Metabolic Engineering of Amino Acid Production in *Corynebacterium glutamicum*

by

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B.S.E. Chemical Engineering
University of Pennsylvania, 1988

Submitted to the Department of Chemical Engineering in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy
in Chemical Engineering

at the

Massachusetts Institute of Technology
June, 1995

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ABSTRACT

The primary goal of this work is to effect a shift of metabolic flux towards a preferred pathway through the coordination of enzymatic activities. The specific system under consideration is the biosynthesis of aspartate family amino acids by *Corynebacterium glutamicum*. For example, to achieve threonine accumulation using a lysine producer, the carbon flux must be redistributed away from lysine biosynthesis at the aspartic-ß-semialdehyde (ASA) branchpoint. Furthermore, the activities of enzymes in the pathway must be coordinated to minimize accumulation of intermediates such as homoserine.

Amplification of the operon containing a feedback-insensitive homoserine dehydrogenase and a wild-type homoserine kinase in a lysine-producing strain resulted in both homoserine and threonine accumulation, with some residual lysine production. It was postulated that homoserine accumulation could be due to competitive inhibition of homoserine kinase activity by threonine. A plasmid enabling separate transcriptional control of each gene was constructed to determine the effect of varying enzyme activity ratios on metabolite accumulation. By using this plasmid (pGC42) to increase the activity of homoserine kinase, homoserine accumulation in the medium was essentially eliminated. Furthermore, a fortuitous result of the cloning strategy was an unexplained increase in the homoserine dehydrogenase activity. The combination of these factors resulted in an approximately 120% increase in the final threonine titer.

Isoleucine production was achieved in a lysine producer by modification of pGC42 to include the gene encoding threonine dehydratase. This enzyme initiates a five-step pathway from threonine to isoleucine, and is inhibited by the latter. A molar carbon balance indicated that most of the carbon flow into the threonine branchpoint was directed to isoleucine in this organism.

Threonine degradation products, notably glycine and isoleucine, begin accumulating early in the threonine fermentation process and detract from process yield. A stable threonine dehydratase disruption mutant was constructed by marker exchange mutagenesis. Fermentations using pGC42 in the mutant resulted in essentially no isoleucine production and high glycine production, with no significant improvement in final threonine titer. Preliminary kinetic analysis of the two enzymes leading to glycine production from threonine is underway to determine which is the most important immediate target for disruption.

To evaluate the thermodynamic implications of these physiological perturbations, the standard Gibbs free energies of reaction for several steps of the aspartate family amino acid pathway were calculated and used to perform a thermodynamic feasibility analysis on the threonine/isoleucine biosynthetic pathway beginning with the aspartokinase step. When data regarding internal metabolite concentrations was incorporated into the analysis, the findings were consistent with the experimental results.

Maximum theoretical yield calculations based on detailed biochemical pathways, cofactor regeneration and energy requirements were performed for both threonine and isoleucine using glucose as a substrate. Comparison of actual experimental yields with the theoretical yields for both products provided insight on the *in vivo* metabolic scenario.

Thesis Supervisors: Dr. Gregory Stephanopoulos
Dr. Anthony J. Sinskey

Acknowledgements

I would like to thank my parents, Hector and Beth Colón, for their unending and unconditional love, support and understanding. Through all the worrying about me never growing up and evolving from professional studenthood, they were enthusiastic and encouraging and always ready to be there in moments of crisis. The same applies to my sisters and brother, Wandi, Eddie, LizAnn and Mary Ellen, as well as the rest of my very large extended family.

Many thanks go to my advisors, Greg Stephanopoulos and Tony Sinskey, for a very fruitful collaboration and many discussions on the importance and future of metabolic engineering. Greg had a way of always managing to find the bright side of any situation and I usually emerged from our meetings with newly charged enthusiasm about research. Tony's never-ceasing humor and encouragement were invaluable. To him I can only say, "The honeymoon's over? I didn't realize it had started!!!". I would also like to thank the members of my thesis committee, Danny Wang, Charlie Cooney, Arnie Demain, and in particular, Max Follettie, for their time and helpful comments throughout the years. As a member of the Sinskey lab, Max Follettie not only was the impetus behind this work, but he also provided endless encouragement, suggestions and enthusiasm, and helped me to try to find "elegant" solutions for everything.

I would like to thank the members of the Stephanopoulos group, in particular Bob Kiss, who "showed me the ropes" when I first joined the lab; Joe Vallino, for being so willing to answer question and for writing a venerable volume full of information; Cathryn Shaw for being so helpful and always cheerful; John Chung, for a tempestuous and never boring friendship, filled with cynical, but sometimes actually helpful, comments about work and life; and Sung Park, for helpful comments and encouragement, as well as long talks together venting research frustrations. I'd also like to thank the "younger" crew in our group, Roy Kamimura and Troy Simpson, as well as the entire BPEC contingent, for providing an attentive ear and helpful suggestions at group seminars.

I would also like to thank the members of the Sinskey group, without whom I would still be up all night running fermentations with immobilized cells. Mike Jetten was an incredibly helpful colleague, with a very fun sense of humor. Thi Nguyen was a wonderful UROP (earned her name on two papers!), and lots of fun to have around lab. Helen Yeni-Komshian worked hard on her summer project as an REU, and was another great friend gained. I would also like to thank Marcel Gubler for his help with *Corynebacterium* molecular biology, and for being so wonderfully Swiss (!).

My fellow ChemE's, classmates and others, shared the joy (and pain!) of

graduate studenthood. First as officemates, and later housemates, Mark Prausnitz and Tushar Merchant became "partners in crime" by co-hosting many a party at good ol' One Strong Place. I will always treasure their friendship, despite all the water balloons they threw at my guinea pig. Rick Costantino, who taught me how to brew (and drink!) beer, and with whom I sang in Concert Choir and John Oliver's Lieder class, was an endless source of energy and bad jokes. We may still open that brew pub/aerobics gym/karaoke bar yet! I was also lucky to meet Erin and Mark Johnson, who allowed me to sing at their wedding and share that joyous occasion. They are two of the most cheerful and caring people I have met here.

My "other life" at MIT kept my sanity (or lack thereof) intact, and provided various outlets for both creativity and frustration. I have made many, many dear friends in the theater arts community, and have had the opportunity to sing, dance, and act some of my dream roles. I would like to thank the MIT Gilbert and Sullivan Players ("Savoyards"), for allowing me to perform and participate in several G&S operettas and for the ensuing parties and life-long friendships which developed (too numerous to mention here). The MIT Musical Theater Guild, a younger, mostly undergraduate crowd, took me in as an "older sister" and allowed me to play romantic roles opposite people ten years younger (my fountain of youth!).

The MIT Athletics Department (and Gordon Kelly) gave me the opportunity to teach high impact aerobics for three years. Thanks to Dawn Orton, the original aerobics guru, who trained several of us who went on to take over classes after she left. Thanks to Ruth, Muriel, Inge and Karen, with whom I shared many an impromptu aerobics session.

Last, but certainly NOT least, I would like to thank the most special, supportive friend/labmate one could ever hope for. Margaret McCormick, who provided friendship and encouragement both in and out of lab, and was an inspiration for many things, including running the Boston Marathon, was a veritable bastion of strength (how's that for a catch phrase, Margie?!). We hung out so much together that people would often ask us where the other one was. She will be sorely missed when she leaves to join the world of management consulting.

In the interest of brevity, a few hundred people have been omitted. You know who you are, but you probably won't be reading this, so the point is moot. I'd just like to thank MIT for being such a wonderful, exciting, eclectic and unique place. There is such an incredible concentration of interesting, motivated and dynamic people, full of ideas and ideals, that I believe it is possible to accomplish virtually anything here (and virtually anything *does* get accomplished here!!). It even almost makes up for the awful winters.

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Chapter 1. Introduction

Under normal physiological conditions, wild type microbial strains optimize the use of available resources to produce metabolites in direct relation to requirements for cell growth and maintenance. Evolution has resulted in a tightly regulated network of hundreds of enzymatic reactions whose sole driving force is survival and reproduction. To obtain overproduction and high yield of a desired metabolite, it is necessary to circumvent the basic regulatory restrictions by altering native pathways. This is achieved through mutation and screening for the desired phenotype, introduction of novel or deregulated genes, or a combination of these strategies.

In order to successfully apply recombinant DNA technology to enhance the yield of a fermentative process, therefore, the intrinsic metabolic constraints of the organism to be used must be carefully considered. Overproduction of a metabolite is often obtained by introduction of novel or deregulated genes, but this process is rarely accompanied by a systematic evaluation of the effects of modulating the subsequent overexpression on cell growth, productivity, and accumulation of metabolic intermediates. Emphasis on simply implementing the genetic changes has taken precedence over documenting the effects of the genetic alterations. A convergent process of a carefully controlled genetic change, a systematic, quantitative analysis of the consequences, and a design of a further change, is necessary to find a stable, optimized strain/process.

Metabolic engineering is the improvement of cellular activities for purposes of metabolite overproduction by manipulation of enzymatic, transport and regulatory functions of the cell with the use of recombinant

DNA technology. Application of this technology, in conjunction with analysis of the control architectures that have evolved in metabolic pathways can enhance the yield and productivity of fermentation processes.

Feedback inhibition of enzymes initiating a biosynthetic pathway by the end-product(s) of that pathway can be overcome for purposes of metabolite overproduction. Either isolation of an analogue-resistant mutant or amplification of a gene encoding the inhibited enzyme have been used successfully to achieve overproduction of a desired metabolite. Such genetic manipulations are different facets of metabolic engineering exemplified through redistribution of metabolic fluxes. *Corynebacterium glutamicum* and related species, which have been used for over thirty years for industrial production of amino acids (Kinoshita, 1985), are ideal candidates for metabolic engineering due to the relatively simple regulation in their biosynthetic pathways.

1.1 Motivation

The motivation for this work is multi-fold:

• Amino acid metabolism in *Corynebacterium* is an ideal paradigm for metabolic engineering due to the relatively simple nature of the regulatory mechanisms in this organism. For example, fluxes in the aspartate amino acid pathway of *Corynebacterium* seem to be controlled by the relative specific activities and regulatory mechanisms of the unique branchpoint enzymes, rather than by the presence of several isozymes controlled by various allosteric effectors, as is the case in *E. coli* (Jetten *et al.*, 1993). In addition, these pathways and their regulation have been well-

characterized; rate-limiting reactions have been identified as those feedback inhibited by their specific products.

• Many amino acids are economically important, large-volume products. There is an emerging market for many of the secondary limiting amino acids for animal and human nutrition (e.g., threonine, isoleucine and valine). There is potential for complete process engineering, from the genetic to the environmental level.

• Fundamental questions regarding maximum yield of various amino acids from glucose remain unanswered. There is extensive knowledge of the biochemistry of glutamic acid bacteria; there is a need for detailed yield calculations, incorporating stoichiometric and energy constraints to arrive at a feasible maximum value.

• The genetic toolbox for *Corynebacterium* has in recent years been expanded, creating the opportunity to design and engineer controlled expression of one or more genes involved in a given metabolic pathway.

1.2 Objectives

The main objectives of this work are:

• To effect a shift of metabolic flux towards a preferred pathway through the coordination of enzymatic activities. The specific system under consideration is the biosynthesis of aspartate family amino acids by

Corynebacterium glutamicum. The products of interest in this work are the essential amino acids threonine and isoleucine.

• To develop a general methodology for metabolic engineering, combining analytical and theoretical methods to examine and interpret the effects of these manipulations.

• To enhance the intellectual framework for the understanding of *Corynebacterium glutamicum* amino acid metabolism by providing information on the relationship between enzyme expression, cell growth, product formation, and yield.

• To perform a thermodynamic analysis which is consistent with physiological reality.

1.3 Method of approach

Figure 1.1 outlines the general experimental approach to metabolic engineering of threonine and isoleucine production in *Corynebacterium glutamicum*. Using the tools developed for this organism (see section 2.4 - recombinant DNA technology for *Corynebacterium*), a series of steps are taken, many of them simultaneously, to generate an increase in carbon flux towards the desired product.

The first step in engineering of the threonine biosynthetic pathway is the choice of critical pathway enzymes encoded by genes which have been previously cloned in the Sinskey laboratory. The next step is the choice of perturbation to be performed using these genes. Intracellular enzyme

activities can be dramatically increased by amplification; in many cases theactivities can be carefully fine-tuned with the used of highly sensitive, inducible promoters. Another option is elimination of a pathway which competes for carbon flow by disruption of the first gene in that pathway. A lysine producer is an ideal initial genetic background, given that the strain already directs a high percentage of substrate-derived carbon into the aspartate amino acid pathway (see section 2.3.2). The goal is to divert this carbon flux into the desired products, threonine and isoleucine, by manipulation of critical enzyme activities. Information on metabolite accumulation and enzyme activities is gleaned from shake flask fermentations with strains containing desired genetic modifications. The next perturbation to be carried out is chosen based on the results of the previous one; this iterative process results in an optimized strain for production of the desired metabolite. The rationale and strategy behind the perturbations performed are discussed in detail in Chapter 5.

1.4 Thesis organization

Chapter 2 provides an overview of amino acid production. The first section describes the development of the industry and the economic motivation for amino acid production. The subsequent three sections discuss the biochemistry and genetics of glutamic acid bacteria. The chapter concludes with a summary of threonine and isoleucine strain improvement efforts. The first section in Chapter 3 provides detailed yield calculations of threonine and isoleucine from glucose, along with comparison with experimental data and discussion. Section 3.2 uses the thermodynamic bottleneck analysis of Mavrovouniotis (1993) to pinpoint

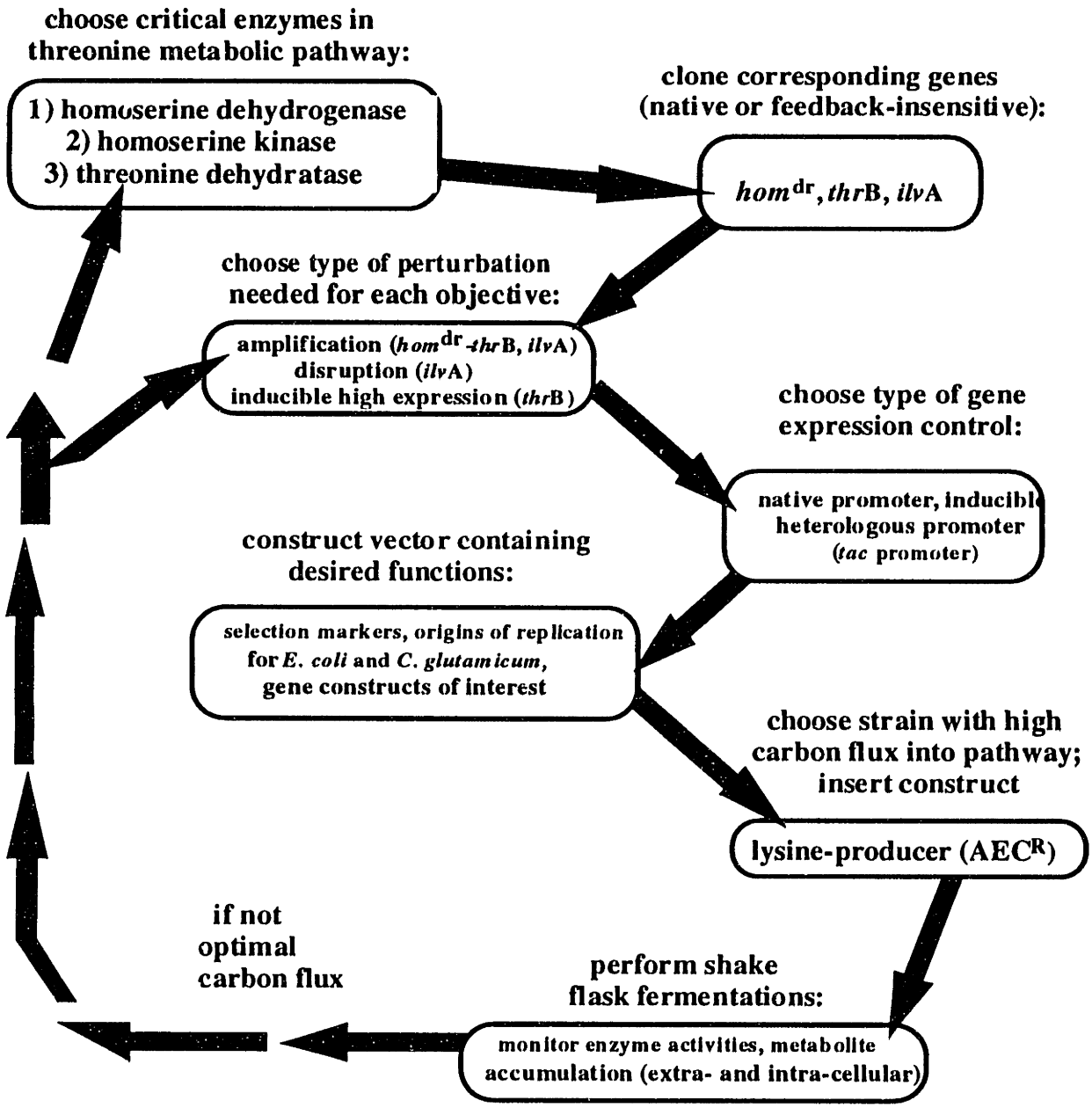

Figure 1.1 Method of approach for metabolic engineering of threonine and isoleucine production in *C. glutamicum*.

thermodynamically difficult steps in the pathways of interest. Chapter 4 describes the materials and methods used, including the develpment of assay techniques for various enzyme activities and for measurement of intracellular metabolites.

Chapter 5 outlines the results of this work, detailing the effects of four genetic perturbations on three critical nodes in the aspartate amino acid pathway. The thermodynamic results are discussed in view of the experimental results. Chapter 6 summarizes the main results and conclusions, and outlines the significance and scope of this research. In addition, suggestions for further metabolic engineering challenges in *Corynebacterium glutamicum* are presented. Chapter 7 defines the symbols and abbreviations used in this work, while Chapter 8 lists the literature references cited. The Appendix contains details on the thermodynamic bottleneck analysis and calculations of the Gibbs free energies of reaction. Studies on inhibition of homoserine kinase and inhibition model parameters are presented.

2. Amino acid production

2.1 The amino acid industry

2.1.1 Economic motivation for amino acid production

Amino acids are used to improve the nutritional value of vegetable protein used in animal and human nutrition. Most natural animal feeds are relatively poor in L-methionine and L-lysine, with L-threonine and L-tryptophan usually being the next limiting amino acids (Kleeman *et al.*, 1985). Amino acids are also used in seasonings, infusions and therapeutic agents, and their derivatives are used in cosmetics, pesticides, and synthetic leather (Hirose and Okada, 1978). In 1980, the annual production of amino acids had a value of about 1.5 billion lbs. or over 500,000 tons (Enei and Hirose, 1984). The predominant product is L-glutamate, followed by L-methionine and L-lysine as feed additives. The latter two are commercially available and relatively inexpensive (approxi. $3.70/kg met; $3.19/kg lys, feed grade, in 1991). In 1991, the prices of feed grade L-threonine and L-tryptophan were $11.50 - 13.00/kg and $69.00/kg, respectively.

When formulating a feed for a given animal, the manufacturer is faced with several options depending on grain availability and current market prices of amino acids. Protein quality, or distribution of essential amino acids, varies with grain type. More efficient production methods of threonine (and lower prices) would provide a wider choice of supplementation strategies for manufacterers and even further increase the

demand (Hirose *et al.*, 1978). The emerging markets may also create a demand for the next limiting amino acids for some animals, such as isoleucine and valine. Another use for some amino acids is supplementation of feed for fermentations with auxotrophic producers.

2.1.2 Development of the amino acid industry

The development of the amino acid industry began in Japan in 1908 with the discovery that the flavor-enhancing properties of konbu, a kelp-like seaweed used for seasoning, were due to L-glutamic acid (Ikeda,1908). Based on this finding, Ajinomoto Co. initiated the industrial production of monosodium L-glutamate by the acid hydrolysis of wheat gluten or soybean protein (Hirose *et al.*, 1978). Production of amino acids through chemical synthesis yields racemic mixtures, and with few exceptions, only the L-amino acids are nutritionally effective. In the 1950's, two groups independently isolated microbial strains which accumulated considerable quantities of L-glutamic acid when cultured in synthetic media containing glucose and ammonia (Asai *et al.*, 1957; Kinoshita *et al.*,1957). *Micrococcus glutamicus* (later renamed *C. glutamicum*) was first successfully used for the direct fermentative production of L-glutamic acid on an industrial scale by Kyowa Hakko Kogyo Co. Ltd.

2.2 Glutamic acid bacteria

Production of glutamic acid as well as amino acids of the aspartic acid family from carbohydrates is carried out by a group of bacteria represented by *Corynebacterium glutamicum* (syn. *Micrococcus*

glutamcus). They include *C. glutamicum*, *C. calcunae*, *C. herculis*, *Brevibacterium flavum*, *B. lactofermentum*, *B. divaricarium*, *B . thiogenitalis*, *Microbacterium ammoniaphilum*, and others. The similarities in guanine and cytosine (G-C) content of the DNA of these bacteria, as well as in morphological and physiological properties, has led researchers to treat them as belonging to the *Corynebactericiae* (Abe *et al.*, 1967; Liebl *et al.*, 1991). In particular, *C. glutamicum*, *B. flavum* and *B. lactofermentum*, which have been extensively investigated in terms of amino acid production, show nearly 100% DNA homology (Kinoshita, 1985). Cell wall peptidoglycan studies have confirmed the close relationship between these species (Schleifer and Kandler, 1972). For the purposes of this work, published data on all three species will be considered valid for *C. glutamicum*.

Glutamic acid bacteria are gram-positive, non-pathogenic, non-motile and non-sporulating ellipsoidal spheres or short rods, varying in length from one to several microns (Abe *et al.*, 1967). All strains require biotin for growth, with some additionally requiring thiamine (vitamin B_1) (Kinoshita, 1972). Biotin is a necessary co-factor in fatty acid syntheis. Under biotin-limiting conditions, the phospholipid composition of the plasma membrane is altered, causing it to become more permeable or "leaky" (Hirose and Okada, 1979). The glutamic acid bacteria have been reported to be deficient in α-ketoglutarate dehydrogenase, which would cause the flow of TCA cycle carbon to be shunted to L-glutamic acid. When the cells are grown in a low biotin environment, the accumulated glutamate is excreted from the cell. In the presence of sufficient biotin (> 0.5 mg/g dry cells), however, the permeability barrier is effective and the glutamate stops its own synthesis when it reaches an internal level of 25 to

50 mg/g dry weight cells (Demain, 1972; Hirose *et al.*, 1978). The high concentration of glutamate may be beneficial to the overproduction of aspartate-derived amino acids since the nitrogen of these amino acids is derived from glutamate by transamination (Shiio and Ujigawa, 1978).

2.3 Amino acid metabolism in *Corynebacterium*

2.3.1 Amino acid metabolic regulation

In this section, the regulation of amino acid biosynthetic pathways will be described, with a focus on strategies to overcome this regulation for purposes of metabolite overproduction.

Most amino acids are synthesized in tightly regulated branched pathways. Some combination of end-product feedback control patterns is usually observed (Figure 2.1) (Shiio, 1982). In sequential control, the end product of one branch will inhibit or repress the first enzyme of that specific branch. In multi-end product control, final products inhibit not only their branches, but act concertedly with other products to control the common pathway enzymes. Other control mechanisms in amino acid biosynthesis include repression and attenuation.

Most high-titer amino acid producing strains have been obtained by isolating auxotrophic and/or analogue-resistant mutants. Various mutagenic events may lead to analogue resistance. For example, increased transcription or translation of a gene via a mutation of the promoter which increases its strength or efficiency, a change in the structural gene which abolishes inhibitor binding at the allosteric site, or increased metabolite flow through the pathway due to blockage of a competing pathway, or a

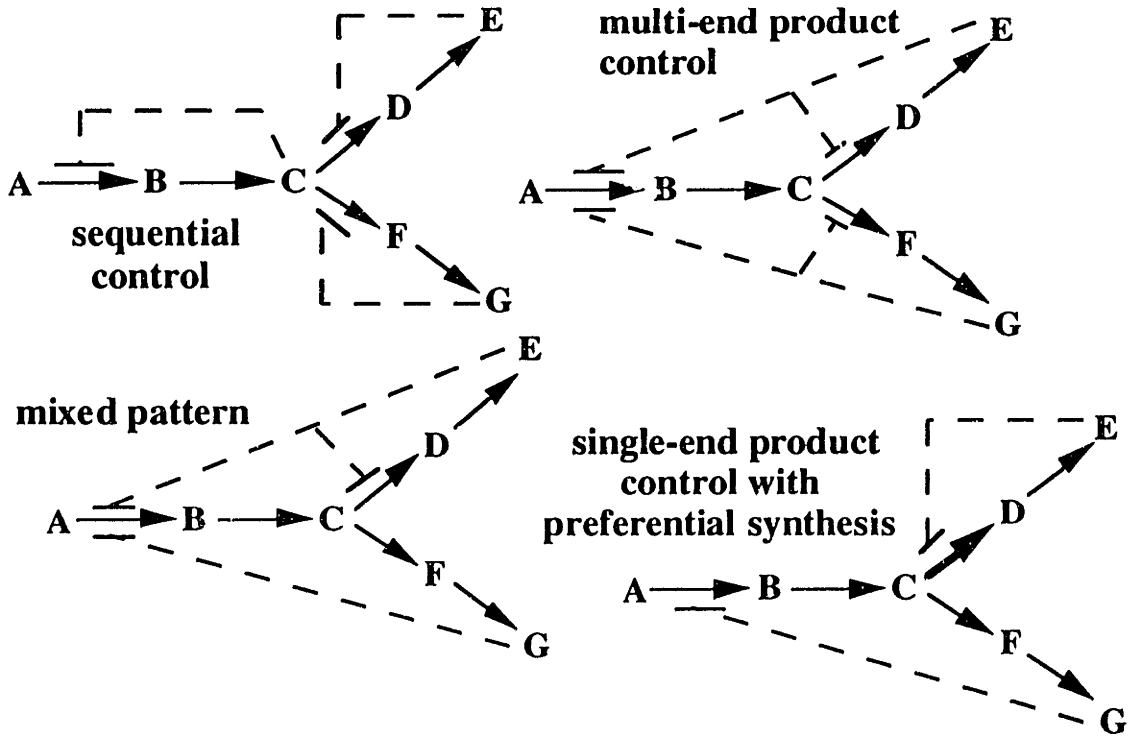

Figure 2.1 Feedback regulation patterns in amino acid biosynthetic pathways (adapted from Shiio, 1982).

combination of these effects may cause a cell to be resistant to normally toxic levels of the analogue.

To produce some intermediate of amino acid biosynthesis, the auxotroph whose pathway in the amino acid synthesis was blocked can be used. The cultivation of this mutant with growth-limiting amounts of nutrient (end product which acts as regulatory effector or co-repressor) renders the cells free of feedback inhibition and/or repression, allowing the intermediate to be overproduced (Hirose *et al.*, 1978; Kiss and Stephanopoulos, 1991). One problem with this approach is reversion, or loss of auxotrophy, with subsequent culture takeover by the faster-growing

revertants (Kiss, 1991). This problem can be avoided to an extent by the use of double auxotrophic mutants and optimizing the limiting nutrient feed ratio (Kiss and Stephanopoulos, 1992). If the gene encoding the first enzyme in the competing pathway is available, another way to avoid this is to isolate a more stable disruption mutant through the use of marker exchange mutagenesis (Gubler *et al.*, 1994).

To accumulate the end product of an unbranched pathway, regulatory mutants can be isolated by selecting for analogue resistance, or for a phototrophic revertant from the auxotroph having a deficiency in a regulatory enzyme (Kinoshita and Nakayama, 1978). Limitations in the primary metabolism can also be overcome or reduced by the introduction of genes with less stringent regulatory characteristics.

2.3.2 Biosynthetic pathways

The basic pathways of the primary metabolism and threonine synthesis of *Corynebacterium glutamicum* are outlined in Figure 2.2. When this organism is grown on glucose, the standard glycolytic pathway and TCA cycle are operative in conjunction with the carboxylation of PEP to oxaloacetate (OAA). OAA is transaminated to form L-aspartate, which is the precursor for the aspartate family amino acids. Further detail on metabolic pathways upstream of aspartate in this organism is provided in the section on yield calculations.

In *C. glutamicum*, as in *E. coli*, diaminopimelate, lysine, methionine, threonine and isoleucine derive part or all of their carbon atoms from aspartate (Cohen, 1985; Shiio, 1982). The scheme for biosynthesis and feedback regulation of aspartate acid family amino acids in *C. glutamicum* is outlined in Figure 2.3. Aspartokinase uses ATP to phosphorylate

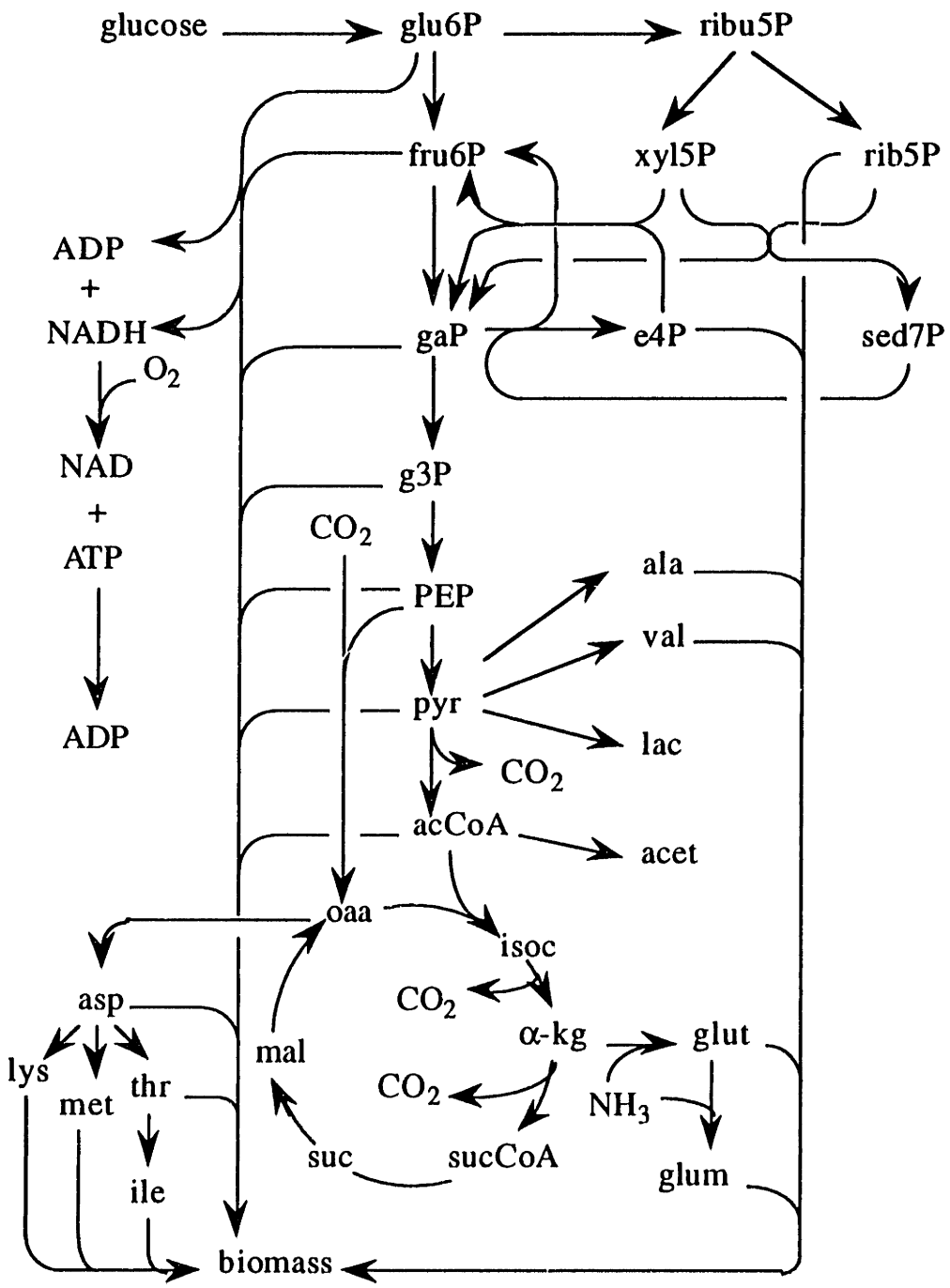

Figure 2.2 Basic pathways of *C. glutamicum* primary metabolism (adapted from Vallino, 1991).

aspartate to aspartyl phosphate, which is subsequently reduced to aspartate-ß-semialdehyde (ASA), the last common intermediate in the aspartate family. In the threonine branch, ASA is reduced to homoserine by homoserine dehydrogenase, which is subsequently phosphorylated to L-homoserine phosphate by homoserine kinase. The final step in the pathway is the dephosphorylation of L-homoserine phosphate to threonine, catalyzed by threonine synthase. Note that threonine is the precursor for isoleucine, homoserine is a precursor for methionine, and that diaminopimelate (DAP) is the immediate precursor of lysine. DAP is required for cell wall biosynthesis (Schleifer and Kandler, 1972; Neidhardt *et al.*, 1990).

E. coli contains three isofunctional aspartate kinases and two homoserine dehydrogenases, each enzyme being independently regulated by inhibition and/or repression of individual end products. In *C. glutamicum*, however, there is yet no evidence of isoenzyme expression, and fluxes in this pathway appear to be controlled by the relative specific activities and regulatory phenomena of the unique branchpoint enzymes. In *C. glutamicum* there is one aspartokinase which is concerted feedback inhibited by lysine and threonine (Shiio and Miyajima, 1969, Kalinowski *et al.*, 1991) and one homoserine dehydrogenase which is threonine sensitive and methionine repressible. The phenomenon of allosteric inhibition of the monofunctional *C. glutamicum* homoserine dehydrogenase enzyme is well characterized (Miyajima and Shiio, 1970; Miyanima and Shiio, 1971; Kase and Nakayama, 1974; Follettie *et al.*, 1988; Section 5.1.1). Wild type homoserine dehydrogenase is inhibited about 75% at 0.1 mM threonine and is completely inhibited at 1 mM threonine. This inhibition is non-competitive with respect to both substrates, ASA and NADPH, indicating that threonine binds at a site different from the substrate binding sites. The

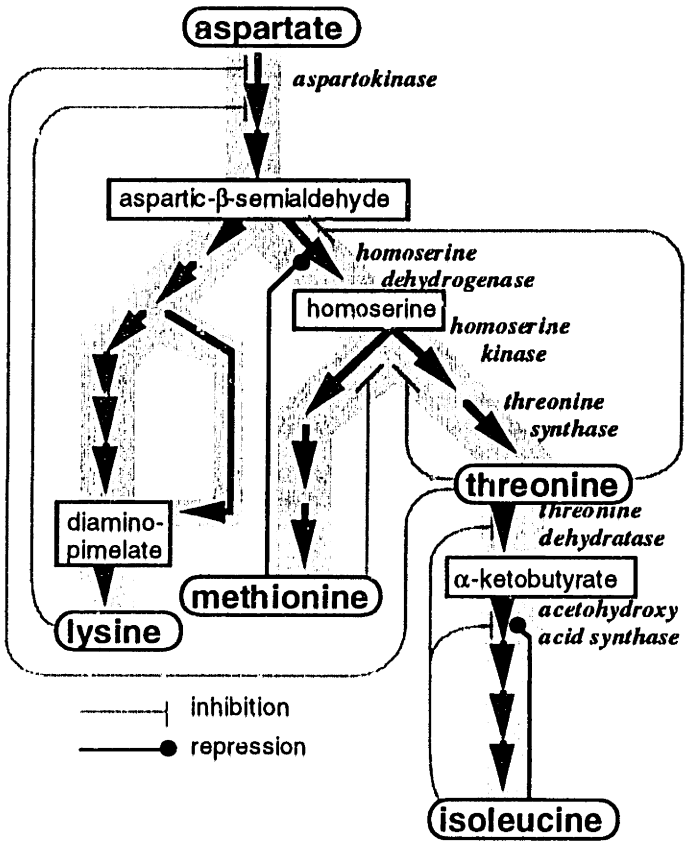

Figure 2.3 Synthesis of aspartate family amino acids in *C. glutamicum.*

repression of *hom-thr*B transcription by methionine in *C. glutamicum* has been studied by Follettie *et al.* (1988). New studies show that this mechanism may have control elements upstream of the hyphenated dyad symmetry region (Margaret M. McCormick, personal communication).

Threonine dehydratase (TDH; EC 4.2.1.16) catalyzes the first of five reactions leading from threonine to isoleucine, and is the only known enzyme specific for isoleucine synthesis in *Corynebacterium.* In contrast, *Escherichia coli*, contains two types of threonine dehydratase, a biosynthetic type and a biodegradative type, each regulated by different effectors (Umbarger and Brown, 1957). The second enzyme in the

pathway, acetohydroxy acid synthase (AHAS; EC 4.1.3.18), is the first enzyme in a series of enzymes that catalyze parallel reactions to isoleucine and valine (Figure 2.4; Tsuchida and Momose, 1975). The penultimate intermediate in the valine pathway, α-ketoisovalerate, is a precursor for leucine. In both *E. coli* and *Salmonella typhimurium*, three isozymes of AHAS occur, each controlled by a different combination of the three branched-chain amino acids (Umbarger, 1987), where as in *Corynebacterium glutamicum*, only one AHAS has been found (Eggeling *et al.*, 1987). In *Corynebacterium* spp., both TDH and AHAS are feedback inhibited by isoleucine (Miyajima and Shiio, 1972). In addition, AHAS is also inhibited by valine and leucine, and its expression is regulated by all three branched-chain amino acids (Tsuchida and Momose, 1975; Eggeling *et al.*, 1987). Both enzymes have been found to be important for controlling the carbon flow to isoleucine (Cordes *et al.*, 1992).

The kinetics of threonine dehydratase from various organisms have been well characterized (Miyajima and Shiio, 1972; Decedue *et al.*, 1975; Hatfield and Umbarger, 1970). Isoleucine is consistently found to inhibit the enzyme whereas valine acts as an activator. Inhibition of *E. coli* threonine dehydratase activity by isoleucine was the first example of end-product feedback inhibition (Umbarger, 1956). The enzyme from *Corynebacterium glutamicum* demonstrates typical cooperativity in substrate saturation kinetics, which is consistent with substrate-promoted conversion of the enzyme to a higher activity conformation (Möckel *et al.*, 1992). The $K_{0.5}$ for threonine was 21 mM, and was increased to 78 mM or reduced to 12 mM in the presence of isoleucine and valine, respectively.

There are two enzymes which use threonine to initiate a glycine formation pathway in *Corynebacterium* (Bell and Turner, 1976).

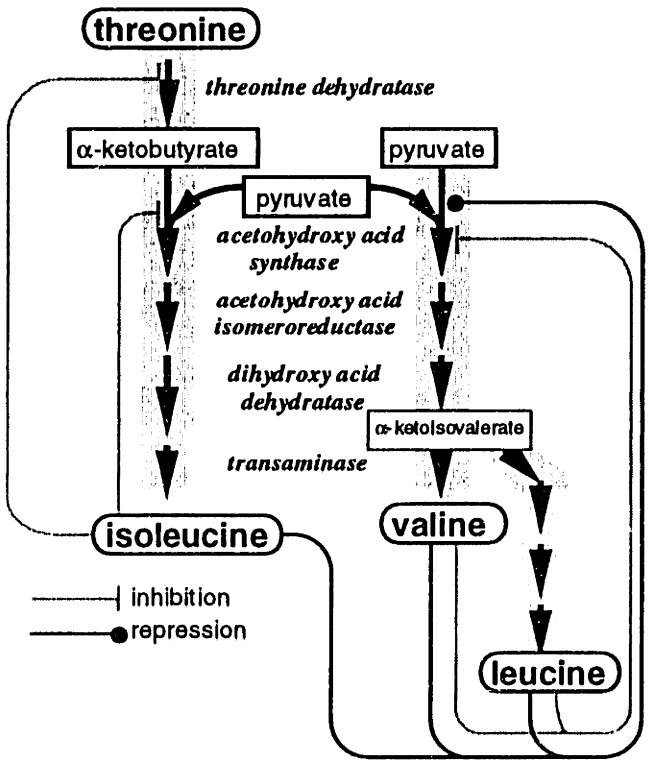

Figure 2.4 Synthesis of branched-chain amino acids in *C. glutamicum.*

Threonine dehydrogenase (TDH; EC 1.1.1.103) converts threonine to amino-oxobutyrate, which either spontaneously decarboxylates to aminoacetone, or is converted to glycine and acetyl CoA by the action of aminoketobutyrate:CoA ligase. Alternatively, the enzyme serine hydroxymethyltransferase (SHMT; EC 2.1.2.1), which interconverts serine and glycine, can also act on threonine to produce acetaldehyde and glycine. Neither of the *C. glutamicum* genes encoding these enzymes have been cloned.

2.3.3 Genetics of amino acid pathways

In this section, a brief review of the genetics of the pathways of interest to this work is given.

2.3.3.1 Threonine pathway

The genes encoding the three enzymes required for conversion of DL-aspartic-ß-semialdehyde (ASA) to L-threonine, *i.e.*, homoserine dehydrogenase (HD; *hom*), homoserine kinase (EC 2.7.1.39; HK; *thr*B), and threonine synthase (EC 4.2.99.2; TS; *thr*C), have been cloned from *C. glutamicum* (Follettie *et al.*, 1988; Peoples *et al.*, 1988; Han *et al.*, 1990). The molecular biology of their expression and regulation has been elucidated (Follettie *et al.*, 1988). The *hom* and *thr*B genes are present in an operon in the *C. glutamicum* genome. The *thr*C gene is unlinked to the other two, but recent data in our laboratory suggests that this gene is adjacent to *gln*, the gene encoding glutamine synthase (Margaret M. McCormick, personal communication).

2.3.3.2 Isoleucine pathway

In *Corynebacterium glutamicum*, threonine dehydratase is encoded by *ilv*A, which is not linked to other isoleucine biosynthetic genes (Cordes *et al.*, 1992). The enzymes catalyzing the subsequent reactions, acetohydroxy acid synthase (AHAS) and isomeroreductase (IR), are encoded by the *ilv*BNC cluster (Keilhauer *et al.*, 1993). The two AHAS subunits are encoded by *ilv*B and *ilv*N; the levels of these transcripts were

found to increase upon addition of α-ketobutyrate to the growth medium. The *ilv*C transcript is regulated independently of the first two and was found to be present in large excess, in agreement with the constant IR activities found in this organism.

2.3.3.3 Threonine degradation reactions leading to glycine

As previously mentioned, the genes encoding threonine dehydrogenase and serine hydroxymethyltransferase in *Corynebacterium* spp. have not been cloned. They have been extensively studied in *E. coli* however (see section 5.4.2.3), and mutants lacking functional forms of these genes are available in this organism. The *Corynebacterium* genes could potentially be isolated by heterologous complementation of these mutants. Alternatively, *Corynebacterium* mutants lacking these activities could be isolated by mutagenesis and screening on minimal medium lacking threonine and glycine. Subsequently, homologous complementation could be used to find the corresponding genes.

2.3.4 Amino acid transport mechanisms

There is limited information on bacterial amino acid transport systems. Active export systems for lysine (Broër and Krämer, 1991a, 1991b and 1993), isoleucine (Ebbighausen *et al.*, 1989a) and glutamate (Hoischen and Krämer, 1989; Krämer, 1994) have only recently been described for *C. glutamicum*. There is evidence for a Na+-dependent threonine transport (uptake) system in *Corynebacterium* (Shiio *et al.*, 1973;

Araki *et al.*, 1973). Excretion could occur by a reversal of the uptake process when the internal concentration of the metabolite exceeds the thermodynamic equilibrium level, but no conclusive studies have yet been carried out.

Isoleucine excretion is carried out by an active efflux carrier system (Ebbighausen *et al.*, 1989a). This excretion system was originally hypothesized in view of data which contradicted simple reversal of the uptake system (a secondary active Na+-coupled symport mechanism; Ebbighausen *et al.*, 1989b). Later work showed quantitative discrimination of this excretion from uptake and diffusion (Zittrich and Krämer, 1994).

2.4 Molecular biology of *Corynebacterium* spp.

2.4.1 Development of recombinant DNA technology for *Corynebacterium*

In the 1980's, the paucity of recombinant DNA technology for food-grade organisms prompted a considerable effort in this area on the part of researchers. The greater part of this technology to this point had focused on techniques for *Escherichia coli,* which, unlike glutamic acid bacteria, has limited potential applicability to the food industry due to its associated endotoxins. In addition, glutamic acid bacteria are part of a larger group which includes animal and plant pathogens, and are thus of considerable interest to the medical and agricultural fields (Batt *et al.*, 1985). The best characterized among the former is *C. diphtheriae* which is the etiologic agent of diphtheria. Plant pathogens, such as *C. michiganese* and *C. fascians*, cause rust and wilt in tomatoes, alfalfa, and other crops.

Many advances established the framework for *Corynebacterium* rDNA technology by providing efficient gene transfer mechanisms. The first step was the screening of numerous coryneform strains for the presence of plasmids for the construction of cloning vectors (Yoshihama *et al.*, 1985; Martin *et al.*, 1987). The vast majority of the plasmids found were cryptic, i.e., did not encode for a known function such as resistance to antibiotics or heavy metals. A cloning vector is an extrachromosomal element (plasmid or virus) which functions to maintain a desired gene or genes by replicating simultaneously with the host. By incorporating the origin of replication of a different species (e.g., *E. coli*), a shuttle vector can be constructed for gene transfer between the two species.

Numerous methods for the introduction of cloning vectors and exogenous DNA were developed. Several groups simultaneously developed protoplast transformation techniques for amino acid-producing corynebacteria (Katsumata *et al.*, 1984; Yoshihama *et al.*, 1985; Martin *et al.*, 1987). Subsequently, electroporation was found to be a much more efficient and convenient transformation technique (Archer *et al.*, 1989; Follettie and Sinskey, 1989; Liebl, 1991). Restriction-deficient host strains can be used to overcome the natural restriction/modification systems found in corynebacteria (Liebl, 1991; Follettie and Sinskey, 1986). Finally, an important advance in this area was the development of a system for transconjugation of mobilizable shuttle plasmids from *E. coli* to various corynebacterial strains (Schäefer *et al.*, 1990). This ability created the opportunity to stably and specifically disrupt and replace genes in the *Corynebacterium* chromosome (Schwarzer and Pühler, 1991).

2.4.2 Genetic toolbox

The elucidation of the genetic structure, organization and regulation of amino acid biosynthetic genes in *Corynebacterium* through the use of the techniques described above has provided a wealth of opportunities for metabolic engineering at the genetic level. Two main strategies were used to clone these genes. Biosynthetic genes in the tryptophan, lysine, threonine and isoleucine pathways were cloned by heterologous complementation of *E. coli* auxotrophs by a gene bank of *C. glutamicum* made in *E. coli* using either plasmid or cosmid vectors (Del Real *et al.*, 1985; Follettie *et al.*, 1988; Follettie *et al.*, 1993; Cordes *et al.*, 1992). Several genes of the phenylalanine pathway were obtained by direct cloning of the genes by complementation of *C. glutamicum* auxotrophs (Ozaki *et al.*, 1985; Follettie and Sinskey, 1986).

Table 2.1 lists the *Corynebacterium* biosynthetic genes that have been cloned and are available for performing specific physiological perturbations. In particular, genes encoding enzymes which are feedback-inhibited or repressed are of interest, since generally these are rate-limiting steps in the wild-type organisms.

2.5 Amino acid-producing strain improvement

Conventional strain improvement techniques have relied on random mutagenesis followed by selection and screening of desirable candidates. These techniques have successfully produced dramatically improved amino acid production strains; however, unidentified mutations often results in ill-defined phenotypes. Recently, researchers have increasingly relied on

Table 2.1 Genes cloned from *C. glutamicum* and related spercies (from Jetten and Sinskey, 1995 and Malumbres *et al.*, 1995).

Gene	Function/gene product[a]	Reference
*ace*A	Isocitrate lyase	Reinscheid *et al.*, 1994b; Lee *et al.*, 1994
*ace*B	Malate synthase	Reinscheid *et al.*, 1993; Lee *et al.*, 1994
*ace*D	AEC lyase	Rossol and Puehler, 1991
*arg*S	Arginyl-tRNA synthetase	Sharp and Mitchel, 1993; Oguiza *et al.*, 1993
*aro*A	DAHP synthase	Chen *et al.*, 1993
*aro*B	Dehydroquinate synthase	Matsui et al., 1988
*aro*E	Shikimate dehydrogenase	Matsui et al., 1988
*aro*L	Shikimate synthase	Matsui et al., 1988
ask	Aspartokinase	Kalinowski *et al.*, 1990; Follettie *et al.*, 1993
ask^{fbr}	Feedback-resistant *ask*	Kalinowski *et al.*, 1991; Follettie *et al.*,1993; Jetten *et al.*,1993
asd	ASA dehydrogenase	Kalinowski *et al.*, 1990; Follettie *et al.*, 1993 Jetten *et al.*,1994a
cspl	Major secreted protein	Joliff *et al.*, 1992.
*dap*A	DHP synthase	Bonnassie *et al.*, 1990b; Cremer *et al.*,1988; Pisabaro *et al.*, 1993; Yeh *et al.*, 1988
*dap*B	DHP reductase	Cremer *et al.*, 1988; Yeh *et al.*, 1988
ddh	DAP dehydrogenase	Ishino *et al.*, 1988; Yeh *et al.*, 1988
*dap*E	DAP epimerase	Wehrmann *et al.*, 1993
fda	Fructose-diP_i aldolase	von der Osten *et al.*, 1990
gap	Glycerol-3P_i, dehydrogenase	Eikmanns, 1992
gdh	Glutamate dehydrogenase	Takeda *et al.*, 1990; Bormann *et al.*, 1992
*glt*A	Citrate synthase	Schwinde *et al.*, 1993
gnd	Exonuclease	Liebl, 1991; Gubler, unpublished results
hom	Homoserine dehydrogenase	Follettie *et al.*, 1988
hom^{dr}	Feedback-resistant hom	Archer *et al.*, 1992; Reinscheid *et al.*, 1991
*ilv*A	Threonine dehydratase	Cordes *et al.*, 1992
*ilv*BN	Acetohydroxy acid synthase	Möckel *et al.*, 1992; Keilhauer *et al.*, 1993
*ilv*C	Isomeroreductase	Möckel *et al.*, 1992; Keilhauer *et al.*, 1993
leu	Leucine biosynmesis	Patek *et al.*, 1994
*lys*A	DAP decarboxylase	Marcel *et al.*, 1990; Yeh *et al.*, 1988
*lys*I	Lysine uptake	Seep-Feldhaus *et al.*, 1992
pgk	Phosphoglycerate kinase	Eikmanns, 1992
*phe*A	Prephenate denydratase	Follettie and Sinskey, 1986
ppc	PEP carboxylase	Eikmanns *et al.*, 1989; O'Regan *et al.*, 1989
*pst*II	Enzyme II PTS system	Yoon *et al.*, 1993
pyk	Pyruvate kinase	Gubler *et al.*, 1994b; Jetten *et al.*, 1994c
*thr*B	Homoserine kinase	Follettie *et al.*, 1988; Peoples *et al.*, 1988
*thr*C	Threonine synthase	Han *et al.*, 1989
tpi	Triosephosphate isomerase	Eikmanns, 1991
*trp*EDCBA	Tryptophan biosynthesis	Del Real *et al.*, 1985; Follettie and Sinskey, 1986; Heery and Dunican, 1993; Katsumata and Ikeda, 1993

[a] AEC, *S*-(2-aminoethyl) L-cysteine; DAHP, 3-deoxy-D-arabinoheptulosonate-7-phosphate; ASA, aspartic-β- semialdehyde; DHP, dihidrodipicolinate; DAP, diaminopimelate; PEP, phosphoenolpyruvate.

recombinant DNA technology to perform specific alterations for strain improvement.

2.5.1 Threonine

Efforts towards strain improvement in *Corynebacterium* threonine producers have focused on developing threonine analogue-resistant strains, and more recently, on isolating analogue resistant strains with a defective lysine branch. Various advances in strain improvement for threonine production, as well as a brief description of the strategies, strains, and plasmids involved, are reviewed in Table 2.2.

Because of the tight biosynthetic regulation of threonine in *C. glutamicum*, it was clear that auxotrophy for competing pathways alone could not be used successfully for production of L-threonine in this organism, as it had been for *E. coli*. A regulatory mutant of *C. glutamicum* or a related strain obtained by isolating a threonine analogue-resistant cell would insure that the homoserine dehydrogenase would no longer be feedback inhibited by L-threonine. Thus, several researchers obtained significant improvement in L-threonine production in these strains by isolating analogue-resistant mutants. Combining both analogue resistance as well as auxotrophy for competing pathways yielded even greater improvements. The next step was cloning of the threonine metabolic genes in *E. coli* as well as in *Corynebacterium/Brevibacterium.* Nakamori *et al.* (1987a) showed that amplification of homoserine dehydrogenase in a threonine- and lysine-producer, *B. lactofermentum* M-15, led to threonine accumulation of 25 g/l. However, by-product

Table 2.2 Threonine production: strain improvement history

*Strains/strategy	Threonine (g/l)	**Reference
E. coli (DAP-; DAP- met-)	2-4	1
E. coli (DAP- met- ile-; ile^{rev} of these)	13.5	2
C. flavum B-183 (AHV^R) (resistant to 5 mg/ml AHV)	10.5	3
C. flavum BB-82 (AHV^R) (from B-183; 8 mg/ml AHV)	13.5	3
C. flavum BBM-21 (AHV^R met-)18		4
C. glutamicum (met- S-lys^R)	14	5
C. glutamicum (met- S-lys^R AHV^R)	9 (+ 5 lys)	5
C. lactofermentum; cell fusion between a lys + thr producer and lysine auxotroph	18	6
E. coli; plasmid containing *thr*A, *thr*B, *thr*C from an AHV^R met- ile- *E. coli* strain	13.4	7
C. lactofermentum M-15; plasmid with HD from M-15 (lys- and thr- producer)	25	8
C. lactofermentum M-15; amplification of both HD and HK from M-15	33	9
C. flavum BBIB-19 (AHV^R ile-); plasmid containing thr operon from AHV^R mutant of*E. coli* K-12	27	10
C. flavum (AHV^R, DPS-)	13.7	11

***Abbreviations: lys, lysine; thr, threonine; AEC, *S*-(2-aminoethyl) L-cysteine (lysine analogue); AHV, α-amino-ß-hydroxyvalerate acid (threonine analogue); DAP, diaminopimelate deficient; DPS-, dihydrodipicolinate deficient; HD, homoserine dehydrogenase; HK, homoserine kinase; ile-, isoleucine auxotroph; leu-, leucine auxotroph; ile^{rev}, isoleucine revertant; met-, methionine autotroph**
****References: 1, Huang, 1961; 2, Kase *et al.*, 1971; 3, Shiio *et al.*, 1970; 4, Nakamori and Shiio, 1972; 5, Kase and Nakayama, 1972; 6, Tosaka *et al.*, 1982; 7, Miwa *et al.*, 1983; 8, Nakamori *et al.*, 1987; 9, Morinaga *et al.*, 1987a; 10, Ishida *et al.*, 1989; 11, Shiio *et al.*, 1989.**

formation in this case included 2.8 g L-homoserine and 1.1 g L-lysine per liter. Upon additional insertion of the homoserine kinase (HK) gene from

B. lactofermentum, threonine production was increased to 33 g/l. Analysis of the HD-HK recombinant showed increases in homoserine dehydrogenase and homoserine kinase activities of up to 14-fold and 30-fold, respectively. Another approach was taken by Shiio *et al.* (1989), who investigated dihydrodipicolinate synthase (DPS)-defective mutants of *B. flavum*. DPS is the first enzyme in the lysine-specific branch and competes with homoserine dehydrogenase for ASA as a substrate. During the screening of AHV-resistant threonine producers, they found a novel type which had normal feedback-sensitive HD but was defective in DPS. One mutant produced 13.7 g/l threonine. The next step was isolation of high AHV-resistant, DPS-defective mutants from a HDR strain. One mutant, strain sDB185, produced 16.5 g/l threonine (Shiio *et al.*, 1990). They also derived mutants resistant to AHV and lysine from strain DK330, a DPS-defective mutant with a homoserine kinase (HK) free from methionine repression. One strain thus derived, BD122, produced 16.6 g/l of threonine and showed a 2-fold increase in HK activity over the parent.

2.5.2 Isoleucine

There have been fewer reports of isoleucine-producing strains. Isoleucine production from glucose was obtained by isolating mutants resistant to thiaisoleucine, ethionine, 4-azaleucine and α-aminobutyric acid from a threonine-producing *Corynebacterium glutamicum* strain (Kase and Nakayama, 1977). Isoleucine production in *Corynebacterium* has also been reported using α-ketobutyrate and α-hydroxybutyric acid as substrates (Eggeling *et al.*, 1987; Scheer *et al.*, 1987).

2.5.3 Summary

It is difficult to precisely compare the effects of genetic and environmental manipulations on these strains, given the lack of specific data on biomass concentration and exact compositions of additives such as soybean meal acid hydolysate. In addition, although some threonine hyper-producing strains have been isolated, the exact genetic modifications that lead to such strains remain uncertain due to the isolation techniques used. However, the results of these studies are in good agreement with the concept that the control site of the threonine specific biosynthetic pathway in *Corynebacterium* (at least downstream of aspartate) is homoserine dehydrogenase.

Chapter 3. Theoretical analyses

3.1 Theoretical yield calculations

3.1.1 Introduction

Calculation of maximum theoretical yield based on known biochemical pathways is an important component of metabolic engineering. A detailed pathway analysis may help pinpoint key target nodes for flux manipulations. In addition, ratios of CO_2 released to O_2 consumed may aid in correlations of respiratory quotient to metabolic state of a fermentation. Finally, yield values are vital for bioprocess economics. In this section theoretical yield values of threonine and isoleucine on glucose are estimated.

The first approach used to obtain an absolute maximum theoretical yield uses an elemental balance which assumes complete conversion of substrate into product. A more realistic approach entails using a detailed reaction scheme which accounts for different metabolic scenarios, as well as cofactor and energy requirements. These results are discussed in further detail in Chapter 5, where the experimental yields obtained are compared with the theoretical yields.

3.1.2 Elemental balance

This approach incorporates the elemental constraints in the production of threonine and isoleucine from glucose, oxygen, and ammonia (no biomass formation is assumed). It can be expressed as:

Threonine:

$$aC_6H_{12}O_6 + b0_2 + cNH_3 \text{ ---> } dC_4H_9O_3N + eCO_2 + fH_2O$$

Isoleucine:

$$aC_6H_{12}O_6 + b0_2 + cNH_3 \text{ ---> } dC_6H_{13}O_2N + eCO_2 + fH_2O$$

There are six unknown stoichiometric coefficients (a-f) and four elemental balance equations (carbon, hydrogen, oxygen and nitrogen), leaving two degrees of freedom for solving the system of equations. The coefficient d can be set to one, thus normalizing the remaining coefficients per mole of threonine or isoleucine produced. Leaving the oxygen coefficient unspecified and solving the elemental balance equations we obtain:

Threonine:

$$(2/3+b/3)\ C_6H_{12}O_6 + bO_2 + NH_3 \text{ ---> } C_4H_9O_3N + bCO_2 + (1 + 2b)H_2O$$

The maximum yield is given by 1/[2/3 + b/3]. In the limiting case of no oxygen consumption (b=0), Y_{max} is equal to 1.5 mol thr/mol glu consumed. Note that in the case of threonine production, the respiratory quotient (carbon dioxide evolution rate/oxygen uptake rate) will always be equal to one. This is the same as for biomass synthesis, due to the similarity in the degree of reduction of biomass and threonine (16.03 vs. 16, as defined by Erickson *et al.*, 1979). Figure 3.1 shows the maximum yield on glucose and respiratory quotient (RQ) as a function of oxygen consumed in the threonine fermentation.

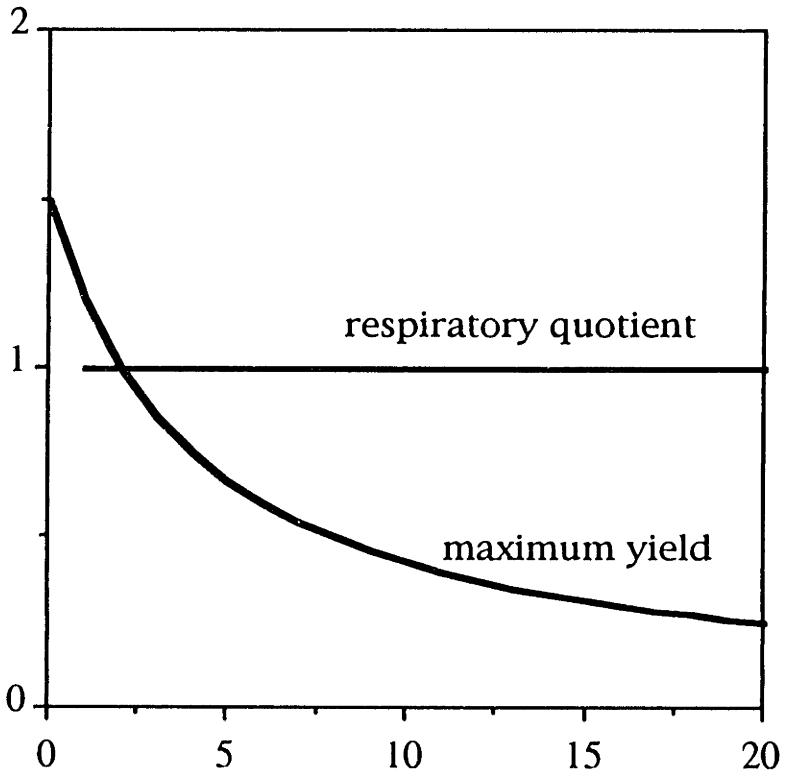

Figure 3.1 Maximum yield on glucose and respiratory quotient as a function of oxygen consumed for pure product synthesis in the threonine fermentation.

Isoleucine:

$(5/4 + b/6)\ C_6H_{12}O_6 + bO_2 + NH_3 \text{ ---> } C_6H_{13}O_2N + (b+3/2)CO_2 + (b+5/2)H_2O$

The maximum yield is thus given by 1/[5/4 + b/6]. In the limiting case of no oxygen consumption, Y_{max} is equal to 0.8 mol ile/mol glu consumed. Figure 3.2 shows a plot of maximum yield and RQ as a function of oxygen consumed in the isoleucine fermentation.

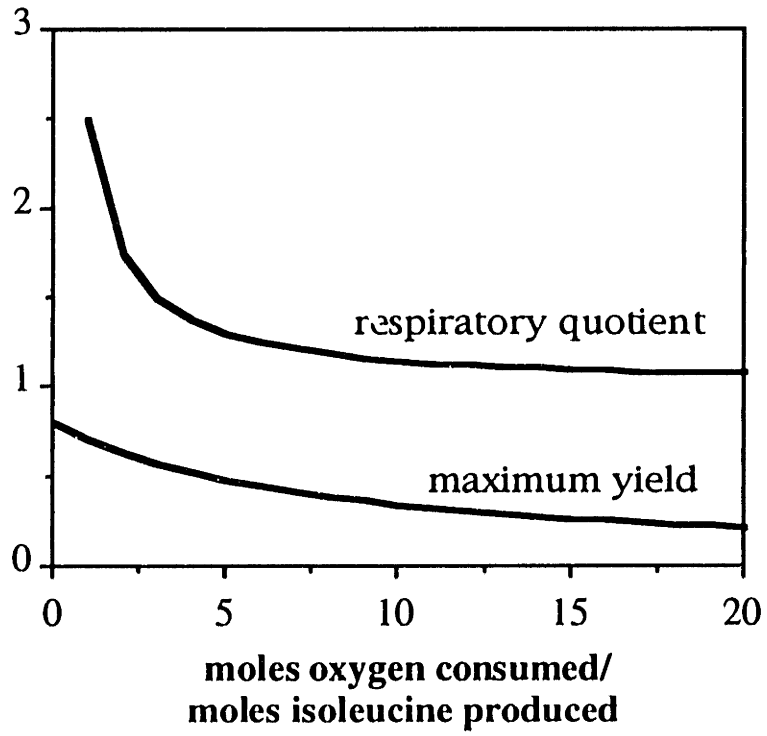

Figure 3.2 Maximum yield on glucose and respiratory quotient as a function of oxygen consumed for pure product synthesis in the isoleucine fermentation.

The above calculations assume no biomass production and make no provision for the regeneration of currency metabolites and intermediate precursors. Figure 3.3 shows a plot of respiratory quotient as a function of yield for both amino acids. These graphs indicate that RQ may be an important parameter in process control for the isoleucine fermentation, but that it is not helpful for the threonine fementation. Because there is no difference between the RQ's for production of threonine and production of

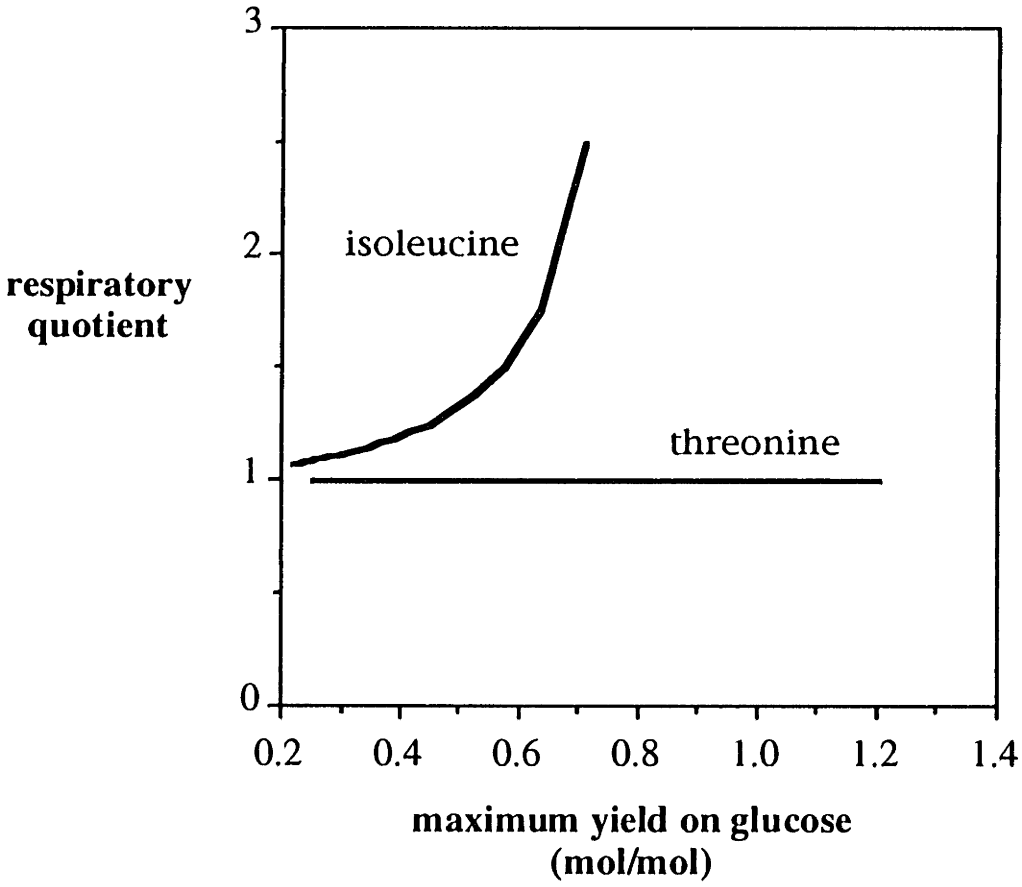

Figure 3.3 Respiratory quotient as a function of yield on glucose for pure product synthesis in the threonine and isoleucine fermentations.

biomass, this parameter cannot be used as a gauge to indicate high threonine production, as in the case of the lysine fermentation (Kiss and Stephanopoulos, 1991). In this case it may be appropriate to use a control scheme based on a growth-limiting nutrient to force carbon flux towards threonine rather than cell growth.

3.1.3 Detailed stoichiometric approach

The detailed reaction scheme approach involves incorporation of metabolic pathway constraints including cofactor and energy requirements, as well as pathway reactions specific to the production strain. In order to provide a useful estimation of theoretical yield, this method requires a detailed knowledge of the biochemistry of the organism. There is now an extensive body of literature regarding the biochemistry of glutamic acid bacteria, enabling us to trace the fates of a glucose molecule with a fair amount of certainty. The results and analysis of several researchers in specifying the exact pathway for aspartate amino acid synthesis in this organism have been followed (see Figure 2.2). The maximum theoretical yield was calculated by incorporating reactions involved in the most direct path to threonine and isoleucine and by accounting for energy and cofactor requirements for its production. Next, loss of carbon as CO_2 through the TCA cycle was incorporated to estimate yield under those conditions.

The following assumptions and equations apply to all the calculations:

• Recycling of biosynthetic intermediates (e.g., α-ketoglutarate, glutamate, and sucCoA) is one hundred percent efficient

• In *C. lactofermentum*, Kawahara *et al.* (1988) found that only two energy-coupling sites translocate protons, implying a maximum P/O ratio of two for oxidative phosphorylation in glutamic acid bacteria:

$$2\ NADH + O_2 + 4\ ATP \text{ ---> } 2\ H_2O + 4\ ATP + 2\ NAD^+$$

$$2\ FADH + O_2 + 2\ ATP \text{ ---> } 2\ H_2O + 2\ ATP + 2\ FAD^+$$

Because of the above assumption about the efficiency of oxidative phosphorylation, the glucose requirement for ATP production can be accounted for by:

$$glucose + 6\ O_2 + 26\ ADP \text{ ---> } 6\ CO_2 + 26\ ATP + 6\ H_2O$$

• The existence of the pentose phosphate pathway has been supported by ^{13}C NMR studies (Ishino *et al.*, 1986; Yamaguchi *et al.*, 1986), and so glucose utilization for regeneration of $NADP^+$ can be represented as follows:

$$glucose + ATP + 12\ NADP^+ + 6H_2O \text{ ---> } ADP + 12\ NADPH + 6\ CO_2$$

• Transport of glucose into *B. flavum* has been shown to be mediated by the PEP:glucose PTS which uses PEP to drive both active transport and phosphorylation of glucose (Mori and Shiio, 1987; Shiio *et al.*, 1990). One mole of PEP is hydrolyzed to pyruvate and is used to provide the phosphate group for the first intermediate in glycolysis, glucose-1-phosphate. The latter yields two moles of PEP via glycolysis and thus the net reaction is:

glu_{out} + 2 NAD+ + ADP ---> PEP_{in} + pyr_{in} + 2 NADH + 6 NADP + ATP

Central carbon metabolism reactions:

- phosphoenolpyruvate carboxylase:

  PEP + CO_2 ---> oaa + P_i

- pyruvate kinase:

  PEP + ADP ---> pyr + ATP

- phosphoenolpyruvate synthetase:

  pyr + ATP + H_2O ---> PEP + AMP + P_i

- pyruvate dehydrogenase complex:

  pyr + CoA + NAD ---> AcCoA + CO_2 + NADH

- net tricarboxylic acid (TCA) cycle:

  AcCoA + 2 NAD + FAD + NADP + ADP + 2 H_2O --->
  2 CO_2+ 2 NADH + FADH + NADPH + ATP + CoA

Aminotransferase reactions:

- aspartate aminotransferase reaction:

  oaa + glut ---> asp + α-kg

- glutamate dehydrogenase:

  NH_3 + α-kg + NADPH ---> glut + NADP + H_2O

Aspartate amino acid family reactions:

• threonine production from aspartate:

$$asp + 2\ NADPH + 2\ ATP + H_2 \text{ ---> } thr + 2\ NADP + 2\ ADP$$

• isoleucine production from aspartate:

$$asp + 4\ NADPH + 2\ ATP + pyr \text{ --->}$$

$$ile + 2\ NADP + 2\ ADP + CO_2 + 4\ H_2O$$

Case 1: maximum carbon recovery

The most direct path to aspartate is through carboxylation of phosphoenolpyruvate (PEP) to oxaloacetate by phosphoenolpyruvate carboxylase (PPC). This route bypasses the tricarboxylic acid (TCA) cycle and avoids loss of carbon through CO_2. This can be achieved if pyruvate formed as a result of the PEP:glucose PTS system is converted to PEP by PEP synthetase.

Thus, for threonine, the synthesis equation becomes:

$$81/52\ glu + 35/26\ O_2 + 2\ NH_3 \text{ ---> } 2\ thr + 35/26\ CO_2$$

and Y_{max} = 2 mol threonine/(81/52 mol glucose), or 1.28 mol thr/mol glu (0.85 g thr/g glu)

In the case of isoleucine, the pyruvate formed by the PTS system can be recovered directly into the isoleucine branch by the action of acetohydroxyacid synthase, the second enzyme in the threonine to isoleucine pathway. Thus, the synthesis equation becomes:

$$17/12\ glu + O_2 + NH_3 \text{ ---> } ile + 5/2\ CO_2$$

Y_{max} = 1 mole isoleucine / 17/12 mole glucose = 0.71 mol ile/mol glu

(0.51 g ile/g glu)

Case 2: some loss of carbon through TCA cycle:

In this case, one mole of pyruvate is subsequently directed to the TCA cycle by the pyruvate dehydrogenase complex (PDC), allowing only the PEP to enter the aspartate branchpoint:

For threonine:

$$7/6\ glu + 3\ O_2 + NH_3 \text{ ---> } thr + 3\ CO_2$$

Y_{max} = 0.86 mol thr/mol glu (0.56 g thr/ g glu).

isoleucine:

If we allow half of the carbon in the form of PEP and pyr to enter the TCA cycle by way of pyruvate kinase and the pyruvate dehydrogenase complex, the synthesis equation becomes:

$$9/4\ glu + 6\ O_2 + NH_3 \text{ ---> } ile + 15/2\ CO_2$$

Y_{max} = 0.44 mol thr/mol glu (0.32 g ile/ g glu).

These two cases present a range of possible yields depending on which reactions are dominant. Since the energetic requirements for production of threonine and isoleucine can be met without the need to go throught the TCA cycle, these calculations underlie the importance of central carbon metabolism perturbations to maximize flow of carbon into the aspartate family amino acid family pathway.

3.2 Thermodynamic analysis

3.2.1 Introduction

Genetic and environmental perturbations performed on a given biochemical system may have thermodynamic implications, due to the possible resultant changes in intracellular metabolite concentrations. Kinetic changes in a system may result in a new set of steady-state metabolite concentrations, thus creating a new system from a thermodynamic perspective. Conversely, identifying thermodynamic bottlenecks of a given system may yield insight into possible targets for optimizing the flow of carbon to a desired product.

To evaluate the thermodynamic feasibility of a metabolic pathway, the permissible ranges of concentrations of metabolites need to be taken into account. Determination of the standard free energies of reaction for each biochemical step does not suffice to detemine which step, if any, may be a thermodynamic constraint. The *actual* Gibbs free energies of reactions (calculated from known, feasible metabolite concentrations, which differ greatly from standard conditions) provide a much more accurate description of the system.

Mavrovouniotis (1993) states that the quantity $\Delta G^{\circ\prime}$ is fundamentally inadequate as a measure of thermodynamic feasibility or reversibility due to the following issues:

- The standard conditions $\Delta G^{\circ\prime}$ refers to are arbitrary and biologically irrelevant.
- $\Delta G^{\circ\prime}$ is dependent on the absolute magnitudes of stoichiometric coefficients. If all coefficients are multiplied by a constant, $\Delta G^{\circ\prime}$ is

multiplied by the same constant, rendering its magnitude meaningless for determining the direction of a reaction.

- It is indifferent to the flexibility introduced by the number and concentration ranges of the metabolites.

Mavrovouniotis developed an analytic algorithm which tests the thermodynamic feasibility of a network of biochemical reactions. The algorithm checks for the existence of real concentrations within specified concentration ranges that satisfy the criteria for reaction feasibility. If the network is infeasible, the algorithm constructs the subpathways which cause and comprise the thermodynamic infeasibility, or "bottleneck". The author presents this work as complementary to a pathway synthesis algorithm performing sequential constraint-satisfaction, which combines biochemical reactions into increasingly comprehensive partial pathways, until a pathway satisfying all the stoichiometric constraints is obtained (Mavrovouniotis, 1992; Mavrovouniotis *et al.*, 1990).

In this section, the standard Gibbs free energies of reaction are used to perform a thermodynamic feasibility analysis on the threonine/isoleucine biosynthetic pathway beginning with the aspartokinase step (Figures 3.4, 3.5 and 3.6). The calculations of the Gibbs free energies of reaction, as well as a brief summary of the notation, equations and significance of the method are presented in the Appendix.

3.2.2 Identification of localized and distributed bottlenecks

This analysis attempts to pinpoint localized and distributed bottlenecks in the pathway based on permissible ranges of metabolite

concentrations. A method derived by Mavrovouniotis (1993) in which a set of scaled quantities are defined which reformulate the themodynamic feasibility problem is used. Table 3.1 lists the metabolic intermediates involved in the pathways of interest: formation of threonine from aspartate, formation of isoleucine from threonine, and threonine degradation reactions leading to glycine. Table 3.2 lists the reactions involved, along with the standard Gibbs free energy of each reaction, (calculations for these numbers are detailed in the Appendix).

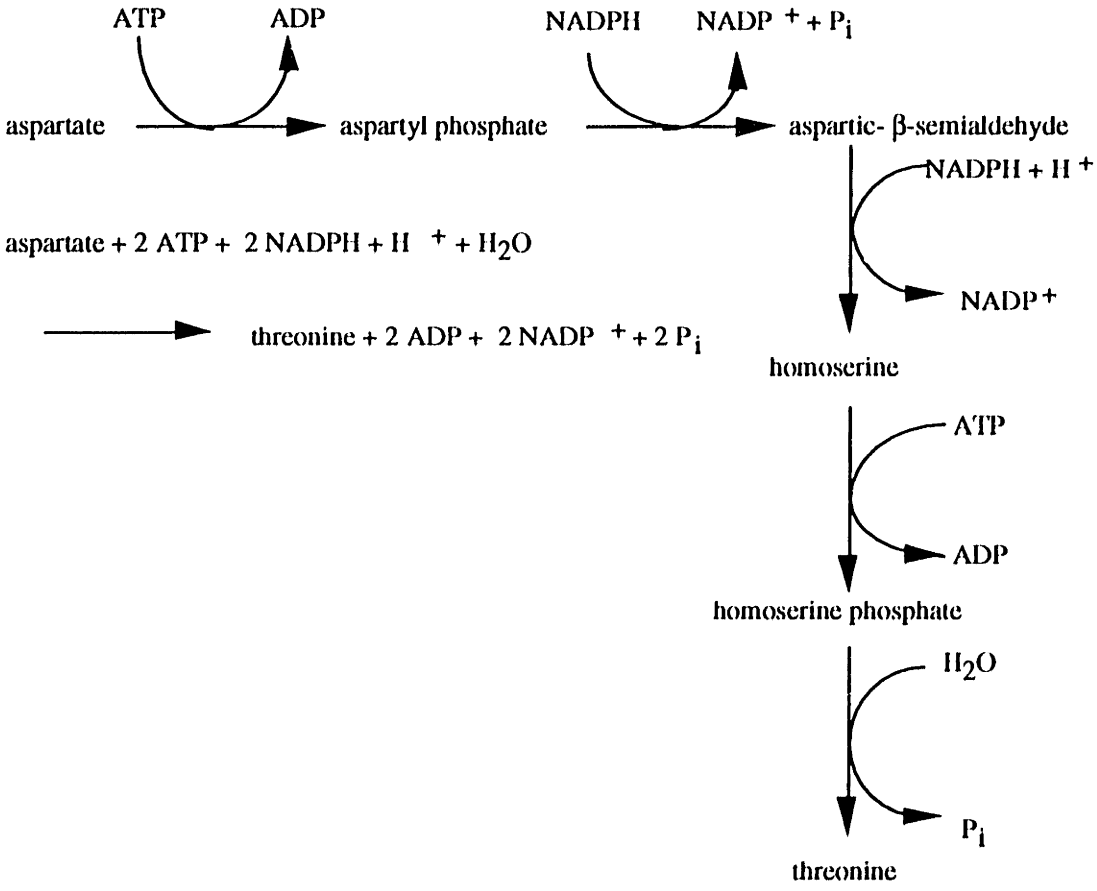

Figure 3.4 Biosynthetic reactions from aspartate to threonine

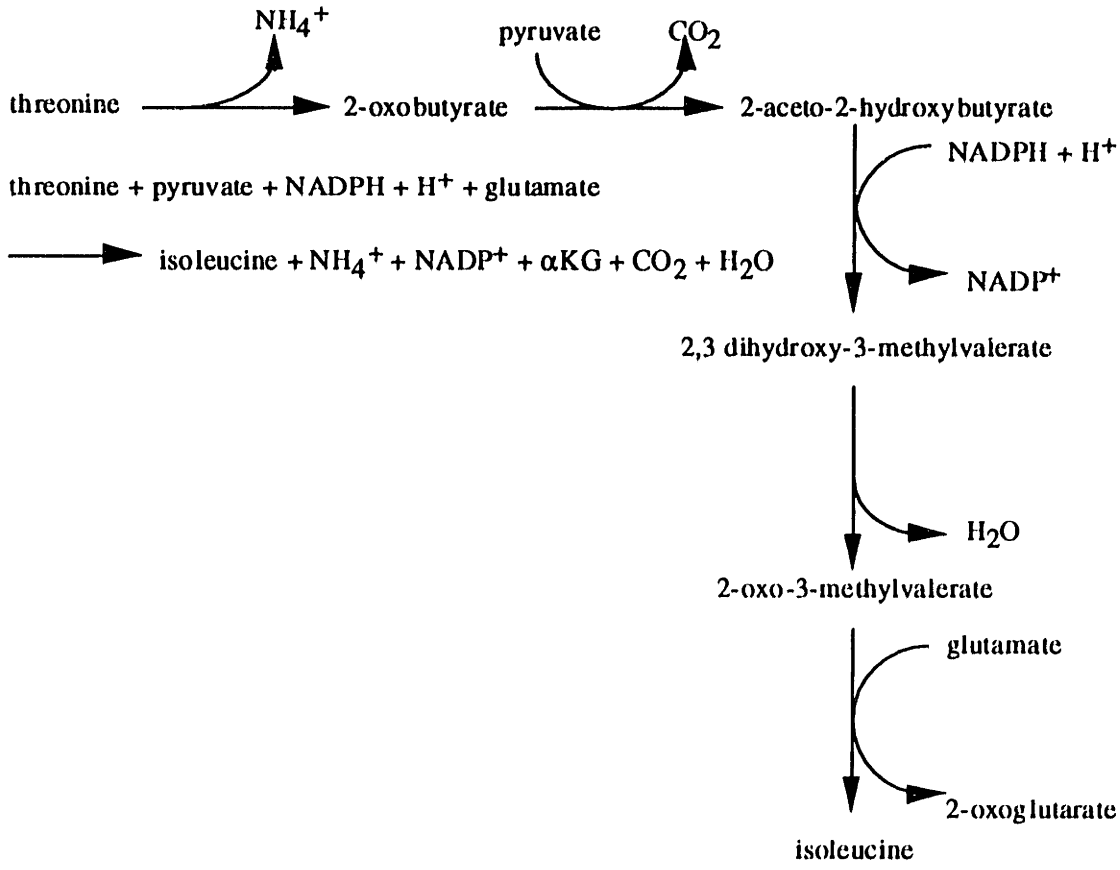

Figure 3.5 Biosynthetic reactions from threonine to isoleucine

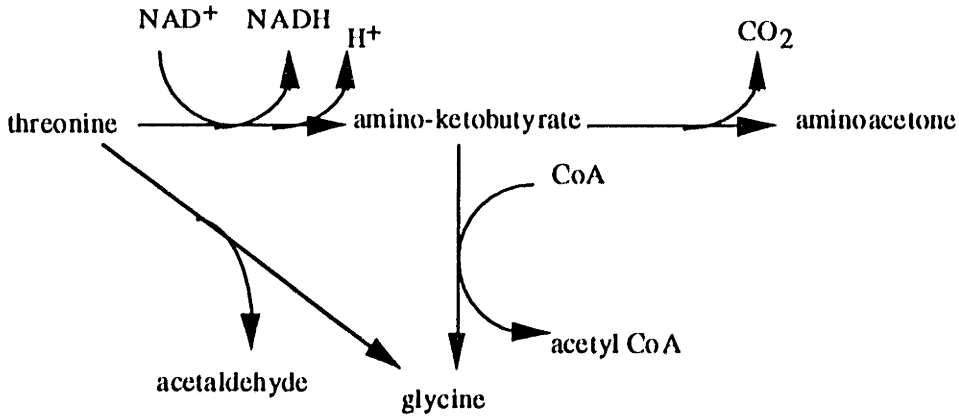

Figure 3.6 Threonine degradation reactions leading to glycine

Table 3.1 Metabolic intermediates in the aspartate/threonine/isoleucine pathways

Index	Abbreviation	Metabolite
1	ASP	aspartate
2	ASPP	aspartyl-ß-phosphate
3	ASA	aspactic-ß-semialdehyde
4	HOM	homoserine
5	HOMP	homoserine phosphate
6	THR	threonine
7	AKB	α-ketobutyrate
8	NH_4^+	ammonium
9	PYR	pyruvate
10	CO_2	carbon dioxide
11	AHB	2-aceto-2-hydroxybutyrate
12	HMV	2,3 dihydroxy-3-methylvalerate
13	OMV	2-oxo-3-methylvalerate
14	GLUT	glutamate
15	AKG	α-ketoglutarate
16	ILE	isoleucine
17	GLY	glycine
18	ACET	acetaldehyde
19	NKB	amino-ketobutyrate
20	AAC	aminoacetone
21	ACA	Acetyl-CoA
22	COA	coenzyme A
23	ATP	adenosine triphosphate
24	ADP	adenosine diphosphate
25	P_i	phosphate
26	NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
27	NADP+	nicotinamide adenine dinucleotide phospahte, oxidized form
28	NADH	nicotinamide adenine dinucleotide, reduced form
29	NAD+	nicotinamide adenine dinucleotide, oxidized form

Table 3.2 Biotransformations

Index	Stoichiometry	ΔG°'(kcal/mol)
threonine		
1	ASP + ATP --> ASPP + ADP	4.7
2	ASPP + NADPH --> ASA + $NADP^+$ + P_i	-0.1
3	ASA + NADPH + H^+ --> HOM + $NADP^+$	-5.0
4	HOM + ATP --> HOMP + ADP	5.0
5	HOMP + H_20 --> THR + P_i	-5.8
isoleucine		
6	THR --> AKB	-6.4
7	AKB + PYR --> AHB + CO_2	-17.6
8	AHB + NADPH --> HMV + NADP+	-4.8
9	HMV ---> OMV	-7.0
10	OMV + GLUT ---> ILE + AKG	0
degradation reactions leading to glycine		
11	THR --> GLY + ACET	1.9
12	THR + NAD^+ --> NKB + NADH + H^+	4.7
13	NKB --> AAC + CO2	-15.8
14	NKB + COA --> ACA + GLY	-7.3

The following assumptions apply for these calculations: pH = 7, T = 30°C, or 305 K, the optimal growth temperature for *Corynebacterium glutamicum*. All activity coefficients are assumed to be constant and equal to 1. The concentrations of ATP, ADP, and P_i are assumed constant for all intervals, since the energy charge is known to be tightly regulated for all organisms at about 0.85-0.95. The values are taken for *C. glutamicum* from Krämer and Lambert (1990): [ATP] = 5mM, [ADP] = 1mM and [P_i] = 5mM.

All other metabolites are varied in several intervals until all bottlenecks are identified. In the first two cases, these concentration

intervals are set at 0.1 mM - 1 mM and 0.05 mM - 5 mM. In the last two cases, known feasible values of some metabolites are chosen, based on either experimental results from this work or known literature values, or information about the chemical nature of the compounds. This is done to examine and compare the results obtained when little is known about a system to results obtained when a significant amount of information is available. By incorporating realistic physiological values, results with a higher metabolic significance can be obtained.

Printouts from a program implementing the thermodynamic feasibility algorithm of Mavrovouniotis (1993), written by Pedro Pissara and Eric Olson are shown below. The list of metabolites, and their concentrations (in M) is followed by the values of H_{min} and H_{max} for that particular interval (for details on the calculations, see Appendix). Reactions where $H_{max} < 0$ in the interval are always feasible; reactions where $H_{min} > 0$ in the interval are always infeasible. Reactions with $H_{min} < 0$ and $H_{max} > 0$ are designated "undetermined", and are feasible in some subset of the interval concentration space. A "distributed bottleneck" for a given interval describes a case where two undetermined reactions are combined such that one intermediate metabolite is eliminated; subsequent calculation of the free energy changes for the overall new reaction yields $H_{min} > 0$. This indicates that although viewed in isolation each reaction is feasible for some portion of the concentration interval, there is no overlap of regions of feasibility; i.e. the overall reaction combination is infeasible in the postulated concentration space. Therein lies the particular utility of this analysis.

Case 1:

Metabolite list:

ASP	C_{min}= 0.0001	C_{max}= 0.001
ATP	C_{min}= 0.005	C_{max}= 0.005
ASPP	C_{min}= 0.0001	C_{max}= 0.001
ADP	C_{min}= 0.001	C_{max}= 0.001
NADPH	C_{min}= 0.0001	C_{max}= 0.001
ASA	C_{min}= 0.0001	C_{max}= 0.001
NADP	C_{min}= 0.0001	C_{max}= 0.001
P	C_{min}= 0.005	C_{max}= 0.005
HOM	C_{min}= 0.0001	C_{max}= 0.001
HOMP	C_{min}= 0.0001	C_{max}= 0.001
THR	C_{min}= 0.0001	C_{max}= 0.001
AKB	C_{min}= 0.0001	C_{max}= 0.001
NH^4	C_{min}= 0.0001	C_{max}= 0.001
PYR	C_{min}= 0.0001	C_{max}= 0.001
AHB	C_{min}= 0.0001	C_{max}= 0.001
CO_2	C_{min}= 0.0001	C_{max}= 0.001
HMV	C_{min}= 0.0001	C_{max}= 0.001
OMV	C_{min}= 0.0001	C_{max}= 0.001
GLUT	C_{min}= 0.0001	C_{max}= 0.001
ILE	C_{min}= 0.0001	C_{max}= 0.001
AKG	C_{min}= 0.0001	C_{max}= 0.001
GLY	C_{min}= 0.0001	C_{max}= 0.001
ACET	C_{min}= 0.0001	C_{max}= 0.001
NAD	C_{min}= 0.0001	C_{max}= 0.001
NKB	C_{min}= 0.0001	C_{max}= 0.001
NADH	C_{min}= 0.0001	C_{max}= 0.001
AAC	C_{min}= 0.0001	C_{max}= 0.001
COA	C_{min}= 0.0001	C_{max}= 0.001
ACA	C_{min}= 0.0001	C_{max}= 0.001

Reaction list:

1) ASP+ATP->ASPP+ADP	H_{min}= 4.1	H_{max}= 8.7	Always Infeasible
2) ASPP+NADPH->ASA+NADP+P	H_{min}= -10	H_{max}=-0.86	Always Feasible
3) ASA+NADPH->HOM+NADP	H_{min}= -13	H_{max}= -3.9	Always Feasible
4) HOM+ATP->HOMP+ADP	H_{min}= 4.6	H_{max}= 9.2	Always Infeasible
5) HOMP->THR+P	H_{min}= -17	H_{max}= -13	Always Feasible
6) THR->AKB+NH^4	H_{min}= -22	H_{max}= -15	Always Feasible
7) AKB+PYR->AHB+CO_2	H_{min}= -34	H_{max}= -25	Always Feasible
8) AHB+NADPH->HMV+NADP	H_{min}= -13	H_{max}= -3.5	Always Feasible
9) HMV->OMV	H_{min}= -14	H_{max}= -9.6	Always Feasible
10) OMV+GLUT->ILE+AKG	H_{min}= -4.6	H_{max}= 4.6	Undetermined

Reaction list, Case 1 (cont.):

11) THR->GLY+ACET	H_{min}= -8.3	H_{max}= -1.4	Always Feasible
12) THR+NAD->NKB+NADH	H_{min}= 3.4	H_{max}= 13	Always Infeasible
13) NKB->CO_2+AAC	H_{min}= -38	H_{max}= -31	Always Feasible
14) NKB+COA->GLY+ACA	H_{min}= -17	H_{max}= -7.8	Always Feasible

In the concentration interval defined in Case 1, reactions 1, 4 and 12 are always infeasible, whereas reaction 10 is undetermined. No possible distributed bottlenecks can be constructed from this set.

Case 2

The concentrations of the following metabolites are maintained constant as follows:

ATP	C_{min}= 0.005	C_{max}= 0.005
ADP	C_{min}= 0.001	C_{max}= 0.001
P	C_{min}= 0.005	C_{max}= 0.005

All others metabolites are now allowed to vary between:

C_{min}= 0.00005 C_{max}= 0.005

Reaction List

1) ASP+ATP->ASPP+ADP	H_{min}= 1.8	H_{max}= 11	Always Infeasible
2) ASPP+NADPH->ASA+NADP+P	H_{min}= -15	H_{max}= 3.7	Undetermined
3) ASA+NADPH->HOM+NADP	H_{min}= -18	H_{max}= 0.74	Undetermined
4) HOM+ATP->HOMP+ADP	H_{min}= 2.3	H_{max}= 11	Always Infeasible
5) HOMP->THR+P	H_{min}= -20	H_{max}= -11	Always Feasible
6) THR->AKB+NH^4	H_{min}= -25	H_{max}= -12	Always Feasible
7) AKB+PYR->AHB+CO_2	H_{min}= -39	H_{max}= -21	Always Feasible
8) AHB+NADPH->HMV+NADP	H_{min}= -17	H_{max}= 1.1	Undetermined
9) HMV->OMV	H_{min}= -16	H_{max}= -7.3	Always Feasible
10) OMV+GLUT->ILE+AKG	H_{min}= -9.2	H_{max}= 9.2	Undetermined
11) THR->GLY+ACET	H_{min}= -11	H_{max}= 2.5	Undetermined
12) THR+NAD->NKB+NADH	H_{min}= -1.2	H_{max}= 17	Undetermined
13) NKB->CO_2+AAC	H_{min}= -41	H_{max}= -27	Always Feasible
14) NKB+COA->GLY+ACA	H_{min}= -22	H_{max}= -3.2	Always Feasible

With these new relaxed concentration intervals, previously feasible reactions (2, 3, 8, 11) have now become undetermined, indicating that this new concentration space may contain regions of infeasibility for these reactions. Conversely, a reaction that was always infeasible in the first concentration interval (12) has now become undetermined, indicating that for this reaction, there are now regions of feasibility.

Reactions 2 and 3, both undetermined, can be combined to test for the presence of a distributed bottleneck:

ASPP+2NADPH->HOM+2NADP+P G^o= -5.1 H_{min}= -27.6 H_{max}= 4.6 Undetermined

Therefore, these reactions do not comprise a distributed bottleneck. There is a range of concentrations for this combination which allows it to occur in the forward direction.

Using available intracellular concentrations

This algorithm can thus provide some information about thermodynamically difficult steps in a pathway when little is known about a particular system, or it can predict possible problems when synthesizing a new pathway. However, when information is available regarding actual *in vivo* conditions, a much more accurate picture can be obtained. For example, certain metabolites (such as amino acids) reach extremely high intracellular concentrations (100-200 mM), whereas other, highly unstable compounds never accumulate beyond the μmolar range. These factors drastically shift the thermodynamic situation from one in which all metabolites exist within the same range. Therefore two more cases have

been analyzed, incorporating values obtained from a typical threonine fermentation with *C. lactofermentum* 21799(pGC42).

Glutamic acid bacteria accumulate very high levels of glutamate (see section 2.2). Intracellular metabolite measurements show that glutamate concentration reaches up to about 100 mM and remains at this level throughout the threonine fermentation. Therefore, a constant glutamate concentration is used in the next iterations. Threonine, isoleucine, and homoserine levels remain elevated midway through the fermentation, and drop sharply towards the end (see section 5.6). Glycine concentration, however, is very low midway through the fermentation, and increases sharply towards the end. Case 3 incorporates the numbers for midway throughout the fermentation, and holds the concentrations of threonine (150 mM), homoserine (100 mM) and isoleucine (50 mM) constant. Case 4 incorporates variable, low concentrations for threonine, isoleucine and homoserine, while maintaining a high concentration of glycine. Also, compounds known to be highly unstable (phosphorylated compounds such as ASPP and HOMP; ASA; and NKB, or amino-ketobutyrate) are treated in both cases as constant at a concentration of 0.05 mM. Finally, data from typical lysine fermentations with *C. glutamicum* shows the concentration of ammonium ion remains high throughout the fermentation (approximately 300 mM; Cathryn Shaw, personal communication). This number is applied to both cases. The remaining metabolites are varied between 0.1 - 1.0 mM.

Case 3

Metabolite List:

ASP	C_{min}= 0.0001	C_{max}= 0.001
ATP	C_{min}= 0.005	C_{max}= 0.005
ASPP	C_{min}= 1e-05	C_{max}= 1e-05
ADP	C_{min}= 0.001	C_{max}= 0.001
NADPH	C_{min}= 0.0001	C_{max}= 0.001
ASA	C_{min}= 1e-05	C_{max}= 1e-05
NADP	C_{min}= 0.0001	C_{max}= 0.001
P	C_{min}= 0.005	C_{max}= 0.005
HOM	C_{min}= 0.1	C_{max}= 0.1
HOMP	C_{min}= 1e-05	C_{max}= 1e-05
THR	C_{min}= 0.15	C_{max}= 0.15
AKB	C_{min}= 0.0001	C_{max}= 0.001
NH^4	C_{min}= 0.3	C_{max}= 0.3
PYR	C_{min}= 0.0001	C_{max}= 0.001
AHB	C_{min}= 0.0001	C_{max}= 0.001
CO_2	C_{min}= 0.0001	C_{max}= 0.001
HMV	C_{min}= 0.0001	C_{max}= 0.001
OMV	C_{min}= 0.0001	C_{max}= 0.001
GLUT	C_{min}= 0.1	C_{max}= 0.1
ILE	C_{min}= 0.05	C_{max}= 0.05
AKG	C_{min}= 0.0001	C_{max}= 0.001
GLY	C_{min}= 0.0001	C_{max}= 0.001
ACET	C_{min}= 0.0001	C_{max}= 0.001
NAD	C_{min}= 0.0001	C_{max}= 0.001
NKB	C_{min}= 1e-05	C_{max}= 1e-05
NADH	C_{min}= 0.0001	C_{max}= 0.001
AAC	C_{min}= 0.0001	C_{max}= 0.001
COA	C_{min}= 0.0001	C_{max}= 0.001
ACA	C_{min}= 0.0001	C_{max}= 0.001

Reaction List

1) ASP+ATP->ASPP+ADP	H_{min}= 1.8	H_{max}= 4.1	Always Infeasible
2) ASPP+NADPH->ASA+NADP+P	H_{min}= -7.8	H_{max}= -3.2	Always Feasible
3) ASA+NADPH->HOM+NADP	H_{min}= -1.6	H_{max}= 3	Undetermined
4) HOM+ATP->HOMP+ADP	H_{min}= -2.3	H_{max}= -2.3	Always Feasible
5) HOMP->THR+P	H_{min}= -5.5	H_{max}= -5.5	Always Feasible
6) THR->AKB+NH^4	H_{min}= -19	H_{max}= -17	Always Feasible
7) AKB+PYR->AHB+CO_2	H_{min}= -34	H_{max}= -25	Always Feasible
8) AHB+NADPH->HMV+NADP	H_{min}= -13	H_{max}= -3.5	Always Feasible
9) HMV->OMV	H_{min}= -14	H_{max}= -9.6	Always Feasible
10) OMV+GLUT->ILE+AKG	H_{min}= -3	H_{max}= 1.6	Undetermined

Reaction List, Case 3 (cont.):

11) THR->GLY+ACET	H_{min}= -13	H_{max}= -8.7	Always Feasible
12) THR+NAD->NKB+NADH	H_{min}= -4	H_{max}= 0.65	Undetermined
13) NKB->CO_2+AAC	H_{min}= -34	H_{max}= -29	Always Feasible
14) NKB+COA->GLY+ACA	H_{min}= -12	H_{max}= -5.5	Always Feasible

Only one reaction is infeasible under these conditions, the aspartokinase reaction. The remaining reactions are all feasible over some or all of the concentration range. Since the concentrations of ATP and ADP are fixed, and aspartyl-phospate (ASPP) is an unstable compound (and is thus maintained at a low concentration), this indicates that a high concentration of aspartate is needed to drive carbon into this pathway. Once the carbon has entered the first reaction, there are no more thermodynamic bottlenecks.

At the high threonine concentrations which are known to exist intracellularly in these fermentations, the threonine dehydrogenase reaction (reaction 12) is undetermined rather than infeasible, as in case 1. This indicates that this seemingly thermodynamically difficult reaction can proceed in the forward direction under physiological conditions which would call for threonine degradation (e.g., growth on threonine as a carbon source, or excess threonine production in the cell).

It is interesting that in *E. coli*, the genes encoding the threonine dehydrogenase and amino-ketobutyrate:CoA ligase enzymes (reactions 12 and 14) are present in an operon, under the control of a single promoter; there is strong evidence that this is the case in *Corynebacterium* spp. as well (Bell and Turner, 1976; see section 5.4.2). The amino-ketobutyrate:CoA ligase reaction (14) is highly thermodynamically favorable, and remains so even in the presumably extremely low intracellular concentrations of the substrate, aminoketobutyrate (NKB).

There is a competing reaction which consumes NKB and is also highly thermodynamically favorable (reaction 13, spontaneous decarboxylation to aminoacetone; see figure 3.7). For the cell to derive the metabolic benefits of acetylCoA and glycine from threonine, it would be valuable for the cell to be able favorably direct threonine carbon towards the first reaction. The evolution of an operon to direct the simultaneous synthesis of the genes encoding the enzymes catalyzing reactions 12 and 14 is consistent with the thermodynamic situation. In this manner, once a high intracellular level of threonine is reached, both genes are expressed concurrently. The aminoketobutyrate derived from the threonine (by the action of threonine dehydrogenase) will thus have a greater chance of being converted to acetylCoA (by the action of amino-ketobutyrate:CoA ligase) and used for anabolic purposes than of being spontaneously converted to aminoacetone and thus serve no useful purpose for the cell.

Case 4

Towards the end of the fermentations with *C. lactofermentum* 21799(pGC42), intracellular threonine, isoleucine and homoserine concentrations drops drastically, and glycine concentration increases (see section 5.6). Incorporating these changes yields the following results:

Metabolite List:

HOM	C_{min}= 0.0001	C_{max}= 0.001
THR	C_{min}= 0.001	C_{max}= 0.01
ILE	C_{min}= 0.0001	C_{max}= 0.001
GLY	C_{min}= 0.2	C_{max}= 0.2

Reaction List

1) ASP+ATP->ASPP+ADP	H_{min}= 1.8	H_{max}= 4.1	Always Infeasible
2) ASPP+NADPH->ASA+NADP+P	H_{min}= -7.8	H_{max}= -3.2	Always Feasible
3) ASA+NADPH->HOM+NADP	H_{min}= -8.5	H_{max}= -1.6	Always Feasible

Reaction List, Case 4 (cont.):

4) HOM+ATP->HOMP+ADP	H_{min}= 2.3	H_{max}= 4.6	Always Infeasible
5) HOMP->THR+P	H_{min}= -11	H_{max}= -8.2	Always Feasible
6) THR->AKB+NH^4	H_{min}= -17	H_{max}= -12	Always Feasible
7) AKB+PYR->AHB+CO_2	H_{min}= -34	H_{max}= -25	Always Feasible
8) AHB+NADPH->HMV+NADP	H_{min}= -13	H_{max}= -3.5	Always Feasible
9) HMV->OMV	H_{min}= -14	H_{max}= -9.6	Always Feasible
10) OMV+GLUT->ILE+AKG	H_{min}= -9.2	H_{max}= -2.3	Always Feasible
11) THR->GLY+ACET	H_{min}= -3	H_{max}= 1.6	Undetermined
12) THR+NAD->NKB+NADH	H_{min}= -1.2	H_{max}= 5.7	Undetermined
13) NKB->CO_2+AAC	H_{min}= -34	H_{max}= -29	Always Feasible
14) NKB+COA->GLY+ACA	H_{min}= -4.8	H_{max}=-0.17	Always Feasible

There are two bottlenecks under these conditions, reactions 1 and 4, both of which involve phosphorylation reactions. In addition, the two threonine degradation reactions are feasible over some concentration range, even with the low threonine concentration present at this stage. The thermodynamic implications of these results are further discussed in Chapter 5.

Several important conclusions can be drawn from these results. The importance of high aspartate flow into the overall pathway is stressed by the highly unfavorable thermodynamics of the aspartate kinase reaction. Only by insuring that a high concentration of aspartate is maintained is flux into the pathway permitted. For example, based on the value of the standard Gibbs free energies of reaction and the typical internal concentrations of ATP and ADP in *Corynebacterium*, there must be a 588:1 ratio of aspartate to aspartyl-ß-phosphate. This means that for a concentration of aspartyl-ß-phosphate of about 0.1 mM, there needs to be an aspartate concentration of 58 mM for the reaction to proceed. This fact underscores the importance of controlling the central metabolism reactions to divert the carbon flux towards oxaloacetate and subsequently aspartate. Similarly, for the homoserine kinase reaction to proceed, there needs to be

a 1000:1 ratio of homoserine to homoserine phosphate. These calculations may provide valuable information for the analysis of limiting factors in the production of a desired metabolite. Conversely, the decision to attempt overproduction of a metabolite may be supported by favorable thermodynamics. For example, the five reactions leading from threonine to isoleucine are all thermodynamically favorable under standard conditions. If there is already a high carbon flux into the pathway (for example, in the threonine producer 21799(pGC42); section 5.4.3), there are then at least no thermodynamics constraints limiting metabolite overproduction. These considerations provide a more complete picture of cell metabolism for purposes of flux redirection and are thus an important component of any metabolic engineering program.

Chapter 4. Materials and Methods

4.1 Strains, plasmids and media

Bacterial strains and plasmids used are listed in Table 4.1. *C. lactofermentum* ATCC 21799 (a lysine producer resistant to *S*-2-aminoethyl-L-cysteine (AEC), a lysine analogue) was used as the base strain. Several types of complex media were used. LB (Lennox, 1955) contains, per liter: yeast extract, 5 g; tryptone, 10 g; NaCl, 5 g. 2xTY medium contains, per liter: yeast extract, 10g; tryptone, 16 g; NaCl, 5 g. MB medium contains, per liter, yeast extract, 5 g; tryptone, 15 g; soytone, 5 g; NaCl, 5 g.

For enzyme assays, cells were cultured in *C. glutamicum* minimal medium adapted from von der Osten *et al.* (1989) and Kiss (1991) containing (per liter): glucose, 20 g; Na_3•citrate•$2H_20$, 1.1 g; NaCl, 1 g; $MgSO_4$•$7H_2O$, 200 mg; Na_2EDTA•$2H_2O$, 75 mg; $FeSO_4$•$7H_2O$, 25 mg; $CaCl_2$•$2H_2O$, 50 mg; K_2HPO_4, 8 g; KH_2PO_4, 1 g; $(NH_4)_2SO_4$, 5 g; biotin, 1 mg; thiamine, 1 mg; calcium pantothenate, 5 mg; 100X mineral salts, 10 ml (per liter, $MnSO_4$, 200 mg; $Na_2B_4O_7$•$10H_2O$, 20 mg; $(NH_4)_6Mo_7O_{24}$•$4H_2O$, 10 mg; $FeCl_3$•$6H_2O$, 200 mg; $ZnSO_4$•$7H_2O$, 50 mg; $CuCl_2$•$2H_2O$, 20 mg), pH 7.0. In addition, threonine, methionine and leucine were supplemented at 150 mg/l for assays, 400 mg/l for fermentations. For studies involving 21799*ilv*A- strains, corresponding amounts of isoleucine were also added.

For fermentations, minimal medium was used; however, glucose was

Table 4.1 Bacterial strains and plasmids[a]

strain or plasmid	genotype or description	source or reference
strains		
C. glutamicum		
AS019	Rf^r derivative of ATCC 13059	Yoshihama *et al.*, 1985
AS019-E12	restriction deficient derivative of *C. glutamicum* AS019	Follettie, 1989
C. lactofermentum		
21799	L-lysine producing strain, AEC^R	ATCC
21799*ilv*A-	*ilv*A-, Cm^r	Colón *et al.*, 1995b
E. coli		
DH5α	*ac*ZΔM15, *hsd*R-, *rec*A-	Hanahan, 1983
AB1255	*ilv*A-	CGSC #1255
5076	*thr*B-	CGSC #5076
plasmids		
pMG108	Km^r, Ap^r, *lac*I^q, *tac*	Gubler and Sinskey, 1993
pM2	*E. coli-C. glutamicum* shuttle vector	Follettie *et al.*, 1993
pFS3.6	3.6 kb *Sal*I fragment containing *hom-thr*B operon cloned in a *C. glutamicum-E. coli* shuttle vector	Follettie *et al.*, 1988
pJD4	Km^r *hom*^{dr}-*thr*B operon	Archer *et al.*, 1991
pCP1	Km^r, Ap^r, *lac*I^q, *tac*::*thr*B	this work
pGC18	Km^r, Ap^r, *lac*I^q, *tac*::*thr*B	this work
pGC42	Km^r, Ap^r, *lac*I^q, *hom*^{dr}, *tac*::*thr*B	this work
pGC77	Km^r, Ap^r, *lac*I^q, *hom*^{dr},*ilv*A, *tac*::*thr*B	this work
pUC18	Cloning vector, Ap^r	Vieira and Messing, 1982
p18-HIB1, p18-HIB2	3.3 kb *Hind* III fragment ligated in both orientations in pUC18	Colón *et al.*, 1995b
pSUP 301	mobilization vector, Km^R, Ap^R	
pSH3-*ilv*A	pSUP 301 with 3.3 kb *Hind* III fragment containing *ilv*A	Colón *et al.*, 1995b
pSH3*cat*6	pSH3-ilvA with a *Bam*HI fragment conatainiing the *cat* gene replacing an internal 400 bp portion of *ilv*A	Colón *et al.*, 1995b

[a] Km^r, Ap^r, Cm^r, and Rf^r indicate resistance to kanamycin, ampicillin, chloramphenicol and rifampicin, respectively. AEC^R indicates resistance to S-2-aminoethyl-L-cysteine, a lysine analogue. ATCC, American Type Culture Collection, Rockville, Md., USA. CGSC, *E. coli* Genetic Stock Center, New Haven, Conneticut, USA.

increased to 80 g/l, and ammonium sulfate was increased to 50 g/l. Calcium carbonate (30 g/l) was added for buffering. For fermentation pre-cultures (50 ml in 100 ml flasks), the following seed medium was used (per liter): sucrose, 50 g; polypeptone, 20 g; urea, 3 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; K_2HPO_4, 1.5 g; KH_2PO_4, 0.5 g; pH adjusted to 7.0.

For the preliminary fermentation studies involving *C. lactofermentum* ATCC 21799(pJD4), the following fermentation medium was used (per liter): glucose, 70 g; $(NH_4)_2SO_4$, 50 g; KH_2PO_4, 0.1 g; $MgSO_4 \cdot 7H_2O$, 40 mg; $MnSO_4$, 1 mg; biotin, 1 mg; thiamine, 1 mg; corn steep liquor (obtained from Archer Daniels Midland, Decatur, Ill.), 200 ml; $CaCO_3$, 25 g; pH adjusted to 7.4.

The organisms were cultured aerobically at 30°C as 100-ml cultures in 500-ml baffled Erlenmeyer flasks on a rotary shaker at 250 rpm. When appropriate, kanamycin (50 mg/l), ampicillin (50 mg/l) and/or chloramphenicol (15 mg/l) were added. Isopropyl ß-D-thiogalactopyranoside (IPTG) was used as an inducer when appropriate.

4.2 DNA manipulations and cell transformations

Isolation of plasmid DNA, preparation of DNA fragments, ligations and other DNA manipulations were performed according to procedures developed by Yoshihama *et al.* (1985) and Sambrook (1989). *Corynebacterium* cells were transformed by electroporation as described by Follettie *et al.* (1993). All restriction enzymes and related reagents for DNA manipulations were obtained from New England Biolabs, Bethesda

Research Laboratories, or Boehringer Mannheim, and used as per the instructions of the manufacturer.

4.2.1 Isolation of plasmid DNA from *Escherichia coli*

10 ml overnight cultures of cells were harvested and the pellets were transferred to 1.5 ml Eppendorf tubes. The cells were resuspended in 200 µl of solution A (50 mM glucose, 2 mM EDTA, 50 mM Tris•Cl, pH 8.0), and the solution was allowed to sit at room temperature for five minutes. 400 µl of solution B (9.1 ml dH_20, 0.5 ml 10% SDS (sodium dodecyl sulfate), 0.4 ml 5 M NaOH) were added, and the cells were placed on ice for 5-10 minutes. Next were added 300 µl of solution C (7.5 M ammonium acetate) and the cells were placed on ice for 5 minutes, with occasional mixing. The cells were centrifuged at 13,000 rpm for 20 minutes, the supernatant was transferred to fresh tubes, and the centrifugation was repeated. 500 µl of isopropanol were added to 800 ml of the supernatant, and the solution was centrifuged for 20 minutes at 13,000 rpm. The pellets were washed in 70% ethanol, allowed to dry, resuspended in 200 ml TE buffer with 1 µl of a 10 mg/ml RNase solution and allowed to remain at room temperature for 5 minutes. The solution was then extracted once with 100 µl phenol, and once with 50 µl chloroform. Nucleic acid was precipitated by the addition of 20 µl of 3 M sodium acetate and 400 µl of cold ethanol. The solution was placed on ice for at least 20 minutes, then centrifuged for 20 minutes. The pellets were washed with 100-200 µl 70% ethanol, then allowed to air dry prior to resuspension in 20 µl TE (10 mM Tris•Cl (Tris (hydroxymethyl) aminomethane), 1mM EDTA).

4.2.2 Preparation of *E. coli* competent cells

E. coli cells were cultured aerobically overnight in tubes containing 10 ml LB broth. 1 ml of the overnight culture was used to inoculate 100 ml of LB or 2X TY broth. Cells were cultured aerobically at 37°C for 1.5-2 hr until the cells reached an absorbance at 600 nm of 0.4. The cells were transferred to two cold 50 ml Falcon centrifuge tubes and harvested at 4°C. After decanting the broth, 20 mls of calcium chloride buffer (70 mM $CaCl_2$, 5 mM $MgSO_4$) were added to each tube and these were placed on ice in the 4°C constant temperature room for at least 15 minutes. After centrifuging and decanting once more, each pellet was resuspended in 1 ml calcium chloride buffer. The tubes were set overnight on ice in the 4°C constant temperature room. The cells can be used immediately for transformation or else stored by diluting 1:1 with sterile calcium chloride/glycerol solution (36% glycerol, 70 mM $CaCl_2$, 5 mM $MgSO_4$).

4.2.3 Calcium chloride transformation of *E. coli* competent cells

The competent cells were thawed on ice, and from 0.01 to 0.1 µg of DNA were added to 100-200 µl cells. The cells were placed on ice for 30 minutes, and subsequently heat shocked for 90 seconds at 42°C. The cells were immediately placed on ice, and 0.8 ml 2 x TY broth were added. The cells were allowed to recover at 37°C for 30 - 60 minutes, and were plated on LB agar plates (100 µl/ plate) containing 50 µg/ml of ampicillin. A control with no DNA was used each time.

4.2.4 Isolation of plasmid DNA from *Corynebacterium*

10 ml LB overnight cultures were harvested, resuspended in 500 µl solution E (40 mM Tris, 2 mM EDTA, pH adjusted to 7.9 with acetic acid) with 3 mg/ml lysozyme, and transferred to 1.5 ml Eppendorf tubes. 10 µl of a 1 mg/ml solution of mutanolysin were added to each tube, and these were placed in a 37°C water bath for 1 hr. EDTA (50 µl of a 0.5 M solution) and sodium dodecyl sulfate (100 µl of a 10% solution) were added to each sample, and complete lysis was achieved by placing the tubes in a boiling water bath for 2 minutes. 100 µl of 5 M sodium chloride were added to each tube, and these were placed on ice for 30 minutes. Cellular debris was removed by centrifugation at 13,000 rpm for 30 minutes. The supernatant was recovered and extracted once with phenol and once with chloroform. Nucleic acid was precipitated by the addition of 600 µl of isopropanol and centrifugation at 13,000 rpm for 15 minutes. The pellets were allowed to air dry before the addition of 100 µl TE and 1 µl RNase. The tubes were placed in a 37°C water bath for 5 minutes. The samples were extracted once with phenol and once with chloroform. Nucleic acid was precipitated with the addition of 20 µl 3 M sodium acetate and 400 µl ethanol. The tubes were placed on ice for at least 20 minutes, then centrifuged at 13,000 rpm for 15-20 minutes. The pellets were washed once with 100-200 µl 70% ethanol, centrifuged for 20 minutes at 13,000 rpm, and resuspended in 20 µl TE.

4.2.5 Preparation of *Corynebacterium* competent cells for electroporation

C. glutamicum cells were transformed via electroporation, following treatment to facilitate the procedure. One ml of an overnight 10 ml LB culture was inoculated into 100 ml MB broth (or 2 x TY), and incubated with aeration at 30°C for 3-4 hours, until the cells reached an absorbance at 600 nm of 0.6-0.8. At this point, 1-2 µl of a 50 mg/ml solution of ampicillin were added (to aid in permeabilizing the cell wall), and the cells incubated for an additional 1-1.5 hours. The cells were then harvested and washed twice with 30 ml of sterile Electroporation Buffer #1 (5% glycerol, 20 mM HEPES, pH 7.2), and resuspended in 1.5 ml of sterile Electroporation Buffer #2 (15 % glycerol, 10 mM HEPES). The cells were either used immediately for electroporation, or were stored in 150 µl aliquots at -80°C.

4.2.6 Electroporation of *Corynebacterium* competent cells

150 µl competent cells were thawed on ice, and < 1 µl DNA was added. The cells were incubated on ice with the DNA for 5 minutes. The mixture was transfered to chilled electroporation cuvettes (Bio-Rad Gene Pulser/*E. coli* Pulser™, 0.2 cm elecrode gap, Bio-Rad laboratories, Herculse, CA), and all air bubbles were carefully removed. The cells were shocked at 2.5 kV, 200 Ω, and 25 µFD, with a typical time constant between 3.5 and 5. As rapidly as possible, 0.75 ml of recovery broth (40 g/l brain heart infusion, 30 g/l sorbitol, 10 g/l sucrose) were added. The cells were incubated for 1-1.5 hrs at 30°C. 100 µl of cells were plated on

each BHI plate (40 g/l brain heart infusion, 30 g/l sorbitol, 10 g/l sucrose, 17 g/l agar, 15 mg/l kanamycin).

4.2.7 DNA fragment extraction from agarose gels

DNA fragments were fractionated by 1% agarose gel electrophoresis and purified by using the QIAEX method (QIAGEN, Chatsworth, CA.). 300 µl QX1 buffer were added per 100 mg. gel and vortexed until a homogeneous system was obtained. 10 µl of the QIAEX suspension were added, the solution was mixed and placed in a 50°C water bath for 10 minutes, with mixing every 2 minutes. The mixture was centrifuged for 30 seconds (DNA is bound to QIAEX at this point) and the supernatant was discarded. The mixture was resuspended in 500 µl QX2 buffer, vortexed, centrifuged for 30 seconds, and the supernatant was discarded (twice). This step was repeated with buffer QX3. The mixture was centrifuged again for 30 seconds, and any trace of ethanol was immediately removed by pipetting. The pellet was resupended in 20 µl TE buffer and placed in a 50°C water bath for 5 minutes to elute the DNA. After a 30 second centrifugation step, the supernatant (containing DNA) was transferred into a clean tube.

4.2.8 DNA modifying treatments

4.2.8.1 Calf intestine phosphatase

Calf intestine phosphatase (CIP) catalyzes the removal of 5'-phosphate residues from DNA, RNA, rNTP's and dNTP's. It is used to remove 5' phosphastes from fragments of DNA to prevent self-ligation and thus minimize the number of transformants not containing insert after a ligation.

For this step, one µl of CIP was added to the DNA solution, which was then placed in a 37°C waterbath for 30 minutes, followed by a 55°C waterbath for 15 minutes. The phosphatase was then inactivated in a 68°C water bath for 20 minutes in the presence of EDTA (50 µl of 0.5M EDTA). The samples were vortexed for one minute after addition of 50 µl dH_2O and 100 µl phenol. An additional 100 µl dH_2O were added, the samples vortexed for 30 seconds, then centrifuged for 15 minutes at 13,000 rpm. The aqueous phase was extracted once with phenol (100 µl) and once with chloroform (50 µl). The DNA was precipitated with 20 µl 3M sodium acetate and 400 µl ethanol. The tube was then chilled at 20°C for at least 20 minutes. After a 15-minute centrifugation step, the supernatant was decanted. After addition of 0.5 ml of 70% EtOH, the tube was centrifuged for five minutes, decanted and the pellet was allowed to dry prior to resuspension in 20 µl TE.

4.2.8.2 Klenow fragment of DNA polymerase I

5' --> 3' DNA polymerase activity is used to fill in recessed 3'-termini and create blunt ends. To a 20 µl digestion reaction were added 5 µl of a 0.2 mM solution of all four dNTPs and 1 µl of the Klenow fragment of DNA polymerase I were added. The tube was placed in a 37°C waterbath for 30 minutes. The enzyme was heat-inactivated by placement in a 68°C waterbath for 10 minutes. The DNA was then ethanol precipitated and resuspended in TE.

4.2.9 Ligations

Ligation of a DNA fragment to a linearized plasmid involves the formation of new bonds between 5' termini phosphate residues and adjacent 3'-hydroxyl moieties. When the plasmid DNA has been treated with CIP, only two new phosphodiester bonds are formed. The two single-strand nicks in the resulting molecule are repaired after the plasmid has been introduced into competent bacteria.

Ligations were carried out at 16°C in a total volume of 15 µl. For blunt-end ligations, the reaction mixture contained 100-200 ng vector and approximately five times (in molar ratio) fragment, 1 µl T4 DNA ligase, 1.5 µl 5X T4 DNA ligase buffer and dH_20. For ligation of cohesive termini ("sticky ends"), a smaller amount of fragment could be used (about twice the molar ratio), along with a higher concentration of 5X T4 DNA ligase buffer (3 µl in a total ligation volume of 15 µl). The amounts of vector and fragment were estimated from an agarose gel by comparison to λ *Hind*III standards.

4.3 Enzymatic activity assays

4.3.1 Preparation of crude extracts from cell samples

For determination of enzyme activities in cells harboring the various plasmids, and for the kinetic studies, cells were cultured in minimal medium to the late exponential phase, harvested at 10,000 rpm for 15 minutes and washed twice with salts buffer (40 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$ and 1 mM $NaHCO_3$). For determination of specific activities during shake flask fermentations, a 1-2 ml sample of cells was removed from the culture flask, washed twice with salts buffer. Crude extracts for all strains were prepared by resuspending harvested cells in 100 mM KH_2PO_4, 150 mM NaCl and 1 mM dithiothreitol (DTT), pH 7.0 and homogenizing with glass beads (212-300 microns; Sigma, St. Louis, MO) in a #5100 Mixer/Mill (SPEX Industries, Edison, NJ) for two minutes (250-500 µl glass beads per 500 µl cell suspension). The solutions were centrifuged at 20,000 rpm for 30 min, and the protein concentration of the supernatants were determined by the method of Bradford (1976). This method was used for both *Corynebacterium* and *E. coli* cells. For *Corynebacterium*, a typical protein yield was 3-4 mg/ml protein when starting with 100 mls of cells grown to an optical density at 600 nm (OD_{600}) of 5 and resuspending the pellet in 1 ml of phosphate/DTT solution.

4.3.2 Enzyme assays

Enzyme assays were performed in methylacrylate cuvettes (Fisher, Pittsburgh, PA) at room temperature (22 ± 2°C) using a Hewlett-Packard

8452A diode-array spectrophotometer. Kinetic parameters were estimated using the non-linear regression analysis program GRAFIT from Erithacus Software.

4.3.2.1 Homoserine dehydrogenase

Activity was determined by the decrease in absorbance at 340 nm due to the oxidation of NADPH in a reaction mixture containing 3 mM DL-aspartic-ß-semialdehyde (ASA), 0.5 mM NADPH, and 100 mM KH_2PO_4 buffer, pH 7.0 and crude extract in a total volume of 1 ml. The absorbance decrease in the absence of the substrate, ASA, was determined and found to be negligible. One unit of enzyme activity corresponds to the oxidation of 1 µmol of NADPH per minute. DL-ASA was synthesized by the ozonolysis of DL-allyl glycine according to the procedure of Black and Wright (1955) (courtesy of Dr. Hiroaki Suga). The concentration in the ASA fractions was measured by following the total decrease in absorbance due to oxidation of NADPH in samples containing various volumes of ASA fractions and crude extracts of 21799(pJD4) cells.

4.3.2.2 Homoserine kinase

Activity was measured by a coupled assay in a reaction mixture containing 5 mM ATP, 1 mM NADH, 5 mM phospho*enol* pyruvate, 10 mM L-homoserine, 10 mM $MgCL_2$, 10 units pyruvate kinase (Sigma), 25 units lactate dehydrogenase (Sigma), 250 mM KCl, 100 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) buffer (pH 7.8) and crude extract in a total volume of 1 ml. The decrease in absorbance at 340

nm due to the oxidation of NADH was followed. The absorbance decrease in the absence of the substrate L-homoserine was determined as a control and subtracted from values obtained with the complete reaction mixture. One unit of enzyme activity corresponds to the oxidation of 1 μmol of NADH per minute. For the kinetic studies, homoserine kinase activity as a function of L-homoserine concentration was measured in crude extracts of 21799 (pJD4) in the presence of various concentrations of threonine (0 mM, 10 mM and 25 mM). The concentration of L-homoserine was varied from 0.1 mM to 40 mM. The concentration of ATP was maintained at saturating conditions (5 mM).

4.3.2.3 Threonine dehydratase

For determination of threonine dehydratase activity, the assay mixture contained 40 mM threonine, 1 mM pyridoxal phosphate, crude extract, and 100 mM potassium phosphate buffer, pH 8.2, in a final volume of 0.8 ml. The reaction was started with the addition of crude extract, and terminated by the addition of 1 ml of a solution containing 1% semicarbazide and 0.9% sodium acetate. After a 15-minute incubation at room temperature, the amount of α-ketobutyrate formed at various intervals was measured as its semicarbazone derivative at 254 nm (Möckel *et al.*, 1992). Relevant standards and controls were carried out in the same manner.

4.3.2.4 Acetohydroxy acid synthase

Activity was determined according to Eggeling, *et al.* (1987). The reaction mixture contained, in a final volume of 1 ml, 100 mM sodium pyruvate, 10 mM $MgCl_2$, 0.2 mM thiamine pyrophosphate, crude extract, and 100 mM potassium phosphate buffer, pH 7.8. The reaction was started by addition of crude extract, and 250 µl samples were taken. The reaction was terminated by the addition of 0.1 ml 10% $ZnSO_4$ and 0.1 ml of 1 N NaOH. The acetolactate formed was decarboxylated to acetoin by placement in a 65°C waterbath for 15 minutes. The acetoin was determined by the method of Westerfeld (1945). After centrifugation of the samples, the supernatants were combined with 0.5 ml of 0.5% creatine, 0.5 ml of 5% α-naphtol, and 2.25 ml H_2O. The reaction was carried out at room temperature in the dark for 60 minutes, at which point the extinction was read at 530 nm. Relevant controls and standards were carried out in the same manner.

4.3.2.5 Serine hydroxymethyltransferase

Activity was assayed at room temperature according to the method of Komatsubara *et al.* (1978) with the following modifications. The reaction mixture contained 0.2 µmol pyridoxal phosphate, 1 µmol to 400 µmol L-threonine, and crude extract in a 200 mM Tris buffer, pH 8.6. The total reaction volume was 1 ml and 250 µl samples were removed every 15 minutes for a total of 45 minutes. The reaction was stopped by the addition of 250 µl of 0.3M trichloracetic acid to each sample. Protein was removed by centrifugation. Acetaldehyde in the supernatant was

measured according to the spectrophotometric method of Paz et al (1965). 400 µl of 0.1M glycine buffer and 100 µl of 0.1% N-Methyl benzothiazolone hydrazone were added to each sample and incubated at 42° for 30 minutes. Absorbance was measured at 306 nm. Water was used as a blank.

It should be noted that Newman *et al.* (1976) pointed out that the MBTH reaction is not specific for aldehydes, but reacts also with other carbonyl groups, including keto acids. To eliminate the signal due to activity of threonine dehydratase, the authors added small amounts of isoleucine, which completely inhibited the activity of this enzyme. When using this assay to measure the kinetic parameters of SHMT in this work, this became a non-issue since the assays were performed using an *ilv*A- strain, which lacks threonine dehydratase. To further ensure that there was no interference from this activity, test assays were performed in the presence and absence of isoleucine, with no significant difference in activity being detected.

4.3.2.6 Threonine dehydrogenase

Activity was measured according to the method of Green and Elliot (1964). Reaction mixtures contained 120 µmol L-threonine, 300 µmol KCl, 5 µmol NAD^+, up to 100 µl of enzyme preparation and 200 mM Tris-HCl in a total volume of 1 ml. The reduction of NAD^+ by threonine dehydrogenase was monitored by the change in absorbance at 340nm. Endogenous NADH oxidase activity was subtracted by an L-threonine control. The other potential problem with this assay was previously described by Morris (1969). In crude extracts of *Arthrobacter* sp.,

collaborative action of threonine aldolase (same activity as SHMT) and aldehyde dehydrogenase results in an L-threonine-dependent reduction of NAD+ that could be miscontrued as L-threonine dehydrogenase activity. Therefore, acetaldehyde dehydrogenase activity was measured according to Morris (1969). In a final volume of 1 ml, the reaction mixture contained 200 µmoles potassium phosphate buffer, pH 7.5, 0.5 µmole NAD+ and 15 µmoles redistilled acetaldehyde. The reaction was started with the addition of crude extract, and the absorbance increase due to reduction of NAD+ was followed. Under conditions tested, it was found that this activity contributed a negligible amount to the overall threonine-dependent reduction of NAD+.

4.4 Abiotic phase measurements

4.4.1 Amino acid analysis

Amino acids were determined spetrophotometrically after precolumn derivatization with *ortho*-phthaldialdehyde (OPA) reagent (Hewlett Packard, Waldbronn, Germany) and separation by an AminoQuant 2.1 bore reversed phase column with a Hewlett Packard series 1050 high-pressure liquid chromatography system. The column was run at 40°C with a two-buffer gradient program at a flow rate of 0.45 ml/min. Buffer A consisted of 20 mM sodium acetate, 50 mM tetrahydrofuran and 2 mM triethylamine in MilliQ water at pH 7.2 (TEA and THF obtained from Fluka Chemie, Buchs). Buffer B consisted of 100 mM sodium acetate, methanol and acetonitrile in a 20/40/40 volumetric ratio (HPLC-grade MeOH and CH_3CN obtained from Mallinckrodt Specialty Chemicals, Paris,

Kentucky). In order to achieve an adequate separation between the glycine, homoserine and threonine peaks the gradient program parameters were optimized as follows: 0-2 min, 100% A, 0% B; 2-7 min, 0-10% B; 7-15 min, 10-15% B; 15-30 min 15-60% B. Samples were diluted to a final concentration of < 0.5 g/l amino acid. Table 4.2 lists the program used with the autosampler for automatic pre-column OPA derivatization.

Table 4.2 Program for automatic pre-column OPA derivatization used with the HP autosampler

Line #	Command		Vial content
10	Draw 4 µl	from vial 1	borate buffer
20	Draw 1 µl	from vial 2	OPA
30	Draw 0 µl	from vial 3	water (wash)
40	Draw 2 µl	from sample	sample
50	Draw 0 µl	from vial 3	water (wash)
60	Mix in seat 10 X		
70	Wait 2 minutes		
80	Inject		

4.4.2 Organic acid analysis

Organic acids and glucose were determined using a BioRad Aminex HPX-87H reverse phase column with a Hewlett Packard series 1050 high-pressure liquid chromatography system. The column was run at 40°C with a mobil phase consisting of 5 mM H_2SO_4 and a flow rate of 0.6 ml/min, and the peaks were detected with an HP 8457A Refractive Index Detector. A sample injection volume of 30 µl was used. Samples were diluted to a final concentration of 1-10 g/l of organic acid. Standards for pyruvate, trehalose, acetate, lactate and α-ketobutyrate were run.

4.4.3 Glucose assay

Glucose was quantified using an enzymatic assay kit (Glucose Trinder, Proc. # 315, Sigma, St. Louis). The enzymatic reactions involved in the assay are as follows:

$$\text{glucose} + H_2O + O_2 \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{4-aminoantipyrine} + \text{p-hydroxybenzene sulfonate} \xrightarrow{\text{peroxidase}} \text{quinoneimine dye} + H_2O$$

The quinoneimine dye has an absorbance maximum at 505 nm. The reagent was reconstituted with dH_20 according to the instructions of the manufacturer, and 1 ml of solution was aliquoted into methylacrylate cuvettes (Fisher, Pittsburgh, PA). Standards ranging from 1-10 g/l glucose were prepared, and samples were diluted to an expected concentration within that range. 5 µl of either water, sample or standard were added to each cuvette, and the solutions thoroughly mixed by inversion. The reactions were allowed to proceed at room temperature (22 ± 2°C) for 20 minutes. The absorbance at 505 nm was determined using a Hewlett-Packard 8452A diode-array spectrophotometer. A typical glucose standard curve is shown in Figure 4.1.

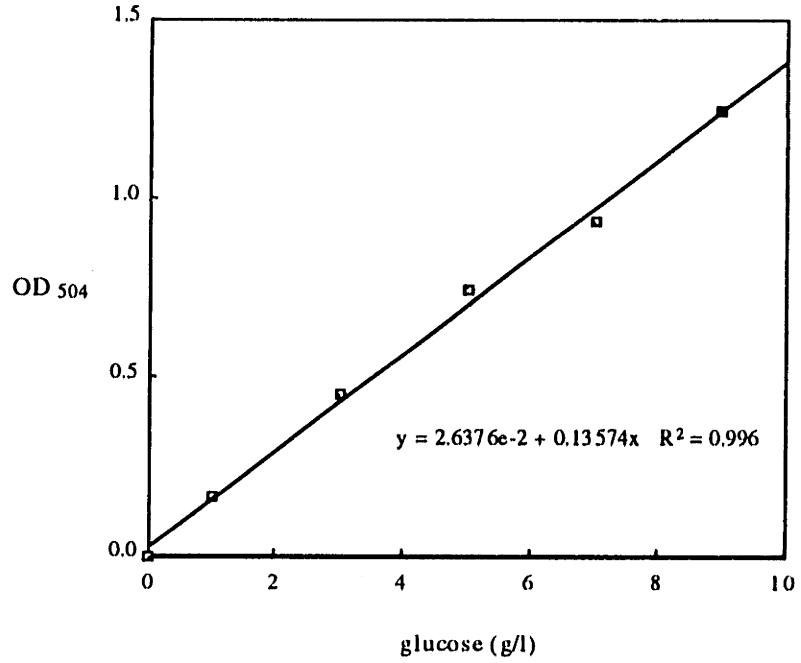

Figure 4.1 Typical glucose standard curve using the glucose Trinder assay.

4.5 Intracellular metabolite measurements

In order to better understand intracellular phenomena during overproduction of amino acids, an assay was developed to estimate intracellular metabolite concentrations at various times during the fermentation. This information, along with kinetic parameters determined *in vitro*, will hopefully provide us with the tools to further direct metabolic activities towards accumulation of the desired amino acids.

The assay used is a modification of that used by Ebbinghausen *et al.* (1989) which uses silicone oil layer centrifugation (Klingerberg and Pfaff, 1967) to separate cells from the medium and perchloric acid to quench metabolic reactions and preserve the instantaneous levels of metabolites.

The authors wash the cells prior to separation by the oil. We found pre-washing introduces a large error in the determinations (resulting in erroneously low values), due to seeping out of some amino acids during the washing step (these were detected in the washes). Therefore, to eliminate that step, a variety of combinations of oils were investigated with the goal of obtaining the right properties. Silicone oil lacked the appropriate density for our purposes, since the elimination of the wash step required that a highly dense fermentation sample be used directly. Therefore, an oil with a density between that of a fermentation sample and the perchloric acid was needed. After an extensive search, a combination of oils which were miscible and had the necessary resultant density was found. The following procedure was thus used.

Eppendorf tubes were prepared containing a layer of of 26% perchloric acid (150 µl), topped by 300 ul of a solution containing a 35:65 volumetric ratio of Beckmann direct drive vacuum pump oil (no. 341661) to methyl salicylate (oil of wintergreen; Sigma, M-6752). A 0.5 ml sample was taken in duplicate directly from a fermentation and carefully placed on top of the layers. In the case of shake flask fermentations, a brief interval of a few seconds sufficed to let the calcium chloride settle. The tubes were immediately centrifuged for 30 seconds, during which the cells traversed the oil layer and were pelleted in the acid layer. The top layer was carefully pipetted out, and saved for HPLC analysis. The oil layer was removed and discarded. The bottom layer was sonicated for five minutes at the highest allowable intensity using an Ultrasonic Liquid Processor/Cell Disruptor equipped with a standard tapered microtip, cat. #419 (Heat Systems Ultrasonics, Farmingdale, New York) placed directly into the Eppendorf to eliminate error associated with the transfer of the

solution. Following sonication, 150 µl of 2.5 M potassium acetate, pH 6.0 were added, and the tubes were vortexed and placed on ice for 10 minutes, then centrifuged for 10 minutes. The extract supernatant was saved for HPLC analysis.

From the measure of dry cell weight, both the extracellular volume of liquid that was cosedimented with the cells during the oil layer centrifugation and the total internal volume of the cells were estimated and used to calculate the internal amino acid concentration. An intracellular volume of 1.5 µl/mg dry cell weight and an extracellular volume of 4.0 µl/mg dry cell weight were used, taken from the literature on *Corynebacterium* and other related species (Brown and Stanley, 1972; Luntz *et al.*, 1986; Bröer *et al.*, 1993) Using these numbers, as well as the volumes used above, by simple algebraic manipulation the final equation for intracellular metabolite concentration becomes:

$$I = E\,[\,(166.7/x) + 3.7] - 2.7T$$

where: I = intracellular concentration of amino acid (in mg/l)

E = concentration of amino acid in final extract (in mg/l)

T = concentration of amino acid in top layer (in mg/l)

x = cell mass in extract (mg dry cell weight)

4.6 Batch fermentation

A batch fermentation was carried out using the strain 21799 (pGC42) in order to perform preliminary characterization of the strain behavior in a reactor.

A five-liter Microferm™ glass bioreactor with stainless steel headplate (New Brunswick Scientific, Edison, NJ) and a three-liter working volume was used. The unit contains temperature and pH control, agitation and dissolved oxygen probe, and was run at a head pressure of 3 psig. The headplate has several ports through which air, base, antifoam, antibiotic, isopropyl ß-D-thiogalactopyranoside (IPTG), and supplementary components can be added by hypodermic needles. The vessel, headplate, and tubing can be removed as a unit for sterilization. The entering gas and off-gas was analyzed on-line by a Dycor quadrupole mass spectrometer. On-line data acquisition and control was accomplished through the use of a Wyse 1400-02 pc+ computer running a modified version of a data acquisition and control program written in Microsoft™ QuickBASIC version 3.0 (Copella and Dhurjati, 1987). Respiratory data (oxyged uptake rate (OUR), carbon dioxide evolution rate (CER) and respiratory quotient (RQ = CER/OUR)) can thus be monitored throughout the fermentation. The above equipment configuration and computer monitoring system was set up by Joe Vallino (Ph.D, 1991) and Bob Kiss (Ph.D., 1992). The medium used for this fermentation is described in Table 4.3. Dry cell weight was measured by washing cells once and drying to a constant weight at 80°C (24 hrs.).

Table 4.3 Medium composition for batch fermentation

Component	Concentration		Total
Portion A (in vessel - 2 liters):			
D-Glucose	120	g/l	360 g
citric acid	0.5	g/l	1.5 g
NaCl	2	g/l	6 g
$MgSO_4 \cdot 7H_2O$	600	mg/l	1.8 g
$Na_2EDTA \cdot 2H_2O$	75	mg/l	225 mg
$FeSO_4 \cdot 7H_2O$	50	mg/l	150 mg
$CaCl_2 \cdot 2H_2O$	1	g/l	3 g
100 x Mineral Salts	20	ml/l	60 ml
poly (propylene glycol) MW 2000	1	ml/l	3 ml
Portion B (1 liter - separately):			
K_2HPO_4	4	g/l	12 g
KH_2PO_4	2	g/l	6 g
L-Threonine	1000	mg/l	3 g
L-Methionine	300	mg/l	0.9 g
L-Leucine	1000	mg/l	3 g
$(NH_4)_2SO_4$	40	g/l	120 g
After autoclaving add to B aseptically the following filter-sterilized components:			
Biotin	1	mg/l	
Thiamine	1	mg/l	
Calcium pantothenate	10	mg/l	
Combine B with A.			

5. Results and Discussion

5.1 Introduction

5.1.1 Rationale

Figure 5.1 outlines the aspartate amino acid family pathways of interest in this work. Also shown are the metabolites that may be detected extracellularly by analysis of the supernatant. Intracellular analysis of metabolite accumulation and *in vitro* enzyme activity measurements provide a more complete picture of biochemical processes during a fermentation.

The main control site of the threonine specific biosynthetic pathway in wild type *Corynebacterium* is the enzyme homoserine dehydrogenase due to the strong feedback inhibition of this enzyme by threonine. Wild-type homoserine dehydrogenase from this organism is completely inhibited at concentrations of 1-5 mM threonine (Archer *et al.*, 1991; Eikmanns *et al.*, 1991; Miyajima *et al.*, 1968). A secondary control element in this pathway is homoserine kinase, which is also inhibited by threonine, but to a lesser extent, by about 60% at 30 mM threonine (Miyajima *et al.*, 1968). Homoserine kinase from *E. coli* and *Saccharomyces cerevisiae* are also inhibited by threonine (45% at 10 mM threonine and 38% at 25 mM threonine, respectively) (Thèze *et al.*, 1974). The same studies have shown

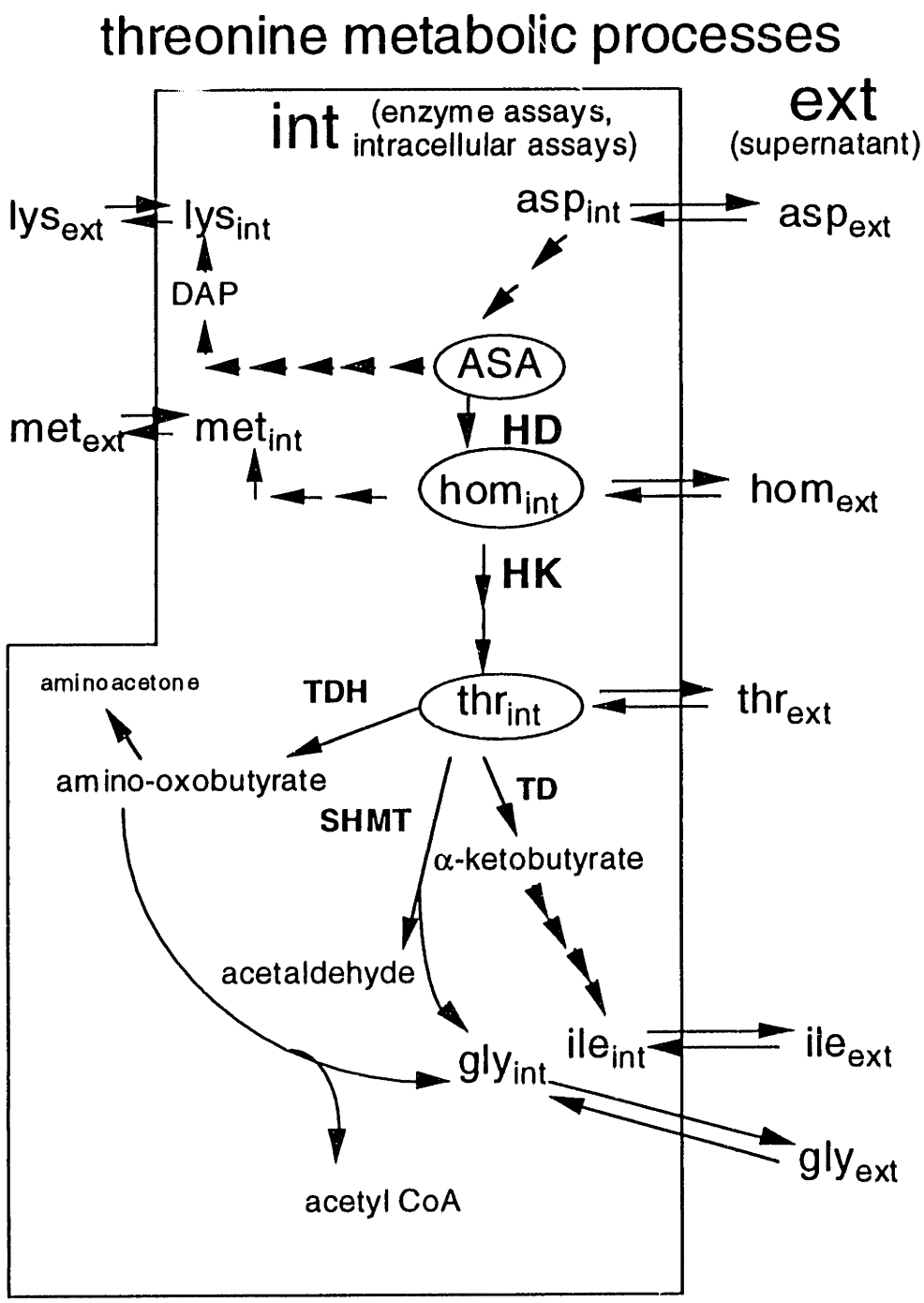

Figure 5.1 Schematic of threonine metabolic processes of interest in *Corynebacterium* spp.

little or no inhibition of threonine synthase activity by members of the aspartate amino acid family.

An obvious strategy for obtaining threonine over-production in this system would be to abolish threonine inhibition of the first two enzymes in this pathway. Threonine over-production by DL-α-amino-ß-hydroxyvaleric acid (AHV, a threonine analogue)-resistant mutants of *Corynebacterium* has been reported (Shiio and Nakamori, 1970; Kase and Nakayama, 1972; Nakamori and Shiio, 1972). A *C. glutamicum* strain containing a threonine-insensitive, AHV-resistant allele of the *hom* gene *hom*^{dr}) was isolated by UV irradiation of *C. glutamicum* AS019 whole cells and designated R102 (Archer *et al.*, 1991). The *hom*^{dr} contains a frameshift mutation which radically alters the C-terminal moiety of the protein, resulting in ten amino acid changes and a deletion of the last seven residues (Figure 5.2). Reinscheid *et al.* isolated a feedback-insensitive *hom* gene in which a 0.23 kb region close to the 3' terminus was shown to be responsible for deregulation (Reinscheid *et al.*, 1991). Furthermore, they found that this region displayed remarkable structural similarity to what are thought to be threonine-binding regions of several procaryotic threonine dehydratases as well as several eucaryotic threonine or serine kinases, supporting the hypothesis that this region may indeed be responsible for the allosteric inhibition by threonine.

The availability of a *hom* allele encoding a threonine-insensitive homoserine dehydrogenase provided an opportunity for a perturbation at the ASA node. The effect of amplifying such a gene in a lysine producer was therefore of interest. The choice of the next perturbation to perform naturally evolved from the results of these experiments.

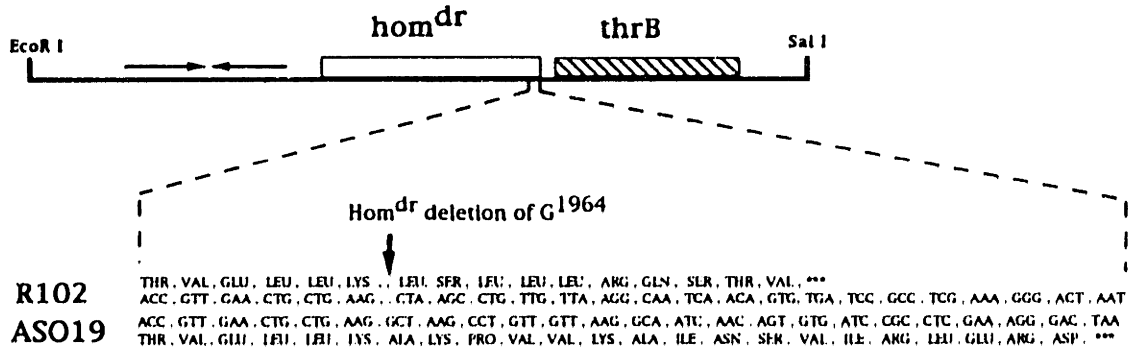

FIGURE 5. Sequence analysis of deregulated *hom*^{dr}-*thrB* of *C. glutamicum*. Abbreviations: ASO19, wild-type *C. glutamicum*; *hom*^{dr}, homoserine dehydrogenase; R102, mutant *C. glutamicum* strain containing chromosomal copy of *hom*^{dr}; *thrB*, homoserine kinase.

Figure 5.2 Sequence of *hom*^{dr}-*thr*B from *C. glutamicum* R102 (reprinted from Jetten *et al.*, 1993).

5.1.2 Description of strain *C. lactofermentum* ATCC 21799

Lysine-producing *Corynebacterium* strains constitute ideal hosts for these studies, given that the strains are metabolically predisposed to direct a significant portion of glucose-derived carbon to the aspartate amino acid family branch.

Corynebacterium lactofermentum ATCC 21799 is resistant to *S*-2-aminoethyl-L-cysteine ((AEC), a lysine analogue) due to an aspartokinase which is not significantly inhibited in the presence of lysine and threonine (US Patent Office, patent #3,825,472). Therefore, there is a high flux of carbon through the aspartate node, resulting in high lysine titer in this strain. Our ability to redirect that high flow away from the lysine branch at the ASA (aspartic-β-semialdehyde) node thus hinges upon the activities of the two enzymes at that branchpoint, homoserine dehydrogenase and dihydrodipicolinate synthase. The ability to maintain that carbon flow

through to threonine depends on a variety of kinetic and thermodynamic effects linking the various related enzymes and intermediate metabolites (Section 5.3).

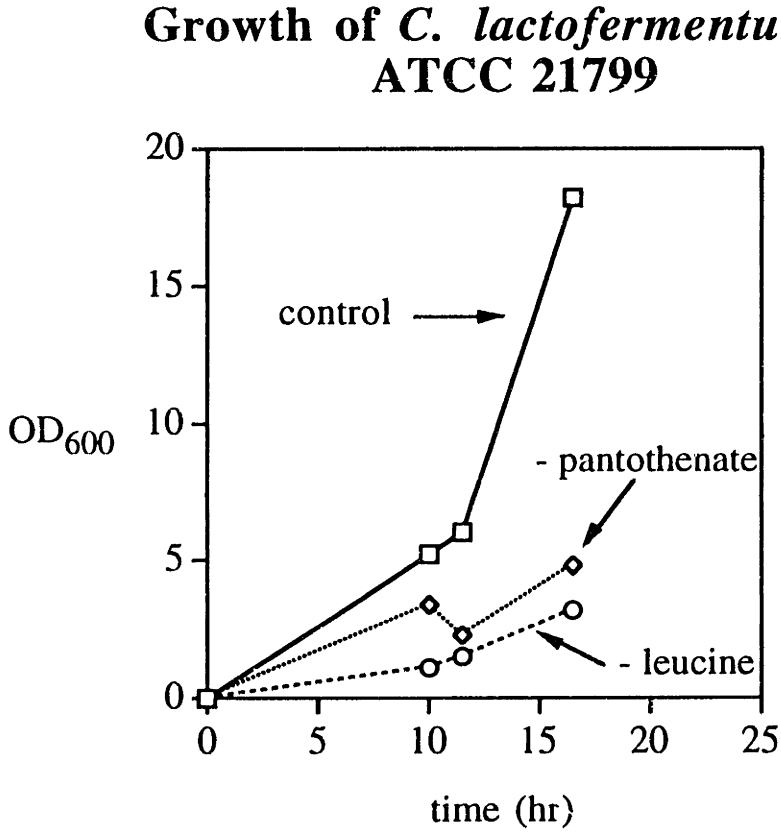

Figure 5.3 *C. lactofermentum* growth profile in BJ minimal medium with leucine and panthothenate (control) and lacking one of the two nutrients.

C. lactofermentum ATCC 21799 was derived from *C. lactofermentum* ATCC 13869 after treatment by X-ray irradiation and nitrosoguanidine (NTG). The strain requires calcium pantothenate and leucine in addition to the usual nutrients for *Corynebacterium* spp. (Figure 5.3).

5.2 Node 1: Aspartic-β-semialdehyde

5.2.1 Introduction

The first perturbation involved inducing a "metabolic shift" of carbon at the aspartate β-semialdehyde branchpoint (Figure 5.4) by introducing the deregulated *hom* gene, thereby not only increasing by about 10-fold the levels of the enzyme, but circumventing threonine regulation of homoserine dehydrogenase as well.

A 3.6 kb restriction fragment encoding the *hom*^{dr}-*thr*B operon was subcloned into a broad host range *C. glutamicum-E. coli* plasmid and this construct was designated pJD4 (Archer *et al.*, 1991; figure 5.5).

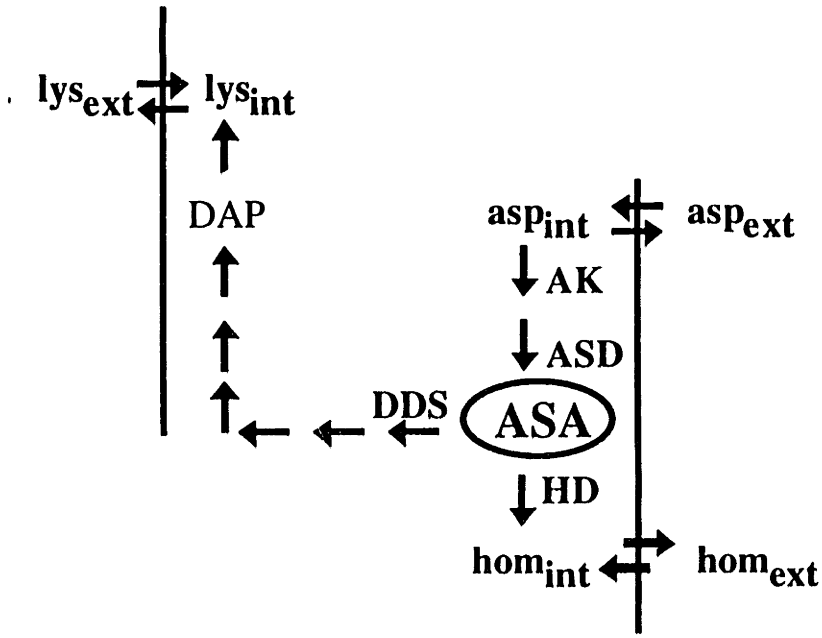

Figure 5.4 Schematic of reactions at the aspartate-β-semialdehyde (ASA) node. lys, lysine; asp, aspartate; hom, homoserine; DAP, diaminopimelate; AK, aspartokinase; ASD, aspartate semialdehyde dehydrogenase; HD, homoserine dehydrogenase; DDS, diaminopimelate synthase; int, intracellular; ext, extracellular.

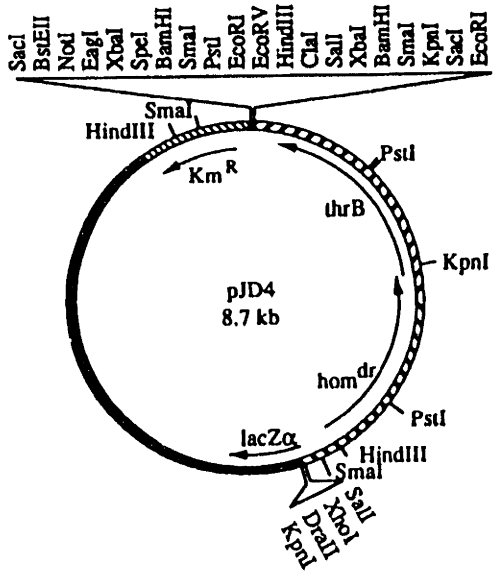

Figure 5.5 Plasmid pJD4 containing *hom*^{dr} (from Archer *et al.*, 1991).

5.2.2 Amplification of *hom*^{dr}-*thr*B operon in 21799

In order to gain familiarity with threonine-insensitive homoserine dehydrogenase expression in *C. glutamicum*, the lysine hyper-producing strain *C. lactofermentum* 21799 was transformed with pJD4, and shake flask experiments were performed with the strain. Four candidates were isolated and studied in a 3-day shake flask fermentation. The control strain was 21799 tranformed with pMF1014α, a *C. glutamicum-E. coli* shuttle vector (Follettie, 1989). Overnight cultures grown in seed medium were used to inoculate 40 ml. fermentation medium containing calcium carbonate for pH control. The results of a three-day shake flask experiment in fermentation medium are shown in Figures 5.6, 5.7 and 5.8.

The control strain accumulated 27 g/l of lysine•HCl, while all four 21799 (pJD4) candidates accumulated only up to 1 g/l lysine•HCl. However, 21799 (pJD4) produced up to 15 g/l of combined homoserine/threonine.

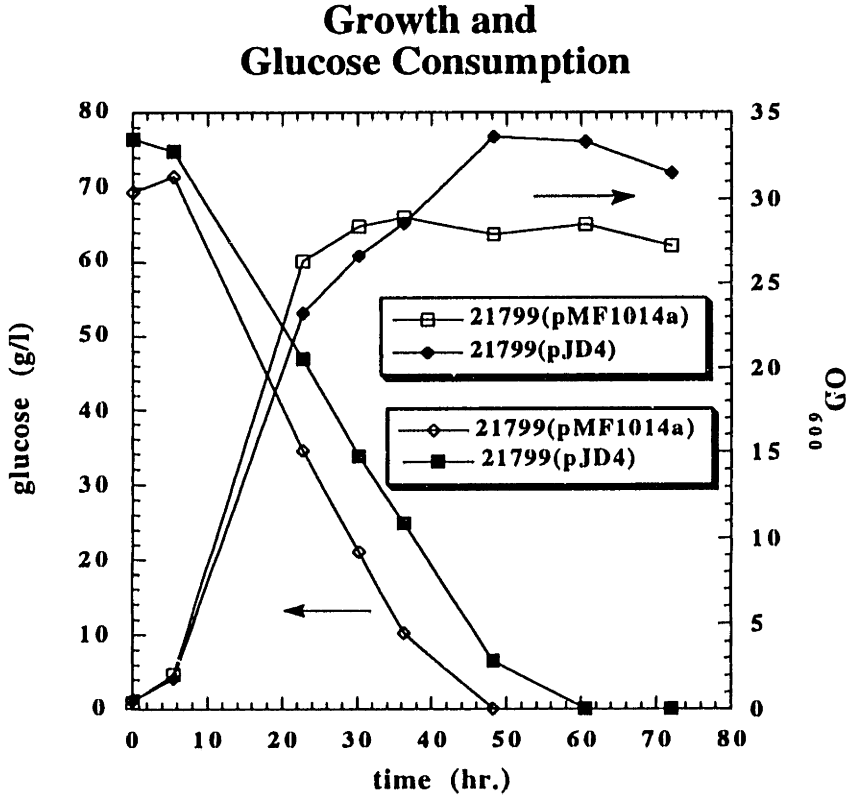

Figure 5.6 Growth and glucose consumption profiles of *C. lactofermentum* 21799(pMF1014α) and 21799(pJD4) during a shake flask fermentation in fermentation medium.

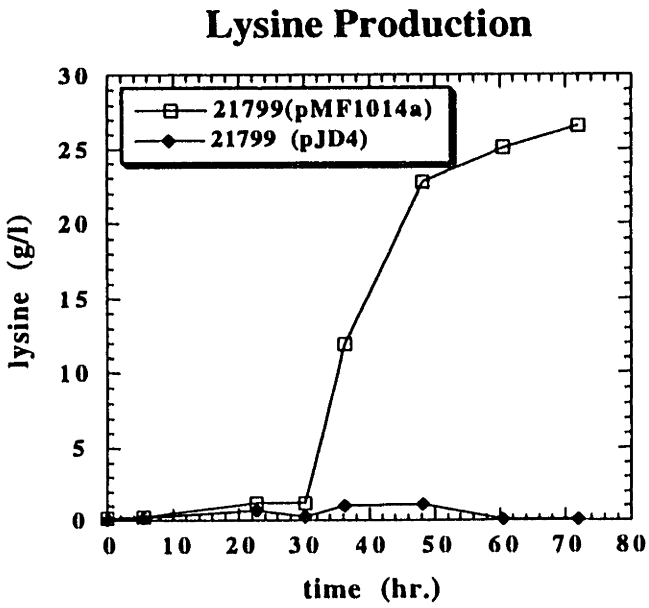

Figure 5.7 Lysine production profiles of *C. lactofermentum* 21799(pMF1014α) and 21799(pJD4) during a shake flask fermentation in fermentation medium.

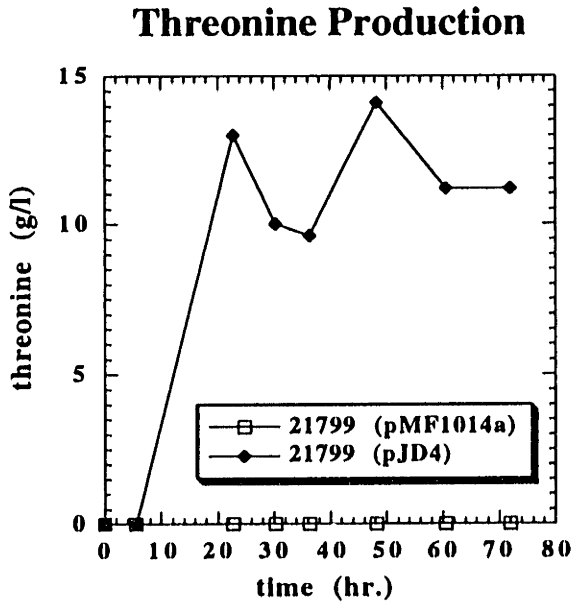

Figure 5.8 Threonine/homoserine production profiles of *C. lactofermentum* 21799(pMF1014α) and 21799(pJD4) during a shake flask fermentation in fermentation medium.

5.2.3 Discussion

The initial method used for HPLC analysis of the samples (Aminex column HPX-87C, 1 ml/min flow rate, 5 mM $CaSO_4$ mobile phase) was unable to achieve consistent resolution of the threonine and homoserine peaks. Later analysis of the final samples under different conditions (0.7 ml/min flow rate) indicated that the mixture varied from approximately 46%/54% to 71%/29% ratios of threonine/homoserine. Regardless of the distribution of these two products, the results indicate that a shift in carbon flux did indeed occur and that overexpression of the deregulated homoserine dehydrogenase diverted the ASA to the threonine biosynthetic pathway. Furthermore, plasmid structural stability was confirmed at the end of the experiment by extracting plasmid DNA from several colonies isolated from each candidate and treatment with the restriction enzyme *Eco*RI. Restriction analysis revealed no structural alterations in any of the individual plasmid preparations. Therefore, the presence of the *hom*^{dr} gene did not seem to affect cell growth or plasmid stability. These results were unexpected, as the plasmid pJD4 was shown to be structurally unstable in *C. glutamicum* AS019E12 (Archer *et al.*, 1991).

One possible explanation for the increased stability of the plasmid pJD4 in ATCC 21799 may be the resistance of this strain to the lysine analogue *S*-(2-aminoethyl) L-cysteine (AEC). The strain AS019E12 contains the wild-type aspartokinase which is concertedly feedback-inhibited by threonine and lysine. The increased activity of the *hom* gene in AS019 cells containing pJD4 would presumably give rise to increased synthesis of threonine, which together with the lysine produced by the cell would subsequently stop the flow of carbon to the aspartate amino acid

family. This could create a strong selective pressure in favor of deletions in the plasmid. In an AEC^R strain, however, this would not be a problem, since the aspartokinase in such a strain would not be subject to inhibition by lysine, and threonine alone does not suffice to completely inhibit the enzyme. Testing of this hypothesis is beyond the scope of this thesis; however, this can be done by comparing plasmid stability, growth, homoserine dehydrogenase activities, and threonine and lysine production in two different *Corynebacterium* genetic backgrounds, one of which is AEC^R and one which is not, and both of which contain the hom^{dr} gene on a plasmid.

5.3 Node 2: Homoserine

5.3.1 Introduction

The shift to threonine production by introduction of the feedback-insensitive *hom* operon was accompanied by accumulation of a metabolic intermediate, homoserine (Figure 5.9). One possibility is that the activities of the first two enzymes in the threonine-specific pathway were imbalanced so that the flow of carbon through the pathway was limited at the homoserine kinase step. The initial hypothesis was that the frameshift

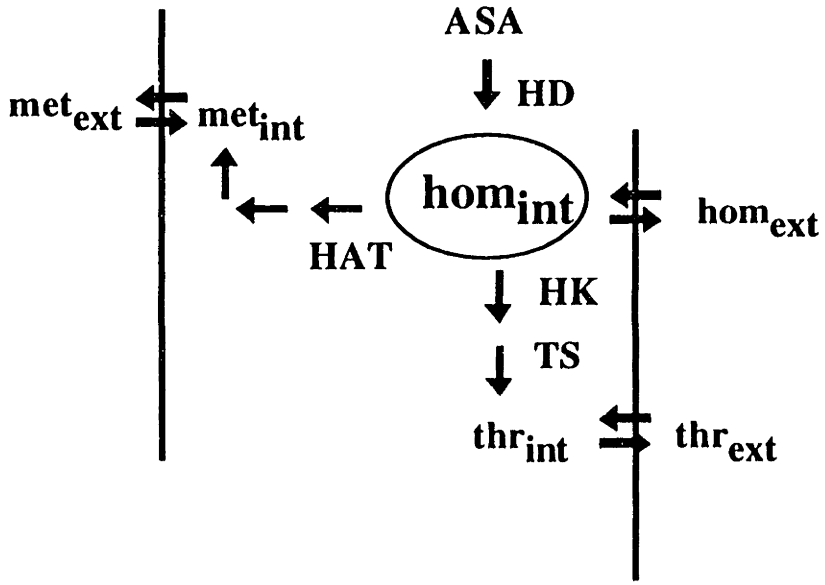

Figure 5.9 Schematic of reactions at the homoserine node. ASA, aspartate-β-semialdehyde; hom, homoserine; met, methionine; thr, threonine; HD, homoserine dehydrogenase; HK, homoserine kinase; TS, threonine synthase; HAT, homoserine acetyltransferase; int, intracellular; ext, extracellular.

mutation in the *hom* gene which gave rise to *hom*^{dr} also exerted a polar effect on the expression of the downstream *thr*B gene, thus reducing the intracellular specific activity of homoserine kinase (Figure 5.10). A polar mutation can interfere with either continued transcription or translation of the polycistronic mRNA, thus reducing or eliminating the expression of genes that are located downstream from the mutation. To est this hypothesis, assays of both enzymatic activities as expressed by both the wild-type and deregulated operons in *Corynebacterium* were performed. The following section outlines the results of these assays.

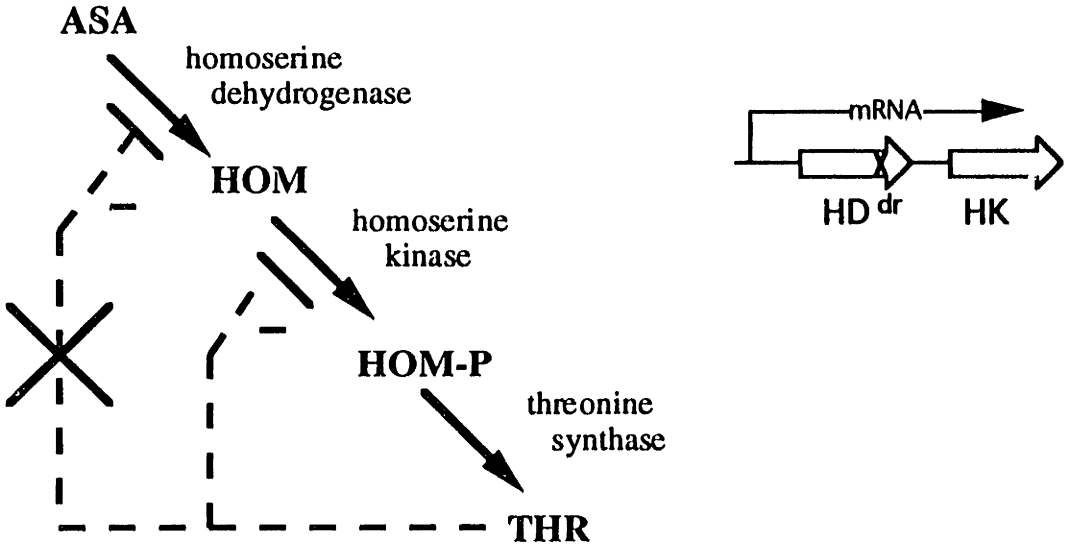

Figure 5.10 Schematic of *hom*^{dr}-*thr*B operon, indicating direction of mRNA transcription and location of point mutation in *hom*^{dr}.

5.3.2 Specific activity measurements

Homoserine dehydrogenase and homoserine kinase activities were measured in *C. lactofermentum* 21799(pFS3.6) and 21799(pJD4) (Table 5.1). No decrease in homoserine kinase activity relative to homoserine dehydrogenase was observed in 21799(pJD4). This clearly demonstrates

Table 5.1 Specific activities of homoserine dehydrogenase and homoserine kinase in *C. lactofermentum* 21799 containing wild-type (*hom-thr*B; pFS3.6) and deregulated (*hom*^{dr}-*thr*B; pJD4) operons.

Strain	Specific Activites* (μmoles/ min-mg protein)	
	HD	HK
C. lactofermentum 21799(pFS3.6)	5.8 ± 0.1	0.68 ± 0.01
C. lactofermentum 21799(pJD4)	6.1 ± 0.3	0.75 ± 0.01

* HD, homoserine dehydrogenase; HK, homoserine kinase

that the expression of the *thr*B gene has not been disrupted by the upstream frameshift mutation in *hom*^{dr} and is not directly responsible for the homoserine accumulation.

5.3.3 Threonine inhibition of homoserine kinase

Threonine inhibition of homoserine kinase has been observed in several species. Miyajima *et al.* (1968) showed that HK activity was inhibited by about 60% at 30 mM threonine. HK's from *Escherichia coli* and *Saccharomyces cerevisiae* are also inhibited by threonine (45% at 10 mM threonine and 38% at 25 mM threonine, respectively) (Wormser and Pardee, 1958). The structural similarity of homoserine to threonine could be one reason why the latter may compete for the active site of this enzyme. It was therefore of interest to study the kinetics of this enzyme in further detail.

Elucidating the nature of the homoserine kinase inhibition by threonine is important in order to provide insight as to what type of perturbation would be useful next. If this inhibition was of a competitive nature, as has been shown for other strains, including *C. glutamicum*, then using a strategy of isolating an analogue-resistant mutant would have a low probability of success. Competitive inhibition signifies that the inhibitor binds at the active site; any mutation in this site which would reduce or abolish inhibitor binding would have a high chance of drastically reducing enzyme activity as well.

Homoserine kinase activity as a function of L-homoserine concentration was measured in crude extracts of ATCC 21799(pJD4) in the presence of various concentrations of threonine (0, 10 and 25 mM) (Figure

5.11). The concentrations of homoserine was varied from 0.1 to 40 mM. The concentration of ATP was maintained at saturating conditions (5 mM). The Michaelis-Menten constant of homoserine for HK was estimated by the nonlinear regression analysis program GraFit from Erithacus software Ltd. It can be seen that increasing concentrations of threonine do indeed inhibit the activity of HK and that this inhibition is relieved as the homoserine concentration is increased. This behavior is characteristic of competitive inhibition. Parameters for a competitive inhibition model were determined by a non-linear regression fit as follows: K_m for homoserine = 0.7 mM, K_i for threonine = 7.7 mM, V_{max} = 1.1 μmol min^{-1} mg of protein $^{-1}$.

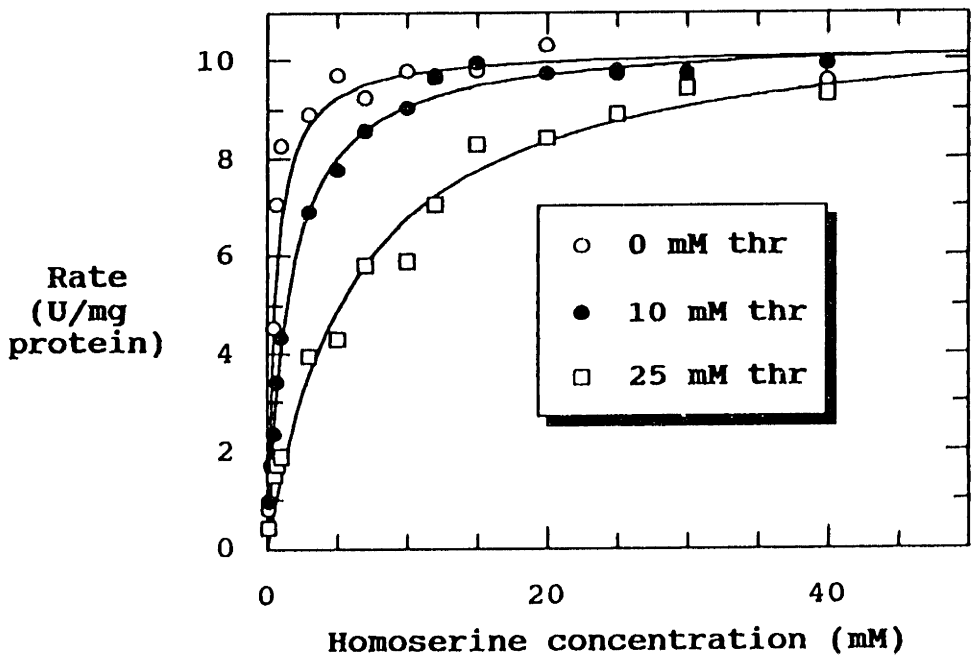

Figure 5.11 Homoserine kinase activity as a function of homoserine at various concentrations of threonine.

5.3.4 Construction of inducible homoserine kinase vectors

The first limiting step in the ASA to threonine pathway is the reaction catalyzed by homoserine dehydrogenase, which is inhibited non-competitively by threonine. This limitation was overcome by isolating a mutant version of the enzyme which contains a destroyed threonine binding site. In the case of homoserine kinase, this tactic is unlikely to work, since the inhibitor (threonine) competes with the substrate (homoserine) for the active site of the enzyme. Any mutation which results in decreased binding of threonine is very likely to result in decreased binding of homoserine. Therefore, rather than attempting to isolate a mutant containing a threonine-insensitive version of homoserine kinase, an approach involving increasing the expression of *thr*B was attempted. Increased expression of the gene would raise the *in vivo* concentration of homoserine kinase. This higher enzyme concentration could potentially support a higher flux of carbon through that pathway, reducing or eliminating accumulation of homoserine and rather, converting it to homoserine phosphate and subsequently threonine by the action of the next enzyme, threonine synthase.

Because it was not obvious what level of homoserine kinase expression would be optimal in terms of efficiently directing flux of carbon completely to threonine, a strategy involving an inducible promoter was used. In this manner, homoserine kinase expression could be finely tuned and the effects of each induction level could be studied. For this purpose the artificial, isopropyl-ß-thiogalactopyranoside (IPTG)-inducible *tac* promoter was used (de Boer *et al.*, 1983). The *tac* promoter is a hybrid of the *E. coli trp* and *lac* promoters which has been shown to function

efficiently in *Corynebacterium* strains (Morinaga *et al.*, 1987; Gubler and Sinskey, 1993).

5.3.4.1 Construction of pCP1

In order to increase expression of the *thr*B gene and increase the *in vivo* concentration of homoserine kinase, a plasmid enabling separate control of the expression of the *Corynebacterium thr*B and *hom*^{dr} genes was constructed. For construction of pCP1, the *E. coli -C. glutamicum* shuttle vector pMG108 (Gubler and Sinskey, 1993) was restriction digested with *Sma*I and *Sal*I and subsequently treated with calf intestine phosphatase. In addition to the *tac* promoter, pMG108 contains the *lac* I^q allele of the *lac* repressor gene (Calos, 1978), as well as the genes for kanamycin and ampicillin resistance. The plasmid pFS3.6 containing the *hom-thr*B operon (Follettie *et al.*, 1988) was restriction digested with *Nhe*I, treated with the Klenow fragment of DNA polymerase I, and digested with *Sal*I. The 1.5kb *Nhe*I-*Sal*I fragment was isolated from a 1% agarose gel by the QIAEX system after size fractionation by electrophoresis. *E. coli* CGSC #5076 (*thr*B-) was used to screen for positive candidates. The mutation in the strain was first confirmed by plating on mimimal medium (MM) plates supplemented with homoserine (no growth) and threonine (growth). *E. coli* 5076 was transformed with pCP1. No stable transformants were obtained when plated on MM plates with and without IPTG. Therefore, the transformation was repeated and the cells were plated on LB plates containing 50 μg/ml ampicillin. Approximately forty candidates were obtained, six of which were screened for insert, using pMG108 as a size marker. Candidates 1-4 were restriction digested with *Sal* 1 and size

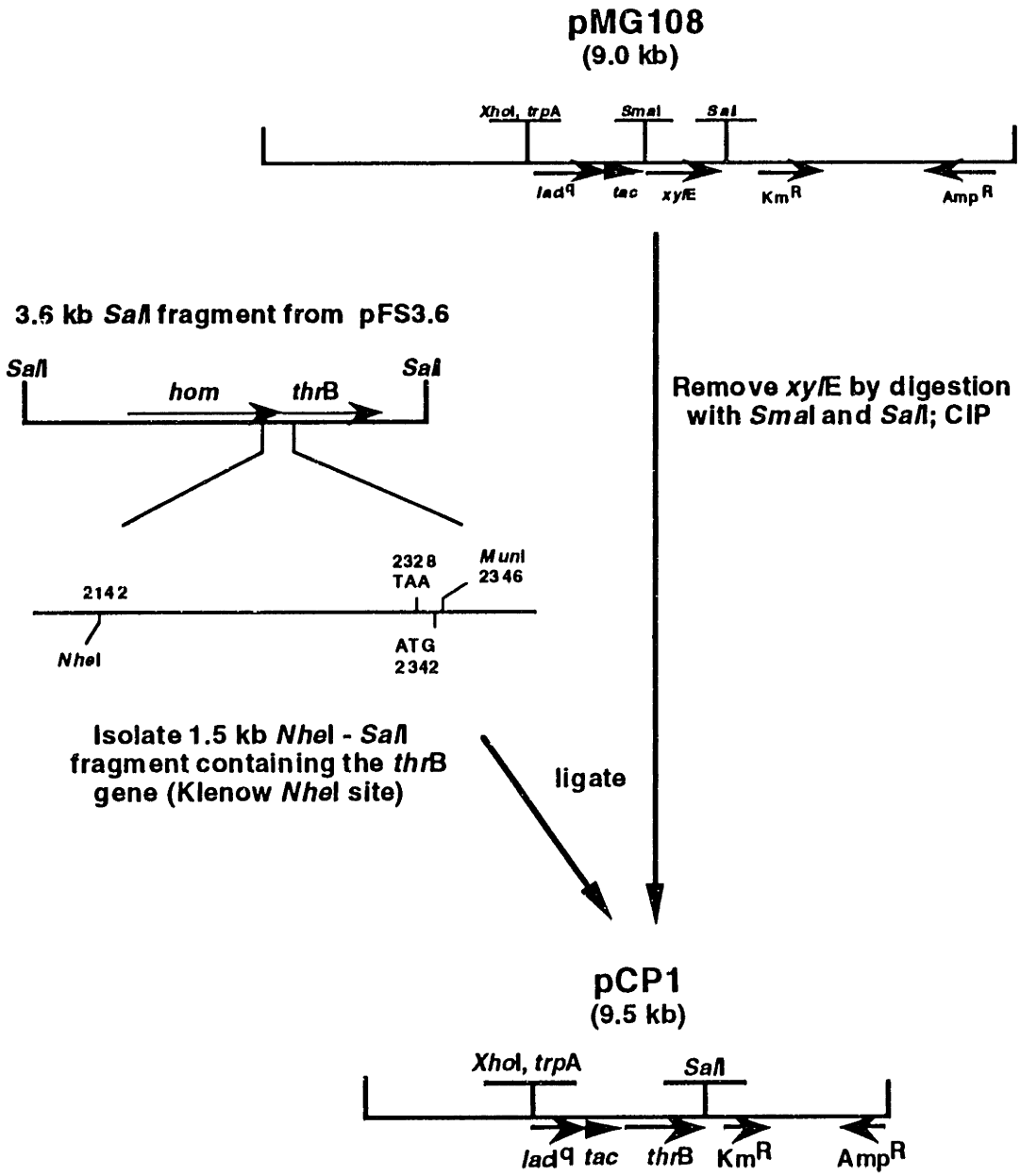

Figure 5.12 Construction of pCP1.

fractionated on a 1% agarose gel. All appeared to have insert of the appropriate size. Homoserine kinase activity in the four *E. coli*(DH5α) candidates cultured in LB broth with and without 1mM IPTG was measured. The results shown in Table 5.2 are averages of 2-3 assays on a single crude extract.

Table 5.2 Homoserine kinase activity in *E. coli* DH5α(pCP1) candidates

Strain	Homoserine kinase activity* (nmoles / min-mg protein)	
	O mM IPTG	1mM IPTG
E. coli DH5α(p1)	46	285
E. coli DH5α(p2)	11	231
E. coli DH5α(p3)	n.d.	220
E. coli DH5α(p4)	56	213

***n.d., not detected**

There was therefore an average 8-fold induction upon addition of 1mM IPTG. The specific activities found in these candidates were all lower than those found in *C. glutamicum* harboring the plasmids pFS3.6 (wild type *hom-thr*B operon) and pJD4 (*hom*^{dr}-*thr*B operon), which are on the order of 600-700 nmoles/mg/min.

The plasmid DNA from candidates 1 and 2 was isolated and used to transform *C. glutamicum* E12 by electroporation. Approximately forty transformants of each were obtained. Several candidates were analyzed for presence of insert and homoserine kinase activity in cells cultured in LB broth with and without 1mM IPTG was measured. The results shown in Table 5.3 are averages of 2-3 assays on a single crude extract.

Table 5.3 Homoserine kinase activity in *C. glutamicum* (pCP1) candidates

Strain	Homoserine kinase activity* (nmoles / min-mg protein)	
	O mM IPTG	1mM IPTG
C. glutamicum E12(1a)	186	328
C. glutamicum E12(1b)	723	1565
C. glutamicum E12(1c)	645	1835
C. glutamicum E12(2a)	912	2230
C. glutamicum E12(2b)	242	726
C. glutamicum E12(2c)	n.d.	1290
C. glutamicum E12(2d)	n.d.	1190

***n.d., not detected**

There was therefore a fairly high level of uninduced expression, with a 2-3 fold increase upon addition of 1 mM IPTG. The level of uninduced activity found in these candidates was comparable to that found in *C. glutamicum* harboring the plasmids pFS3.6 (wild type *hom-thr*B operon) and pJD4 (*hom*^{dr}-*thr*B operon) grown in minimal medium, which is on the order of 600-700 nmoles/mg/min (Follettie *et al.*, 1988; Follettie, 1989).

The results of some other studies involving homoserine dehydrogenase showed that expression levels might vary significantly from cells grown in minimal or complex medium. Therefore, the activity of HK in the strain 2b was measured in minimal medium. The results are shown in Table 5.4 (average of 3-5 measurements). The uninduced activity of HK of strain 2b grown in MM is higher than that of the strain grown in LB with addition of 1mM IPTG.

Table 5.4 Homoserine kinase activity in *C. glutamicum* (pCP1) grown in minimal medium supplemented with various amounts of isopropyl-β-thiogalactopyranoside (IPTG).

Strain	Homoserine kinase activity (minimal medium) (nmoles/ min-mg protein) ----------IPTG----------		
	0 mM	0.05 mM	0.5 mM
C. glutamicum E12(p2b)	1040	1330	2440

5.3.4.2 Construction of pGC18

Due to the available information on restriction enzyme sites at the time of the design of the construct, pCP1 contains not only the *thr*B gene, but about 200 base pairs (bp) of the C-terminal end of the *hom* gene. Because of the possibility of this extra mRNA interfering with proper translation of the *thr*B DNA, a new construct was designed. At the time of the latest mapping of the *hom-thr*B operon, a recently purified and newly available enzyme, *Bpu*1102I, was found to have a convenient site located at about only 50 bp from the ATG start codon of the *thr*B gene. Therefore, pFS3.6 and PMG108 plasmid DNA was isolated from *E. coli*. The plasmid pFS3.6 containing the *hom-thr*B operon (Follettie *et al.*, 1988) was restriction digested with *Bpu*1102I, treated with the Klenow fragment of DNA polymerase I, and digested with *Sal*I. The 1.4 kb *Bpu*1102I-*Sal*I fragment was isolated from a 1% agarose gel after size fractionation by electrophoresis. The plasmid pMG108 was restriction digested with *Sma*I and *Sal*I and subsequently treated with calf intestine phosphatase. The *Bpu*1102I-*Sal*I fragment was ligated into pMG108, placing the gene

downstream of the *tac* promoter in this vector and creating the plasmid pGC18 (see Figure 5.13). *E. coli* DH5 cells were transformed with pGC18. Cells were cultured in LB broth and the homoserine kinase activity was measured. In cells grown with 0 and 1 mM IPTG added to the medium, the average HK activity was 0.1 and 1.0 μmol/min•mg protein, respectively. *C. glutamicum* ASO19-E12 cells were also transformed with the plasmid pGC18 and the activity of the cells grown in minimal medium supplemented with various amounts of IPTG was determined. At 0 mM IPTG, the HK activity averaged 0.3 μmol/min•mg protein. Cells grown in minimal medium supplemented with 0.1mM IPTG showed an average HK activity of 4 μmol/min•mg protein. Upon supplementation of the growth medium with 0.5 to 1.0 mM IPTG, the homoserine kinase activity increased to an average of 6.0 - 6.2 μmol/min•mg protein, respectively. By performing these inducibility studies with this plasmid in ATCC 21799, it was determined that the homoserine kinase activity could be increased to an average of 6.0 - 6.2 μmol/min•mg protein. These values are approximately ten to twentyfold higher than the activities observed in cells harboring plasmids containing the *hom-thr*B operon under the control of its native promoter (Follettie, 1989; Follettie *et al.*, 1988). This was considered to be a satisfactory range with which to conduct these studies.

E. coli DH5α cells were transformed with the ligation mixture. 24 candidates were screened for insert, and the homoserine kinase activity of candidates 18 and 19 when grown in LB broth the presence or absence of 1mM IPTG was measured. The results shown in Table 5.5 are averages of 2-3 assays on a single crude extract.

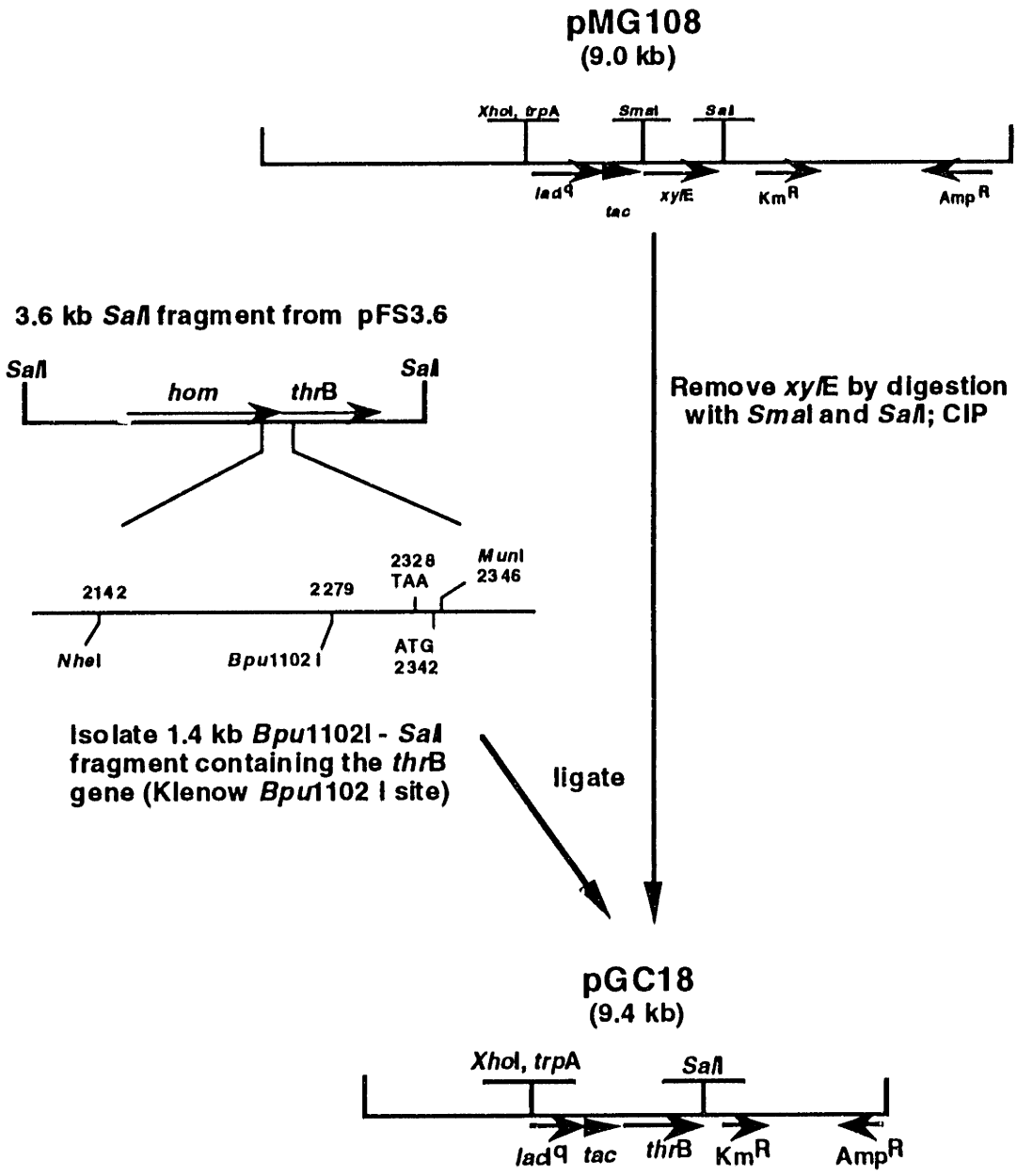

Figure 5.13 Construction of pGC18.

Table 5.5 Homoserine kinase activity in *E. coli* DH5α(pGC18) candidates

Strain	Homoserine kinase activity (nmoles / min-mg protein)	
	O mM IPTG	1mM IPTG
E. coli DH5α(18)	121	1052
E. coli DH5α(19)	128	963

This represents about 4 times the activity found in DH5α containing the previous construct, with about a 9-fold increase in activity upon induction.

C. glutamicum E12 cells were transformed with the DNA from candidates 18 and 19 and the activity of HK in candidates 18a, 19a and 19b when grown in LB broth the presence or absence of 1mM IPTG was measured. The results shown in Table 5.6 are averages of 2-3 assays on a single crude extract. HK enzyme activity levels are not higher than in E12 harboring pCP1. However, the background is reduced in this case.

Table 5.6 Homoserine kinase activity in *C. glutamicum* (pGC18) candidates

Strain	Homoserine kinase activity (nmoles / min-mg protein)	
	O mM IPTG	1mM IPTG
C. glutamicum E12(18a)	70	1352
C. glutamicum E12(19a)	70	956
C. glutamicum E12(19b)	193	945

Finally, the HK activity was measured in 18a grown in minimal medium supplemented with various amounts of IPTG. The results shown in Table 5.7 are averages of 2-3 assays on a single crude extract. In minimal medium there is significant induction of HK activity in this strain.

Table 5.7 Homoserine kinase activity in *C. glutamicum* (pGC18) grown in minimal medium supplemented with various amounts of isopropyl-β-thiogalactopyranoside (IPTG).

Candidate	Homoserine kinase activity (minimal medium) (nmoles/ min-mg protein)			
	IPTG			
	0 mM	0.1 mM	0.5 mM	1mM
C. glutamicum E12(18a)	296	3888	6071	6255

It is noteworthy that even under no induction there is significant amount of background HK activity in these strains. This could be due to inefficient expression of the repressor gene. The promoter of the gene encoding the *lac* repressor (*lac*I) is inefficient in *E. coli*, and consequently the *lac* promoter is not tightly regulated in this organism. To overcome this, Calos (1978) developed the *lac*Iq gene, which contains a mutation increasing the efficiency of LacI production and better regulates *lac*-based promoters in *E. coli*. This allele is commonly used in conjunction with the *tac* promoter to render the vectors autorepressed and to expand their host range to non-*lac*Iq strains (Amman *et al.*, 1988). Tsuchiya and Morinaga (1988) found that inactivation of *lac* repressor is incomplete in *C. lactofermentum*. A version of *lac*I, designated *lac*I^{18k}, differs from lacIq in that the promoter, ribosome binding site, and GTG initiation codon have

been replaced with a promoter, ribosome binding site, and ATG codon from the 18-kDa antigen gene from *Mycobacterium leprae* (Booth *et al.*, 1988). Utilization of *lac*I^{18k} in *C. glutamicum* led to markedly reduced levels of transcription in uninduced cells containing a recombinant ovine gamma interferon - glutathione *S*-transferase gene fusion (GST-IFN-γ) under the control of the *tac* promoter (Billman-Jacobe *et al.*, 1994). These results seem to confirm that the *lac*Iq promoter may not be highly efficient in *Corynebacterium*, a fact consistent with the above results. However, this background activity is low enough to not interfere with the studies undertaken in this work.

5.3.4.3 Construction of pGC42

Having confirmed that the *tac-thr*B construct functioned as desired in *Corynebacterium*, addition of the deregulated homoserine dehydrogenase gene was carried out. In order to add the feedback-insensitive homoserine dehydrogenase gene, pGC18 isolated from DH5α was restriction digested with *Xho*1, treated with the Klenow fragment of DNA polymerase I and subsequently treated with calf intestine phosphatase. The *hom*^{dr} gene was isolated as a 2.0 kb *Sal*1-*Mun*1 fragment from pJD4 (Archer *et al.*, 1991) and ligated into pGC18 after treatment with the Klenow fragment of DNA polymerase 1, creating the 11.4 kb plasmid pGC42 (Figure 5.14). *E. coli* DH5α cells were transformed with pGC42, and homoserine dehydrogenase activities were measured in this strain. The activity of this enzyme was not detected. This is consistent with previous observations where it was found that this gene is not well-expressed in *E. coli*. Therefore, *C. lactofermentum* 21799 was transformed with pGC42 and homoserine

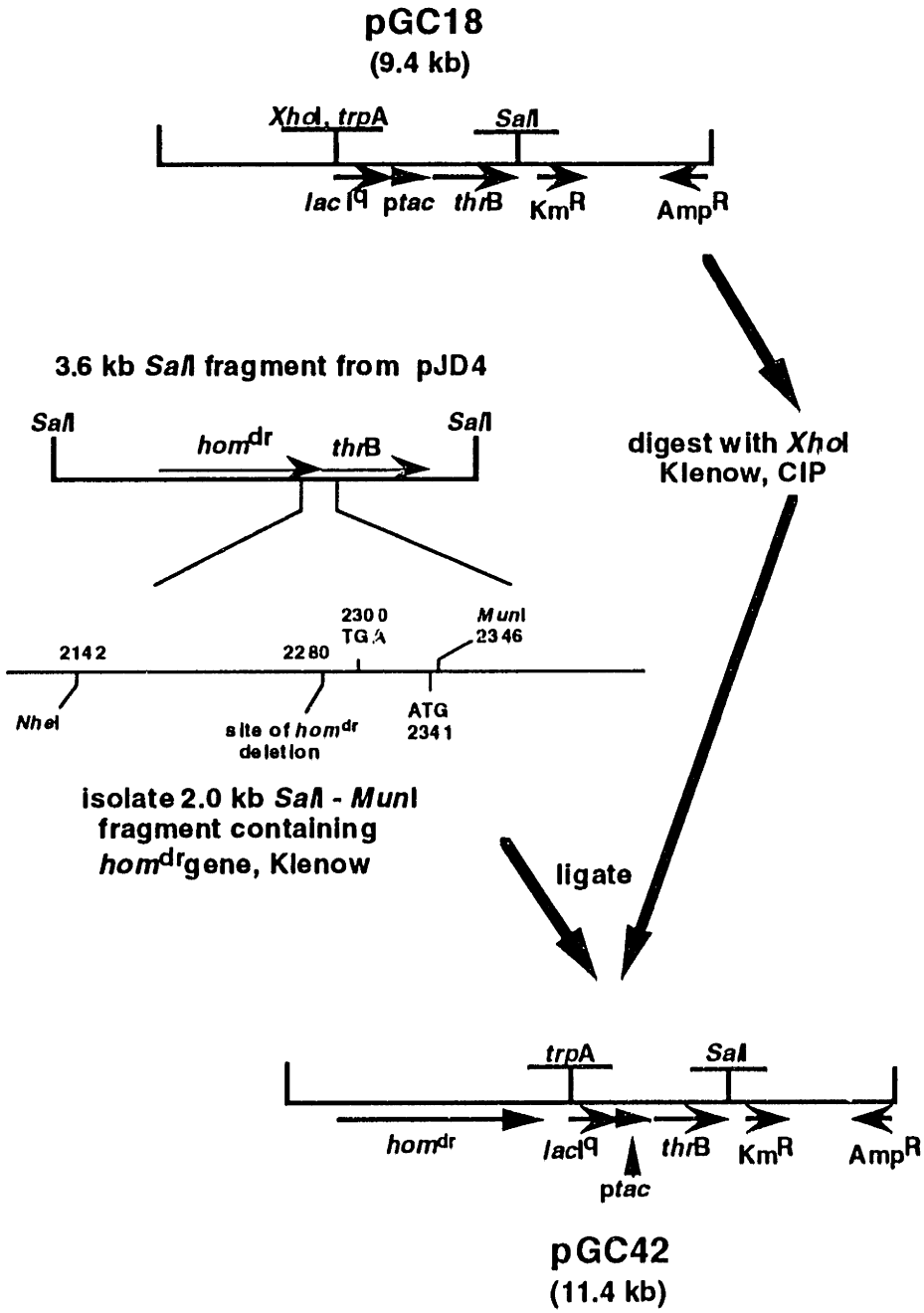

Figure 5.14 Construction of pGC42.

dehydrogenase and homoserine kinase activities were measured in cells grown in minimal medium supplemented with various amounts of IPTG (Table 5.8). Surprisingly, levels of HD activity were detected which were significantly higher than those in either 21799(pJD4) and 21799(pFS3.6). With no IPTG added to the medium HD activity was on the average about 80-100% higher. As IPTG induction was increased, there was an increase in HK activity which levelled off at about 5-6 μmoles/min•mg protein. HD activity decreased drastically as higher amounts of IPTG were added. These results were used to choose an induction level for preliminary shake flask experiments.

Table 5.8 Homoserine dehydrogenase and homoserine kinase activities in *C. lactofermentum* 21799(pGC42) grown in minimal medium (μmoles/min•mg protein).

IPTG (mM)	HD	HK
0	9.53	0.28
0.1	4.56	5.87
0.5	2.25	5.28
1.0	1.73	1.44

5.3.5 Preliminary shake flask experiments with pGC42

A five-day shake flask experiment was conducted to determine the effects of increases in HD and HK activities on the distribution of accumulated metabolites. Cells were cultured in 100 ml BJ minimal medium with 100 g/l glucose. Growth, glucose consumption, and

metabolite accumulation were compared in three strains: *C. lactofermentum* 21799(pMF1014α), *C. lactofermentum* 21799(pJD4) and *C. lactofermentum* 21799(pGC42). Upon inoculation, 0.1 mM IPTG was added to all flasks. Table 5.9 compares the specific activities of homoserine dehydrogenase and homoserine kinase at approximately 26 hrs. into the fermentation. Table 5.10 compares the distribution of accumulated metabolites in the three strains at the point of glucose exhaustion.

C. lactofermentum 21799(pJD4) accumulated 5.9 g/l threonine, 3.1 g/l homoserine and 8.5 g/l lysine, in addition to about 3 g/l each of the threonine degradation products isoleucine and glycine. *C. lactofermentum* 21799(pGC42), however, accumulated about 70% more threonine and 80% less lysine than 21799(pJD4). The activities of HD and HK were highest in 21799(pGC42) and this is consistent with a lower lysine and higher threonine titer in this strain. However, there was still about 2 g/l homoserine accumulated in this strain, indicating that the induction strategy needs to be optimized. Most of the homoserine was excreted towards the end of the fermentation, suggesting that repeated induction might be a good strategy.

Table 5.9 Homoserine dehydrogenase and homoserine kinase activities (mmoles/min•mg protein) in three strains during a shake flask fermentation (t = 26 hrs).[a]

strain	HD	HK
C. lactofermentum 21799(pMF1014α)	0.05	.20
C. lactofermentum 21799(pJD4)	3.44	1.24
C. lactofermentum 21799(pGC42)	11.43	5.28

[a] Cultures were induced by addition of 10 μmoles of IPTG to the 100 ml culture upon inoculation.

Table 5.10 Comparison of final products of shake flask fermentation with *C. lactofermentum* 21799 containing various plasmids (g/l).[a]

Product	Strain		
	21799(pα)	21799(pJD4)	21799(pGC42)
Glycine	-	3.1	4.2
Homoserine	-	3.1	2.1
Threonine	-	5.9	9.8
Isoleucine	-	2.9	1.3
Lysine	15.3	8.5	1.8

[a] Cultures were induced by addition of 10 μmoles of IPTG to the 100 ml culture upon inoculation.

Several conclusions can be obtained from these results. First, carbon flux can be almost completely shifted from the lysine branch to the threonine branch by high, deregulated homoserine dehydrogenase activity. Second, homoserine accumulation in threonine fermentations can be diminished by increasing the level of homoserine kinase. However, a better induction strategy is needed to try to completely eliminate the accumulation of homoserine. A smoother flow of carbon down the branchpoint could, in addition, allow more of the carbon to preferentially flow down the threonine pathway rather than the lysine pathway.

5.3.6 Shake flask fermentations with 21799(pGC42) using various levels of induction

Corynebacterium lactofermentum 21799(pM2) was used as a lysine-producing control. 21799(pM2), 21799(pJD4) and 21799(pGC42) were pre-cultured in 50 mls seed medium. 1 ml of exponentially growing cells was inoculated into 100 mls of mineral salts medium containing 80 g/l

glucose. After approximately 30 hours, and twice more at about 30-hour intervals, various amounts of IPTG were added to the 21799(pGC42) cells. The duration of a typical fermentation was approximately 100 hours with the cells reaching a typical OD_{600} of about 50. The specific activities of homoserine dehydrogenase and homoserine kinase were followed throughout the fermentation (Table 5.11). In 21799(pGC42) the homoserine dehydrogenase activity was independent of IPTG addition, and the average activity ranged from about 7.3 - 9.0 μmole/min•mg protein. Homoserine kinase activity increased with addition of IPTG reaching up to over 7 μmole/min•mg protein. Higher addition of IPTG did not significantly increase the HK activity (data not shown). The culture fluid was analyzed for amino acid content at the point of glucose exhaustion (Table 5.12). 21799(pM2) produced 22 g/l lysine, whereas 21799(pJD4) produced 4.5 g/l lysine, 5.4 g/l threonine and 2.0 g/l homoserine, as well as some glycine and isoleucine. In 21799(pGC42), for all cases, irrespective of IPTG addition, about 1 g/l of lysine was produced. In the case of no induction, a significant amount of threonine and homoserine was produced. In the case of low induction, accumulation of homoserine was not observed, but up to 11.8 g/l of threonine was produced (1.5 μmoles IPTG). Higher amounts of IPTG in fact resulted in a lower final threonine titer. In all cases there was significant accumulation of glycine and isoleucine. Due to concerns about possible plasmid instability (Reinscheid *et al.*, 1994), the activity of homoserine dehydrogenase was also measured in the presence of 50 mM threonine to determine if any structural instability had occurred in the plasmids containing the *hom*^{dr} gene. After 80 hours the enzyme still retained 75-95% of its activity in the presence of 50 mM threonine.

Table 5.11 Average specific activities of homoserine dehydrogenase and homoserine kinase during shake flask fermentations of various 21799 recombinants

C. lactofermentum strain[a]	Specific activity (μmole/min•mg protein)[b]	
	HDH	HK
21799(pM2)	0.2	0.1
21799(pJD4)	3.6	2.0
21799 (pGC42) with:		
no induction	8.9	0.5
0.5 μmol IPTG (0.005 mM)	7.9	1.0
1.0 μmol IPTG (0.010 mM)	7.8	1.5
1.5 μmol IPTG (0.015 mM)	8.0	3.8
5.0 μmol IPTG (0.050 mM)	7.3	4.6
8.3 μmol IPTG (0.083 mM)	9.0	5.8
20 μmol IPTG (0.200 mM)	7.4	7.3

[a] 21799(pGC42) cultures were induced by addition of the stated amount of IPTG to the 100 ml culture at approximately 30 hour intervals during the course of the fermentation; values in parenthesis are instantaneous mM concentrations of IPTG in culture
[b] average specific activity during the fermentation

Table 5.12 Amino acids accumulated in culture medium by various 21799 strains

C. glutamicum strain[a]	Amino acid concentration in supernatant (g/l)[b]				
	lys	thr	hom	gly	ile
21799(pM2)	22.0	< 0.1	< 0.1	< 0.1	< 0.1
21799(pJD4)	4.5	5.4	2.0	2.0	1.0
21799(pGC42) with:					
no induction	0.9	5.6	6.7	1.3	1.0
0.5 μmol	0.8	9.0	< 0.1	4.5	1.1
1.0 μmol	1.1	11.3	< 0.1	4.5	1.4
1.5 μmol	0.8	11.8	< 0.1	4.6	1.3
5.0 μmol	0.9	9.3	< 0.1	3.4	0.9
8.3 μmol	0.7	5.8	< 0.1	2.9	0.5
20.0 μmol	0.8	7.9	< 0.1	2.3	0.8

[a] 21799(pGC42) 100 ml cultures were induced by addition of the stated amount of IPTG at approximately 30 hour intervals during the course of the fermentation.
[b] Amino acid concentrations are given at the point of glucose exhaustion from the medium.

5.3.7 Discussion

To effect threonine accumulation in a lysine producer, carbon flux must be redistributed away from lysine biosynthesis at the aspartic-ß-semialdehyde branchpoint. Furthermore, the activities of enzymes in the threonine pathway must be coordinated to minimize accumulation of intermediates such as homoserine. Thus in this case the simple over-expression of the *hom*^{dr}-*thr*B operon resulted in the expected reduction in lysine and increase in threonine production, along with accumulation of homoserine (Nakamori *et al.*, 1987; Reinscheid *et al.*, 1994), due to an apparent imbalance in enzyme activities under these conditions.
Therefore, the homoserine accumulation is probably due to threonine inhibition of homoserine kinase that is of a competitive nature.

Table 5.11 shows that under optimal induction, the activities of homoserine dehydrogenase and homoserine kinase in 21799(pGC42) are approximately doubled with respect to the base case of 21799(pJD4). The obtained higher final threonine titer and lack of homoserine accumulation (Table 5.12) result from a) further enhancement of carbon flux towards the threonine branch and b) reduced *in vivo* threonine inhibition of homoserine kinase due to higher intracellular enzyme levels. The combination of these effects apparently results in optimal intracellular conditions for smooth carbon flow to threonine.

Although under standard conditions the reduction of DL-aspartic-ß-semialdehyde to homoserine is thermodynamically favorable ($\Delta G^{\circ\prime}$ of reaction = -5.0 kcal/mol; see section 3.2), a high enough concentration of homoserine can reverse the direction of the reaction. In strains with an amplified *hom*^{dr}-*thr*B operon, the intracellular concentration of homoserine may reach up to 74-100 mM during periods of the

fermentation (Reinscheid *et al.*, 1994; section 5.8). Thermodynamic analysis using the method of Mavrovouniotis (1993; see Section 3.2) shows that taking into account the permissible concentration ranges of the metabolites involved, this range is well within that necessary to drive the reaction towards formation of ASA. A higher concentration of ASA may allow some of the ASA to be captured for the lysine pathway. Although, in general, increasing the *in vivo* enzyme activity simply accelerates the rate at which the participating reactants and products reach thermodynamic equilibrium, changing these activities may result in new steady state levels of metabolites, which in turn affect the equilibrium. In *C. lactofermentum* 21799(pGC42), the high homoserine kinase activity may have contributed to a reduced intracellular level of homoserine and allowed the flux of carbon to proceed through to threonine. This situation, however, is precarious since by reducing the homoserine concentration, the equilibrium of the homoserine kinase reaction (which is highly unfavorable at standard conditions; $\Delta G^{\circ\prime}$ of reaction = +5.0 kcal/mol) is affected. A high flux towards threonine is thus contingent upon a low internal concentration of homoserine phosphate, the reaction product. This low concentration can be maintained if the threonine synthase reaction, which converts homoserine phosphate to threonine, is not limiting. Therefore, both thermodynamic and kinetic effects need to be considered when contemplating metabolic perturbations.

A molar amino acid balance performed on the accumulated metabolites derived from aspartate in the various strains (Figure 5.15) shows that more than the molar equivalent of the carbon entering the lysine pathway in the control strain enters the threonine pathway under optimal induction conditions in 21799(pGC42). About 180 mmoles/ liter of amino

acids are excreted in 21799(pGC42) vs. about 150 mmoles/liter of lysine in 21799(pM2). In other words, not only is most of the carbon entering the aspartate pathway diverted to the threonine pathway in 21799(pGC42) under optimal conditions, but these new conditions allow more carbon in the cell to enter the aspartate pathway.

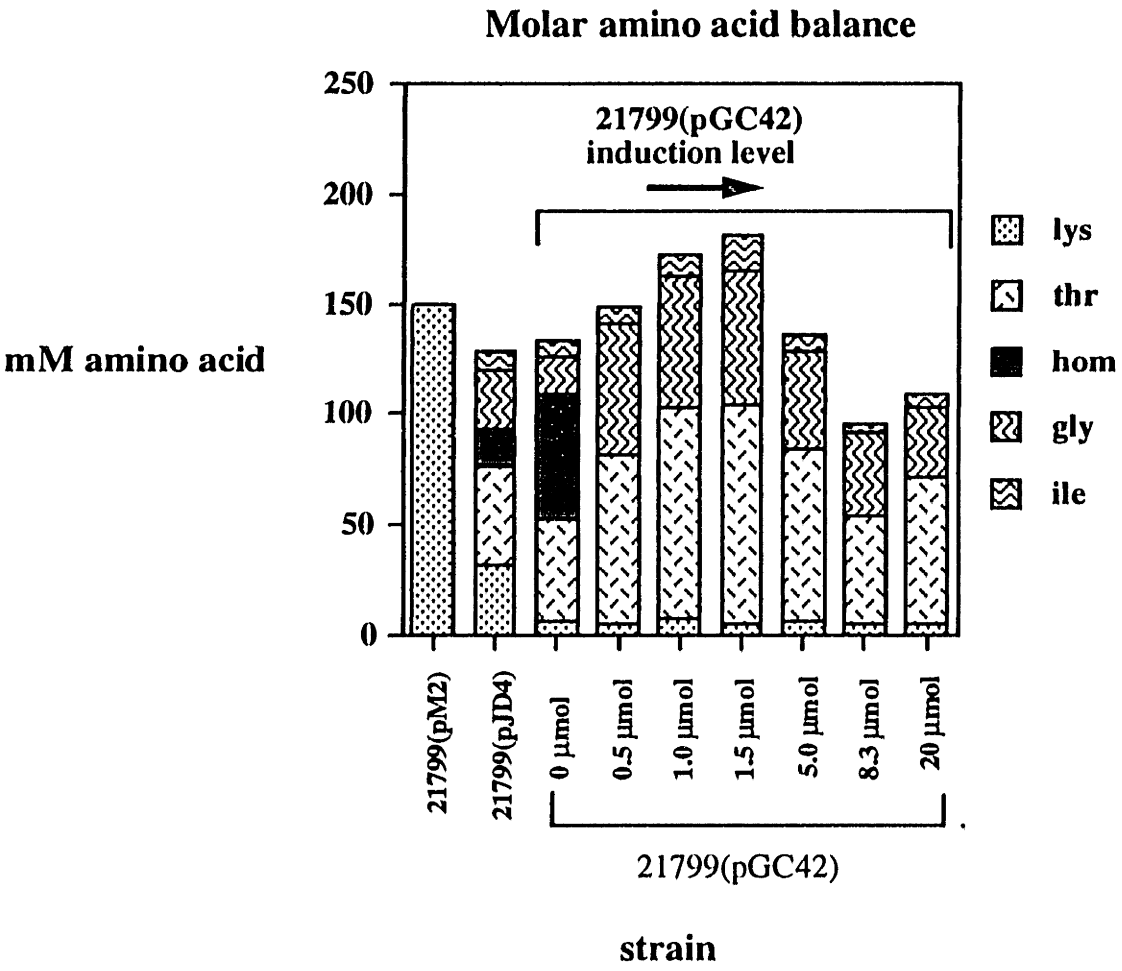

Figure 5.15 Molar amino acid balance for various 21799 strains and under various induction conditions.

The simplest Michaelis-Menten kinetic model illustrates the effect of increasing the intracellular concentration of an enzyme subject to competitive inhibition. Figure 5.16 shows two families of curves of reaction velocity versus substrate concentration (for equation, assumptions and parameters used, see Appendix). In both cases, the activity of the enzyme in the presence of increasing inhibitor concentrations is reduced, with the inhibition being relieved at higher concentrations of the substrate. However, by simply increasing the intracellular concentration of enzyme ([E]), the family of curves is shifted upward. At a given inhibitor concentration, a much higher reaction velocity is supported in the case of higher [E]. Although many factors affect *in vivo* enzyme activity, the fermentation results add validity to the principles illustrated by this model

The increased activity of feedback-insensitive homoserine dehydrogenase activity in ATCC 21799(pGC42) relative to that of ATCC 21799(pJD4) could be possibly due to differences in DNA conformation, transcription readthrough of another gene present on the plasmid, or some other structural effect. Because other plasmids containing a deregulated homoserine dehydrogenase gene in a *Corynebacterium* lysine producer have previously resulted in significant residual lysine accumulation in the medium, (Reinscheid *et al.*, 1994), these increased levels of HD^{dr} are likely to be important for diverting a higher percentage of the carbon flow at the ASA branch for threonine production. In fact, one study in which a hom^{dr} gene was amplified in an AEC^R, AHV^R strain, *C. glutamicum* DM368-3, HD activities were comparable to those found in 21799(pGC42) (Eikmanns *et al.*, 1991). In this case, residual lysine production in this strain was nearly negligible.

There was considerable accumulation of glycine and isoleucine in

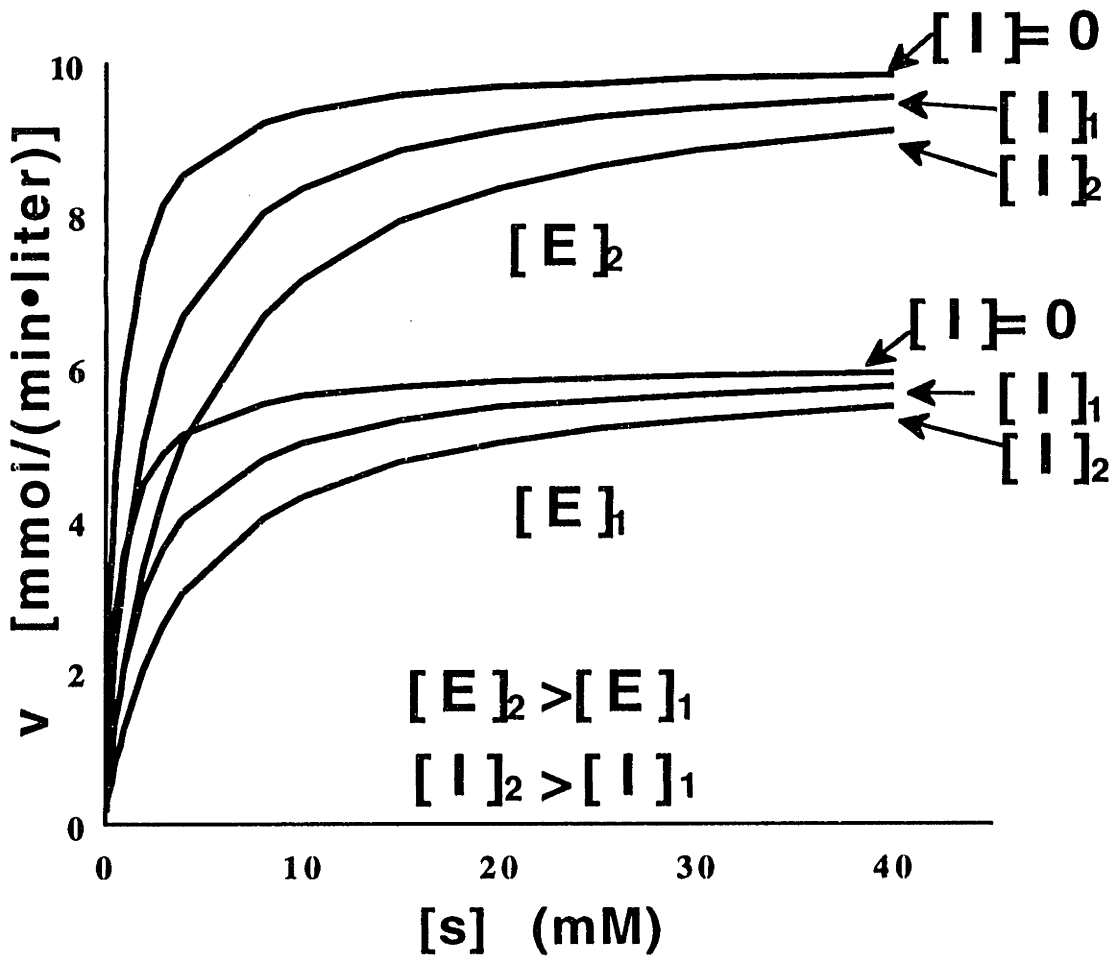

Figure 5.16 Effect of increasing enzyme concentration on family of inhibition curves.

fermentations with all threonine producers. There are three routes to threonine degradation in *Corynebacterium*: threonine dehydrogenase (EC 1.1.1.129; *tdh*), threonine dehydratase (EC 4.2.1.16; *ilv*A) and serine hydroxymethyltransferase (EC 2.1.2.1; *gly*A) (Bell and Turner, 1976). The first two lead to production of glycine. Threonine dehydratase initiates a five-step pathway from threonine to isoleucine, and is feedback-inhibited by isoleucine (Miyajima and Shiio, 1972). Obviously, this inhibition did not suffice to prevent isoleucine accumulation in our threonine producers. These results prompted the next two perturbations performed on this sytem.

The induction of cloned genes by IPTG has been shown to have an inhibitory effect on cell growth and metabolite production in both coryneform and enteric bacteria due to over-expression of the cloned gene product (Bentley *et al.*, 1991; Tsuchiya and Morinaga, 1988). We observed that there was an optimal level of IPTG addition at which HK activity sufficed to minimize homoserine accumulation and beyond which threonine production was even hampered. At these levels, growth was not significantly affected.

5.4 Node 3: Threonine

5.4.1 Introduction

By performing the two previous perturbations, the carbon flow at the ASA node was essentially completely redirected to threonine in a lysine producer, with minimal or no accumulation of the intermediate, homoserine. However, there was considerable accumulation of the

threonine degradation products, isoleucine and glycine, detracting from product yield in a threonine fermentation. It was therefore of interest to attempt to minimize or eliminate the degradation reactions in this case. Conversely, one of these degradation products, isoleucine, is of interest itself. It is, like threonine, an essential amino acid; the fact that it accumulates in a threonine producer prompted the question of how to overproduce it by further redirecting the threonine carbon to isoleucine. Therefore, the next perturbations involved reactions at the threonine node (Figure 5.17). Specifically, the gene encoding threonine dehydratase (*ilv*A), was used.

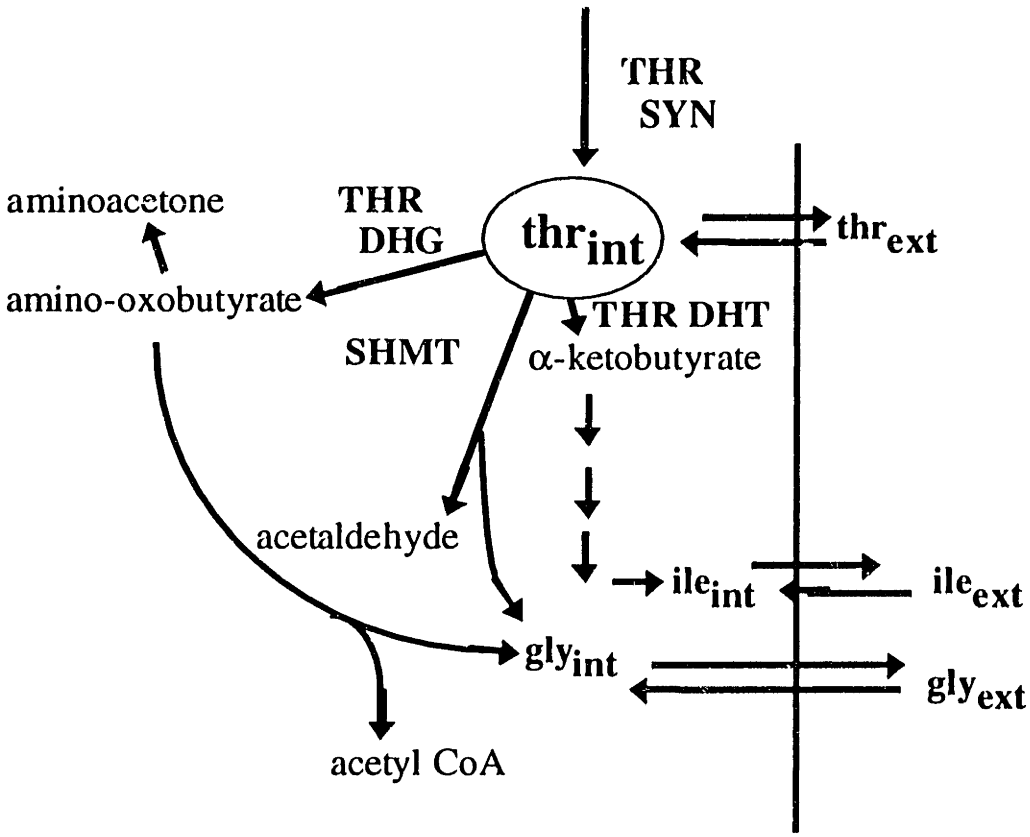

Figure 5.17 Schematic of reactions at the threonine node. THR SYN, threonine synthase; THR DHG, threonine dehydrogenase; SHMT, serine hydroxymethyltransferase; THR DHT, threonine dehydratase.

Threonine dehydratase (TD; EC 4.2.1.16) catalyzes the first of five reactions leading from threonine to isoleucine, and is the only known enzyme specific for isoleucine synthesis in *Corynebacterium*. The gene encoding this enzyme, *ilv*A, has recently been cloned in *C. glutamicum* (Cordes *et al.*, 1992). The gene was isolated by Dr. Mike Jetten in the Sinskey laboratory from a genomic *C. glutamicum* library by complementation of *E. coli ilv*A mutant 1255 (Colón *et al.*, 1995b).

There are two enzymes which use threonine to initiate a glycine formation pathway in *Corynebacterium* (Bell and Turner, 1976). Threonine dehydrogenase (TDH; EC 1.1.1.103) converts threonine to amino-oxobutyrate, which either spontaneously decarboxylates to aminoacetone, or is converted to glycine and acetyl-CoA by the action of amino-ketobutyrate:CoA ligase (AKL; EC 2.3.1.29). Alternatively, serine hydroxymethyltransferase (SHMT; EC 2.1.2.1), which interconverts serine and glycine, can also act on threonine to produce acetaldehyde and glycine. Although the genes encoding these enzymes have been cloned in *E. coli* (see section 5.4.1.2), they have not been cloned in *C. glutamicum*.

The availability of marker exchange mutagenesis technology, developed for strains of *Corynebacterium* (Schwarzer and Pühler, 1991), introduces new opportunities for metabolic engineering in this organism. Stable mutants which lack specific enzyme activities can be constructed for a wide variety of applications (Gubler *et al.*, 1994a; Gubler *et al.*, 1994b). In particular, disruption of genes encoding enzymes which degrade products of interest can be used as a methodology for both studying the effects of specific network perturbations and for yield improvement. In the case of threonine production, the three degradation enzymes described above are potential targets for manipulation.

5.4.2 Disruption of threonine dehydratase

To construct stable threonine dehydratase deletion mutants, a 3.3 kb *Hind*111 *Corynebacterium* chromosomal fragment containing the *ilv*A gene was subcloned into mobilizable vector pSUP301 as follows. Plasmid pSUP301 was digested with *Sca*I and *Hind*III/*Sma*I adapters were ligated to the blunt ends. The 5.1 kb fragment was isolated and ligated with the 3.3 kb *Hind*III fragment containing the *ilv*A gene. The resulting plasmid pSH3-*ilv*A was transformed into *E. coli ilv*A mutant 1255. Replica plating onto minimal medium without isoleucine showed that the plasmid could complement the isoleucine auxotrophy. Thereafter pSH3-*ilv*A was digested with *Bgl*II to replace an 400 bp internal fragment of the *ilv*A gene with a 1.4 kb *Bam*H1 fragment from pMG110 containing Cm^R marker. S17-1 was transformed with the resulting plasmid pSH3-*ilv*A-*cat*. This colony containing plasmid pSH3cat6 was used for transconjugation with strain 21799. For gene disruption, a double cross-over, which deletes a portion of the *ilv*A gene, is desired. This mutation can be screened by isolating Cm^R colonies which remain sensitive to kanamycin. For 21799, 125 candidates were obtained and their phenotype investigated by growth on MB/Kn and MB/Cm/Nx plates. One candidate was obtained (21799*ilv*A) which showed the desired Cm^RKn^S phenotype. This strain did not grow on minimal medium plates lacking isoleucine or 2-oxobutyrate, thereby confirming its *ilv*A- phenotype.

5.4.2.1 Shake flask fermentation results

Strain 21799*ilv*A- was made competent and transformed with

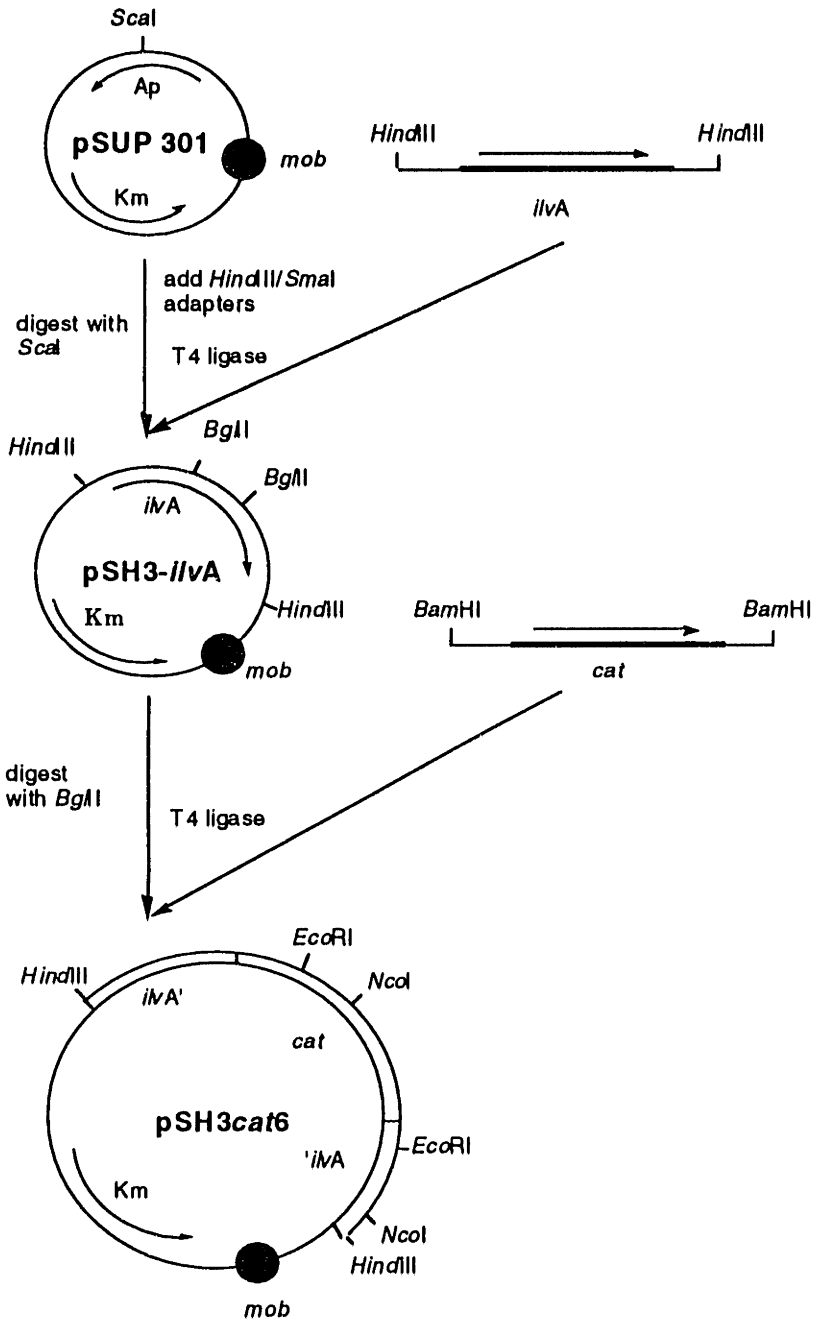

Figure 5.18 Construction of *ilv*A disruption vector (courtesy of Dr. Mike Jetten).

plasmid pGC42. The results of shake flask fermentations with 21799*ilv*A-(pM2) and 21799*ilv*A-(pGC42) are shown in Table 5.13, along with data from the corresponding strains not containing the *ilv*A deletion. Strain 21799*ilv*A-(pGC42) produced about 1 g/l of lysine, but the production of isoleucine was reduced from 1.9 g/l to 0.8 g/l. The levels of threonine were similar in both strains containing pGC42, but glycine was observed in higher concentration in the threonine dehydratase mutant (5.5 g/l vs. 4.6 g/l). A molar amino acid balance (Figure 5.19) shows that the flow of carbon into the threonine branchpoint remained the same. There was no change in threonine excretion; however the decrease in isoleucine production was accompanied by an increase in glycine production. The fact that there is more glycine accumulation in the threonine dehydratase deletion mutant seems to indicate that in this strain there is a higher driving force for threonine degradation to glycine, and subsequent glycine export, rather than for export of threonine. Glycine begins to accumulate soon after threonine is detected extracellularly, increasing monotonically with

Table 5.13 Amino acids accumulated in culture medium by various 21799 strains

C. glutamicum strain[a]	Amino acid concentration in supernatant (g/l)[b]				
	lys	thr	hom	gly	ile
21799(pM2)[c]	22.0	< 0.1	< 0.1	< 0.1	< 0.1
21799*ilv*A-(pM2)	23.5	< 0.1	< 0.1	< 0.1	< 0.1
21799(pGC42)[c]	0.8	11.8	< 0.1	4.6	1.9
21799*ilv*A-(pGC42)	1.0	11.6	< 0.1	5.5	0.8

[a] 21799(pGC42) and 21799*ilv*A-(pGC42)100 ml cultures were induced by addition of 1.5 µmoles of IPTG at approximately 30 hour intervals during the course of the fermentation.
[b] Amino acid concentrations are given at the point of glucose exhaustion from the medium.
c Data from Colón *et al.*, 1995a.

no significant increase at the point of glucose exhaustion. However, extracellular threonine concentration begins to decline dramatically at the point of glucose exhaustion, as does homoserine concentration. Comparison of extracellular and intracellular threonine concentration profiles show that intracellular/extracellular threonine ratios are extremely high throughout the fermentation up until the point of glucose exhaustion (section 5.6). This seems to indicate that upon glucose exhaustion, the cells shift to a new physiological state in which threonine is used as a carbon and energy source for cell maintenance. The kinetics of the threonine degradation reactions leading to glycine were investigated to try to determine the next useful target for perturbations.

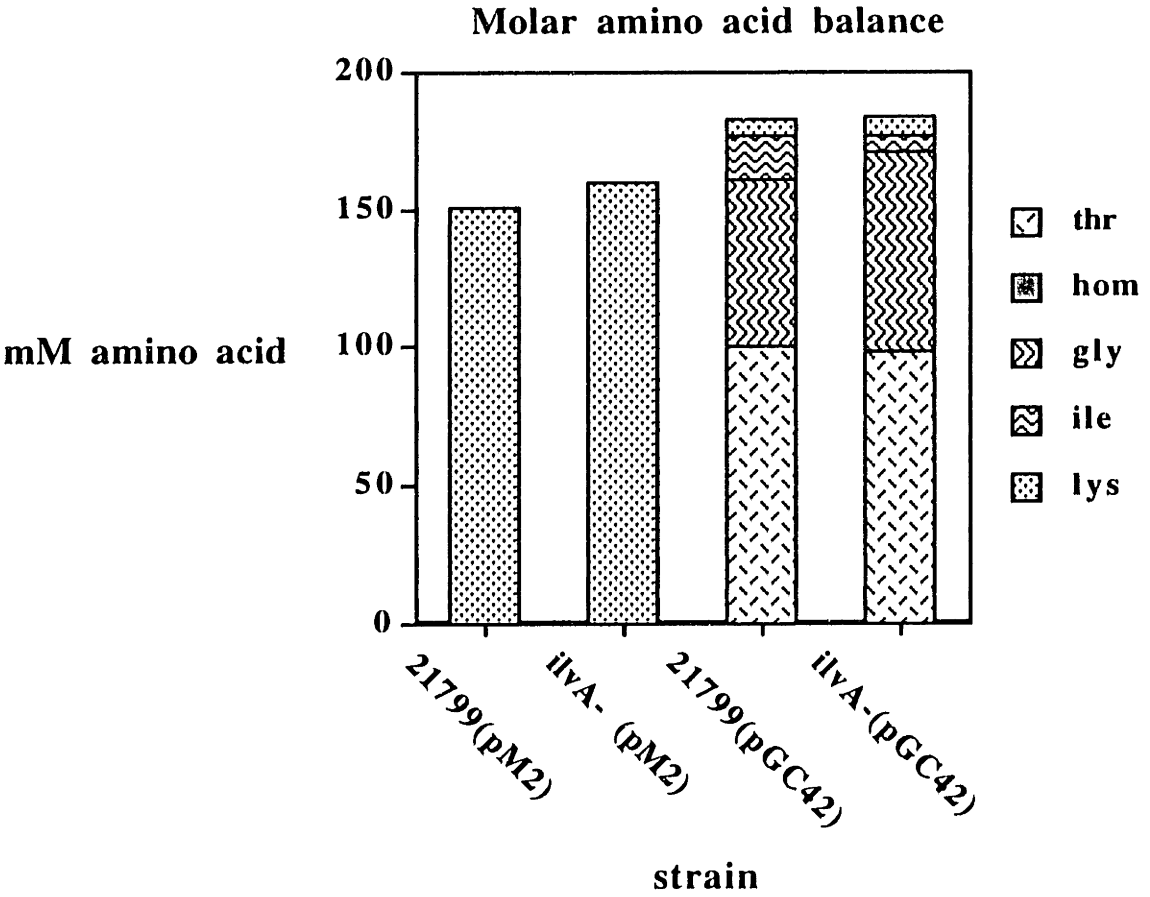

Figure 5.19 Molar amino acid balance for various 21799 strains.

5.4.2.2 Kinetic parameters of glycine-forming enzymes

Serine hydroxymethyltransferase (encoded by *glyA* in *E. coli*) catalyzes the following reactions (Schirch and Gross, 1968):

SHMT
L-serine + tetrahydrofolate <--> glycine + 5,10-methylene tetrahydrofolate

SHMT
L-threonine <--> glycine + acetaldehyde

This enzyme was purified from *E. coli* by Schirch *et al.*, (1985), and the corresponding gene sequence was determined by Plamman *et al.* (1983).

Threonine dehydrogenase (TDH; encoded by *tdh* in *E. coli*) catalyzes the conversion:

TDH
L-threonine <--> amino-oxobutyrate

This enzyme was purified from *E. coli* K-12 by Boylan and Dekker (1981) and the corresponding gene was cloned by Ravnikar and Somerville (1986).

Amino-oxobutyrate is an extremely unstable compound which can either be spontaneously decarboxylated to aminoacetone, or be converted to acetyl-CoA and glycine by the action of the enzyme 2-amino-3-ketobutyrate CoA ligase (AKL). The *E. coli* enzyme was purified and characterized by Mukherjee and Dekker (1987). The gene encoding this enzyme (*kbl*) was cloned in *E. coli* by Aronson *et al.* (1988).

$$\text{amino-oxobutyrate} \xrightarrow{\text{(spont.)}} \text{aminoacetone} + CO_2$$

$$\text{amino-oxobutyrate} \xrightarrow{\text{AKL}} \text{acetyl-CoA} + \text{glycine}$$

All three enzyme activities (TDH, AKL and SHMT) have been detected in *Corynebacterium* spp. and may play roles in the production of glycine from threonine in *C. lactofermentum* 21799(pGC42) and *C. lactofermentum* 21799*ilv*A-(pGC42). In addition, as the other intermediates involved in these reactions are not excreted, it is difficult to know *a priori* which of the two enzyme systems is more important in the formation of glycine from threonine in these strains, and thus which should be a first target for cloning and disruption in strain improvement for threonione production. In order to address this question, kinetic analysis of these enzymes in crude extracts of this strain was carried out. The assay conditions used are described in Chapter 4. Table 5.14 lists the kinetic constants of threonine dehydrogenase and serine hydroxymethyltransferase in crude extracts of *C. lactofermentum* 21799*ilv*A-(pGC42).

Table 5.14 Kinetic parameters of threonine dehydrogenase and serine hydroxymethyltransferase with respect to threonine in crude extracts of *C. lactofermentum* ATCC 21799 *ilv*A-(pGC42)

	TDH	SHMT[a]
$K_{m\ (threonine)}$ (mM)	4.4 ± 1.2	139 ± 21
V_{max}(nmol/min•mg protein)	7.3 ± 0.5	2.3 ± 0.2

[a]estimated; activity increased continuously up to solubility limit of threonine in the reaction mixture

5.4.2.3 Discussion

Bell and Turner (1976) performed a survey of a large number of strains (including those of *Corynebacterium* and *Brevibacterium*) isolated from soil samples by virtue of their ability to grow on threonine as a sole or major source of carbon and nitrogen and found that the majority possessed inducibly synthesized L-threonine 3-dehydrogenase of high activity but low dehydratase and aldolase activities. Presumably, the acetyl CoA derived from this pathway enters cell growth and maintenance pathways (e.g., the TCA cycle). The glycine derived is further degraded by serine hydroxymethyltransferase and the glycine cleavage pathway (EC 1.4.4.2 and EC 2.1.2.10) to generate serine and essential one-carbon units. The isolated strains were also able to grow well on defined basal medium containing glycine, L-serine, pyruvate, succinate or acetate as sole source of carbon and energy. However, in all strains tested, only the activities of threonine dehydrogenase and 2-amino-3-oxobutyrate-CoA ligase increased significantly when the cells were grown using threonine as a sole source of carbon. In addition, for each *Corynebacterium* or *Brevibacterium* strain, the ratio of these activities remained approximately constant under the conditions used (table 5.15). This is consistent with both genes being present in an operon, as is the case for *E. coli*.

Values of $[S]_{0.5}$ for threonine for threonine dehydrogenase (or K_m, as defined in this work) for the three *Corynebacterium* species ranged between 13.6 - 20.0 mM (table 5.16). The value of 20 mM was for partially purified enzyme from *Corynebacterium* sp. B6. The authors did not measure the kinetic constants for serine hydroxymethyltransferase with respect to threonine. However, values from other sources are shown in

table 5.17 for comparison. The K_m for threonine for this enzyme is invariably much higher than the K_m for serine, which is consistent with the primary function of this enzyme.

Ravnikar and Somerville (1987) found that *E. coli* serine (*ser*A, *ser*B and *ser*C; see Figure 5.20) and *gly*A mutants were able to grow on minimal medium without serine or glycine provided the medium contained elevated levels of threonine, as well as leucine, arginine, lysine and methionine. Amino acid analysis of spent culture medium after growth of these mutants showed that all of the threonine had been exhausted, while the other amino acids were used to lesser and various extents. The authors proposed that the other four amino acids most likely enhanced the growth of the mutants either because they acted as inducers of necessary enzymes or else spared a drain of some essential metabolites. Double mutants with lesions in one of the *ser* genes plus a second lesion in *gly*A, *tdh* or *gcv* (glycine cleavage

Table 5.15 Enzyme activities (nmol/min per mg prot) under various growth conditions (data from Bell and Turner, 1976).

Strain	Growth substrates	Enzyme activities*	
		TDH	AKL
Brevibacterium sp. P2	L-threonine	870	65
	Glycine	16	1
	Succinate	24	2
Brevibacterium sp. B10	L-theonine	968	242
	Glycine	28	9
	Succinate	40	14
Corynebacterium sp. B6	L-threonine	1040	364
	Glycine	42	14
	Succinate	75	25

*TDH, threonine dehydrogenase; AKL, 2-amino-3-oxobutyrate CoA ligase

Table 5.16 Threonine dehydrogenase - K_m with respect to threonine

K_m (mM)	strain/source	description/reference*
1.43	*E. coli* K12	p., Boylan and Dekker, 1981
13.6	*Brevibacterium* sp. P2	c.e., Bell and Turner, 1976
14.3	*Brevibacterium* sp. B10	c.e., Bell and Turner, 1976
20.0	*Corynebacterium* sp. B6	c.e., Bell and Turner, 1976

* c.e., measured in crude extracts; p., purified or partially purified enzyme

Table 5.17 Serine hydroxymethyltransferase - K_m with respect to threonine

K_m (mM)	strain/source	description/reference*
40	rabbit liver cytosol	Schirsh *et al.*, 1985
33	rabbit liver mitochondria	Schirsh *et al.*, 1985
**	*E. coli*	Schirsh *et al.*, 1985

* c.e., measured in crude extracts; p., purified or partially purified enzyme
** For *E. coli*, the rate with L-threonine was so slow and the K_m so large that the authors were unable to determine the kinetic parameters even with purified SHMT.

system) were unable to grow on this medium, supporting the hypothesis of an alternate pathway of serine and glycine biosynthesis through threonine. The authors proposed that a series of reactions already present in the cell could function as a cycle which would support growth of these mutants on threonine as a carbon source, provided there is a high activity of threonine dehydrogenase; they called this the "Tut" cycle, for Threonine utilization (Figure 5.21). In fact, this cycle provides an energetically favorable alternative for serine synthesis, as it yields a net of 5 mol of ATP, rather than the one mole consumed in the conventional cycle. The net reaction is conversion of 1 mol of threonine to serine and CO_2. If the glyoxylate cycle is not functional under these conditions, the net reaction is the conversion of 2 mol of threonine to 1 mol of serine and 2 mol of acetate.

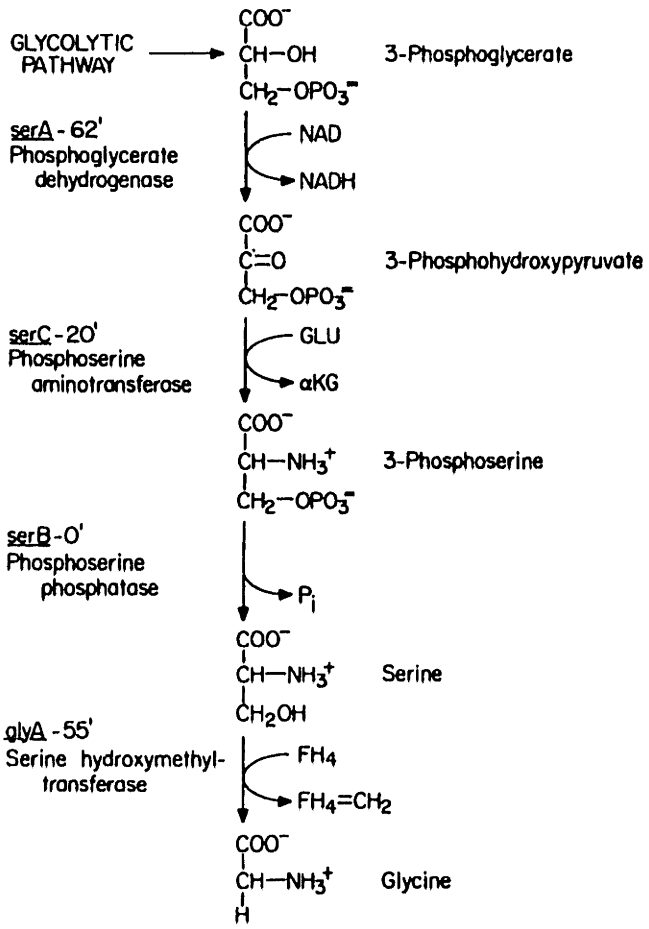

Figure 5.20 Conventional serine biosynthetic pathway (from Ravnikar and Somerville, 1987).

This analysis is applicable to threonine, serine and glycine synthesis in *Corynebacterium* as well, since these reactions are present in this strain. This situation complicates the cloning of *tdh* and *glyA* in *Corynebacterium* spp., as mutations in one gene can thus be complemented by the other.

In conclusion, although preliminary kinetic studies of the enzymes responsible for glycine production from threonine suggest that although the activity of threonine dehydrogenase may be responsible for most of the

glycine accumulation, serine hydroxymethyltransferase could also play a role in degradation of threonine to glycine. At low threonine concentrations, this reaction has a thermodynamic advantage over threonine dehydrogenase (see section 3.2). At higher threonine concentrations, the activity of serine hydroxymethyltransferase reaches a high enough level to contribute a significant amount to the overall threonine to glycine conversion. To obtain greatly improved threonine yields, it would be useful to isolate and disrupt the genes encoding both of these enzymes. However, based on the results of the kinetic studies, it is likely that disruption of *tdh* (encoding threonine dehydrogenase) alone would suffice to eliminate most of the loss of threonine carbon to glycine. Therefore, this gene should be the first target for disruption to decrease degradation of threonine to glycine.

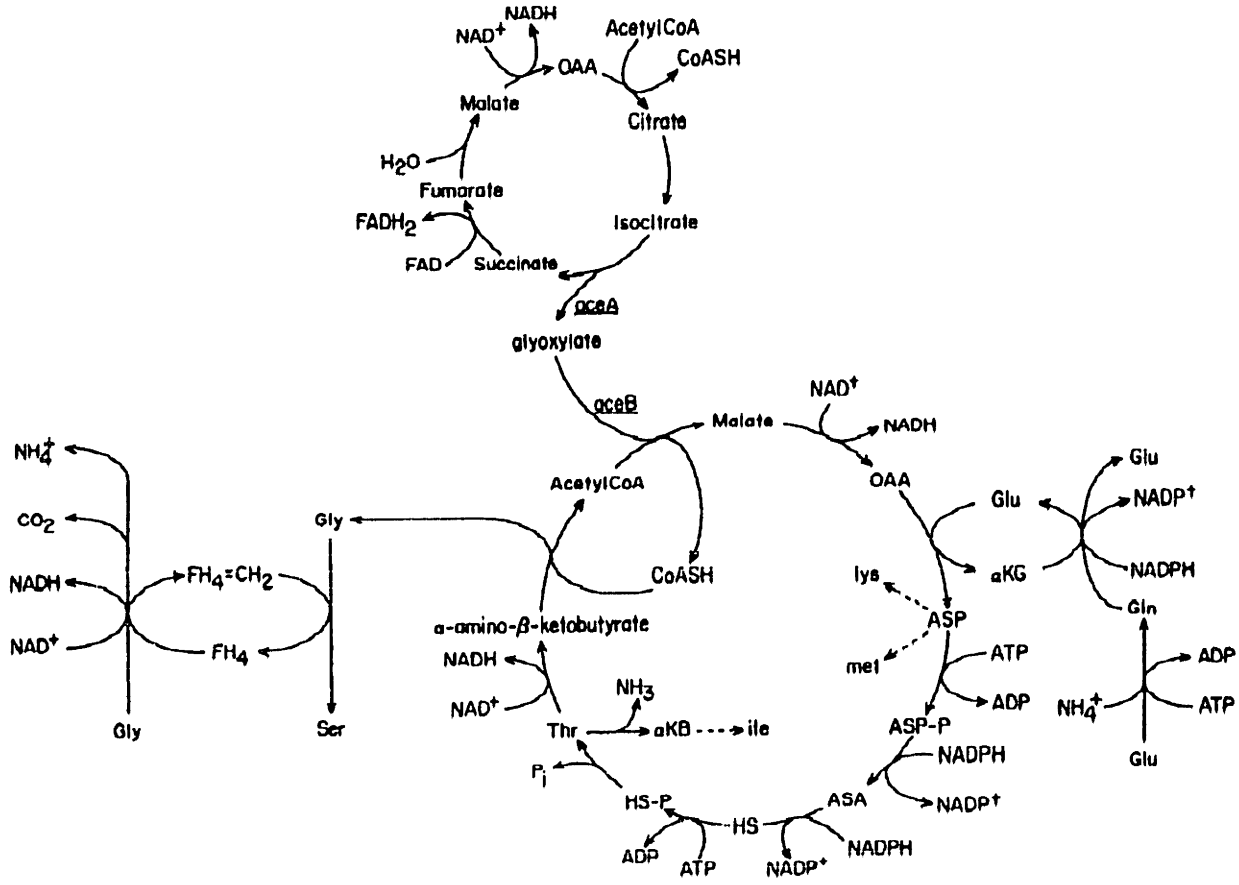

Figure 5.21 Proposed threonine utilization (Tut) cycle in *E. coli*, used in serine mutants as an alternate serine pathway when grown on minimal medium supplemented with threonine and at least three out of the four amino acids leucine, arginine, lysine and methionine (from Ravnikar and Somerville, 1987). Final reaction of the cycle is thr + 3 ATP + FAD + 2 NAD+ ---> ser + CO_2 + 3 ADP + $FADH_2$ + 2 NADH + 2 H^+ + 3 P_i.
Abbreviations: *ace*A, isocitrate lyase gene; *ace*B, malate synthase gene; OAA, oxaloacetate, Glu, glutamate; Gln, glutamine; αKG, α-ketoglutarate; ASP, aspartate; AspP, β-aspartyl phosplate; ASA, aspartate β-semialdehyde; HS, homoserine; HS-P, homoserine phosphate; αKB, α-ketobutyrate; FH_4, tetrahydrofolate; FH_4=CH_2, N^5,N^{10}-methylenetetrahydrofolate.

5.4.3 Amplification of the threonine dehydratase gene: Production of isoleucine

The excretion of isoleucine in shake flask fermentations with 21799(pGC42) prompted a new question - what effect would the amplification of a threonine degradation enzyme have? The gene encoding threonine dehydratase in *Corynebacterium* (*ilv*A) was recently cloned (Cordes *et al.*, 1992). Dr. Mike Jetten obtained the gene by complementation and used it to construct a disruption vector to interrupt the gene's function in the lysine producer 21799 (section 5.4.2). The other perturbation at the threonine node thus consisted of amplification, rather than disruption, of *ilv*A in the threonine-producing strain *C. lactofermentum* 21799(pGC42), with the aim of investigating the extent of isoleucine production in the new strain.

The rationale behind this strategy is multi-faceted. First, the threonine-producing strain *C. lactofermentum* 21799(pGC42) excreted 1-2 g/l of isoleucine in addition to the other metabolites, in spite of the fact that in this strain there is feedback inhibition of threonine dehydratase by isoleucine. Second, formation of acetohydroxy acid synthase, the second enzyme in the pathway, is highly inducible by α-ketobutyrate, the product of the threonine dehydratase reaction and one of its substrates (Eggeling *et al.*, 1987). Third, thermodynamic analysis of the pathway from threonine to isoleucine shows that under standard physiological conditions, not only is the entire pathway highly favorable (ΔG° = - 36 kcal/mole), but all five individual reactions are favorable (see section 3.2). Finally, as was seen in the case of threonine inhibition of homoserine kinase, end product inhibition of a pathway enzyme can be overcome for purposes of

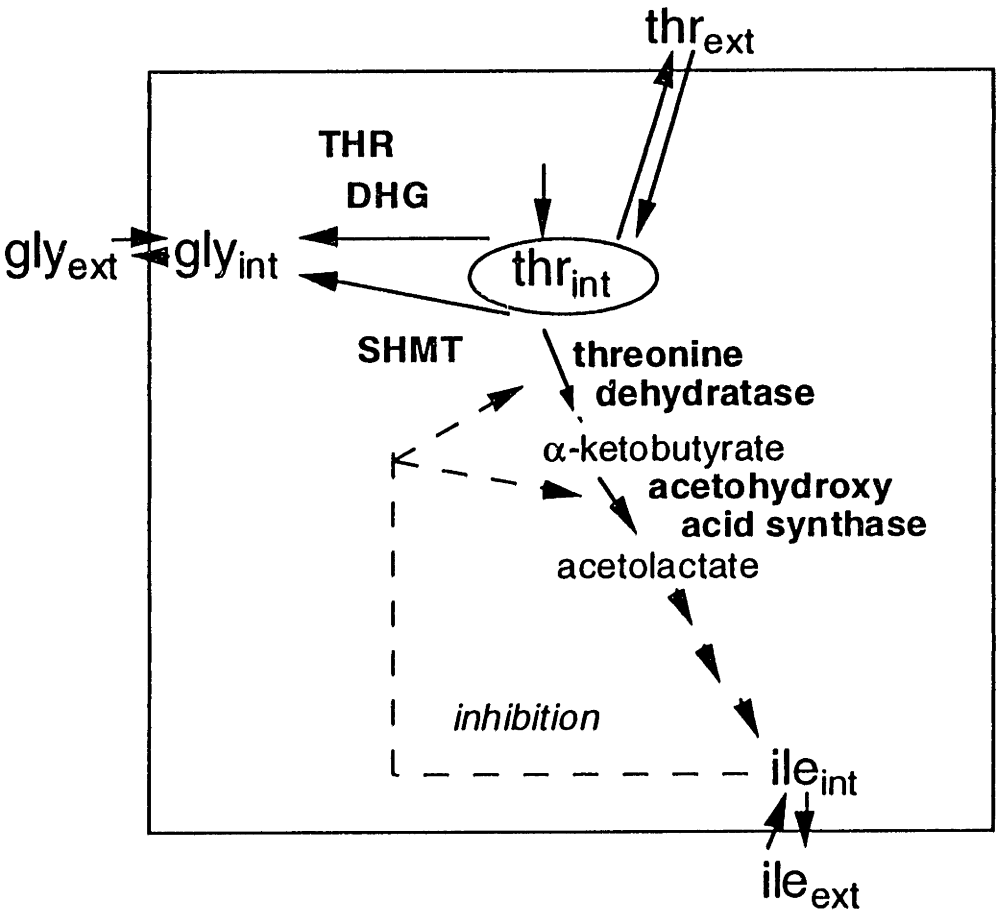

Figure 5.22 Isoleucine metabolic processes

metabolite overproduction by increasing *in vivo* enzyme activity. It seems reasonable that a high intracellular concentration of threonine (such as that found in our 21799 (pGC42) threonine producer), together with a high intracellular level of threonine dehydratase (achieved by amplifying *ilv*A from *Corynebacterium*) would provide the conditions necessary for isoleucine overproduction. Therefore, the next perturbation involved investigating the extent of isoleucine accumulation in a threonine producer containing an amplified wild type *ilv*A gene.

5.4.3.1 Construction of pGC77

In order to construct an isoleucine producer, the gene encoding threonine dehydratase was isolated from *Corynebacterium glutamcium* and ligated into the plasmid pGC42. The *Corynebacterium ilv*A gene encoding threonine dehydratase was isolated from a pM2-based genomic *C. glutamicum* library by heterologous complementation of *E. coli ilv*A mutant CGSC 1255. The gene library was prepared as previously described (Follettie and Sinskey, 1986). *E. coli* 1255 was transformed with the library, transformants were selected on LB supplemented with 50 μg/ml kanamycin and subsequently screened on minimal medium plates. Plasmid DNA from complemented cell lines was purified and screened twice. Plasmid DNA was purified and digested with *Hind*III for subcloning of the *ilv*A gene. A 3.3 kb *Hind*III *Corynebacterium* chromosomal fragment ligated to pUC18 in both orientations (p18-HIB1 and p18-HIB2) complemented strain 1255. Enzyme assays confirmed the presence of the *ilv*A gene on the 3.3 kb *Hind* III fragment (data not shown). For construction of pGC77, pGC42 was restriction digested with *Sma*I and subsequently treated with calf intestine phosphatase (Figure 5.24). The 3.3 kb *Hind*III fragment containing the *ilv*A gene was ligated into pGC42 after treatment with the Klenow fragment of DNA polymerase 1, creating the 14.7 kb plasmid pGC77. *C. lactofermentum* 21799 was transformed with pGC77 by electroporation.

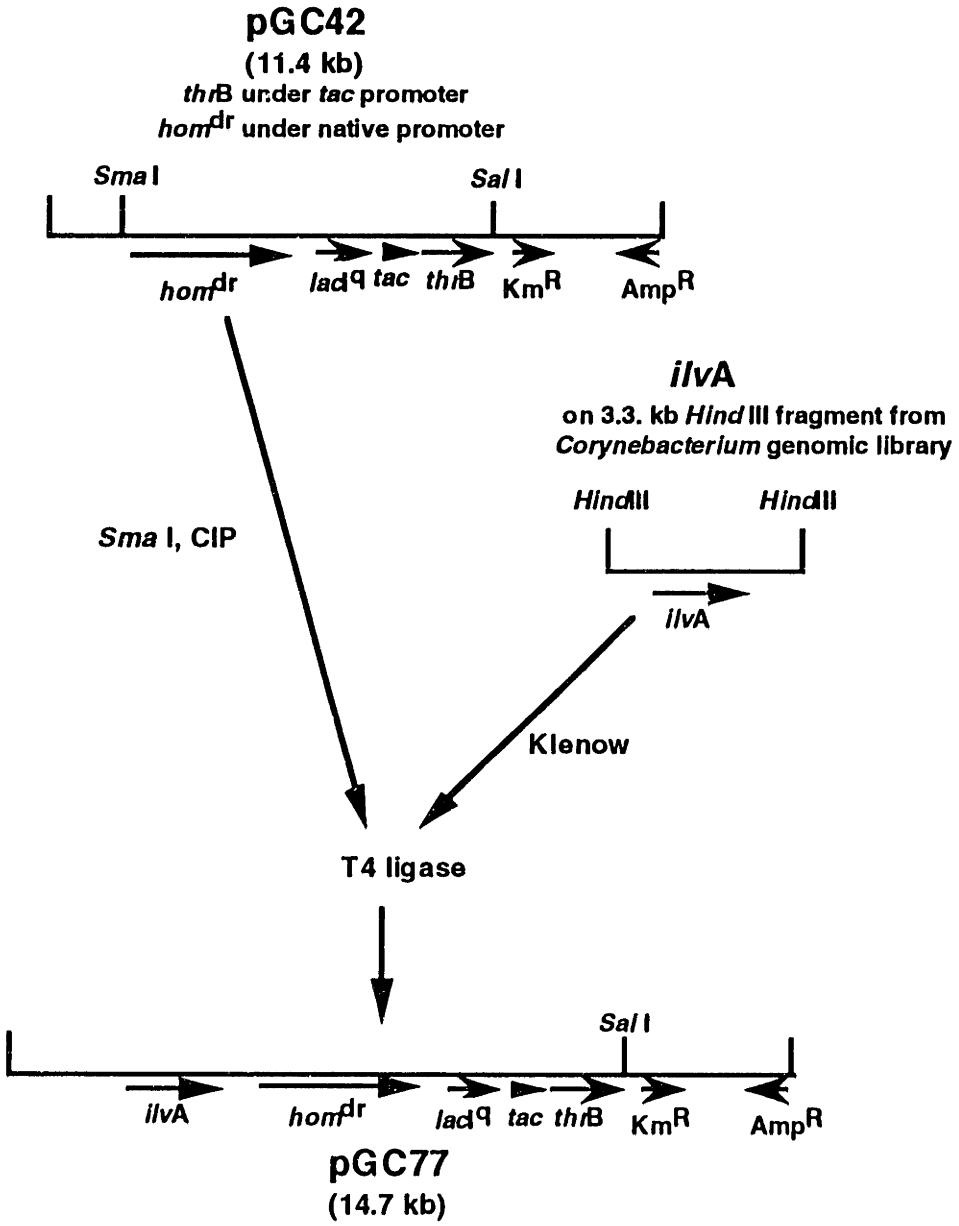

Figure 5.23 Construction of pGC77.

5.4.3.2 Enzymatic activities

Table 5.18 lists the activities of threonine dehydratase (TDH) and acetohydroxy acid synthase (AHAS) in the three 21799 strains of interest. The strains were cultured in minimal medium, with or without the addition of 50 mM α-ketobutyrate (AKB). The activity of TDH was not significantly affected by growth in medium supplemented with AKB. Activity of TDH in 21799(pGC77) was approximately five times higher than in strains without the amplified *ilv*A gene. AHAS activity was induced about 10-fold in 21799(pM2) and in 21799(pGC42) cultured with AKB. However, AHAS activity in 21799(pGC77) cultured without AKB was about two to three times higher than that in 21799(pM2) and 21799(pGC42), and was unaffected by growth with AKB.

Table 5.18 Specific activities of threonine dehydratase (TDH) and acetohydroxy acid synthase (AHAS)

C. lactofermentum strain[a]	Specific activity (μmole/min•mg protein)[a]			
	TDH		AHAS	
	-	+	-	+
21799(pM2)	0.374	0.389	0.028	0.276
21799(pGC42)	0.248	0.268	0.022	0.392
21799(pGC77)	1.734	1.444	0.072	0.073

[a] Activtities in cells grown with (+) or without (-) 50 mM α-ketobutyrate.

5.4.3.3 Shake flask fermentation results

21799(pGC77) cells were cultured in BJ minimal medium with 80 g/l glucose. After approximately 30 hours, and twice more at about 30-hour intervals, 1.5 µmoles of IPTG were added. The cells reached a typical OD_{600} of 50. The culture fluid was analyzed for amino acid content at the point of glucose exhaustion (approximately 80 hours). Table 5.19 shows the amino acids accumulated in the culture medium at the point of glucose exhaustion. Data from 21799(pM2) and 21799(pGC42) fermentations are shown for comparison (see section 5.3.6). 21799(pM2) produced exclusively lysine, and 21799(pGC42) produced almost 12 g/l of threonine, 4.6 g/l of glycine, 1.9 g/l of isoleucine, and 0.8 g/l of lysine. 21799(pGC77) produced 15 g/l isoleucine, as well as about 0.5 g/l of lysine and 0.4 g/l of glycine. No threonine was detected in the supernatant at any point in the fermentation.

Table 5.19 Amino acids accumulated in culture medium by various 21799 recombinants

C. glutamicum strain[a]	Amino acid concentration in supernatant (g/l)[b]				
	lys	thr	hom	gly	ile
21799(pM2)	22.0 ± 1.0	< 0.1	< 0.1	< 0.1	< 0.1
21799(pGC42)	0.8 ± 0.1	11.8 ± 0.6	< 0.1	4.6 ± 0.2	1.9 ± 0.2
21799(pGC77)	0.4 ± 0.1	< 0.1	< 0.1	0.5 ± 0.1	15.1 ± 0.2

[a] 21799(pGC42) and 21799(pGC77) 100 ml cultures were induced by addition of 1.5 µmoles IPTG at approximately 30 hour intervals during the course of the fermentation.

[b] Amino acid concentrations are given at the point of glucose exhaustion from the medium.

5.4.3.4 Discussion

The first two enzymes in the threonine to isoleucine pathway (TDH and AHAS) in *C. glutamicum* have been shown to be limiting in isoleucine biosynthesis. However, threonine producers usually accumulate significant amounts of isoleucine as well, indicating that feedback inhibition in the isoleucine pathway in this organism may be partially overcome under conditions of high threonine production. In addition, the second enzyme in the pathway, AHAS, has been shown to be highly inducible in the presence of its substrate, α-ketobutyrate (Eggeling *et al.*, 1987).

Activity measurements of the two limiting enzymes (TDH and AHAS) in crude extracts of 21799(pGC77) yielded some insight into the results. Amplifying the *ilv*A gene in 21799 resulted in a five-fold increase in threonine dehydratase activity, which was not significantly affected by elevated concentrations of AKB in the growth medium. AHAS synthesis in *Corynebacterium glutamicum* was previously shown to be subject to multivalent repression by all three branched chain amino acids, as indicated by the fact that individual starvation of each amino acid resulted in increased levels of AHAS (Eggeling *et al.*, 1987; Scheer *et al.*, 1987). The 10-fold increase in AHAS activity observed in cultures grown with AKB has been attributed to the kinetic characteristics of the enzyme: A lower K_m for AKB compared to pyruvate would lead to a kinetic preferential partitioning of pyruvate to the isoleucine branch. This stripping of pyruvate precursor would cause an L-valine and L-leucine deficiency and derepress AHAS synthesis. This was further supported by later work which showed that growth in medium supplemented with AKB leads to an increased steady-state level of the *ilv*BNC transcript (corresponding to

AHAS and isomeroreductase, or IR, the next enzyme in the pathway; Keilhauer *et al.*, 1993). Enzyme activity data measurements showed an increase in acetohydroxy acid synthase activity in 21799 (pM2) cells grown with 50 mM AKB from 0.028 to 0.276 μmoles/min•mg protein, comparable to previous results in *Corynebacterium glutamicum* (from 0.021 to 0.200 μmoles/min•mg protein; Eggeling *et al.*, 1987). The effect of AKB on 21799(pGC42) cells was even greater, with AHAS activity increasing to about 0.4 μmoles/min•mg protein. However, AHAS activity in 21799(pGC77) was unaffected by growth in medium supplemented with AKB.

Isoleucine and valine are synthesized by parallel pathways (Figure 2.4) which are catalyzed by the same enzymes. Acetohydroxy acid synthase converts either two moles of pyruvate to α–acetolactate, or one mole of pyruvate and one of AKB to one of α–acetohydroxybutyrate. As mentioned above, the regulation of acetohydroxy acid synthase (also known as acetolactate synthase) is highly complex. Flow into the isoleucine pathway is controlled by the relative affinity and rate constants of acetohydroxy acid synthase for pyruvate and AKB. The K_m of this enzyme for pyruvate is 8.3 mM and for AKB is 4.8 mM. The V_{max} of AHAS with pyruvate is 0.37 μmol/min•mg prot, while with AKB it is 0.58 μmol/min•mg prot. (Eggeling *et al.*, 1987). In the presence of AKB, there is therefore a kinetic preferential partitioning of pyruvate towards isoleucine. This preferential partitioning is advantageous in view of the dual functionality of the enzymes leading to isoleucine and valine: it is not possible in this case to disrupt "competing" pathways leading to valine, since the same enzymes lead to isoleucine.

The results are consistent with the above picture of AHAS regulation

in *Corynebacterium*. Measurements of AHAS activity in *C. glutamicum* grown with 100 mM AKB and various combinations of one, two or all three of the branched chain amino acids showed that valine and leucine, but not isoleucine, abolished the promotive effect of AKB on AHAS synthesis (Eggeling *et al.*, 1987). The Leu- phenotype of *C. lactofermentum* 21799 has been confirmed in growth studies (see section 5.1.2), so any cancelation of the promotive effect of AKB may be presumed to be due to valine. It is then possible that the three-fold increase in AHAS activity observed sufficed to increase the flux of pyruvate into valine, leading to repression of any further AHAS synthesis (Figure 2.4). The complexity of the multivalent repression observed could certainly result in a dynamic situation in which AHAS activity may vary widely throughout the cell's growth. Moreover, AHAS is weakly inhibited by all three branched-chain amino acids (Tsuchida and Momose, 1975), which may further affect the *in vivo* activity of this enzyme under production conditions.

The main isoleucine degradation pathway for isoleucine is transamination back to its immediate precursor, α-oxo-β-methyl valeric acid, by a non-specific aminotransferase (Figure 5.26), and subsequent oxidative carboxylation and condensation with CoA. Several subsequent reactions result in the formation of acetyl CoA and propionyl CoA. Since disruption of the final isoleucine synthesis step is not desired, we need to examine the equilibrium of this reaction to gauge the importance of the reverse reaction. Under standard conditions, this reaction has a standard Gibbs free energy (ΔG°_{Rx}) of 0 kcal/mol, indicating a reversible reaction. However, intracellular metabolite analysis shows that *in vivo* concentrations of glutamate in *Corynebacterium* throughout cell growth remain high (50-100 mM, see section 5.7; >150 mM, Ebbighausen *et al.*,

1989a). This high glutamate concentration shifts the equilibrium towards isoleucine, which is then excreted, presumably by an active efflux carrier system (Ebbighausen *et al.*, 1989a). This excretion system was hypothesized in view of data which contradicted simple reversal of the uptake system (a secondary active Na+-coupled symport mechanism; Ebbighausen *et al.*, 1989b). Ebbighausen *et al.* also showed that isoleucine concentration in the cytosol exceeds the extracellular concentration of isoleucine during the early part of an isoleucine fermentation from α-ketobutyrate. Towards the latter part of the fermentation, however, isoleucine concentration in the supernatant exceeds the cytosolic concentration. These results are consistent with the results of this work (section 5.7). The complexity of isoleucine uptake and excretion systems warrants careful monitoring of intracellular isoleucine concentrations under different experimental conditions to determine if manipulation of the transport systems is necessary for yield improvement.

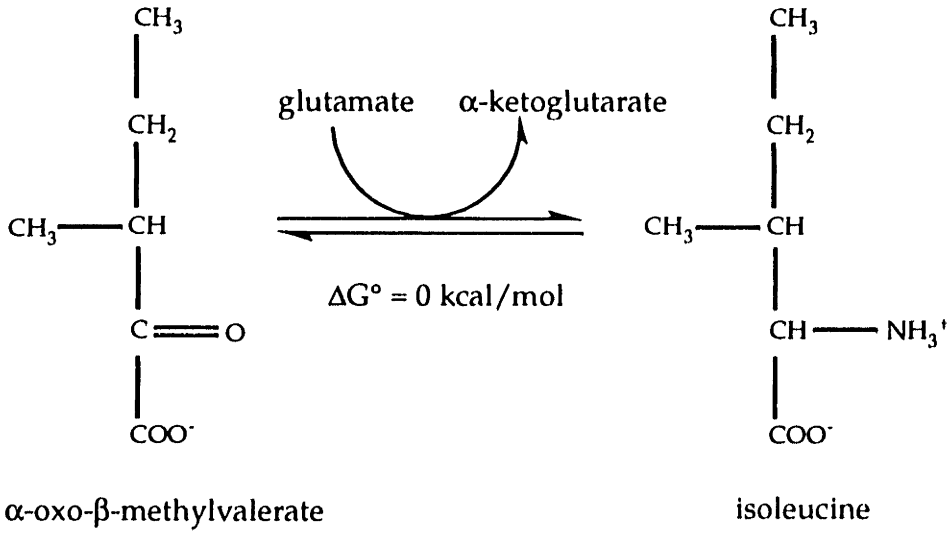

Figure 5.24 Final isoleucine biosynthetic reaction

Fermentations with 21799(pGC77) result in a much lower glycine titer than those with 21799(pGC42), indicating that threonine flows preferentially to the isoleucine pathway rather than to the glycine-producing pathways due to the increased specific activity of TDH in the former.

A molar carbon balance comparing the flow of carbon into different aspartate family amino acids in *C. lactofermentum* strains suggests that most of the carbon available for the threonine pathway in *C. lactofermetum* 21799 (pGC42) has been converted into isoleucine (Figure 5.25). Thus, in

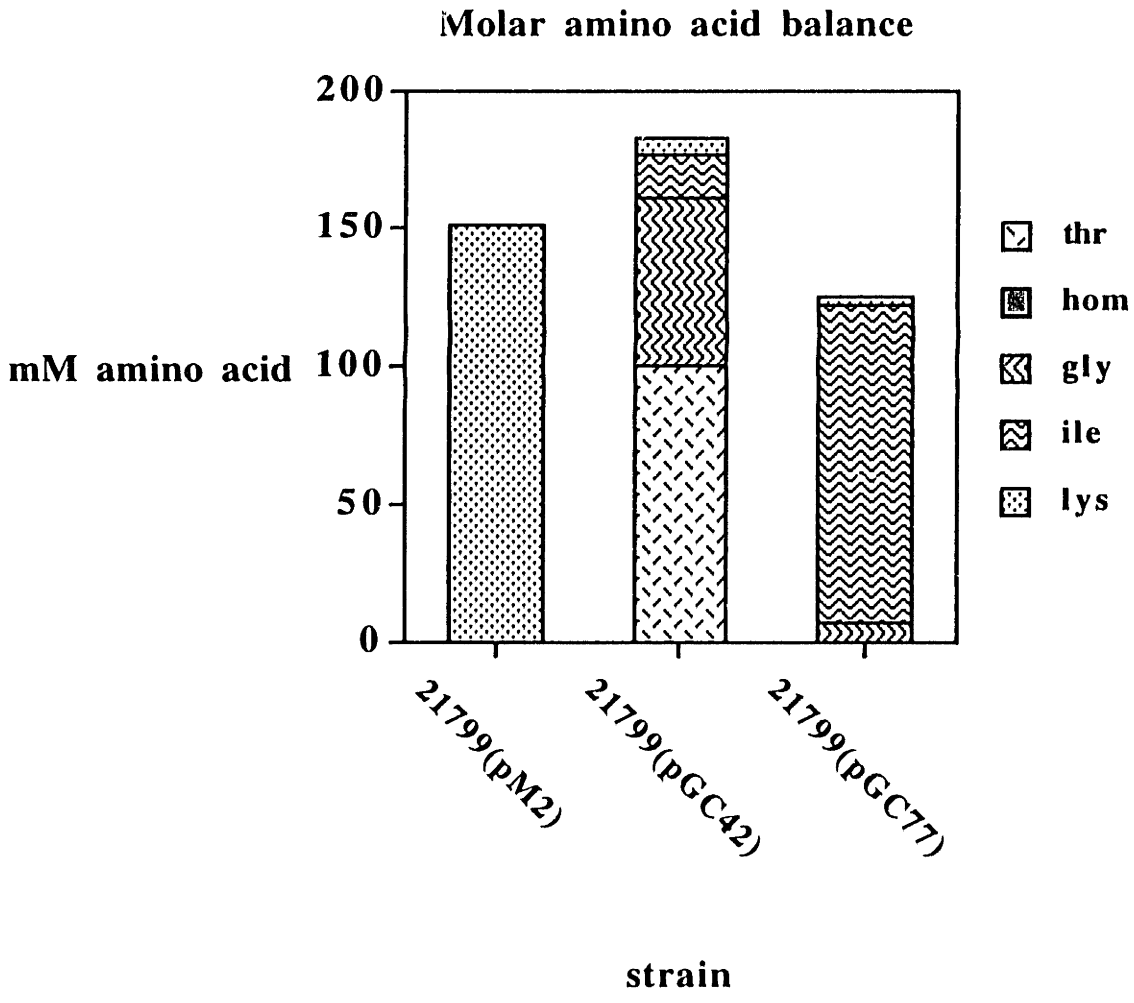

Figure 5.25 Molar amino acid balance in various 21799 strains.

this strain, approximately 80% of the carbon available for the threonine pathway was converted to isoleucine. Any improvements achieved with regards to metabolic engineering of the central carbon metabolism of *Corynebacterium* may in the future lead to increased carbon flux to aspartate. Combining the downstream results with increased aspartate availability from oxaloacetate could lead to a closer approach to maximum theoretical yield.

Production of L-isoleucine in *Corynebacterium glutamicum* by fermentation from glucose has also previously been achieved by isolating mutants resistant to isoleucine analogues and related compounds (Kase and Nakayama, 1977). Threonine dehydratase in these mutants is no longer inhibited by the end-product, isoleucine, and thus a high carbon flux through this pathway is supported. In contrast, this work has shown that high isoleucine production is possible by simply amplifying the wild-type threonine dehydratase gene in a threonine producer.

5.5 Extension of threonine productivity by a fed-batch strategy

Threonine degradation begins in the middle of the fermentation, prior to glucose exhaustion. Intracellular threonine reaches very high levels, driving degradation reactions forward to glycine and isoleucine. Upon the point of glucose exhaustion, there is a sharp decrease in extracellular threonine, implying threonine uptake and subsequent degradation. As discussed in detail in section 5.4.2, the cellular infrastructure capable of supporting growth (or at least maintenance) on threonine exists. Therefore, it was of interest to investigate the potential sparing effect of glucose on threonine uptake and degradation. This information is also useful for scale-up strategies to a large fermentation.

A shake flask fermentation with *C. lactofermentum* 21799(pGC42) was run with the objective of mimicking a "fed-batch" scenario, in which additional glucose is added upon the point of glucose exhaustion. Figure 5.26 compares growth, glucose consumtion and threonine, glycine and isoleucine production in the base case with the case in which an addition 20 g/l glucose were added at the point of glucose exhaustion. The glucose in this case was quickly used up, and a reduction in extracellular threonine was not observed until the additional glucose was consumed. Threonine concentration reached up to 16 g/l, compared to 12 g/l in the base case (30% molar yield on glucose vs. 26%). Glycine and isoleucine accumulation and growth profiles were virtually identical in both cases, indicating that availability of glucose seemed to prevent loss of carbon through threonine degradation.

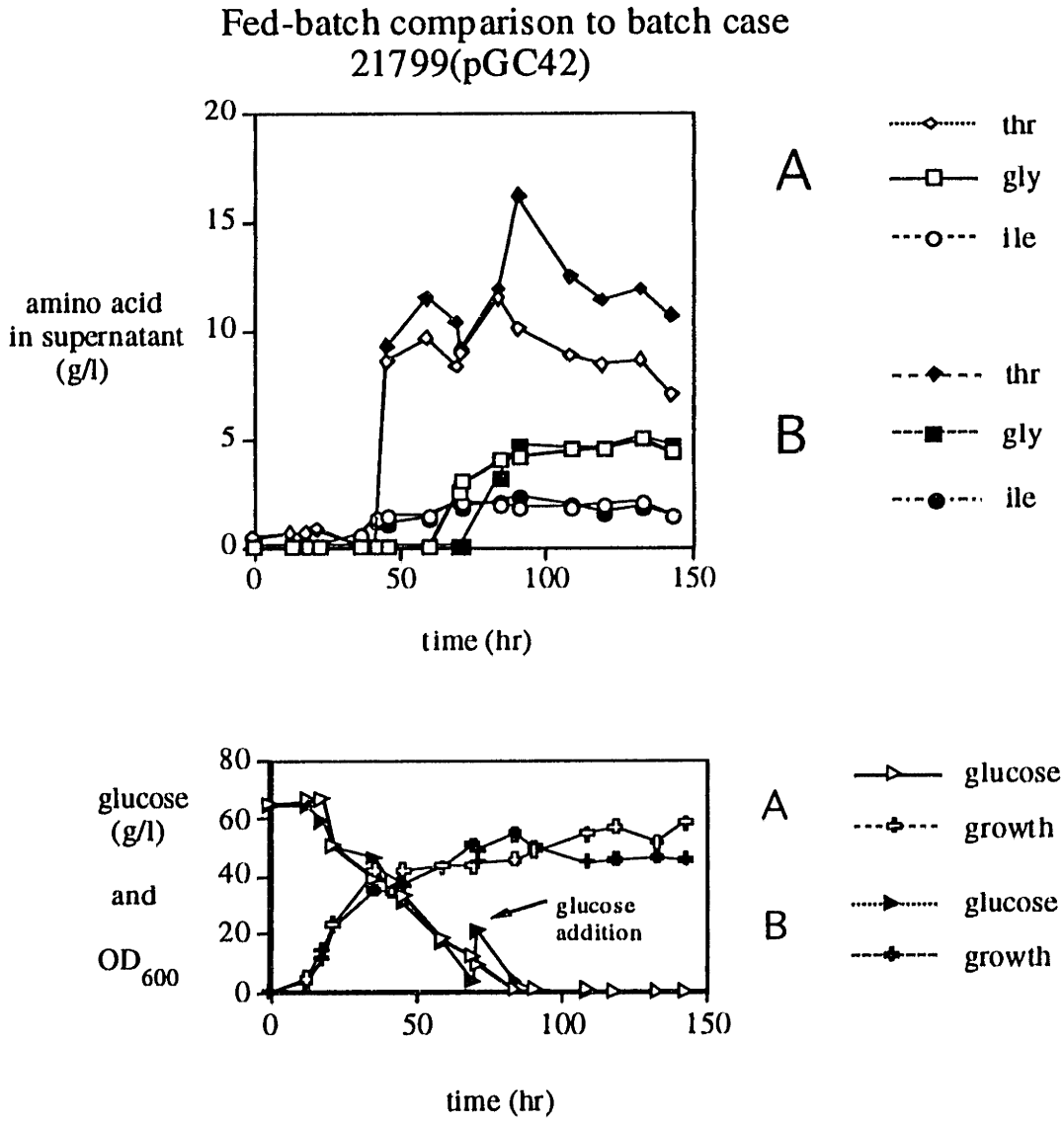

Figure 5.26 Comparison of amino acid accumulation, growth and glucose consumption in *C. lactofermentum* 21799(pGC42) in the base shake flask case and in a simulated "fed-batch" case in which 10 g/l glucose were added at the point of glucose exhasution.

5.6 Intracellular metabolite measurements

A batch fermentation was carried out using the strain *C. lactofermentum* 21799 (pGC42) in order to obtain intracellular metabolite concentration values in a reactor. As the main focus of this thesis was not fermentation control strategies but rather metabolic engineering for strain development, further optimization of the fermentation was not carried out. The data obtained, however, may be used as a basis for future work on large-scale threonine fermentations. Valuable insight was gained from estimates of intracellular metabolite concentrations. A 3-liter working volume Microferm fermentor was used. For a description of the reactor, medium and conditions used, see Chapter 4.

Because the cell density, oxygen level, and general conditions are so different from those in a shake flask, it was unclear what IPTG induction levels would be necessary to achieve sufficient homoserine kinase activity for optimal threonine production. Therefore, the culture was induced by addition of 300 µl 100 mM IPTG at the onset of exponetial phase (t = 40 hr.). Measurement of enzyme activities 8 hours later showed that homoserine kinase activity was extremely low (0.5 µmoles/(min•mg prot)). The cells were induced once more during mid-exponential phase with the addition of 350 µl of 100 mM IPTG.

Figure 5.27 shows glucose consumption as a funcion of time during the fermentation. Figure 5.28 shows a plot of cell growth vs. time, measured by both absorbance at 600 nm and dry cell weight. There is an excellent correlation between the two types of measurement. The long lag phase exhibited could be the result of too low of an inoculum/fermentor ratio.

The threonine yield and productivity were significantly reduced compared to the shake flask (only up to approimately 3 g/l of threonine were excreted). The activities of HD and HK were measured during the fermentation and averaged 7.3 μmoles/(min•mg prot) and 1.0 μmoles/(min•mg prot), respectively. Although this activity level is

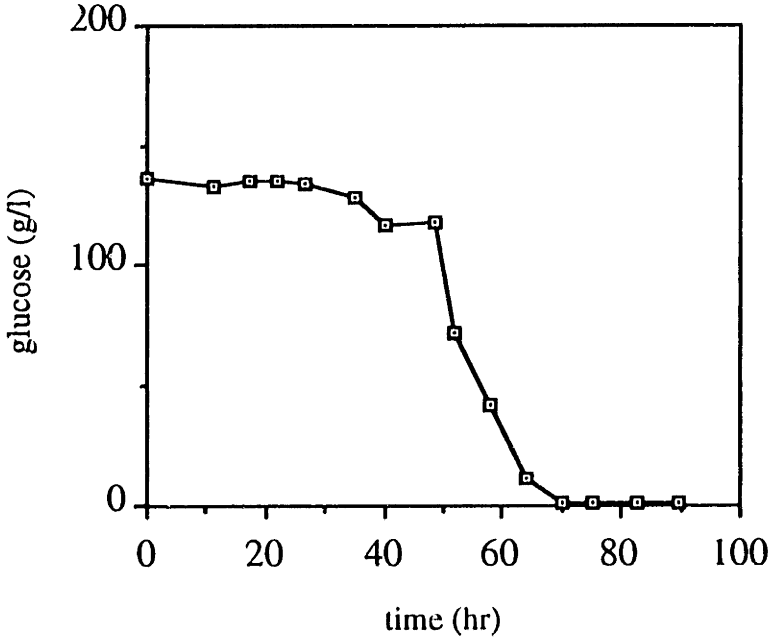

Figure 5.27 Glucose consumption as a function of time during batch fermentation with *C. lactofermentum* 21799(pGC42).

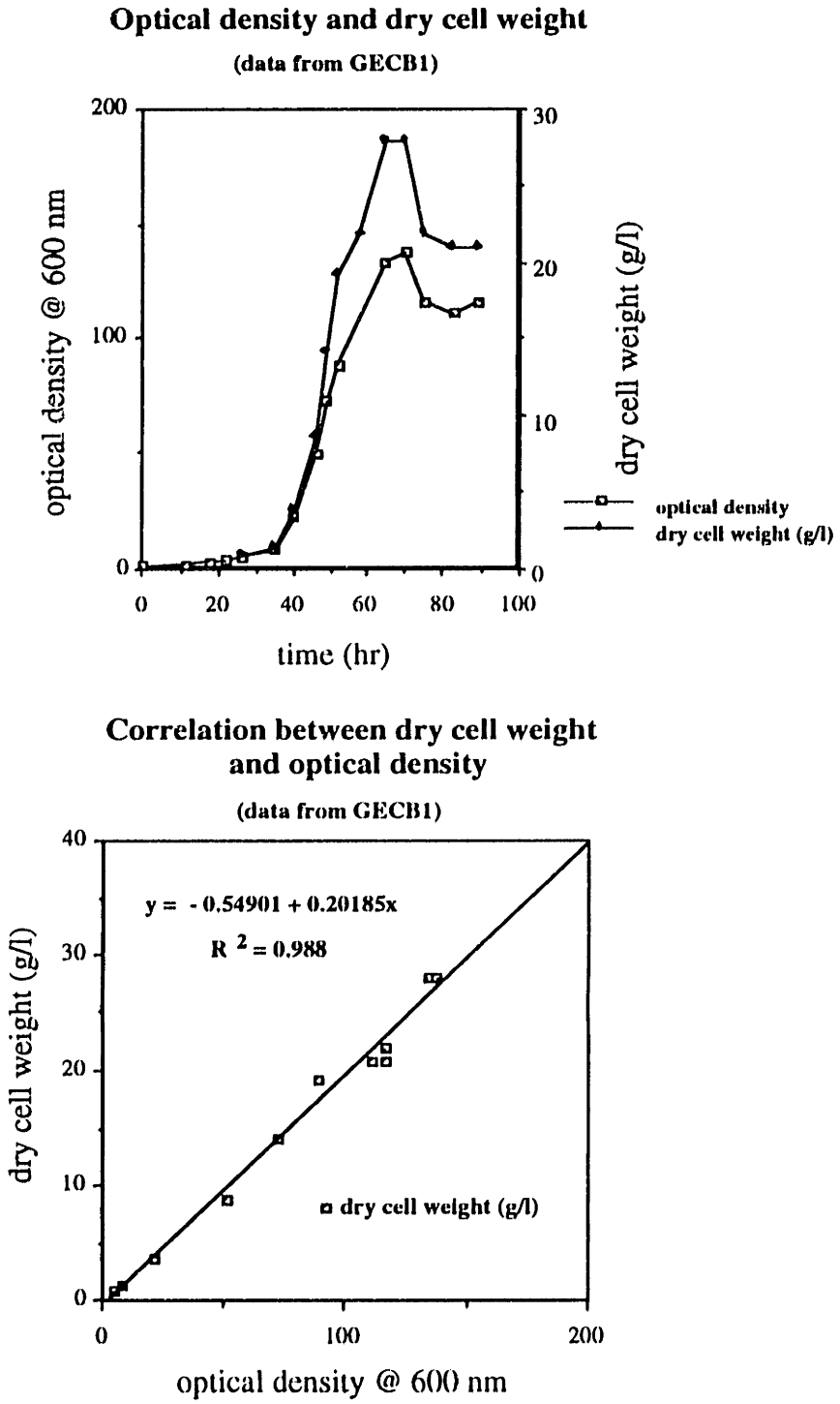

Figure 5.28 a-b. Optical density at 600 nm and dry cell weight as a function of time during batch fermentation with *C. lactofermentum* 21799(pGC42)(above). Correlation between the two (below).

typical for HD at the shake flask level, homoserine kinase activity was considerably lower than that achieved in shake flask fermentations. This factor may have contributed to low overall carbon flux into the pathway. Molar yield on glucose was only 3.3%, considerably lower than the optimal yields obtained in shake flask fermentations (Section 5.7).

Intracellular metabolite concentrations were monitored from the point at which cell density was high enough to obtain a meaningful sample. Intracellular and extracellular profiles of threonine, homoserine and isoleucine are compared in Figures 5.29-5.30. No glutamate was detected extracellularly, but the intracellular level remained between 60-90 mM during the measurement period (Figure 5.31), consistent with the literature (Chapter 2). There is very high accumulation of threonine and homoserine during the fermentation, which drops sharply at the point of glucose exhaustion from the medium (approx. 70 hr.); this is consistent with a physiological switch to threonine degradation for metabolic maintenance (Section 5.4.2.3). Internal threonine and homoserine concentrations reached up to about 150 mM and 100 mM, respectively. Internal isoleucine concentration peaked at about 80 mM late in the fermentation, before sharply dropping to about 0 at the point of glucose exhaustion.

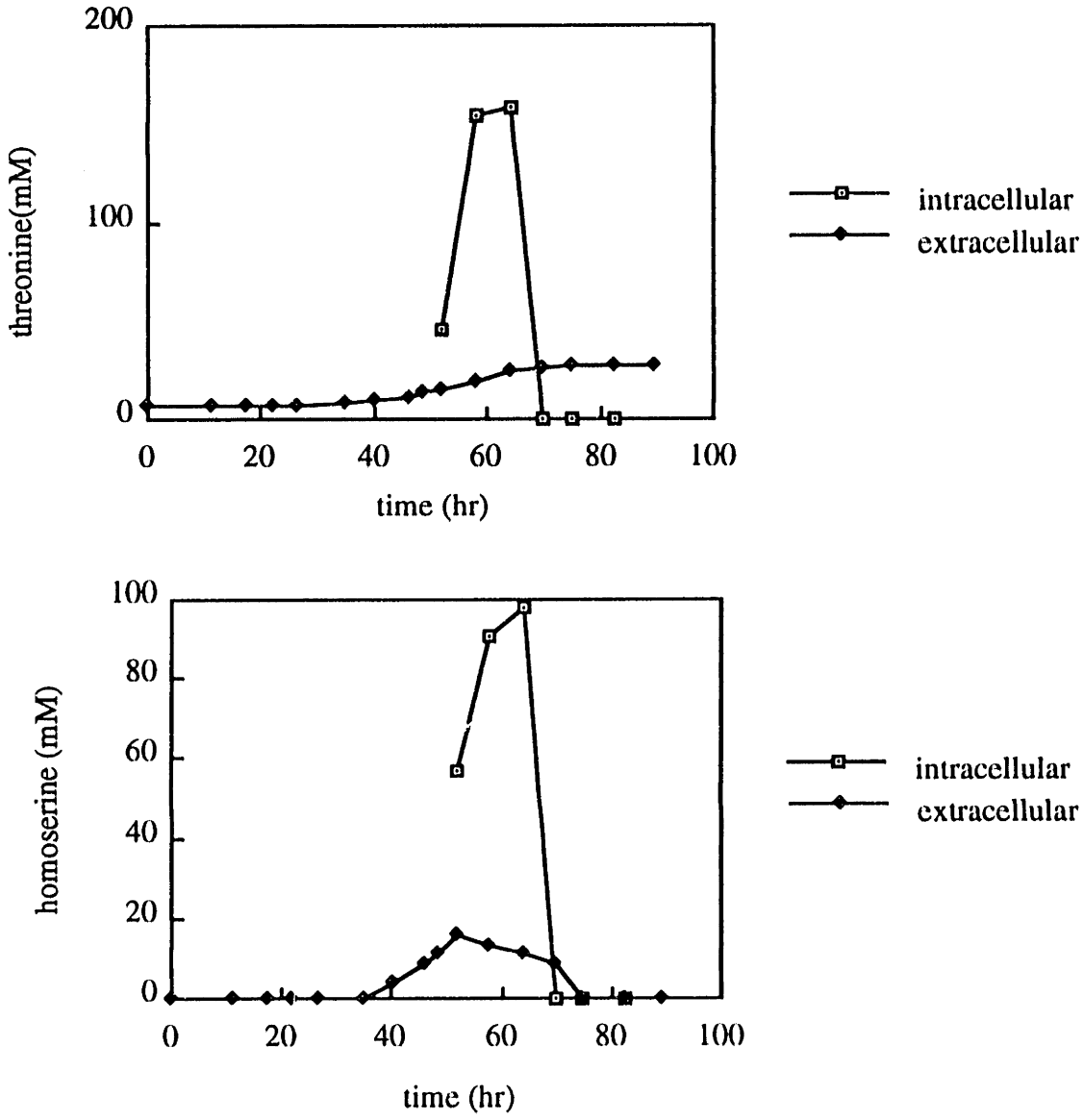

Figure 5.29 a-b. Comparison of intracellular and extracellular accumulation of threonine (above) and homoserine (below) during batch fermentation with *C. lactofermentum* 21799(pGC42).

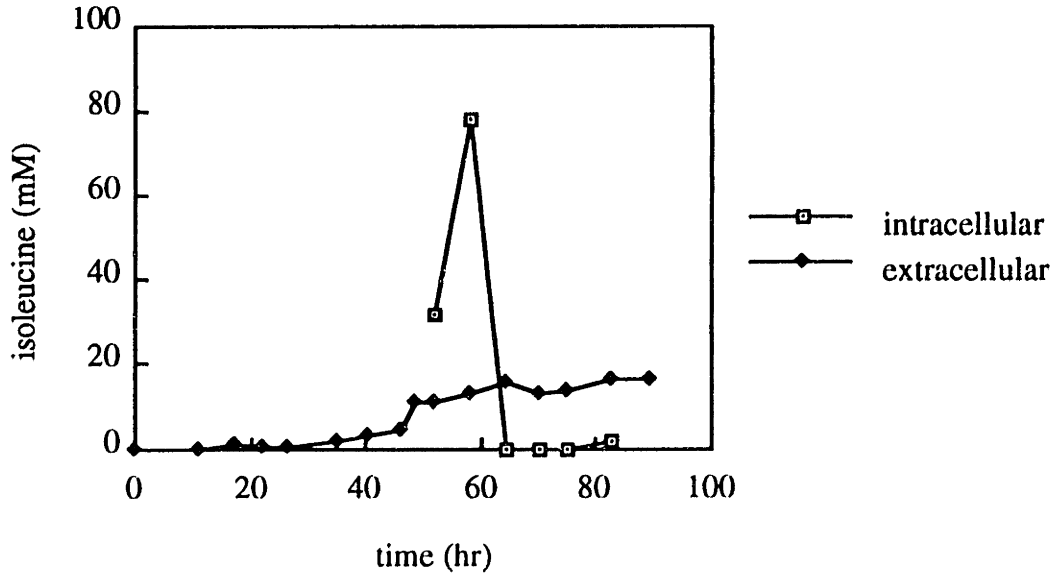

Figure 5.30 Intracellular and extracellular accumulation of isoleucine during batch fermentation of *C. lactofermentum* 21799(pGC42).

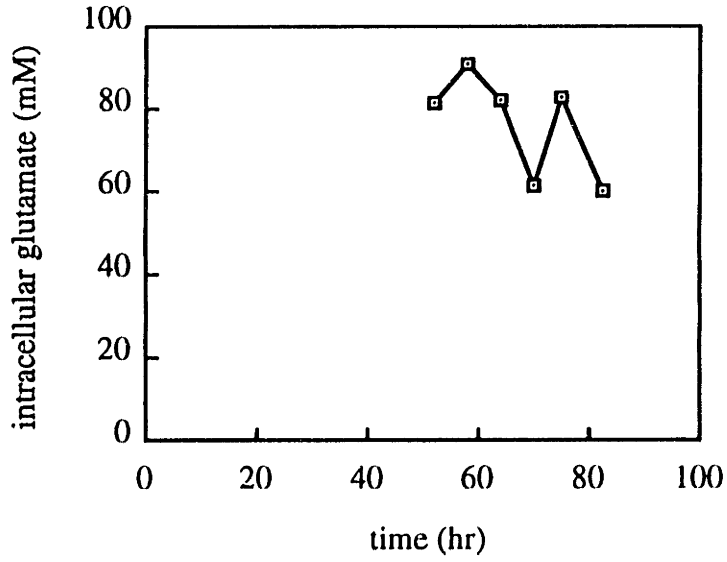

Figure 5.31 Intracellular accumulation of glutamate during batch fermentation of *C. lactofermentum* 21799(pGC42).

In order to test whether the high ratios of intracellular to extracellular levels of threonine and homoserine were maintained under different conditions, a 21799 (pGC42) shake flask in typical fermentation medium was run. To encourage homoserine accumulation, no IPTG was added. Intracellular and extracellular values of threonine and homsoserine and shown in Table 5.20. Samples were taken at t = 43 hr. and t = 68.5 hr., corresponding to mid-exponential and early stationary phases of the culture, respectively. Internal threonine levels reached 87-91 mM, while homoserine levels reached up to 232 mM, later dropping to 137 mM. During mid-exponential phase, the intracellular/extracellular ratios of both homoserine and threonine were approximately 11, and dropped to 3.3 and 4.6, respectively. These high ratios of intra to extracellular threonine are expected; the fact that the homoserine ratios are similar is not inconsistent with the possibility of a transport system capable of recognizing both. Further studies on these phenomena were not undertaken in the course of this work, but may be important for future work on threonine production in *Corynebacterium*.

Table 5.20 Extracellular and intracellular accumulation of threonine and homoserine in a 100 ml minimal medium culture of *C. lactofermentum* 21799(pGC42).[a]

	Extracellular		**Intracellular**		
	(mM)	(g/l)	(mM)	(g/l)	**Ratio (Int./Ext.)**
homoserine					
43 hr	21	2.5	232	27.6	11.0
69 hr	42	5.0	137	16.3	3.3
threonine					
43 hr	8	1.0	87	10.3	10.8
69 hr	20	2.4	91	10.8	4.6

[a] No IPTG was added to the flasks.

5.7 Comparison of theoretical and experimental yields

The maximum shake flask yields of threonine and isoleucine on glucose are first compared with the maximum theoretical yields, and then corrected with the numbers for glucose conversion into biomass. In the shake flask fermentations the optical density at 600 nm typically reaches 50-60, or about 15 g/l (for correlation, see section 5.6). Using the value of intrinsic biomass yield on glucose for *Corynebacterium* of $Y_{B/G} = 0.64$ (Kiss, 1991), this means approximately 23 g/l glucose were used for biomass production, leaving 57 g/l for product synthesis (maintenance requirement is neglected for this estimate). The subsequent estimate further constrains the calculation by using the low end estimate of aerobic biomass production due to the relatively poor aerobic nature of shake flask fementations. A value of $Y_{B/G} = 0.4$ is used, which leaves 42.5 g/l glucose for product synthesis.

Threonine

The maximum experimental yield was 0.22 mol thr/mol glu, or 17.5% of maximum theoretical yield based on the detailed stoichiometric approach.

Subtracting for biomass production ($Y_{B/G} = 0.64$):

$Y_{T/G} = 0.31$ (24% of maximum theoretical yield)

Subtracting for biomass production ($Y_{B/G}$ = 0.4):

Y = 0.41 (32% of maximum theoretical yield)

Isoleucine

In our shake flask fermentation, the molar yield of isoleucine on glucose was approximately 0.24, or 34% of the maximum theoretical yield.

Subtracting for biomass production ($Y_{B/G}$ = 0.64):

Y = 0.36 (51% of maximum theoretical yield)

Subtracting for biomass production ($Y_{B/G}$ = 0.4):

Y = 0.49 (69% of maximum theoretical yield)

It is significant that a much closer approximation to maximum theoretical yield is reached for isoleucine than for threonine. A likely explanation is the incorporation of one mole of pyruvate into the pathway. There is a strong drive in central carbon metabolism towards pyruvate formation and subsequent utilization in the TCA cycle. For threonine production, this pyruvate is wasted carbon, unless it can be converted to PEP and subsequently aspartate through oxaloacetate (see section 3.1). For isoleucine production, however, one mole of pyruvate is incorporated at the acetohydroxy acid synthase step, thereby recovering that carbon for product formation.

Chapter 6. Conclusions and Recommendations

6.1 Summary

The lysine producer *Corynebacterium lactofermentum* ATCC 21799 was used in these studies. This strain is resistant to *S*-2-aminoethyl-L-cysteine ((AEC), a lysine analogue) due to an aspartokinase which is not significantly inhibited in the presence of lysine and threonine. Therefore, there is a high flux of carbon through the aspartate node, resulting in high lysine titer in this strain. This flux was redirected to the threonine and isoleucine pathways in a series of genetic manipulations at three critical nodes in the threonine metabolic pathway.

Node 1 - Aspartic-β-semialdehyde (ASA):

• Amplification of the operon containing a feedback-insensitive homoserine dehydrogenase gene and a wild-type homoserine kinase gene in a lysine-producing strain resulted in both homoserine and threonine accumulation, with some residual lysine production. In addition, there was significant accumulation of the threonine degradation products, isoleucine and glycine. It was postulated that homoserine accumulation could be due to competitive inhibition of homoserine kinase activity by threonine. Homoserine kinase kinetic parameters (K_m, V_{max}) were determined and characterization of threonine inhibition kinetics with respect to homoserine confirmed the competitive nature of this phenomenon.

Node 2 - Homoserine:

• A plasmid enabling separate transcriptional control of the genes encoding homoserine dehydrogenase and homoserine kinase was constructed to determine the effect of varying enzyme activity ratios on metabolite accumulation. By using this plasmid (pGC42) to increase the activity of homoserine kinase, homoserine accumulation in the medium was essentially eliminated and the final threonine titer was increased by about 120%. Furthermore, a fortuitous result of the cloning strategy was an unexplained increase in homoserine dehydrogenase activity. This resulted in a further decrease in lysine production along with a concomitant increase in threonine accumulation. Therefore, to achieve threonine accumulation using a lysine producer, the carbon flux must be redistributed away from lysine biosynthesis at the aspartic-ß-semialdehyde (ASA) branchpoint and the activities of enzymes in the pathway must be coordinated to minimize accumulation of intermediates such as homoserine.

Node 3 - Threonine:

• Threonine degradation products, notably glycine and isoleucine, begin accumulating early in the threonine fermentation process and detract from process yield. There are three enzymatic routes to threonine degradation in *Corynebacterium*: threonine dehydratase, threonine dehydrogenase and serine hydroxymethyltransferase. Threonine dehydratase initiates a five-step pathway from threonine to isoleucine, and is feedback-inhibited by isoleucine. Obviously, this inhibition did not

suffice to prevent isoleucine accumulation in our threonine producers. The other two enzymes lead to glycine production from threonine.

• A stable threonine dehydratase disruption mutant (*C. lactofermentum* 21799 *ilv*A-) was constructed by marker exchange mutagenesis. Fermentations using pGC42 in the mutant resulted in reduced isoleucine production and higher glycine production, with no improvement in final threonine titer. Preliminary kinetic analysis of the two enzymes leading to glycine production from threonine shows that threonine dehydrogenase has a lower K_m for threonine and a higher specific activity than serine hydroxymethyltransferase in this strain. This suggests that threonine dehydrogenase may be the most important immediate target for disruption.

• Isoleucine production was achieved in 21799 by modification of pGC42 to include the gene encoding threonine dehydratase, forming the plasmid pGC77. A molar carbon balance indicated that most of the carbon flow into the threonine branchpoint was directed to isoleucine in this organism. The next likely target for amplification is the second enzyme in the pathway, acetohydroxy acid synthase. This enzyme is both inhibited and repressed by all three branched-chain amino acids, and is highly inducible by its substrate, α-ketobutyrate.

• Yield calculations based on elemental balances provided information on maximum theoretical yield on glucose for both threonine and isoleucine. Respiratory quotient and theoretical yield as a function of

moles of oxygen consumed per mole of product formed were also calculated to provide insight into possible fermentation control strategies

• Yield calculations based on detailed biochemical pathways, cofactor regeneration and energy requirements were performed for two cases for both threonine and isoleucine, and can be extended for any possible metabolic scenario (Note: part of this work was first presented in the thesis proposal).

• Carbon balances around the aspartate amino acid pathway based on experimental results were performed to examine the extent of flux redirection.

• Thermodynamic analysis based on the method of Mavrovouniotis was used to explore possible bottlenecks in the pathway from aspartate to threonine and its degradation products.

6.2 Conclusions

Perturbations performed on three critical nodes in the aspartate-derived amino acid pathway resulted in dramatic shifts in carbon flux in the pathway. Carbon entering the aspartate amino acid family in a lysine producer has been almost completely redirected to threonine and isoleucine by using these perturbations.

• carbon flux can be essentially completely redirected from lysine pathway to threonine pathway at the ASA branchpoint by an increase in the activity of feedback-insensitive homoserine dehydrogenase

• disruption of *ilv*A (the gene encoding threonine dehydratase) in 21799(pGC42) results in higher glycine titer and no improvement in threonine titer

• preliminary kinetic analysis of threonine degradation enzymes leading to glycine suggests that threonine dehydrogenase may be most important immediate target for disruption

• amplification of *ilv*A in 21799(pGC42) results in high isoleucine titer

• extension of shake flask fermentation by glucose addition results in improved yield

• theoretical yield analysis generated information regarding fermentation control strategies

• thermodynamic analysis specified possible bottlenecks in pathway

6.3 Recommendations

It is possible that an increase in the flow to aspartate would translate into an increased flow into threonine and isoleucine. Any improvements achieved with regards to metabolic engineering of the central carbon metabolism of *Corynebacterium* may in the future lead to increased carbon flux to aspartate. It is also possible that these new conditions would reveal new limiting steps. However, we have seen that a combination of analytical, kinetic and genetic tools have helped overcome flux limitations.

Several recommendations are presented concerning further perturbations in the threonine and isoleucine pathways for improved production of these amino acids. In addition, a generalized methodology for metabolic engineering of amino acids in *Corynebacterium glutamicum* developed from the results of this work is outlined.

6.3.1 Threonine production

The perturbations performed on the first two nodes (ASA and homoserine) resulted in a smooth shift of carbon from the lysine pathway to threonine. However, accumulation of threonine degradation products contributed to a less than optimal yield. Although the construction of a threonine dehydratase disruption mutant resulted in reduced production of isoleucine, glycine production was enhanced. Kinetic analysis of the glycine-producing enzymes suggested that threonine dehydrogenase may be the enzyme primarily responsible for glycine production from threonine. However, at high intracellular threonine concentrations, serine hydroxymethyltransferase may be responsible for a significant portion of

the threonine to glycine flux. Therefore, isolation and characterization of these genes would be useful for construction of mutants blocked in these pathways. Presumably, the absence of these enzymatic activities would lead to increased threonine excretion rather than degradation.

Escherichia coli mutants lacking these enzyme activities are available; a strategy for isolating the *C. glutamicum* genes by heterologous complementation is one possibility. Alternatively, degenerate primers based on these genes cloned in other bacteria could be used to attempt to isolate the *C. glutamicum* genes by the polymerase chain reaction (PCR).

6.3.2 Isoleucine production

Most of the carbon entering the aspartate pathway was succesfully redirected to isoleucine. However, it is likely that to achieve high isoleucine production, the two limiting steps in the isoleucine pathway need to be removed. Threonine dehydratase and acetohydroxy acid synthase, are both subject to regulation, the former at the protein level and the latter at both the gene and protein level.

Isoleucine analogue-resistant mutants which contain feedback-insensitive threonine dehydratases have been derived from *Corynebacterium glutamicum* (Kase and Nakayama, 1977). Analogues used include thiaisoleucine, ethionine, 4-azaleucine and α-aminobutyric acid. It is thus possible to isolate a feedback-insensitive threonine dehydratase mutant and incorporate the corresponding *ilv*A^{dr} allele into an isoleucine producer.

Tsuchida and Momose (1986) isolated mutants of *C. lactofermentum* which were resistant to β-hydroxyleucine. These mutants contained an

AHAS which was released from inhibition by not only leucine, but of valine and isoleucine as well. These results show that it is likely that analogues of all three branched-chain amino acids may be used to isolate a feedback-insensitive enzyme. Kinetic studies may be undertaken to determine the best choice based on ability to inhibit the activity of the enzyme.

One mole of pyruvate and one mole of phosphoenolpyruvate can be generated with each glucose molecule that is transported into the cell, due

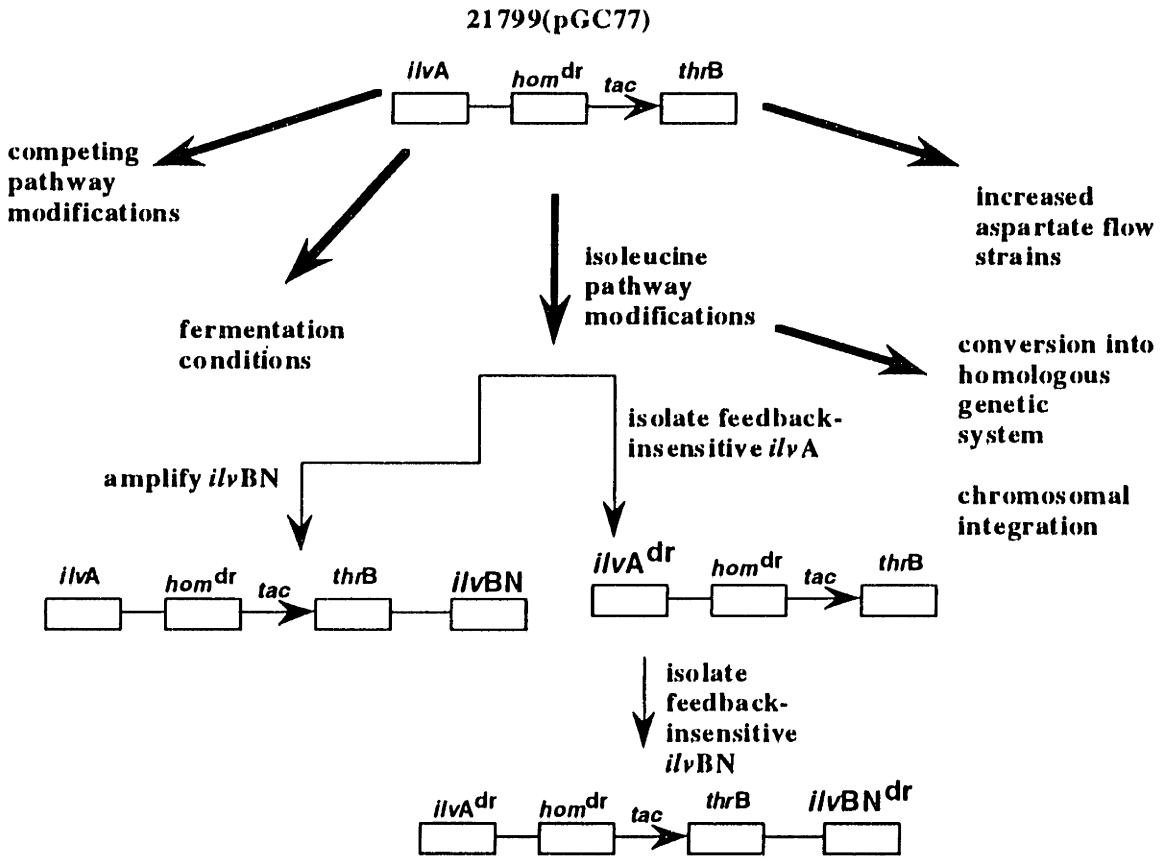

Figure 6.1 Flowsheet for metabolic engineering of isoleucine production.

to the PTS-glucose transport system. These are the exact precursors needed for one mole of isoleucine. Therefore, for optimal isoleucine yield, it is important to minimize competing pyruvate-consuming reactions. This is analogous to the lysine paradigm, where the objective is to recapture as much of the PEP carbon lost to TCA cycle reactions through pyruvate.

Amplification of the AHAS gene, *ilv*BN (either native or deregulated), would presumably capture a higher percentage of the pyruvate available in the cell and redirect it to isoleucine. In order to favor production of isoleucine over the other two branched-chain amino acids, a high flux of α-ketobutyrate will be required. This can be achieved by amplification of the *ilv*A^{dr} allele.

The competing pathways at the threonine node are the ones leading to glycine. Although there was negligible accumulation of glycine in 21799(pGC77), it is possible that this would become more substantial in a higher isoleucine producer. Isolation of *gly*A- and *tdh*- mutants for threonine production would also be useful for isoleucine production.

6.3.3 Generalized methodology for metabolic engineering of amino acids in *C. glutamicum*

Figure 6.2 outlines the general experimental approach to metabolic engineering of threonine and isoleucine production in *Corynebacterium glutamicum*. Using the tools developed for this organism (see section 2.4 - recombinant DNA technology for *Corynebacterium*), a series of steps are taken, many of them simultaneously, to generate an increase in carbon flux towards the desired product.

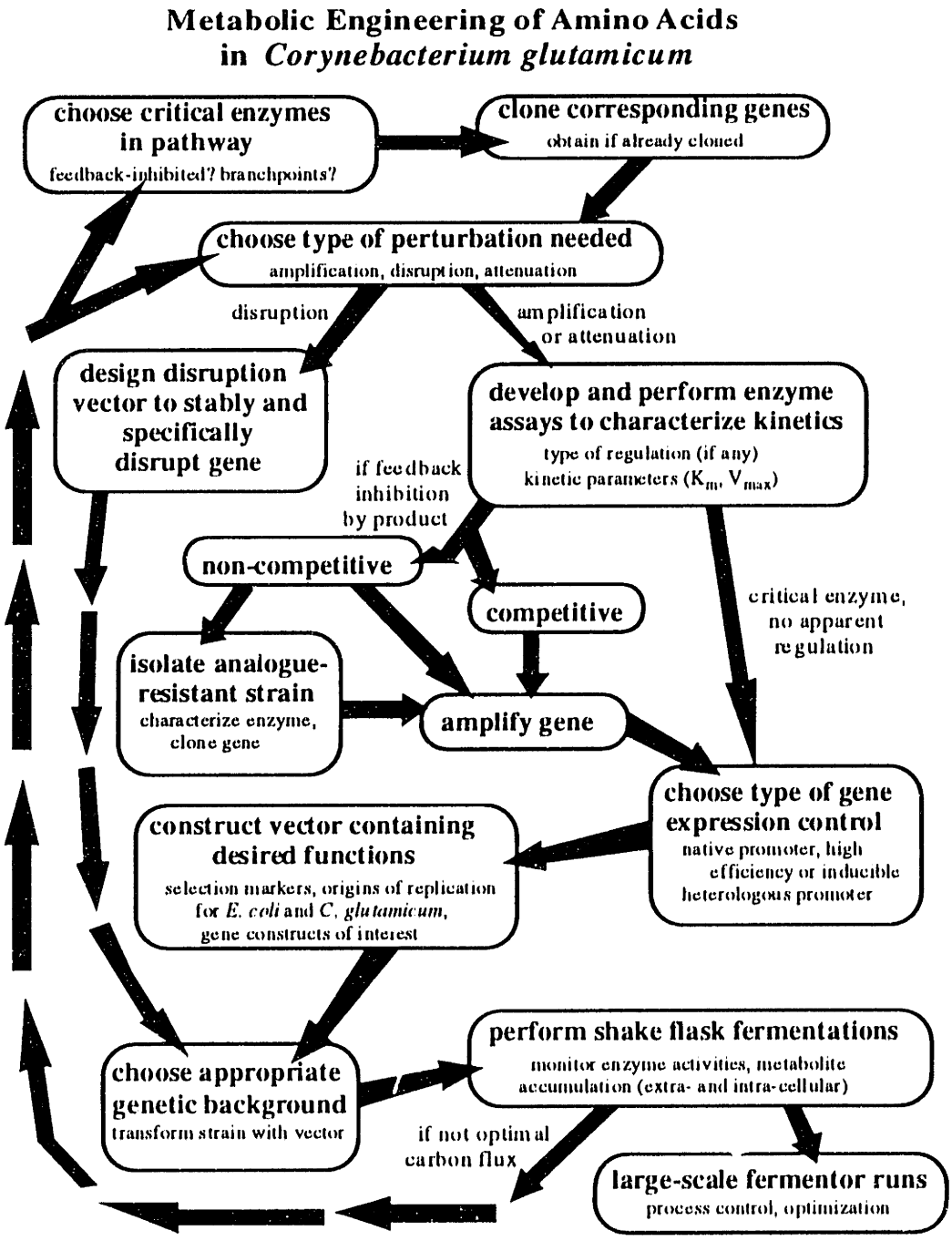

Figure 6.2 Generalized methodology for metabolic engineering of amino acids is *Corynebacterium glutamicum.*

(1) Choose critical enzymes in pathway

Generally, pathway steps that have been defined to be rate-limiting in amino acid metabolism are controlled by feedback inhibition by a final or intermediate product of that pathway. Another type of critical step is at a branchpoint, where vying enzymes compete for carbon to be directed towards their final product. Branchpoint enzymes may or may not be feedback inhibited by their final product; in either case, these are by definition critical because they initiate the flow into a pathway and compete with each other for carbon.

(2) Clone or otherwise obtain corresponding genes

Many genes of interest in the amino acid pathways of *Corynebacterium glutamicum* have been cloned. Techniques have been developed to clone new genes either by a homologous or heterologous systems (see Chapter 2).

(3) Decide on perturbation to be performed

(3a) Amplification

To increase the carbon flow towards a desired product, a critical gene in that pathway can be amplified to increase the *in vivo* activity of the corresponding enzyme. Alternatively, the expression of the enzyme can be more precisely controlled to obtain a desired *in vivo* activity (see 3ab). If there is transcriptional regulation of the

gene (repression or attenuation), this can sometimes be overcome by replacing the native promoter with another, unregulated one or by a synthetic, heterologous promoter (also 3ab).

(3aa) Characterize kinetics

Enzyme assays need to be developed and/or tailored to the system to elucidate the kinetic behavior of the enzyme (K_m, V_{max}, regulation). If the enzyme is feedback-inhibited by an intermediate or product, the type of inhibition should be identified. Although there are many types of inhibition, in general they can be classified as competitive and non-competitive.

(3aaa) Competitive inhibition

If the inhibition is of a competitive nature, the probability of isolating a mutant in which the binding site is altered such as the inhibitor will not bind but the substrate will is very low. Therefore, the strategy is to increase the expression of the gene so that the resultant activity is high enough even with the inhibition to maintain a high flux of carbon through that step.

(3aab) Non-competitive

If the inhibition is of a non-competitive nature, amplification of the gene may suffice to increase the *in vivo* activity enough to overcome the inhibition (3aaa). A simultaneous attempt to isolate an analogue-resistant version of the enyzme, and subsequently cloning the allele may further increase the flux through that step.

(3ab) Choose type of gene expression control

Once the desired gene has been chosed and the kinetics have been characterized, the type of gene expression control can be chosen based on those results. The native promoter may prove to be adequate, or if extremely high activity is needed, a high efficiency and/or controllable promoter (such as the *tac* promoter) may be used.

(3ac) Construct vector containing desired functions

The vector will contain the specific gene constructs designed, origins of replication for both *E. coli* and *C. glutamicum* to facilitate manipulations, and selectable markers.

(3b) Disruption

To halt carbon flow through a competing pathway or a degradation pathway, the gene encoding those enzymes may be stably disrupted through the use of marker exchange mutagenesis.

(4) Choose appropriate genetic background

The ideal strain already possesses a high flow of carbon towards the pathways of interest.

(5) Perform shake flask fementations

Shake flask fermentations with the constructed strains are performed to gauge the effect of the genetic perturbation on carbon flow. Enzymatic assays, and analysis of intracellular and extracellular metabolite accumulation yield insight into the metabolic state of the strain. A preliminary carbon balance comparing the new strain with the control (not containing the vector, or non-disrupted) indicates whether any steps need to be fine-tuned. In that case, the next iteration can begin at step 1, 3, 3b, 3ab or any of the above.

(6) Perform large-scale fermentor runs

Once the strain has been optimized (constrained perhaps by biochemical steps not yet characterized), large-scale fermentor runs can be performed to obtain a better carbon balance, as well as to monitor the

metabolic state of the strain under more controlled conditions. Respiratory data may provide insight into other reactions detracting from yield (see section 3.1), and higher cell densities may drive the system closer to the maximum theoretical yield.

6.4 Significance and major contributions of this work

Although traditional genetic engineering techniques were used to bring about changes in carbon flux, it is the innovative approach taken in the analysis and decision-making process that is the major contribution of this research. The synthesis of information obtained from the fields of microbiology, kinetics, thermodynamics and biochemistry, among others, was used successfully to make specific alterations and analyze the results. There are several conclusions, methodologies and strategies of this work which are relevant to the field of metabolic engineering:

I. Importance of iterative approach.

This is a seemingly obvious yet important fact which has been confirmed by these studies. Due to the non-linear nature of the cellular responses, the results of a given perturbation are needed to design the next one. For example, the accumulation of homoserine led to investigation of the kinetics of homoserine kinase and the subsequent design of an inducible construct to greatly overexpress the gene encoding this enzyme. In the same manner, accumulation of the threonine degradation product isoleucine

led to strategies for both the elimination and overproduction of isoleucine using the gene encoding the first enzyme in the pathway.

II. Importance of enzyme kinetics in approach taken for ovecoming a limiting step.

The strategy used to overcome kinetic limitations is dictated by the enzyme kinetics. For example, competitive inhibition kinetics is indicative of the likely proximity of the enzyme active site to the inhibitor binding site. Any tampering with the latter by mutagenesis for purposes of isolating a feedback-insensitive version is likely to result in reduced enzyme activity. In many cases, simple amplification of the gene encoding the wild-type enzymes suffices to overcome regulatory restrictions, as in the amplification of *thr*B (encoding homoserine kinase) to overcome homoserine accumulation, which is due to threonine inhibition to the enzyme.

III. Importance of "fine-tuning" gene expression.

There is an optimal level of *in vivo* enzyme activity which permits "smooth" carbon flow without hampering cell growth or productivity. The use of the inducible *tac* promoter and the range of expression levels tested confirmed this. It was also shown that new fermentation conditions warrant testing of induction strategies to obtain the desired amount of expression for optimal carbon flow. For example, in higher cell density cultures (such as in a reactor), greater or more frequent addition of inducer may be necessary to maintain high enzyme activity.

IV. Ability to shift carbon flow by quantitative manipulations.

This illustrates the difference between "rigid" and "flexible" metabolic nodes (Stephanopoulos and Vallino, 1991). Rigid nodes are insensitive to perturbations, whereas flux can be manipulated at flexible nodes by removing regulatory restrictions and/or increasing enzyme concentrations. The nodes at ASA, homoserine and threonine (to isoleucine) are flexible. However, the two reactions leading to glycine from threonine are rigid with respect to the third reaction (leading to isoleucine).

V. Potential for extrapolation to higher titers: Importance of genetic background.

Although this work has emphasized the intellectual considerations of metabolic flux redirection, practical sense dictates that high final titers need to be achieved for an industrial process. For example, 12 g/l threonine and 15 g/l isoleucine may not be industrially impressive numbers; however, the base case for this work was a laboratory strain, a "poor" lysine producer by industrial standards (22 g/l lysine). The importance of the results are shown in the histograms comparing the molar amounts of lysine versus molar amounts of these other amino acids and their derivatives. It was shown that *given the amount of carbon flow* into the aspartate pathway, the manipulations were successful in diverting all or most of the *available* carbon. It is very likely that under higher aspartate flow conditions (e.g., use of higher lysine producers), the final titers will greatly increase.

VI. The role of thermodynamic analysis.

Thermodynamic considerations play an important part in engineering of metabolic pathways. It was shown that thermodynamic limitations may become significant under certain conditions. For example, the highly unfavorable phoshphorylation reactions in the threonine pathway may proceed in the forward direction only under conditions of high substrate accumulation (e.g., aspartate and homoserine). Estimation of the concentrations necessary for this to occur yields insight into possible pathway limitations. Conversely, favorable thermodynamics may encourage exploration of new pathways and products, as in the case of isoleucine production.

The principles behind this work can be extended to all metabolic pathways, whether in microbial, plant or animal systems. The underlying rationale is the integration of analytical and theoretical techniques to simultaneously understand and optimize the flow of carbon towards a desired metabolite.

Chapter 7. Abbreviations and Nomenclature

AAC	aminoacetone
ACA, AcCoA	acetyl coenzyme A
acet	acetaldehyde
ADP	adenosine 5'-diphosphate
AEC	*S*-(2-aminoethyl) L-cysteine (lysine analogue)
AHB	2-aceto-2-hydroxybutyrate
AHV	α-amino-β-hydroxyvaleric acid (threonine analogue)
AMP	adenosine 5'-monophosphate
ASA	aspactic-ß-semialdehyde
asp	aspartate
aspP	aspartyl-ß-phosphate
ATP	adenosine triphosphate
ATCC	American Type Culture Collection
CER	carbon dioxide evolution rate
CO_2	carbon dioxide
CoA	coenzyme A
DAP	diaminopimelate
DNA	deoxyribonucleic acid
DO	dissolved oxygen
EDTA	(ethylenedinitrilo)-tetra acetic acid
e4P	erythrose-4-phosphate
FAD	flavin adenine dinucleotide (oxidized)
FADH	flavin adenine dinucleotide (reduced)

glu	glucose
glu6P	glucose-6-phosphate
glut	glutamate
gly	glycine
HMV	2,3 dihydroxy-3-methylvalerate
hom	homoserine
homP	homoserine phosphate
ile	isoleucine
IPTG	isopropyl-β-D-thiogalactopyranoside
leu	leucine
mDAP	*meso*-diaminopimelate
NAD+	nicotinamide adenine dinucleotide, oxidized form
NADH	nicotinamide adenine dinucleotide, reduced form
NADP+	nicotinamide adenine dinucleotide phospahte, oxidized form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NH_4^+	ammonium
Nkb	amino-ketobutyrate
NTG	nitrosoguanidine
oaa	oxaloacetate
OD	optical density
OMV	2-oxo-3-methylvalerate
OUR	oxygen uptake rate
PC	pyruvate carboxylase
PDC	pyruvate dehydrogenase complex
PEP	phospho*enol*pyruvate

P_i	inorganic phosphate
PK	pyruvate kinase
PPC	PEP carboxylase
PS	pyruvate synthase
pyr	pyruvate
rib5P	ribose5P
ribu5P	ribulose-5-phosphate
RQ	respiratory quotient (CER/OUR)
TD	threonine dehydratase
TDH	threonine dehydrogenase
thr	threonine
TS	threonine synthase
val	valine
xyl5P	xylose-5-phosphate
Y_{max}	maximum theoretical yield
α-kb	α-ketobutyrate
α-kg	α-ketoglutarate

Genes

asd	aspartic-β-semialdehyde dehydrogenase
ask	aspartokinase
*gly*A	serine hydroxymethyltransferase
hom	homoserine dehydrogenase
*ilv*A	threonine dehydratase
*ilv*BN	acetohydroxy acid synthase
*ilv*C	isomeroreductase
kbl	2-amino-3-ketobutyrate CoA:ligase
*ser*A	phophoglycerate dehydrogenase
*ser*B	phosphoserine phosphatase
*ser*C	phosphoserine aminotransferase
tdh	threonine dehydrogenase
*thr*B	homoserine kinase

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Appendix

A.1 Thermodynamic bottleneck analysis

Summary of "Identification of localized and distrubuted bottlenecks in metabolic pathways", by Michael L. Mavrovouniotis. 1993. Proceedings of the International Conference on Intelligent Systems for Molecular Biology. AAAI Press. pp 275-283.

A.1.1 Objective and Description

The purpose of this analysis is to determine whether a pathway is thrermodynamically feasible and, if it is not feasible, to determine where in the pathway the infeasibility occurs. The basic objectives of this analysis are therefore to:

- **define** a range of concentrations within which reactions may occur
- **pinpoint** thermodynamically difficult reactions
- attempt to **reconcile** thermodynamic results with physiological reality

For this analysis, we need:

- description of pathway in the form of **reaction stoichiometries**
- **thermodynamics** of each reaction (ΔG_i°)

- description of the **permissible intracellular concentrations** of each metabolite

This analysis goes beyond the traditional thermodynamic evaluation, where the concentrations of metabolites are not taken into account when determining the feasibility of a reaction. This is physically unrealistic, since intracellular metabolite concentrations are almost always much smaller than 1 M, the standard state for ΔG_i°.

A.1.2 Notation and Equations

A metabolites of involved in a general biochemical reaction pathway (designated a_1, a_2,...a_j...a_A)

S bioreactions in the pathway (designated s_1, s_2,...s_i...s_S), each of which accomplishes a specific biotransformation $r_i = R(s_i)$

α_{ij} stoichiometric coefficient of metabolite **j** in step s_i. $\alpha_{ij} > 0$ if a_j is a product, $\alpha_{ij} < 0$ if a_j is a reactant (substrate) of s_i and $\alpha_{ij} = 0$ if a_j does not participate in s_i.

C_j concentration of metabolite **j**

ϕ_j activity coefficient of metabolite **j**

ΔG_i Gibbs Energy of Reaction. Gibbs energy gained when the extent of reaction r_i increases by 1 mole, i.e., when the reaction causes the amount of each metabolite a_j to change by α_{ij} moles.

ΔG_i°' Standard Gibbs Energy of Reaction - Gibbs Energy of Reaction in a prescribed standard state (independent of actual reaction conditions).

The stoichiometry of the biotransformation $\mathbf{R(s_i)}$ can thus be written as:

$$r_i = R(s_i) = \sum_{j=1}^{A} \alpha_{ij} a_j$$

To evaluate the thermodynamic feasibility of the reaction $\mathbf{r_i}$, one needs to calculate $\Delta \mathbf{G_i}$. The reaction is thermodynamically feasible in the forward direction if this quantity is negative.

However, in evaluating the thermodynamic feasibility of an entire pathway, one must consider that several bioreactions share metabolites. In specifying bounds for their concentrations, a coupling of the thermodynamic characterization of the bioreactions is created. If different combinations of metabolite concentrations can be selected to make each bioreaction feasible separately but cannot make them feasible simultaneously, a distributed bottleneck exists.

When all actual conditions, except metabolite concentrations, are the same as the standard conditions, we can write:

$$\Delta G_i = \Delta G_i^{\circ\prime} + \sum_{j=1}^{A} [\alpha_{ij} RT \ln(\phi_j C_j)]$$

where $\mathbf{R}$ is the ideal-gas constant, $\mathbf{T}$ is the temperature, $\mathbf{ln}$ is the natural logarithm, $\mathbf{C_j}$ is the concentration of $\mathbf{a_j}$ (expressed in M) and ϕ_j is the activity coefficient (equal to 1 for ideal systems; in dilute systems is

assumed to be ~ 1).

In this analysis, a range of concentrations is defined, bounded by the products $\phi_j^{max} C_j^{max}$ and $\phi_j^{min} C_j^{min}$. The concentrations of certain metabolites are fixed, i.e., $\phi_j^{max} C_j^{max} = \phi_j^{min} C_j^{min}$, due to either the defined standard conditions (e.g., pH) or strict metabolic regulation, as in the case of the currency metabolites ATP and ADP. Dividing the concentration terms into constant and variable metabolite concentrations, the expression for ΔG_i can be written:

$$= \Delta G_i^{o'} + \sum_{\substack{A \\ j \text{ (with} \\ \text{const. conc.)}}} [\alpha_{ij} RT \ln(\phi_j C_j)] \quad + \sum_{\substack{A \\ j \text{ (with} \\ \text{var. conc.)}}} [\alpha_{ij} RT \ln(\phi_j C_j)]$$

If the first two terms are grouped together and renamed $\Delta G_i^{o''}$ (a constant value for each iteration):

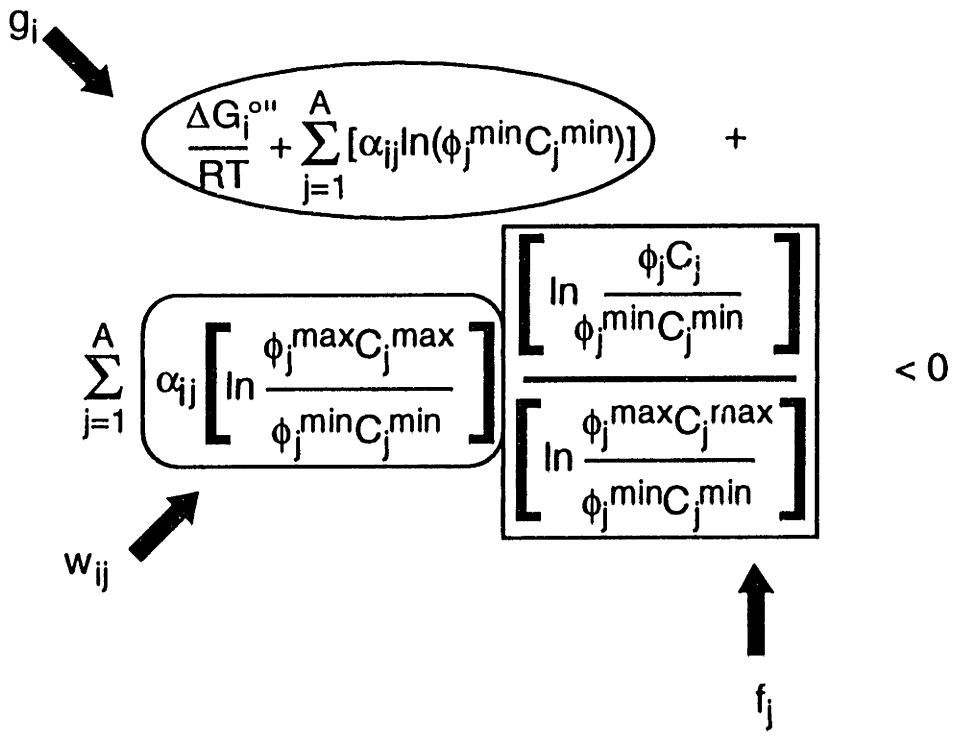
$$= \Delta G_i^{o''} + \sum_{\substack{A \\ j \text{ (with} \\ \text{var. conc.)}}} [\alpha_{ij} RT \ln(\phi_j C_j)]$$

The feasibility condition becomes:

$$\Delta G_i < 0 \longleftrightarrow \frac{\Delta G_i}{RT} < 0 \longleftrightarrow \frac{\Delta G_i^{o''}}{RT} + \sum^{A} [\alpha_{ij} \ln(\phi_j C_j)] < 0$$

$$\Longleftrightarrow \quad \frac{\Delta G_i^{o'''}}{RT} + \sum_{j=1}^{A}[\alpha_{ij} \ln(\phi_j^{min} C_j^{min})] + \sum_{j=1}^{A}\left[\alpha_i \ln \frac{\phi_j C_j}{\phi_j^{min} C_j^{min}}\right] < 0$$

Rescaling once again:



or,

$$g_i = \sum_{j=1}^{A} w_{ij} f_j < 0$$

where g_i then represents the standard free energy of reaction and incorporates all the constant terms in the expression. It effectively becomes a new standard state, based on the lower bound of the allowed range of concentrations. The term w_{ij} can be described as a stoichiometric coefficient corrected to include the range of concentrations, and f_j is simply the scaled concentration range and is bounded by $0 \leq f_j \leq 1$.

A.1.3 Algorithm

The Mavrovouniotis algorithm pinpoints thermodynamic limitations for a given concentration interval of metabolites. If a single step is infeasible, it is classified as a "thermodynamic bottleneck" for that interval. If a group of steps is infeasible, together they comprise a "distributed bottleneck". The algorithm can be summarized as follows:

1) Postulate upper and lower bounds for $\phi_j C_j$ of each metabolite.

2) Identify metabolites with constant concentrations.

3) Initiate set of infeasible reactions: Includes all steps in several pathways.

4) Compute in sequence: $\Delta G_i^{\circ\prime}$, $\Delta G_i^{\circ\prime\prime}$, $\frac{\Delta G_i^{\circ\prime\prime}}{RT}$, g_i, w_{ij}

Defining the function H for an isolated reaction r_i:

$$H(r_i,f) = g_i + \sum_{j=1}^{A} w_{ij}f_j < 0$$

and specifying the following feasibility condition:

$$H(r_i,f) < 0, \quad 0 \le f \le 1$$

For each reaction, f_j can vary between 0 and 1, independent from all other reactions. Therefore, to obtain the maximum value of **H** (H_{max}), f_j is set to 1 whenever $w_{ij} > 0$, and to 0 whenever $w_{ij} < 0$. The converse is true for H_{min}.

Using these criteria, we can write:

$$H_{max}(r_i) = g_i + \sum_{\substack{j \\ (wij>0)}} w_{ij} \quad \text{and} \quad H_{min}(r_i) = g_i + \sum_{\substack{j \\ (wij<0)}} w_{ij}$$

5) Reject from the set of bottleneck pathways all pathways with $H_{max}(r_j) < 0$ (such pathways are always feasible).

6) Retain all pathways with $H_{min}(r_j) > 0$ (always infeasible).

7) For the cases where $H_{min}(r_j) < 0$ but $H_{max}(r_j) > 0$, such

pathways are not always feasible and are thus labelled "undetermined" Construct combinations of two among all remaining subpathways such that an intermediate metabolite is eliminated. If no undecided subpathways exist or no combinations are possible, then END.

go to step 4).

Proven bottlenecks: from step 6).

A.2 Calculations of standard Gibbs free energies of reaction

The standard Gibbs free energy of reaction for reactions containing compounds for which data on heats of formation was unavailable were calculated using group contribution.

1 ASP + ATP --> ASPP + ADP

-166.9 kcal/mole + (-55.2 kcal/mole) - (-166.4 kcal/mole) - (-60.4 kcal/mole)

= 4.7 kcal/mole

2 ASPP + NADPH --> ASA + $NADP^+$ + P_i

-112.2 kcal/mole + (-4.7 kcal/mole) + (-59.6 kcal/mole) - (-166.9 kcal/mole)

- (-9.5 kcal/mole) = -0.1 kcal/mole

3 ASA + NADPH + H^+ --> HOM + $NADP^+$

+ (-122.0 kcal/mol) - (+4.7 kcal/mol) - (-112.2 kcal/mol) - (- 9.5 kcal/mol) = -5.0 kcal/mol

4 HOM + ATP --> HOMP + ADP

By group contribution, the transformation of the $-CH_2OH$ group of homoserine to the $-CH_2OPO_3$ of homoserine phosphate is equivalent to an energy change of -0.2 kcal/mol. Adding this to the ATP --> ADP change:

-0.2 kcal/mol + 5.2 kcal/mol = 5.0 kcal/mol

5 HOMP + H_20 --> THR + P_i

By group contribution, two groups undergo changes in the transformation of homoserine phosphate to threonine. The $-CH_2OPO_3$ of homoserine phosphate is transformed to a $-CH_3$ group (energy change: +7.9 kcal/mol - (-27.8 kcal/mol) = 35.7 kcal/mol). The $-CH_2-$ group of homoserine phosphate is converted to the $-CH_2OH$ group of threonine (-36.8 kcal/mol - (+1.7 kcal/mol) = -38.5 kcal/mol). Factoring in the utilization of one molecule of water and the release of an inorganic phosphate, the total energy of reaction is:

+ (-59.6 kcal/mol) - (-56.6 kcal/mol) + 35.7 kcal/mol - 38.5 kcal/mol = - 5.8 kcal/mol

6 THR --> AKB + NH_4^+

+ (-113.3 kcal/mol) + (-18.1 kcal/mol) - (-125 kcal/mol) = - 6.4 kcal/mol

7 AKB + PYR --> AHB + CO_2

+ (-150.1 kcal/mol) + (-94.3 kcal/mol) - (-113.3 kcal/mol) - (-113.4 kcal/mol)

= -17.7 kcal/mol

8 AHB + NADPH + H^+ --> HMV + NADP+

+ (-159.7 kcal/mol) - (-150.1 kcal/mol) - (-9.5 kcal/mol) - (4.7 kcal/mol) = -4.8 kcal/mol

9 HMV ---> OMV + H_2O

+ (-110.1 kcal/mol) + (-56.6 kcal/mol) - (-159.7 kcal/mol) = -7.0 kcal/mol

10 OMV + GLUT ---> ILE + AKG

+(-83.4 kcal/mol) + (-191.4 kcal/mol) - (-110.1 kcal/mol) - (-164.7 kcal/mol)

= 0 kcal/mol

11 THR + NAD^+ --> NKB + NADH + H^+

+ (-115.4 kcal/mol) + (+4.7 kcal/mol) + (-9.5 kcal/mol) - (-124.9 kcal/mol)

= 4.7 kcal/mol

12 THR --> GLY + ACET

+ (-89.6 kcal/mol) + (-33.4 kcal/mol) - (-124.9 kcal/mol) = 1.9 kcal/mol

13 NKB --> AAC + CO_2

+ (-36.9 kcal/mol) + (-94.3 kcal/mol) - (-115.4 kcal/mol) = -15.8 kcal/mol

14 NKB + COA --> ACA + GLY

+ (-33.1 kcal/mol) + (-89.6 kcal/mol) - (-115.4 kcal/mol) = -7.3 kcal/mol

Note: the value of -33.1 kcal/mol is equal to the Gibbs energy of ACA minus the Gibbs energy of CoA (Mavrovouniotis, 1990)

A.3 Homoserine kinase inhibitor studies

A.3.1 Inhibition by a variety of compounds

A variety of amino acids, threonine stereoisomers and threonine analogues were investigated for their ability to inhibit homoserine kinase activity (Table A.1).

Table A.1 21799 (pJD4) homoserine kinase: inhibition by various compounds*

Substance	Relative activity (%)
no inhibitor	100
amino acids (L form)	
glycine	121
valine	117
lysine	113
alanine	108
isoleucine	99
serine	92
arginine	76
cysteine	75
histidine	72
threonine and threonine stereoisomers	
L-threonine	43
L-allo-threonine (10 mM HS, 200 mM L-allo thr)	90
D-threonine (5 mM HS; 25 mM D-thr)	85
threonine analogues	
L-α-amino-n-butyrate	41
DL-β-hydroxynorvaline (AHV)	78
D-α-amino-n-butyrate	115
γ-aminobutyric acid	88
butyric acid	93
isovaleric acid	88

*(2mM HS; 10 mM test compound, unless otherwise specified)

Amino Acids

Of the amino acids tested, only cysteine, histidine and arginine demonstrated slight inhibitory effects.

Threonine stereoisomers

These studies confirmed the moderate threonine inhibition of homoserine kinase. They also demonstrated that altering the stereochemistry of threonine at either the α-carbon (D-threonine) or the β-carbon (L-allo-threonine) destroys its ability to inhibit homoserine kinase.

Threonine analogues

Only L-α-amino-n-butyrate (ABA) was able to significantly inhibit the homoserine kinase reaction. Preliminary kinetic studies showed that this inhibition is of a competitive nature.

A.3.2 Testing of α-amino-n-butyrate (ABA) as toxic analogue

The high level of inhibition of homoserine kinase exhibited by α-amino-n-butyrate (ABA) prompted the desire to test this compound as a possible toxic analogue should this strategy be of use in the future. In order to test the potential of ABA as a toxic threonine analogue with which to select mutants with a threonine-deregulated homoserine kinase, *C. glutamicum* AS019 was grown on minimal medium with increasing concentrations of ABA. Growth of the cells is completely inhibited at 200

mM ABA, showing that the compound can enter the cell and is not degraded. The inhibition is almost completely overcome at 150 mM ABA by addition of 10 mM threonine to the plates, confirming that ABA toxicity is due to blockage of threonine synthesis. Addition of 10 mM homoserine did not enhance growth at this ABA concentration, confirming that ABA action is directed towards homoserine kinase rather than dehydrogenase. Thus, ABA could be a potential threonine analogue with which to select threonine-resitant mutations in homoserine kinase.

Table A.2 Growth of AS019 on minimal medium plates with glucose supplemented with α-amino-n-butyrate (ABA)*

	Concentration of α-amino-n-butyrate (ABA) (mM)			
	0	**100**	**150**	**200**
	+++++	++	+	-
+ hom	+++++	+++	+	barely det
+ thr	+++++	++++	+++	barely det

***Single colonies at 72 hrs.**

A.4 Classical competitive inhibition kinetic model

$$V = \frac{[S]\, V_{max}}{[S] + K_m\,[\,1 + ([I]/K_i)\,]}$$

where

V = reaction velocity (mmol min^{-1} liter^{-1})

$[S]$ = concentration of substrate (mmol liter^{-1})

V_{max} = maximal reaction rate = $k_{cat}\,[E_o]$ (mmol min^{-1} liter^{-1})

k_{cat} = turnover number (mol substrate mol enzyme min^{-1})

$[E_o]$ = concentration of free enzyme (mol enzyme liter^{-1})

K_m = Michaelis constant (mM)

$[I]$ = concentration of inhibitor (mmol)

K_i = dissociation constant of enzyme-inhibitor complex (mM)

We assume the simplest model of competitive inhibition, with one substrate binding site per enzyme molecule. We assume that a second substrate either does not exist or is saturating. For the curves depicted in Figure 5.16, the following values were used: [S], 0.1 mM to 40 mM, K_m = 0.7 mM, K_i =10.7 mM , $[I]_1$ = 20 mM, $[I]_2$ = 50 mM , V_{max1} (= $k_{cat}E_{o1}$) = 6 mmol min^{-1} liter^{-1}, V_{max2} (= $k_{cat}E_{o2}$) = 10 mmol min^{-1} liter^{-1}.

A.5 Kinetic parameters of homoserine dehydrogenase and homoserine kinase in *C. glutamicum*

Kinetic parameters for homoserine dehydrogenase and homoserine kinase were estimated using assay conditions previously described (see Materials and Methods). These were found to be within the range of constants found in the literature for other strains (Tables A.3 and A.4)

Table A.3 Homoserine dehydrogenase - K_m with respect to ASA

K_m (mM)	strain	reference
0.19	*C. flavum*	Miyajima *et al.*, 1968
0.40	*C. flavum*	"
0.38	*C. flavum*	"
0.37	*C. glutamicum*	this work

Table A.4 Homoserine kinase - K_m with respect to homoserine

K_m (mM)	strain	reference
0.6	*E. coli*	Wormser and Pardee, 1958
1.0	*S. cerevisiae*	"
0.3	*S. cervisiae*	Ramos *et al.*, 1990
2.0	*C. glutamicum*	Miyajima *et al.*, 1968
0.7	*C. glutamicum*	this work