	Tryptophan
82	TYR	Tyrosine
83	UDPNAG	Uridine diphosphate-N-acetyl glucosamine
84	UDPNAM	Uridine diphosphate-N-acetyl muramic acid
85	UMP	Uridine monophosphate
86	VAL	Valine
87	X5P	Xylulose-5-phosphate

Appendix 3 shows the ten most sensitive reaction to changes in the seven measured rate. Each table shows the metabolic function, reaction, and sensitivity to changes in the specified measured flux. Sensitivity analysis is discussed in section 5.4.3.

Appendix 3: Sensitivity Analysis of *S. erythraea* Metabolic Network

GLUCOSE UPTAKE

Metabolic Function	Reaction	Sensitivity
Glycolysis	PG=PEP	1.2928
Glycolysis	GAP=ATP+PG+NADH	1.2508
TCA Cycle	CO_2+PEP=ATP+OAA	0.9220
Erythromycin Production	SucCoA=RMMCoA	0.9118
Erythromycin Production	RMMCoA=SMMCoA	0.7853
TCA Cycle	FUM=NADH+OAA	-0.7515
TCA Cycle	SucCoA=SUC+ATP	-0.7492
TCA Cycle	SUC=FUM+FADH	-0.7469
Cell Maintenance	0.5O_2+NADH=2ATP	0.7391
Cell Maintenance	0.5O_2+FADH=1.33ATP	-0.7201

NITRATE UPTAKE

Metabolic Function	Reaction	Sensitivity
Cell Maintenance	ATP=ADP	-2.3269
Pentose Phosphate Pathway	G6P=CO_2+Ru5P+2 NADPH	1.5427
Glycolysis	G6P=F6P	-1.3108
Pentose Phosphate Pathway	Ru5P=X5P	0.9368
Amino Acid Production	NH_4+GLU+ATP=GLN	0.7284
Pentose Phosphate Pathway	Ru5P=R5P	0.6207
Glycolysis	PEP=ATP+PYR	-0.5947
Pentose Phosphate Pathway	E4P+X5P=F5P+GAP	0.5715
Glycolysis	PG=PEP	-0.5136
Cell Maintenance	FTHF+NADH=MTHF	-0.5007

α-KETOGLUTARATE PRODUCTION

Metabolic Function	Reaction	Sensitivity
Erythromycin Production	SucCoA=RMMCoA	-1.1283
Erythromycin Production	RMMCoA=SMMCoA	-0.9603
TCA Cycle	AKG=SucCoA+CO_2+NADH	-0.9063
Cell Maintenance	ATP=ADP	0.5253
Glycolysis	PEP=ATP+PYR	0.3732
Glycolysis	PG=PEP	0.3701
Glycolysis	GAP=ATP+PG+NADH	0.2931
Cell Maintenance	0.5O_2+FADH=1.33ATP	0.2798
Cell Maintenance	0.5O_2+NADH=2ATP	-0.2450
TCA Cycle	SUC=FUM+FADH	0.2308

PYRUVATE PRODUCTION

Metabolic Function	Reaction	Sensitivity
Glycolysis	PEP=ATP+PYR	0.8272
Erythromycin Production	SucCoA=RMMCoA	-0.6402
TCA Cycle	CO_2+PEP=ATP+OAA	-0.6317
Erythromycin Production	RMMCoA=SMMCoA	-0.5456
Glycolysis	ATP=ADP	0.4299
Glycolysis	0.5O_2+FADH=1.33ATP	0.3666
Glycolysis	0.5O_2+NADH=2ATP	-0.3508
TCA Cycle	SUC=FUM+FADH	0.3443
TCA Cycle	SucCoA=SUC+ATP	0.3423
TCA Cycle	FUM=NADH+OAA	0.3404

SPECIFIC GROWTH RATE

Metabolic Function	Reaction	Sensitivity
Cell Maintenance	ATP=ADP	-2.6103
TCA Cycle	PYR=NADH+CO_2+AcCoA	0.8884
Glycolysis	PEP=ATP+PYR	0.7506
Glycolysis	GAP=ATP+PG+NADH	0.6897
Pentose Phosphate Pathway	G6P=CO_2+Ru5P+2 NADPH	0.6875
Cell Maintenance	0.5O_2+FADH=1.33ATP	-0.6191
Pentose Phosphate Pathway	Ru5P=X5P	0.5851
Glycolysis	PG=PEP	0.5695
Cell Maintenance	0.5O_2+NADH=2ATP	0.5263
TCA Cycle	AKG=SucCoA+CO_2+NADH	-0.4467

CARBON DIOXIDE PRODUCTION

Metabolic Function	Reaction	Sensitivity
Erythromycin Production	SucCoA=RMMCoA	-0.5205
TCA Cycle	CO_2+PEP=ATP+OAA	-0.4950
Cell Maintenance	0.5O_2+FADH=1.33ATP	0.4664
Erythromycin Production	RMMCoA=SMMCoA	-0.4368
Cell Maintenance	0.5O_2+NADH=2ATP	-0.4189
TCA Cycle	SUC=FUM+FADH	0.3995
TCA Cycle	SucCoA=SUC+ATP	0.3936
TCA Cycle	FUM=NADH+OAA	0.3879
Pentose Phosphate Pathway	G6P=CO_2+Ru5P+2 NADPH	-0.2658
Glycolysis	G6P=F6P	0.2645

OXYGEN CONSUMPTION

Metabolic Function	Reaction	Sensitivity
Cell Maintenance	ATP=ADP	-4.4871
Cell Maintenance	$0.5O_2$+NADH=2ATP	-1.9624
TCA Cycle	AKG=SucCoA+CO_2+NADH	-0.4394
TCA Cycle	OAA+AcCoA=NADH+CO_2+AKG	-0.4235
Erythromycin Production	SucCoA=RMMCoA	-0.3685
Glycolysis	G6P=F6P	-0.3488
TCA Cycle	CO_2+PEP=ATP+OAA	-0.3414
Glycolysis	GAP=ATP+PG+NADH	-0.3312
Erythromycin Production	RMMCoA=SMMCoA	-0.3059
Pentose Phosphate Pathway	G6P=CO_2+Ru5P+2 NADPH	0.2969