TRANSCRIPTIONAL REGULATION IN *CORYNEBACTERIUM GLUTAMICUM*

by

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ABSTRACT

Corynebacterium glutamicum has been widely studied due to its ability to produce large quantities of amino acids. Interest has been especially focused on those pathways which lead to the aspartate-derived amino acids and the aromatic amino acids. While many of the genes have been cloned and many of the enzymes have been characterized, the study of transcription and transcriptional regulation has lagged behind. The goal of this research was to study transcription and transcriptional regulation as it pertains to RNA polymerase and methionine repression at the *hom* promoter.

DNA-dependent RNA polymerase is the enzyme which synthesizes RNA using a DNA template. This enzyme has been purified in many prokaryotes and in this thesis the purification of the enzyme from *Corynebacterium glutamicum* is described. The procedure used to purify the enzyme was based on protocols used for the purification of RNA polymerase from other prokaryotes. The *Corynebacterium glutamicum* RNA polymerase was purified 2700-fold from the initial crude extract. Using SDS-PAGE analysis, this enzyme was found to have the same subunit structure as the RNA polymerases from other bacteria. Also present in the SDS-PAGE analysis are several co-eluting bands which have not been identified yet. In addition, initial binding assays and *in vitro* transcription studies were done.

The *hom* promoter drives the expression of the *hom-thrB* operon which encodes the enzymes homoserine dehydrogenase and homoserine kinase, respectively. The *hom* promoter has been shown to be repressed two-three fold by exogenous methionine. To further study this repression, the *hom* promoter was cloned upstream of reporter genes in order to monitor its expression. Two versions of the *hom* promoter were studied. One version was the native form in which the promoter plus a 90 base pair leader sequence was analyzed and in the other only the promoter without the leader sequence was analyzed. It was found that the truncated version was repressed 7.5-fold and the native promoter was repressed 4.7-fold. To further study this repression, a strain of *Corynebacterium glutamicum* was developed which was derepressed at the *hom* locus. This was done by mutagenizing cells which contained a plasmid with the *hom* promoter driving the

expression of the *cat* gene encoding chloramphenicol acetyl transferase. By selecting on medium which contained both methionine and chloramphenicol, a derepressed strain was isolated. Further study of the derepressed strain, E12Rep-2, indicated that the strain is also derepressed at the *metA* locus. *MetA* encodes the enzyme O-acetylhomoserine transferase, the first enzyme in the methionine specific pathway. This finding suggests that a common regulatory element mediates methionine repression in *Corynebacterium glutamicum*.

During the course of the *hom* promoter study, a vector was made which was used to integrate the *hom* promoter: *cat* reporter gene cassette into the *Corynebacterium glutamicum* chromosome. The target site for this integration was the gene *ddh* which encodes the enzyme diaminopimelate dehydrogenase. This enzyme is the bypass pathway to *meso*-diaminopimelate from L-Δ-tetrahydrodipicolinate. In *Corynebacterium glutamicum* E12, the promoter: reporter gene cassette was integrated into the chromosome; repression at the *hom* promoter in this strain was comparable to repression when the *hom* promoter is on a multi-copy plasmid. This same vector was used in another strain, *Corynebacterium glutamicum* ATCC 21253 in order to disrupt the *ddh* gene. Strain 21253 is a lysine-overproducing strain and the disruption of *ddh* was needed to further understanding of the lysine pathway. The disruption of the *ddh* was achieved and that strain is now being further characterized.

A discussion of methionine regulation and the strategy that *Corynebacterium glutamicum* has evolved in order to regulate its gene expression is also presented. This discussion combines the new information that was obtained through this research with what has already been presented in the literature.

Thesis Supervisor:	Anthony J. Sinskey
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Dedicated to Mom, Dad, and Tom

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PREFACE

This thesis is organized into five chapters and two appendices. Each chapter deals with a separate topic. Because of this, each chapter has its own materials and methods section, results, and discussion sections where relevant. Chapter Three is broken down further into three sections each with independent materials and methods, results, and discussion sections where relevant. This organization does lead to some redundancy. It is hoped, however, that by combining related materials together to give a complete picture of each particular subject, the thesis will be easier to read and understand.

CHAPTER 1
INTRODUCTION

INTRODUCTION TO *CORYNEBACTERIUM GLUTAMICUM*

There are many ways to describe the featured bacterium of this thesis, *Corynebacterium glutamicum*. One way is to define an organism in terms of the basic taxonomic features taxonomists use when they are classifying a new species. This description will be given for the general genus of *Corynebacterium*. As any definition becomes more clear when comparisons to other examples are given, the second section will describe *C. glutamicum* in terms of its standing among related prokaryotes.

The literature is filled with species which have been classified as "*Corynebacterium*" when in fact they bear little resemblance to what is now considered a true *Corynebacterium* species. The genus *Corynebacterium* was created in 1896 by Lehmann and Neumann [1]. One of the first distinguishing characteristics of *Corynebacterium* was their shape, for which they are named; "*coryne*"= club, "*bacterium*"= bacterium. Hence many bacterium were placed under *Corynebacterium* when all they had in common was some similarity in shape [1]. As more accurate chemotaxonomical methods were developed and 16S rRNA cataloging was put into place, membership into the genus became more rigorous and many former members were displaced [1, 2]. Sixteen species are now included under the genus *Corynebacterium*. The membership is still quite diverse with saprophytic and non-pathogenic as well as medically important species being included [1]. For the purposes of this thesis, the bacteria which have been designated *C. glutamicum*, *Brevibacterium lactofermentum*, and *B. flavum* will be referred to as *C. glutamicum* because of their near identity [1, 3].

General Features

Corynebacterium are Gram-positive, although sometimes they stain unevenly, especially if they are old. They are typically found in soil, feces, dairy products, animal skin or plant material. They are non-motile, non-sporulating, and facultatively anaerobic. As mentioned earlier, they are often club-shaped, but their morphology is variable and they sometimes appear as straight or slightly curved rods and ovals. Their growth is marked by a V-shaped "snapping" division [1, 4]. Oftentimes metachromatic granules, which are thought to be patches of polyphosphates, are inside [2]. One of the hallmarks of the genus is a high GC content, usually in the range 51-65 mol% [1].

The cell wall composition of *Corynebacterium* is also very distinctive. Its peptidoglycan is directly cross-linked with *meso*-diaminopimelic acid as the cross-linking agent [1]. Arabinose and galactose are the most prevalent sugars in the cell wall [1]. Also present are mycolic acids, α-substituted-β-hydroxy acids with 22-36 carbons in the carbon chains (considered "short-chained") [1]. *Corynebacterium's* cell wall has a lipid component which is comprised of straight-chain saturated or mono-unsaturated fatty acids. Dihydrogenated menaquinones with eight and/or nine isoprene units are also present [1].

Corynebacterium are catalase positive. *C. glutamicum* also exhibits urease activity, tyrosine hydrolysis, and the ability to reduce nitrate to nitrite [1].

Industrial Use of *C. glutamicum*

Amino acids have long been used as additives to both animal feed and human foods. While plant proteins are often used as animal feed, they are often

deficient in essential amino acids such as L-lysine, L-threonine, L-methionine, and L-tryptophan [4]. Approximately 31% of amino acids produced become feed additives and 66% of amino acids go to the food industry. The rest are used in medicine and cosmetics or are used as starting materials in the chemicals industry [4].

C. glutamicum was isolated in 1957. Due to an increased demand for monosodium glutamate as a flavoring agent, bacterial cultures were screened for their ability to excrete glutamate. *C. glutamicum* was found to be the best producer [4]. Many strains of *C. glutamicum* are able to convert 30-50% of glucose consumed into glutamate [5]. Under biotin limitation, the glutamate is excreted easily. If biotin is not limiting, glutamate excretion can be achieved by the addition of sublethal concentrations of penicillin or detergent during the early exponential phase of the fermentation [5].

Presently, Corynebacteria are the main bacteria used for the production of amino acids, although other genera also have commercial interest [4]. Wild type strains of *Corynebacterium* can naturally produce the amino acids glutamate, valine, proline, glutamine, and alanine [5]. In order to produce higher quantities of these amino acids and to produce the other amino acids such as lysine, tryptophan and threonine, modifications to the cell metabolism must be introduced [4, 5]. The modifications include introducing relevant auxotrophies and regulatory mutations. In order to create even better producers, mutations are made which improve the cell's permeability in favor of product excretion and also eliminate the cell's ability to degrade the product [5].

One of the reasons *C. glutamicum* is such a good amino acid producer is because its amino acid degrading activities are lower than those of other organisms. For instance, only serine and threonine have been found to be degraded to any extent. There is some degradation of glutamate depending on the culture conditions and lysine is degraded only a small amount in some strains [5].

Generally, companies do not share information regarding amino acid titers for their industrial strains. Theoretical maximum yields can be calculated by analyzing the metabolic network. For instance, for lysine, the theoretical maximum yield in *C. glutamicum* can be calculated to range from 0.5-0.86 moles lysine/mole glucose depending upon what assumptions are made [6]. While the theoretical maximum yield is difficult if not impossible to achieve, higher titers can be reached by the addition of more carbon and using special feed strategies. Using laboratory strains and controlled fermentations, titers such as 77 g lysine/liter can be achieved [6]. We believe that companies can more than double that titer.

Corynebacterium and Related Genera

As stated above, *Corynebacterium* are in the Gram positive division of the eubacteria [7]. They are part of the Class Actinomycetes [7]. The genera which are most closely related to them include *Rhodococcus, Nocardia,* and *Mycobacterium* (Figure 1.1) [7-9]. All four of these genera share a common cell wall type. All have *meso*-diaminopimelic acid (*meso*-DAP) as the cross-linking agent in the peptidoglycan. Mycolic acids are also present in all of the genera; *Corynebacterium* has the shortest side chains of the group. Finally, all genera are noted for their high GC content [8].

Figure 1.1: Classification of *Corynebacterium*. *Corynebacterium* are of the Class Actinomycetes. While they are classified in the Family Corynebacteriaceae, they are closely related to the genera in the Family Mycobacteriaceae due to such things as similarities in cell wall composition [1].

Division: Gram-positive

Class 2: Actinomycetes

Order 3: Actinomycetales

Family 2: Corynebacteriaceae

Genus: *Corynebacterium*

Family 3: Mycobacteriaceae

Genera: *Mycobacterium, Nocardia, Rhodococcus*

Nocardia

Nocardia strains have been used for many purposes including the production of antibiotics [10, 11], epoxidation reactions used to introduce oxygen into unactivated organic substrates [12], production of enzymes which are used as diagnostics in cholesterol testing [13], and the production of corticoid analogs used for anti-rheumatic and anti-allergic compounds [14].

Nocardia Genetic Toolbox

In recent years, basic genetic tools and procedures have been developed for *Nocardia* which has precipitated the beginning of a collection of cloned and characterized *Nocardia* genes. At this time, several different *Nocardia* cloning systems have been described in the literature. One system described by Yao, *et al.* [15] is based on an *Escherichia coli - Nocardia* shuttle vector. The *Nocardia* origin of replication is taken from a functional plasmid replicon derived from the pathogen *N. asteroides*. In another report, Kumar, *et al.*, described the construction of another *E. coli-Nocardia* shuttle vector [16]. In this case, the origin of replication from the endogenous plasmid pA387 of *Amycolatopsis* sp. strain DSM 43387 was shown to be functional in *Nocardia*. Two transformation systems have also been described. Yao, *et al.*, tested their shuttle vector by electroporation [15]. With DNA prepared from *E. coli* HB101, the transformation efficiency was 10-100 transformants/μg DNA. With DNA prepared from *Nocardia*, the transformation efficiency jumped to 8×10^4 transformants/μg DNA [15]. An alternative transformation procedure was reported by Kumar, *et al.*, which is based on a method developed for *A. mediterranei* which uses a combination of PEG and alkaline cations to transform cells [16]. Transformation efficiencies reached 6.7×10^5/μg DNA when using *Nocardia* DNA or DNA made from methylation deficient *E. coli* strains [16].

Of potential utility in the *Nocardia* genetic tool box is the discovery of the transposable element *IS*204 [17]. *IS*204 is 1452 base pairs long and it includes an ORF of 1134 base pairs encoding a putative transposase [17]. This putative transposase is similar to *IS*1096 from *Mycobacterium smegmatis* ar.d Tn4652 from *Pseudomonas* sp [17].

At this time, several genes have been cloned from *Nocardia* sp. Some of the earlier genes were cloned from *Nocardia* but then expressed in heterologous organisms such as *E. coli* [12] or *Streptomyces lividans*. [13] Now that efficient transformation procedures have been established, further characterization of the genes will be possible in the relevant *Nocardia* strains.

Rhodococcus

In the last two years there has been a veritable explosion in the *Rhodococcus* literature. Much of this stems from an interest in bioremediation and the ability of different species of *Rhodoccocus* to degrade toxic compounds in the environment. Species of *Rhodococcus* have been identified which can break down compounds such as polychlorinated biphenyls (PCBs) [18-20], herbicides like *S*- ethyl dipropylthiocarbamate (EPTC) and atrazine [21, 22], and nitrile-containing compounds [23]. *R. rhodochrous* has been shown to be capable of desulfurizing certain sulfur-containing compounds without degrading the carbon structure [24]. For this reason, it is being studied for its ability to desulfurize coal or petroleum so that sulfur emissions are reduced but the amount of British thermal units (BTUs) of the fuel remains the same [25-27].

Rhodococcus Genetic Toolbox

The first genes to be cloned from *Rhodococcus* generally were identified in *E. coli*. *Rhodococcus* genomic DNA was isolated and used to make *Rhodococcus* genomic libraries in *E. coli* vectors. Different genes were identified in *E. coli* by screening for a specific activity that the *E. coli* strain would gain [18, 20, 23]. Another method was to use the same gene from other organisms as probes to find the gene in the new strain [19, 28]. These probes were then used to isolate either DNA fragments in Southern blots or colonies which had been transformed with a *Rhodococcus* gene library. Several *Rhodococcus/E. coli* shuttle vectors have been developed which allows for *Rhodococcus* genes to be screened for in *Rhodococcus* strains [19, 21, 22, 25]. This of course is an advantage because many genes may not have been identified in *E. coli* since not all *Rhodococcus* promoters or translational signals are functional in *E. coli*. In addition, at least two different *Rhodococcus* phages have been sequenced and characterized to further assist in vector development [29]. Transformation in *Rhodococcus* has been achieved by electroporation [29]. A method for the conjugative transfer of plasmid pT01 from *E. coli* to *Rhodococcus* spp. has also been developed [30].

Mycobacterium

Mycobacterium has been probably the most extensively studied genera of those discussed because of the pathogens, *M. tuberculosis* and *M. leprae*. Because of the interest in prevention and treatment of tuberculosis and leprosy, much of the work in these organisms has been focused on pathways or enzymes which are targets for possible drugs, mutations which arise which render the organism resistant to drugs, and the genes which confer virulence [31, 32]. Another area of study is in the antigenic properties of the *Mycobacterium* cell wall in order to develop assays to determine infections by *Mycobacterium* species [32, 33].

The cell wall has been extensively studied due to the interest in a target site for potential drugs and for serological typing. Due to the importance of *meso*-DAP in the cell wall, some of the same genes from *Mycobacterium* have been isolated as from *C. glutamicum*. The aspartokinase and asparatate semialdehyde dehydrogenase genes from *M. smegmatis* have been isolated and characterized [34]. Like *C. glutamicum*, *ask-asd* form a single operon in *M. smegmatis* and bacille Calmette-Guerin (BCG), a non-pathogenic *M. bovis* derivative [34, 35]. Many of the DAP genes in *Mycobacterium* have also been isolated [32]. These genes encode the enzymes which convert aspartate semialdehyde into *meso*-DAP. There is evidence that *Mycobacterium* also have the same two pathways to *meso*-DAP as *C. glutamicum* does, the succinylase pathway and the diaminopimelate dehydrogenase pathway [32, 36]. In *Mycobacterium*, however, it appears that the gene arrangement may be different than that which has been established for *C. glutamicum* [32, 36]. There is also some evidence that the DapB enzyme of *Mycobacterium* may be bifunctional for both the dihydropicolinate reductase and the diaminopimelate dehydrogenase activity, although this is yet to be proven conclusively [32].

The RNA polymerase (RNAP) of *M. smegmatis* has been purified and characterized [37]. The *Mycobacterium* RNAP appears to have a similar structure to the RNAPs of other prokaryotes. Subunits of comparable size to those in the general $\alpha_2\beta\beta'\sigma$ holoenzyme can be seen by SDS-PAGE analysis. The purification also showed the presence of several extra bands which may be extra σ factors, contaminating proteins, or both [37]. There is evidence of multiple σ factors since a heat shock response has been described in *M. bovis* BCG [38]. The *Mycobacterium* RNAP also seems to be very sensitive to supercoiling; the RNAP

was not able to transcribe linear or relaxed plasmids. This preference for a supercoiled template in *Mycobacterium* has been hypothesized to be due to the extremely high (G+C) content of the organism; i.e the DNA superhelicity is relied upon to unwind the promoter DNA [37].

Mycobacterium Genetic Toolbox

Much work has been done to develop molecular biology techniques in *Mycobacterium* [39]. Some of the problems in studying *Mycobacterium* are the long cultivation times and the virulence [39]. To get around this, much of the study has been in strains such as *M. smegmatis* which is non-pathogenic and has a doubling time of nearly an hour and *M. bovis* BCG which is an avirulent derivative of a pathogenic strain and has a doubling time of approximately 24 hours [37]. Early genes were cloned by expression in *E. coli* or *Streptomyces lividans* [39]. There are also *E. coli /Mycobacterium* shuttle vectors [40]. Furthermore as part of the *M. tuberculosis* genome project, a collection of ordered, cosmid clones is being established [31]. Transformation of *Mycobacterium* is generally accomplished through electoporation [41]. A transposon has also been identified in *Mycobacteria* which will be useful for genetic analyses [42].

Corynebacterium

This thesis will be concerned with many of the particulars of gene expression and regulation in *C. glutamicum.* In order to do this study, a tremendous amount of work was done by the Sinskey lab and others to develop the tools and techniques necessary for understanding the basic molecular biology of *Corynebacterium* [43-45]. Like the genera mentioned above, many of the *C. glutamicum* genes were isolated by the use of genomic libraries of *C. glutamicum* DNA in *E. coli* vectors and the complementation of *E. coli* auxotrophs [44, 46, 47]. Although this is still

done, shuttle vectors have long been developed which allow for the study of a particular gene in a *C. glutamicum* background [1, 43, 44].

Other developments which have aided the study of *C. glutamicum* include the development of transconjugation procedures which are discussed in more detail in Chapter Three [48-50]. Transposons have been isolated which can be used for mutagenesis procedures [51]. Vectors which are described in this thesis and also in the literature allow for the study of promoters in *C. glutamicum* [52]. In addition, the purification of *C. glutamicum* RNA polymerase will enable others to study transcription more thoroughly than what was before possible.

In terms of the progress made in the study of molecular biology in *Corynebacterium, Mycobacterium, Rhodococcus,* and *Nocardia*, *Corynebacterium* and *Mycobacterium* are probably the most advanced. *Rhodococcus* is next followed by *Nocardia*. The advancement in each area is exciting because of the synergies which could potentially develop. For instance, an example was given above in which the genetic organization of the *C. glutamicum* and *M. smegmatis ask* and *asd* genes were the same. By using the intensive studies which have already been done in *C. glutamicum* as a guide, the groups studying these genes in *Mycobacterium* have an advantage over starting from scratch or using *E. coli* as a model. In another case, a major focus of study in *Mycobacterium* is on the cell wall composition and its synthesis. Since all the genera discussed have similar cell wall compositions, a lot of this knowledge is most likely relevant across the board.

In the rest of the thesis, I have made an effort to compare *C. glutamicum* to these more related species since I believe that they are more relevant to *C. glutamicum*

physiology than the much more distantly related *E. coli*. Of course, in most cases, *E. coli* is used as the model because it has been so well studied, whereas similar phenomenon in *Mycobacterium*, *Rhodococcus*, and *Nocardia* have not been studied at all. It is hoped that as progress in the study of the Actinomycetes advances, this will cease to be the case.

RNA POLYMERASE, PROMOTERS, AND TRANSCRIPTION REGULATION

In Chapter Two, the purification of *C. glutamicum* RNA polymerase is presented and in Chapter Three the *C. glutamicum* methionine repressor is discussed. In order to put this work into context, this next section gives background information on general characteristics of prokaryotic RNA polymerase, promoters, and transcriptional regulation. At the end of the section, a summary of transcriptional regulation in *C. glutamicum* is given.

Prokaryotic DNA-Dependent RNA Polymerase

DNA-dependent RNA polymerase (RNAP) has been purified from a variety of bacteria [37, 53-56]. RNAP is a complex formed of a core enzyme which has the subunit structure $\alpha_2\beta\beta'$ [57, 58]. The functional holoenzyme is the core enzyme plus one of a variety of σ factors [57, 58].

The function of RNAP is to catalyze the transfer of a ribonucleoside monophosphate to the 3'-hydroxyl end of a growing RNA chain [57, 58]. Ribonucleoside triphosphates are used as the substrates for this polymerization reaction. Transcription is a complicated process including many steps. The RNAP must first recognize a DNA sequence upstream of the template sequence from whence the RNA is being copied. This DNA sequence is termed a promoter and it will be discussed later. After RNA synthesis is initiated the RNAP must

then incorporate the correct nucleotide into the RNA chain as determined by the sequence of the DNA template. This must be done with complete processivity, i.e. the RNA transcript must be transcribed from beginning to end as the result of one event in which the RNAP bound the DNA. Finally, the RNAP must recognize the signals which indicate the termination of transcription of the particular DNA sequence [57, 58].

The subunits of RNAP have different functions in transcription. It is believed that the β and β' subunits are the main catalytic portions of the polymerase. They are responsible for transcription initiation and elongation [59]. The α subunit was long thought to be only responsible for core assembly, serving as a scaffold for the construction of the rest of the complex [59]. In addition to this, the α subunit has now been implicated in regulation of transcription, especially positive control [59, 60]. Studies with mutants in which the carboxy terminus of the α subunit has been truncated indicate that this subunit plays a role in mediating regulation by several transcription factors such as OmpR and CRP [59]. Another study showed that the α subunit is also involved in the recognition of the promoter [60]. Upstream of many promoters, including the *E. coli rrnB* P1 promoter, is a region which is rich in (A ÷ T). This region occurs around -40 and -60 and has been deemed the upstream (UP) element. It is the α subunit which recognizes this part of the extended promoter region [60].

The final component of the RNAP holoenzyme is the σ factor [61]. The activities of the σ factor include core binding and activation of promoter recognition. Additionally, the σ factor may be included in DNA melting and inhibition of nonspecific transcription [62]. After transcription is initiated and the RNAP moves off of the promoter region, the σ factor falls off of the complex in order to

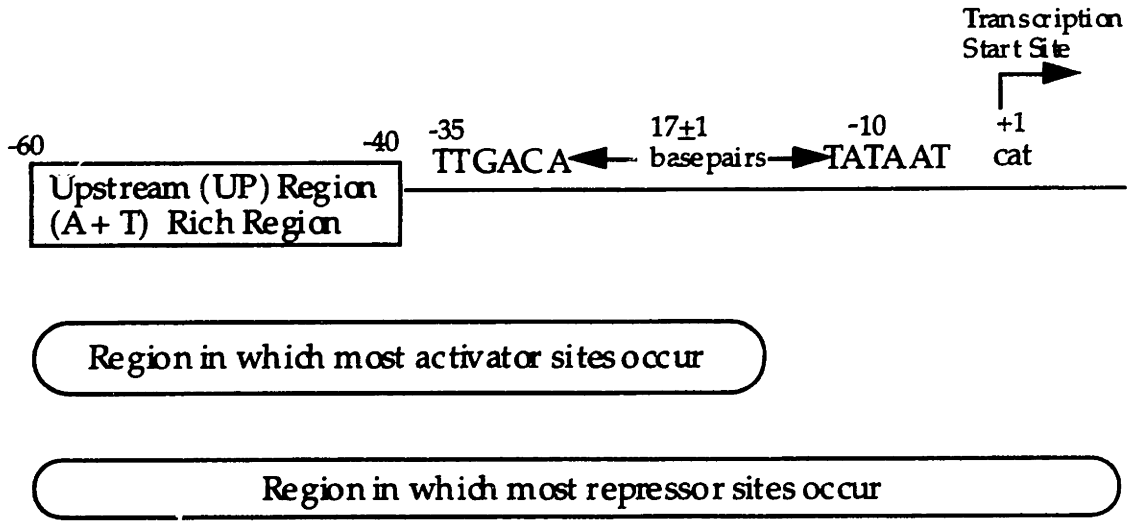
be recycled [61]. Most prokaryotes have been found to have multiple σ factors. *E. coli*, for example, has a major σ factor for most of its maintenance genes, σ^{70}. Then there are other σ factors which recognize different promoters under specific conditions such as heat shock, nitrogen limitation and stationary phase [63]. *Bacillus subtilis*, in addition to a major σ factor, has many other σ factors which control the sporulation of the cell [62-64]. Other cases in which multiple σ factors are used are for chemotaxis and virulence [65].

Promoters

RNAP recognizes and binds to a DNA sequence called the promoter [57, 58]. Many studies have been done on the consensus sequences for various classes of promoters in different bacteria. For instance, the consensus sequences of the major σ factors in both *E. coli* and *B. subtilis* are the same with one conserved hexamer around -10 (where +1 is the transcription start site) of TATAAT and another at -35 of TTGACA (Figure 1.2). The spacing between these hexamers is also important and tends to be 17± 1 bp [57, 58]. Other σ factors recognize promoters with different DNA sequences, and for each class the promoter sequence is conserved. Few promoters have an exact match to the consensus sequence. Substitutions in these promoters which either bring the promoter closer to or further away from the consensus sequence result in either a stronger or weaker promoter, respectively [57, 58, 66].

As stated earlier, both *E. coli* and *B. subtilis* have the same consensus sequence for promoters recognized by their major σ factors [62]. This consensus sequence may be present in other bacteria, but it is not as well established. For instance, some promoters of genes which would generally be considered maintenance genes, are very different from the consensus sequence, while other promoters

Figure 1.2: Schematic of an *E. coli* Promoter Which is Recognized by the Major σ Factor. The schematic below shows the proximal region of a general *E. coli* promoter. The UP element has been found in some promoters. Most of the activator sites are located between -60 and -40. For the repressor locations, there is an increase in the number of sites from -60 to +1, then a decline in sites from +1 to +30. [57, 60, 68, 69]



more closely match. In *Streptomyces* sp., which are also in the order of Actinomycetes like *Corynebacterium,* sequences of 139 promoters have been compiled [67]. Of these 139, only 29 resemble the canonical sequence of *E. coli* and *B. subtilis.* The other 110 sequences either have a close approximation to one or the other conserved hexamer or have a sequence which was widely different [67].

In *C. glutamicum* at least ten promoters have been identified (Figure 1.3). In all of these promoters, the transcription start site was determined by either S1 nuclease mapping or primer extension analysis. As post-transcriptional modification of the RNA transcript may take place, there may be changes in the transcription start site assignment once more rigorous biochemical analyses are done on the promoters. In Chapter Two, the purification of *C. glutamicum* RNAP is described. Using this purified protein, it is now possible to so some of the more rigorous analyses of *C. glutamicum* promoters such as DNA footprinting.

Of the apparent *C. glutamicum* promoters, it is possible to pick out regions which have some homology to the consensus sequences of the *E. coli* and *B. subtilis* major σ factor. Areas which resemble the -10 region are easier to visually determine, although some sequences in the -35 region also have some homology. *C. glutamicum* can recognize many *E. coli* promoters when present in *C. glutamicum*. For instance, the expression from the *tac, trp,* and *lacUV5* promoters are very similar in *E. coli* and *C. glutamicum,* with the *C. glutamicum* expression only slightly lower [52]. In a similar way, many *C. glutamicum* promoters are used in *E coli* which has made the cloning of many *C. glutamicum* genes using *E. coli* auxotrophic strains a very useful strategy.

Figure 1.3: Sequences of 10 Apparent *C. glutamicum* Promoters - Below are the first 40 nucleotides of 10 different *C. glutamicum* apparent promoters. All of the promoters express genes which are either in the glycolysis pathway or in the amino acid biosynthetic pathways. [35, 44, 47, 96, 99, 100, 104, 121, 136]

Promoter	+1
hom	CTTTAAAGCAAAAATGAACAGCTTGGTCTATAGTCGGCTA
fda	TCACGACAAAAGTTGAGTGATGCAGGCATAATTGGCTATA
thrC	GGTGTGGGGGAGTATTGTGTCACCCCTTGGGATAGGGTTA
lysA	CGTATTCTGTGCGACGGGTGTACCTCGGCTAGAATTTCTC
askP1	CTGGCTAGGTAGACACAGTTTATAAAGGTAGAGTTGAGCG
pheA	TTCGGCGGGTTAAGCTGTGTAACCATGAGCGACGCACCAA
askP2	GTGCATTCAATGTGCCACTTCGCGTATGCTCGTCTTATAG
gap	ATCTGCTGCGAAATCTTTGTTTCCCCGCTAAAGTTGAGGAC
pgk	GGTTGCCAGCGCCACACAATGTGTGGCAATCTGGGACAG
gdh	TGTGGTCATATCTGTGCGACACTGCCATAATTTGAACGTG

Transcription Regulation

Transcription is regulated in several ways. Some promoters are repressed by various proteins and some are activated by others [68]. In some cases the same protein may act as a repressor for one promoter and an activator for another. The global control by σ factors is another example of transcription regulation. For example in flagellar gene regulation in *E. coli* and *Salmonella typhimurium*, a novel σ factor activates transcription of flagellin [65]. This σ factor cannot act, however, before the cell has produced a functional flagellum in which to insert the flagellin protein. In this way, the cell controls the synthesis of a costly material, the flagellin protein, until it is assured that it will be used [62]. DNA supercoiling also plays an important role in the regulation of some promoters [37].

There have been many papers on the regulation of *E. coli* promoters. Many studies separate the *E. coli* promoters into two groups, the σ^{70} promoters and the σ^{54} promoters [68, 69]. There are also promoters which are controlled by other minor σ factors, but these tend to be regulated similarly to the σ^{70} group while the σ^{54} promoters are regulated quite differently. Many σ^{70} promoters have been studied and their regulatory regions have been defined. At least two papers have presented a compilation in which 119 promoters were analyzed [68, 69]. Of these 119, 107 are σ^{70} promoters and the other 12 are σ^{54} promoters. Of the 107 σ^{70} promoters, 70 are repressed and 48 are activated. All of the σ^{54} promoters are activated [68, 69].

This compilation of the regulatory features of promoters showed some interesting trends, especially in the distribution of regulatory sites. In almost all of the cases of repression in σ^{70} promoters, the repressor site is proximal to the

promoter, i.e. overlapping the DNA from -60 to +20 (Figure 1.2). As RNAP covers the DNA from -50 to +20, the repressor is almost always close enough to make contact with RNAP. In some cases, the repressor may not touch RNAP directly, but may interact with an activator which is in contact with RNAP. There are examples of repressors which act from a distance, such as the repressor for the *purR* promoter. In this case, a weak operator is located around +100 and +200 and, therefore, the repression may be the result of a block in transcription elongation [68, 69].

Activators of the σ^{70} promoters are typically located immediately upstream of the -35 region. Of the 48 promoters which are activated, 47 have operator sites which are considered proximal and in the remaining promoter the operator site is just over the -60 boundary. Activator sites are located in this position because of the presumed necessity for the activator to interact with the RNAP. The movement of an activator site to a position distant from the RNAP results in a loss of activation [68, 69].

Moving a repressor site generally results in a change in activity. In fact, many repressors act at several promoters and their binding site for each might be different. Repressor sites are usually in one of three positions; upstream of -35, in the spacer region, or downstream of -10. Of the three, the spacer site seems to confer the highest level of repression. It is believed that the binding of a repressor to an operator is not enough to repress transcription; some type of interaction with the RNAP might also be necessary. Repressors bound at variant locations would then have different types of interactions with the RNAP and therefore a different level of repression would be mediated [68, 69].

Some promoter regions have several sites to which a repressor binds. This is especially evident in the MetJ operators, where the different genes in the regulon have anywhere from two to five operator sites [70]. The purpose of these extra operator sites is that it allows greater control over the regulation of expression. In instances where high repression is necessary, more repressor proteins bind in a cooperative manner. When less repression is needed, fewer repressor proteins bind [70].

The other class of promoters are those that are recognized by σ^{54}. These promoters are interesting in that they more closely resemble eukaryotic promoters than prokaryotic promoters [68, 71, 72]. Many of the σ^{54} promoters are for genes which are involved in nitrogen assimilation and fixation in *E. coli, S. typhimurium,* and *Klebsiella pneumoniae*. [68, 71, 72] Not all σ^{54} promoters are involved in nitrogen regulation, however [72]. For example, in *Pseudomonas aeruginosa,* promoters which are recognized by σ^{54} are also involved in flagellin and pilin synthesis [73].

All σ^{54} promoters need to be activated [68, 69]. What contrasts these promoters from the σ^{70} promoters is that the activator site is far upstream of the transcription start site, centered near -110. Furthermore these sites, which function like eukaryotic enhancer elements, can be placed kilobases in *cis* and still retain activation capability. For these activators to work, the intervening DNA must loop in order to allow the activator to interact with the RNAP at the promoter and stimulate transcription. In fact, RNAP which has σ^{54} can form a stable complex with the promoter and does not begin transcription until it is activated. Initiation cannot happen until a molecule of ATP is hydrolyzed in

order to open the transcription start site, which is also reminiscent of eukaryotic promoters but not other prokaryotic promoters [68].

As noted above and alluded to in the discussion of σ^{54} promoters, the different σ factors can also regulate transcription. In many cases, transcription regulation by σ factors results in a complex transcriptional cascade. In cases where a large number of genes need to be expressed, the control by σ factors helps to ensure that the genes which need to be turned on at a later time are not expressed until the earlier genes have been transcribed and their products are functional. This allows the cell to prevent wasting energy on products which will not be used. Alternative σ factors were first described in phage-infected cells of *B. subtilis* and were found to control the expression of middle and late phage genes [62]. More alternative σ factors were later found in uninfected *B. subtilis* cells. Now the *B. subtilis* model of a cascade of alternative σ factors regulating endosporm formation is one of the best studied examples of alternative σ factors regulating a complex cellular event [62, 64].

As most prokaryotes have been found to have σ factors, it is reasonable to assume that *C. glutamicum* will also have them, although no promoter has been described which is recognized by one. Heat shock promoters have been identified in *Mycobacterium,* so it is expected that they will also be found in *Corynebacterium* given the relatedness of the two genera [38]. In the purification of *C. glutamicum* RNAP that will be described in Chapter Two, several bands are present between 31 kd and 45 kd. Although these may be contaminating proteins, they may also represent alternative σ factors. Most of the alternative σ factors which have been identified cluster around 30 kd in size [62].

Another method used by bacteria to regulate transcription is mediated through DNA supercoiling [37]. DNA supercoiling is believed to have an important role in the regulation of virulence genes that are induced by environmental stress. In *Mycobacterium*, supercoiling has been shown to be important in *in vitro* transcription studies in which purified RNAP from *M. smegmatis* transcribes very poorly from a relaxed DNA substrate, but much more efficiently after that same substrate is treated with gyrase in order to supercoil it [37]. While some *E. coli* promoters are more responsive to supercoiling than others, all *Mycobacterium* promoters which were tested were dependent on supercoiling [37]. One hypothesis to explain the stronger dependence on supercoiling in *Mycobacterium* as opposed to *E. coli* is the much greater (G+C)% in *Mycobacterium. M. smegmatis* has a (G+C) content of 65% [37], whereas *E. coli*'s is around 50% [74]. One model is that *Mycobacterium* have evolved transcriptional apparati which depend on DNA superhelicity in order to help unwind the promoter region during transcription initiation [37]. Whether this same dependence on supercoiling is also present in *C. glutamicum* is something which can be explored now that *C. glutamicum* RNAP has been purified. In Chapter Two, data is presented which suggests that *C. glutamicum* is not as reliant on supercoiling as *Mycobacterium.* Although *C. glutamicum* does have a high (G+C) content of 55.5-57.5%, it is not quite as high as *M. smegmatis,* and therefore, the dependence on using the potential energy of negative supercoiling in order to unwind the promoter region may not be as important in *C. glutamicum* as it is in other (G+C)-rich organisms [1].

TRANSCRIPTIONAL CONTROLS IN *CORYNEBACTERIUM*

There are several examples of *Corynebacterium* genes or operons whose expression is controlled by transcriptional regulators. For most of these

examples, however, little is known about the mechanism of regulation other than the molecule(s) which cause the effect. For example, the expression of the gene *ppc* which encodes phosphoenolpyruvate carboxylase has been shown to be repressed by both glutamate and aspartate, but how this effect is mediated is unknown [75]. Described below are other examples of transcriptional controls in *Corynebacterium* in which more is understood.

tox Gene of *C. diphtheria*

Perhaps the best known example of *Corynebacterium* transcriptional control is in the pathogenic bacterium *C. diphtheria* for the expression of the diphtheria toxin. As early as 1936 it had been established that the addition of iron to the growth medium inhibited the toxin production [76]. The *tox* gene itself is actually encoded in the genomes of closely related corynebacteriophages [77]. The control element, however, is encoded in the genome of *C. diphtheria* [76, 77]. Biochemical and genetic evidence support the hypothesis that the *tox* gene is regulated by an aporepressor which in the presence of iron forms a complex which binds to the *tox* operator and thereby blocks transcription. When iron is limiting, the complex disassociates and transcription of *tox* proceeds [76].

The diphtheria *tox* iron-dependent regulatory element, *dtxR*, has been cloned and the sequence reported [76]. The repressor is 25,316 Da and has a predicted amino acid sequence of 226 residues. It has been shown to repress the *tox* promoter in an iron-dependent fashion [76].

The nucleic acid sequence of *tox* contains a 27 bp palindromic sequence which has a 9 bp inverted repeat separated by an intervening sequence of 9 bp. The inverted repeat overlaps the -10 region of the promoter [78]. Binding of the *dtxR*

protein to the operator renders the DNA resistant to DNaseI-digestion [78]. This operator has considerable homology to the Fur (ferric-uptake regulator)-binding sites in *E. coli*. Fur has been shown to bind with iron and act as a global negative controlling element [76, 78]. The Fur and DtxR DNA binding sequences are quite different so no cross-regulation occurs between the Fur repressor and the DtxR operator site and vice versa [76, 78]. The DtxR protein has been crystallized and a preliminary crystallographic characterization has been done. The repressor is asymmetric and is composed of two DtxR monomers [78].

trp Operon of *C. glutamicum*

The *trp* operon of *C. glutamicum* and *Brevibacterium lactofermentum* have been characterized [79, 80]. They are comprised of six structural genes responsible for the seven enzymatic steps from chorismate to tryptophan [79, 80]. The operon is believed to be repressed through tryptophan by two mechanisms; an operator/repressor system and attenuation [79-81].

Evidence for an operator/repressor mechanism has been reported in two separate papers [80, 81]. In both cases, the *trp* promoter was cloned upstream of a reporter gene. The portion of the *trp* operon with the putative attenuator sequence was not included. The level of repression by tryptophan in these constructs varied from 40% to 5-fold, depending on the reporter gene [80, 81]. The operator is believed to be a 14 bp palindrome which is located from positions -15 to -28 of the promoter [80, 81]. A mutation was made in this sequence which changed the conserved adenine adjacent to the axis of symmetry of the operator [81]. The promoter with the mutated operator sequence was no longer repressed by tryptophan and it showed wild type activity when tested in minimal medium. When growth in minimal medium was supplemented with tryptophan, however,

the activity of the reporter gene product was stimulated 3-9-fold. The hypothesis for this stimulation is that the mutation in the operator abolishes repression at the operator. This allows for the exposure of growth rate-dependent expression of the *trp* operator which is usually masked when the *trp* operator is wild type. This growth rate-dependent expression also occurs in *E. coli* and is believed to be caused here because the cultures grow better when tryptophan is added to the medium [81].

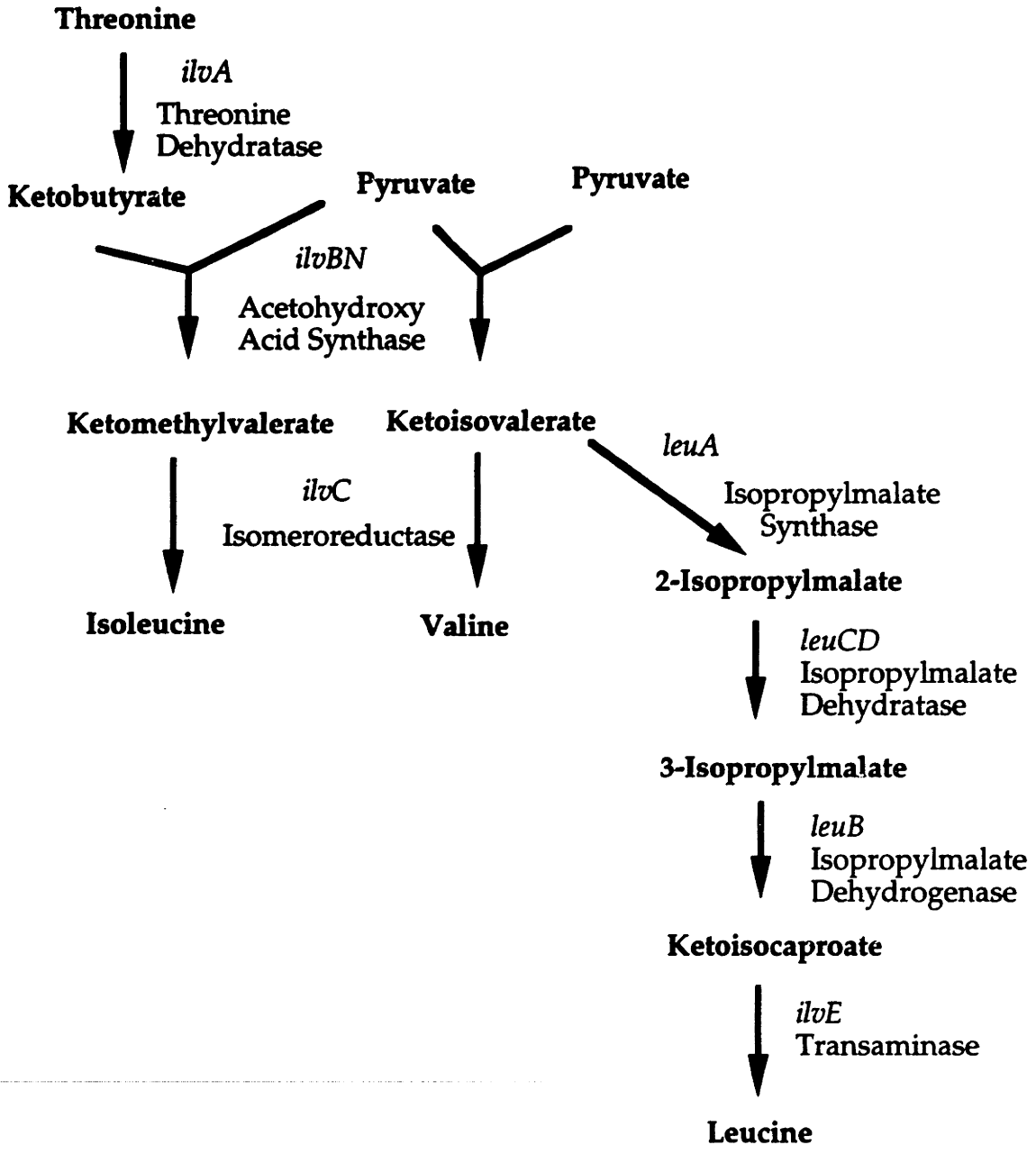
The other mechanism of repression by tryptophan in this operon is due to an attenuator [79]. An analysis of the upstream region of the *trp* operon after the promoter shows remarkable homology to that region in other prokaryotes. A 17-codon open reading frame which contains three consecutive Trp codons is located immediately after the promoter and about 200 bp away from the *trpE* translation start site. The position, length and sequence strongly suggest its role as a leader sequence in an attenuation mechanism. Furthermore, the region from the promoter to the *trpE* gene contains regions of extensive hyphenated dyad symmetry, with sequence motifs which are found in other organisms' *trp* attenuator region [79]. There are different models for which structures in this region constitute the termination and anti-termination stem loops [79]. Besides extensive homology to the *trp* attenuator regions of other bacteria, another strong piece of evidence for repression by attenuation is suggested by a mutation which causes a missense mutation in the third of the tandem Trp codons of the leader sequence. A strain which carries this mutation hyperproduces tryptophan, suggesting that the locus has become derepressed [79].

ilv, val and *leu* Regulation

The biosynthetic pathways of isoleucine, valine and leucine share some of the same enzymes (Figure 1.4) [82]. The last four steps of the valine and isoleucine pathway share the same enzymes which use different substrates for the synthesis of each amino acid [82]. The first enzyme in these parallel pathways is acetohydroxy acid synthase (AHAS) [83, 84]. This enzyme is both feedback inhibited and repressed by all three amino acids [84]. In addition, the expression of the AHAS gene, *ilvBN*, is stimulated by the addition of α-ketobutyrate (the substrate for the isoleucine pathway) to the culture [84]. Although the exact mechanism of regulation of *ilvBN* is not known, it is speculated that some of the regulation is mediated through an attenuation mechanism. Upstream of the *ilvB* coding region, an open reading frame exists which may encode a leader peptide. This leader peptide is 15 residues long and contains 2 isoleucine, 3 valine and 2 leucine residues. Also contained in this region are several areas of potential stemloop structures [84].

Leucine is synthesized from the isopropylmalate (IPM) pathway which starts with the conversion of ketoisovalerate (from the valine pathway) to 2-isopropylmalate by IPM synthase, the product of the *leuA* gene [82]. The activities of IPM synthase as well as IPM dehydratase and IPM dehydrogenase have approximately half the activity if grown in minimal medium with leucine added as opposed to minimal medium without leucine, suggesting that leucine may regulate the expression of their genes. The *leuA* gene has been cloned and sequenced [82]. The upstream region has a sequence which suggests that an attenuation mechanism may be responsible for at least some of the regulation. An open reading frame exists which could encode a leader peptide which

Figure 1.4: Pathways to Isoleucine, Valine and Leucine from Threonine in *C. glutamicum* [82]



contains four tandem leucine codons. There is also extensive dyad symmetry in the area around the leader peptide. Other elements which suggest an attenuation mechanism are the low leucine content (6%) of the IPM synthase, which is the lowest known in *C. glutamicum*, and the strong bias of the *leu* codons towards the most frequently used one so that the less frequent $tRNA^{Leu}$ are not rate limiting for the enzyme's synthesis [82].

METHIONINE

The *hom* promoter of *C.glutamicum* has been shown to be repressed two-three fold by methionine [85]. In order to study this repression, it is best to frame it in terms of the overall regulatory effect of methionine in the cell. To do this I will first summarize what is understood in better characterized prokaryotes such as *E. coli* and *S. typhimurium* before returning to what is known about the methionine pathway in *C. glutamicum*

Methionine is unique in many ways to the rest of the amino acids. Besides it being a component of proteins, the formyl-form of methionine is also responsible for the initiation of protein synthesis. Methionine is a precursor of polyamine spermidine and also S-adenosyl-methionine (S-AM), the universal cell methylating agent. Furthermore, methionine is the most expensive amino acid to make, costing the cell one oxaloacetate, one C-unit, one NH_3, one S-unit, seven ATPs, and eight NADPHs. For these reasons the synthesis of methionine is tightly regulated in the cell [70].

Methionine Biosynthesis in *E. coli*

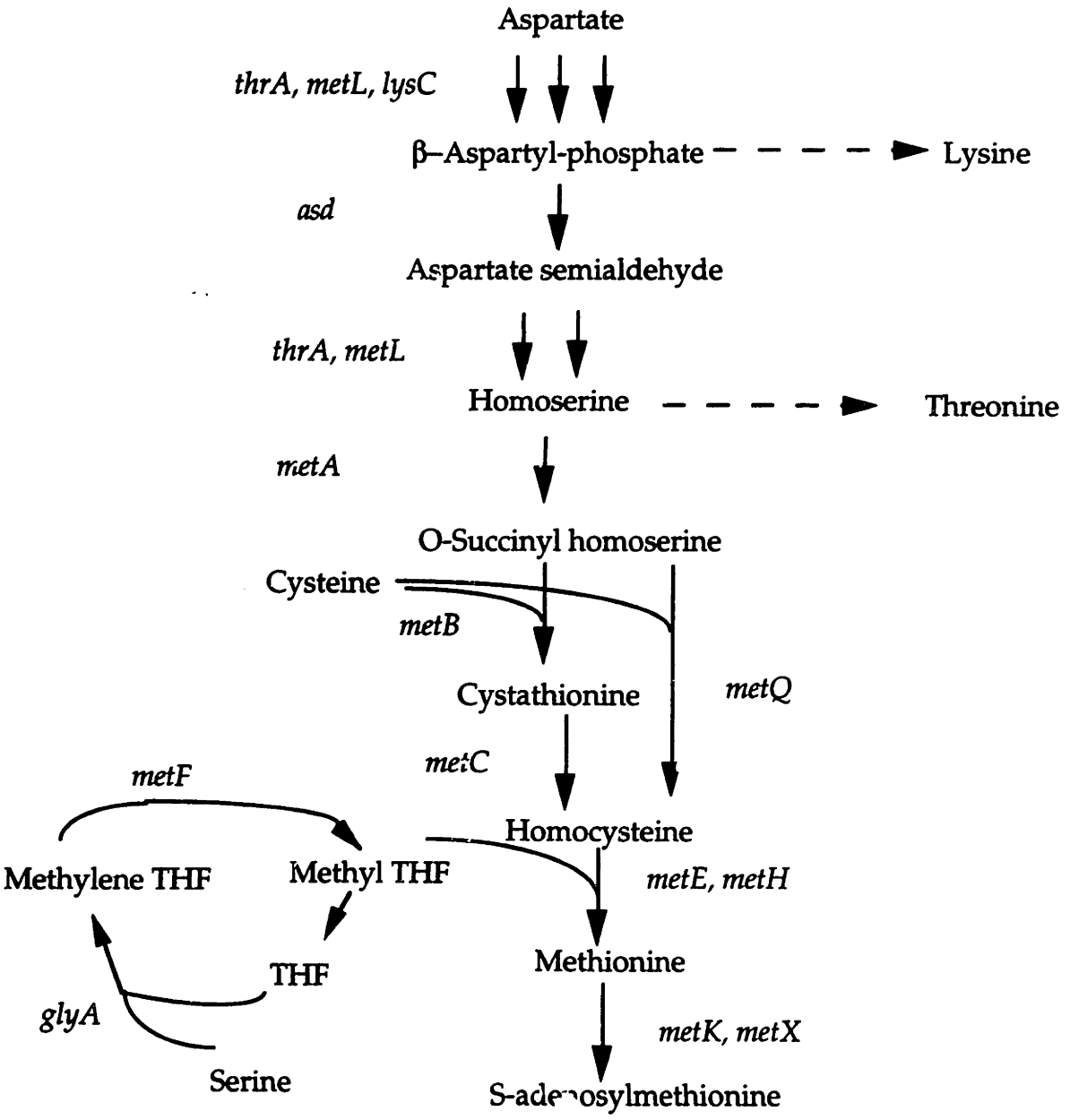
E. coli has evolved a complex regulatory scheme to control the synthesis of methionine [70, 86]. There is repressor-mediated control by the holorepressor

MetJ and the co-factor S-AM, repression by vitamin B_{12}, and activation by MetR [70]. Thus far no evidence of any type of classical attenuation has been detected for methionine biosynthesis, although attenuation is common in the other amino acid pathways.

Methionine is one of several amino acids derived from aspartate. Aspartate is formed by the action of aspartate aminotransferase on oxaloacetate. In *E. coli* there are three isoenzymes of aspartokinase, the enzyme which converts aspartate into aspartyl-ß phosphate, ThrA, MetL, and LysC (Figure 1.5) [70]. Both ThrA and MetL are bifunctional and serve as homoserine dehydrogenase later in the pathway. Both the aspartokinase and homoserine dehydrogenase activities of ThrA are inhibited by threonine. *ThrA* expression is multivalently repressed in an attenuation mechanism by threonine plus isoleucine and also by the product of the *ileR* gene. *MetL* expression is repressed by methionine [70]. *MetL* is part of an operon with *metB* [87]. Although many of the *E. coli met* genes are clustered, this is the only operon. ThrA and MetL are structurally different in that ThrA is a tetramer and MetL is a dimer. LysC is inhibited and its gene is repressed by lysine [70].

The second step is the conversion of aspartyl-ß-phosphate to aspartate semialdehyde by aspartate semialdehyde dehydrogenase, the product of the *asd* gene. This gene is repressed independently by threonine, lysine, and methionine, although derepression is greatest if lysine is the limiting factor. There is also some evidence for regulation by glucose-6-phosphate [70].

Figure 1.5: Methionine Pathway in *E. coli.* Below in the pathway from aspartate to methionine in *E. coli.* Multiple arrows indicate isoenzymes. Dashed lines represent multiple steps. The abbreviation THF stands for tetrahydrofolate. [70]



From aspartate semialdehyde the pathway splits with one branch going towards lysine and the other to threonine and methionine. In the threonine and methionine pathway, the next step is to homoserine via homoserine dehydrogenase. As mentioned earlier, this is catalyzed by the bifunctional ThrA and MetL enzymes [70].

At homoserine, the methionine pathway splits from that of threonine. The next step in methionine biosynthesis is catalyzed by the product of *metA*, homoserine transsuccinylase, to give O-succinyl homoserine. This step is heavily regulated. The enzyme is inhibited by both high levels of methionine or S-AM, or by low levels of both acting synergistically. *MetA* is repressed about 300 fold by MetJ [70]. This step is also sensitive to high temperatures (up to 45°C) in what is believed to be a mechanism which limits growth at temperatures which might be lethal to the cell by blocking a range of metabolic functions. There is also a linkage between methionine synthesis and heat shock. The *metA* gene has a region upstream of its other promoter which has a lot of homolgy to the σ^{32} recognition sequence. As the level of homoserine transsuccinylase increases dramatically upon heat shock and because of the similarity in the promoter sequence, MetA is considered a heat shock protein [70]. *MetA* has two other mapped promoters, one which is sensitive to the intracellular level of methionine and one which is constitutive [70].

The next step, the conversion of O-succinyl homoserine plus cysteine to cystathionine is catalyzed by cystathionine-γ-synthase, the product of the *metB* gene [70]. As mentioned earlier, *metB* is in an operon with *metL* and is repressed by methionine via MetJ [70, 86].

Cystathionine-β-lyase is the next enzyme in the methionine pathway which converts cystathionine to homocysteine, NH_4^+, and pyruvate [70]. Its gene, *metC*, is also repressed by MetJ [70, 86]. MetB and MetC exhibit much homology to one another and it is theorized that they are descendents from the same ancestor [70]. In some organisms, such as *C. glutamicum*, there is actually one enzyme which catalyzes the conversion of O-succinyl homoserine directly to homocysteine [88]. This will be discussed later.

The final step which converts homocysteine to methionine is catalyzed by isoenzymes, MetH (5-methyltetrahydrofolate-homocysteine (Vitamin B_{12}) methyltransferase) and MetE (5-methyltetrahydropteroyltriglutamate homocysteine methyltransferase) [70]. MetH is a vitamin B_{12}-dependent enzyme whereas MetE is not. Most organisms have one or the other enzyme, but *E. coli* and *S. typhimurium* have both [70]. *MetH* is different from the other *met* genes in that it is not regulated directly by MetJ. Its promoter also does not have the typical "-10" and "-35" sites of general *E. coli* housekeeping promoters. Rather its transcriptional start site is found 300 bp upstream of the gene and it and the *metH* promoter are within the coding sequence of the gene *iclR* [70]. The promoter and transcription start site of *S. typhimurium* are much closer to the translational start of *metH*, only about 29 bp upstream. As there is so much homology in the sequences of *E. coli* and *S. typhimurium* in this region, however, the possibility has been suggested that both organisms have two promoters for this gene, a minor one around -29 and a major promoter in the *iclR* gene. Both the *E. coli* and the *S. typhmurium* promoters are activated by MetR [70].

MetE is the alternative, vitamin B_{12} independent enzyme [70]. However, its gene, *metE*, is regulated by vitamin B_{12}. Repression of the expression of *metE* by

vitamin B_{12} is understandable in light of the fact that the MetH enzyme is over 50-fold more efficient than MetE. Therefore, if vitamin B_{12} is present, MetH is active and extra MetE is not necessary. *MetE* is also repressed approximately 60-fold by MetJ [70].

While this is the main pathway to methionine, the actual route is a little more complicated because of the loop through different species of tetrahydrofolate (THF) [70]. In order to provide the methyl group which combines with homocysteine to form methionine, another series of reactions takes place. In this case, the product of the *glyA* gene converts serine and THF into methylene-THF and glycine. Methylene-THF is then converted to methyl-THF by the product of *metF*. It is this methyl group which is transferred to homocysteine. The other product, THF, can then go through the cycle again. The control of *glyA* is very complex with serine, glycine, methionine, thymine, guanine and adenine involved in a cumulative repression. MetR is responsible for the methionine control. The control of *metF* is mediated through both MetJ and vitamin B_{12} [70].

Although the conversion of methionine to S-AM is not part of the pathway to methionine, it is worthy of mention because of the important role S-AM plays in the regulation of the pathway as the corepressor of MetJ [70]. Two genes have been identified which code for methionine adenosyl transferase, *metK* and *metX*. *MetX* is only expressed in rich media while *metK* is only expressed in minimal medium [70].

In the above sections the regulation at each step was briefly described. As mentioned before, there are three major mechanisms of regulation in *E. coli* and *S. typhimurium* for methionine biosynthesis: repression by vitamin B_{12},

activation by MetR, and repression by MetJ [70, 86]. Vitamin B_{12} represses both *MetE* and *MetF*. The addition of vitamin B_{12} to the medium also increases the activity of MetH, but upon further study this was found to be due to the formation of the stable MetH-vitamin B_{12} complex. Since MetH is so much more efficient than MetE, reducing the synthesis of *MetE* makes sense when there is vitamin B_{12} around to act as a co-factor for MetH. The synthesis of *metF* is also reduced because the increased efficiency of MetH makes extra MetF unnecessary. The level of repression of vitamin B_{12} on *metF* is less than that on *metE*, 78% versus 99%, indicating that MetF is still needed in some quantity [70].

MetR plays an important role in the regulation of the last step in methionine biosynthesis, the conversion of homocysteine to methionine [70, 86]. Both *S. typhimurium* and *E. coli* have similar gene arrangements for *metR*. *MetR* is linked to *metE* and the two genes are transcribed from overlapping divergent promoters. *MetR* is repressed approximately 70-80-fold by MetJ and it is also negatively auto-regulated [70]. MetR activates both *metE* and *metH*. For *met E* activation, homocysteine acts as a co-activator with MetR. For *metH*, the presence of homocysteine actually reduces transcription [70]. The model that has been put together to explain this is that upon vitamin B_{12} depletion, homocysteine builds up as MetH is not as active and there is not much MetE around. As homocysteine builds up, expression of *metE* is activated and that of *metH* is slowed down. Thus, MetR acts as a switch to shift from vitamin B_{12} dependent transmethylase to the vitamin B_{12} -independent enzyme [70]. Similar systems as the *metE/metR* switch have been identified in other pathways such as *lysA/lysR* and *ilvC/ilvY*. MetR also plays a role in activation of the *glyA* and *metA* expression. In both these last two examples, the range of activation is about three-fold [70].

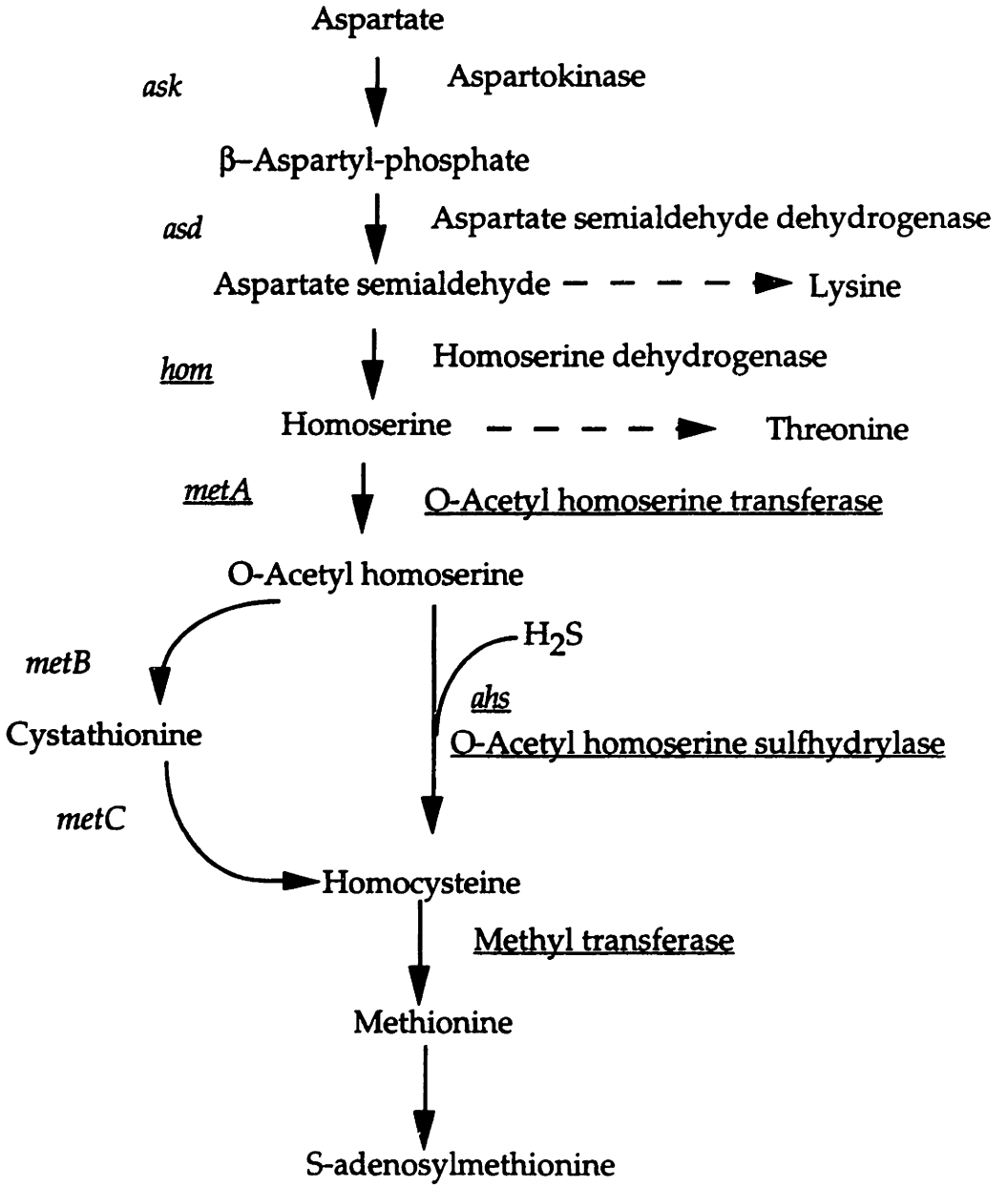
MetJ represses almost all of the *met* genes except for *metH* , *metK* and *asd* [70]. The latter three genes are also repressed by methionine, but do not appear to have the MetJ consensus operator sequence. This sequence, known as the "met box," is a variation of the palindrome AGACGTCT and usually is found in two to five copies in the promoter region of MetJ regulated genes [70]. The *met* genes are repressed over a 3 to 300 fold range by MetJ, with *metJ* itself being repressed 3-fold and *metA* being repressed 300-fold. Studies done in which the homology to the consensus sequence was either increased or decreased led to subsequent increased or decreased repression, respectively [70].

The MetJ repressor has been studied extensively, and its structure has been determined [70]. The repressor is a dimer and the results of experiments with the repressor and the operator sites are consistent with a model in which multiple dimers act cooperatively in order to effficiently repress the operator [70]. The solved crystal structure of the MetJ repressor was the first example of a class of DNA binding proteins with what is known as the "ribbon-helix-helix" motif. Other proteins which have a similar structure are the Arc and Mnt repressors from bacteriophage P22 [70].

Methionine Biosynthesis in *C. glutamicum*

C. glutamicum and *E. coli* share many of the same enzymes in the pathway from aspartate to methionine (Figure 1.6). The genetic arrangement and regulation in *C. glutamicum*, however, is vastly different from that described in *E. coli*.

Figure 1.6: Methionine Pathway in *C. glutamicum* - Below is the pathway from aspartate to methionine in *C. glutamicum*. Not as much is known about the pathway in *C. glutamicum* compared to *E. coli*. It appears that although the *metB* and *metC* enzyme activities are present in *C. glutamicum*, they may not be involved in methionine synthesis. Genes and enzymes which are underlined are regulated by methionine or S-AM. [88, 92, 111]



C. glutamicum also uses aspartokinase to convert aspartate to aspartyl-ß-phosphate. In this case, there is only one aspartokinase as opposed to several isoenzymes [1, 45]. In *C. glutamicum*, the aspartokinase and the homoserine dehydrogenase are separate enzymes stemming from unlinked genes. The *ask* gene is in the same operon as the *asd* gene, coding for aspartate semialdehyde dehydrogenase (ASD) [35]. There is an internal promoter in this operon which directs the synthesis of a transcript which encodes the ß-subunit of aspartokinase and ASD [35]. Neither of these promoters are end-product regulated. The enzyme aspartokinase is regulated by its concerted feedback inhibition via threonine and lysine [1].

The next step is the conversion of aspartyl-ß-phosphate to aspartate semialdehyde by the enzyme ASD. There is no known regulation at this step. As mentioned before, the gene for ASD, *asd*, is in the same operon as *ask* [35].

As in *E. coli*, the pathway splits off at aspartate semialdehyde to go to threonine, methionine, and isoleucine or to lysine. The next step of the common pathway to methionine and threonine is the conversion of aspartate semialdehyde to homoserine by homoserine dehyrogenase. Carbon flow is preferentially directed towards the threonine pathway over the lysine pathway because homoserine dehydrogenase has a 15-fold higher affinity for the common substrate aspartate than dihydropicolinate synthetase (the first enzyme in the lysine branch) [45]. Homoserine dehydrogenase is inhibited by threonine and its gene, *hom*, is repressed two-three fold by methionine [85, 89]. The *hom* gene is in an operon with the next gene in the threonine pathway, *thrB*, encoding the enzyme homoserine kinase [85]. There is also a second promoter within the *hom-thrB* operon that directs a low level of expression of the *thrB* trancript [90].

The *C. glutamicum* and *E. coli* methionine pathways are most different in the branch which converts homoserine ultimately to methionine. Most of the study of the methionine-specific pathway in *C. glutamicum* has been biochemical and genetic in nature. Methionine mutants have been used to establish the intermediates produced on the way to methionine [88, 91]. Some of the proteins have been purified and their properties studied [88, 92]. None of the genes have been reported in the literature. In Project Lab 7.31, 1993, *C. glutamicum* clones were isolated which complement some of the *E. coli* methionine mutants, but these have not yet been fully characterized.

Whereas the first reaction in *E. coli* is the conversion of homoserine and succinyl-CoA to make O-succinylhomoserine, in *C. glutamicum* acetyl-CoA is substituted for the succinyl-CoA in order to make O-acetylhomoserine [92]. This step is highly regulated in *C. glutamicum*. Methionine represses the expression of the *C. glutamicum* approximately 70-fold. Methionine and S-AM also inhibit the activity of acetyl transferase, although there does not seem to be a synergistic effect when both inhibitors are present. It is hypothesized that the level of methionine required to inhibit the acetyl transferase *in vitro* is too high to have any real physiological effect [92].

The next enzymatic reaction is the direct conversion of O-acetylhomoserine to homocysteine. The enzyme which catalyzes this reaction is O-acetylhomoserine sulfhydrylase [88]. Its gene has been shown to be repressed and it is inhibited by methionine. Once again, however, the concentration of methionine required for inhibition may be too high to have any physiological effect [88]. H_2S is the donor of the sulfur atom in *C. glutamicum* [88]. In the *E. coli* pathway, cysteine donates

the sulfur atom [70]. There has been the report of a mutation, *metQ*, in *E. coli* K12 *metC* mutants which also converts O-succinylhomoserine directly into homocysteine. Further investigation revealed that *metQ* was in fact a new gene which is 1149 bp and encodes a polypeptide of 41 kDa [70].

There is evidence that the enzyme activities in the pathway through cystathionine also exist in *C. glutamicum*. Cystathionine-γ-synthase and cystathionine-ß-lyase activities can be measured in *C. glutamicum* [70]. The cystathionine-γ-synthase activity has been studied in a variety of organisms and it was found that *Corynebacterium* sp. have an enzyme which can utilize both O-acetylhomoserine and O-succinlyhomoserine as a substrate [93]. Enteric bacteria utilize mostly O-succinylhomoserine and many Gram-positive organisms such as *B. subtilis* only use O-acetylhomoserine [93]. Furthermore, one of the *E. coli* mutants which was screened and complemented with a *C. glutamicum* library in Project Lab was a *metB* auxotroph. The activity of the next enzyme, cystathionine-ß-lyase, has also been detected in *C. glutamicum*. A *metC* auxotroph of *E. coli* has been complemented with a *C. glutamicum* library. This DNA was partially sequenced and it was found that the complementing activity was due to a gene which had already been isolated due to its ability to confer aminoethylcysteine (AEC) resistance via the activity of its gene product, a C-S lyase [94]. AEC is a lysine analog commonly used for screening lysine-overproducing strains during classical mutagenesis. AEC resistance can be conferred in several ways: by a mutation in aspartokinase which renders the enzyme insensitive to lysine inhibition, by a mutation in the lysine transport enzyme which prevents AEC from entering the cell, and by amplification of the C-S lyase activity, which degrades AEC. The C-S lyase was reported as a non-essential gene which can also use cysteine, cystine, and cystathionine as

substrates [94]. Although the enzyme activities of cystathionine-γ-synthase and cystathionine-ß-lyase are present in *C. glutamicum*, they are not believed to be a bypass pathway to homocysteine because *C. glutamicum* strains deficient in the O-acetylhomoserine sulfhydrylase activity are still homocysteine (and methionine) auxotrophs. Therefore, the activities of cystathionine-γ-synthase and cystathionine-ß-lyase are not directing enough, if any, carbon towards homocysteine [88].

The last step in the pathway is the conversion of homocysteine to methionine. There is not much in the literature about this step, the conversion of methionine to S-AM, or any of the steps in the tetrahydrofolate cycle.

There is currently no description of a *C. glutamicum met* regulator in the literature. The strain described in Chapter 3, Section I, E12Rep-2, is the only known strain which appears to be derepressed. This derepression occurs at two loci, the *hom* operator and the *metA* operator. If the methionine regulator in *C. glutamicum* is similar to that of *E. coli*, this strain may be deficient in a MetJ homolog. It is believed that this mutation is in the regulator rather than a methionine transport protein. This is based on homoserine acetyltransferase assays of cultures of E12Rep-2 and E12 grown in minimal medium without methionine. The activity of the homoserine acetyltransferase is higher in E12Rep-2, presumably because some of the repression caused by endogenous levels of methionine is derepressed in the mutant strain but not in the wild type strain.

CHAPTER 2
PURIFICATION OF *CORYNEBACTERIUM GLUTAMICUM* RNA POLYMERASE

SUMMARY

The RNA polymerase from *Corynebacterium glutamicum* 13059 has been purified over 2700-fold. *Corynebacterium glutamicum* RNA polymerase appears to have the same subunit structure as the RNA polymerases of other bacteria. Also present in the profile are several other co-eluting bands whose identities have not been established. The purified protein has been shown to bind a DNA fragment which contains the *hom* promoter of *Corynebacterium glutamicum*. The purified RNAP has reduced efficiency when using linear substrates in *in vitro* transcription reactions.

INTRODUCTION

DNA-dependent RNA polymerase (RNAP) is the enzyme responsible for the transcription of DNA into RNA [57, 58]. The RNAP from many different prokaryotes has been purified [37, 53-55]. RNAP is composed of several different subunits; the core enzyme is of the form $\alpha_2\beta\beta'$ [57, 58]. The final subunit, σ, is necessary for a functional polymerase [57, 58]. All of the RNAPs studied from other prokaryotes have been found to have several σ factors. There is usually one major σ factor which is responsible for the transcription of the maintenance genes and then many alternative σ factors which recognize promoters of genes which need to be expressed during specific times [62].

Corynebacterium glutamicum is a Gram-positive, non-pathogenic, non-sporulating soil bacterium [1]. It has been extensively used for the industrial production of amino acids and as the model organism for the emerging field of metabolic engineering [1, 4, 95]. One of the reasons that *C. glutamicum* is used for these

purposes is because its biosynthetic pathways are not as complex as those of other prokaryotes, such as *Escherichia coli* [1]. For instance, at key regulatory points in *C. glutamicum*, such as the enzyme aspartokinase, only one enzyme is used and it is regulated through inhibition by endproducts of the pathway [45]. That same enzymatic activity in *E. coli* is catalyzed by three isoenzymes, each regulated by different endproducts [70]. Since *C. glutamicum* only uses one enzyme at these important regulatory spots, it is much easier to manipulate its biosynthetic pathways than it would be a bacterium with more complicated regulatory strategies. This relative simplicity has been exploited since the 1950's in strain development by classical mutagenesis to create better amino acid producing strains of *C. glutamicum* [4]. In the past decade, however, there has been a rapid expansion of molecular biology techniques which have been developed for use in *C. glutamicum*. This has lead to a change from using classical mutagenesis in strain development to using more targeted manipulations through recombinant DNA techniques [45]. In metabolic engineering, the simplicity is exploited in order to understand how the cell controls the carbon flux into different metabolites [95]. The simplicity of the system allows one to make predictions on the effect of perturbing a specific gene or enzyme and then creating a strain in which these predictions can be tested. These perturbations can be in the form of such procedures as gene amplification, gene disruption, or abolishment of regulatory sites in important enzymes.

A full understanding of gene expression in *C. glutamicum* is important to have a complete knowledge of regulation in these pathways. To that end, the purification of *C. glutamicum* RNAP allows the study of transcriptional properties that were not before possible. For instance, several apparent *C. glutamicum* promoters have been determined through S1 nuclease mapping or primer

extension analysis [35, 44, 85, 96-100]. All of these promoters are involved in either glycolysis or different amino acid pathways and would therefore be considered maintenance genes. From this set of promoters, it is difficult to tell whether there are consensus sequences which would describe a canonical motif for *C. glutamicum* promoters. It has not been possible to ascertain whether this collection of promoters were recognized by different σ factors and therefore did not contain a consensus promoter motif, used a consensus sequence that allowed great variability, or had been post-transcriptionally modified so that the apparent transcription start site was false. Using purified *C. glutamicum* RNAP, it will be possible to more accurately map the promoter location and resolve some of these questions.

We have been able to successfully purify *C. glutamicum* RNAP from ATCC strain 13059. We have compared this RNAP to those from other organisms and have found many similarities. We have used the purified enzyme in *in vitro* binding and transcription assays using the *hom* promoter which directs the expression of the *hom-thrB* operon, encoding homoserine dehydrogenase and homoserine kinase, respectively [85]. The *hom* promoter is relatively strong in comparison to the other promoters in the threonine pathway (unpublished observations). In addition, it is one of the few promoters which has been demonstrated to be regulated in *C. glutamicum;* methionine represses the *hom* promoter two-three fold in the native state [85]. Using RNAP to study the *hom* promoter will increase our understanding of the different properties of the *hom* promoter, such as the kinetic parameters which describe transcription initiation. Furthermore, we are able to study effects of other potential transcription regulators. For instance, in *Mycobacterium smegmatis,* DNA superhelicity is a requirement for transcription using *M. smagmatis* RNAP [37]. We have now demonstrated that this

superhelicity is not an absolute requirement in *C. glutamicum*, although transcription efficiency is reduced when a linear template is used.

MATERIALS AND METHODS

Strains, Proteins and Plasmids. *C. glutamicum* ATCC 13059 was grown in Luria-Bertani broth and Brain Heart Infusion (Difco) to exponential phase. The plasmids pBStac and pBSblaZ were used for transcription assays during RNAP purification and have short mRNA transcripts under the control of either the *tac* promoter of *E. coli* or the *blaZ* promoter of *Staphylococcus aureus*. The plasmid pHomcm was used for *in vitro* transcription assays. The RNAPs from other organisms were all purified at Scriptgen (Medford). The antibody to *E. coli* σ^{70} was made by Scriptgen.

RNAP Purification. We harvested over 100 g (wet weight) of *C. glutamicum* 13059. These cells were added to 200 ml TGED (50 mM Tris (pH 7.5), 10% glycerol, 1mM EDTA, 10 mM $MgCl_2$, 3 mM DTT, and 0.1 mM PMSF) plus 0.2 M KCl and 200 ml 100 micron glass beads in a Biospec Products Bead-Beater. The cells were grinded for 30 second periods and then the compartment was set on ice for 90 seconds. Twelve cycles were done. The ground cells were separated from the beads as well as possible by allowing the mixture to settle. After the cell lysate was removed, the beads were washed with TGED to remove as much cell lysate as possible. The cell lysate was centrifuged at 4°C for 20 minutes at 30,000 rpm. The supernatant was the crude extract.

The crude extract was treated with Polymin P such that the final concentration of Polymin P was 0.35% [101]. After stirring for 30 min at 4°C, the mixture was centrifuged at 30,000 rpm for 20 minutes (4°C). The pellet was resuspended and

homogenized in 300 ml TGED + 0.5 M NaCl. This mixture was centrifuged at 30,000 rpm for 20 minutes (4°C) and the pellet was resuspended and homogenized in 300 ml TGED + 1 M NaCl. This was then centrifuged at 30,000 rpm for 20 minutes (4°C) and the supernatant was kept. Ammonium sulfate was added to the supernatant to a final concentration of 55% (w/v) and stirred at 4°C. After centrifugation, the pellet was resuspended to a final volume of 1.5 ml in TGED containing a protein inhibitor (PI) mix (0.1 mM benzamidine, 0.5 mg/l leupeptin, and 0.4 mg/l pepstatin).

The protein mixture was then loaded onto a Bio-Gel A1.5m gel fractionation column. Protein was eluted with 0.1 M KCl in TGED plus PI mix. Fractions were collected and assayed by SDS-PAGE (4-15% gel) analysis and transcription assays using either the *tac* promoter or the *blaZ* promoter from *S. aureus*. The fractions which had the highest activities were pooled and loaded onto a heparin-agarose column (which had been equilibrated with TGED plus 0.1 M KCl). After the column was loaded it was washed with TGED plus 0.1 M KCl and the PI mix and eluted over a gradient of 0.1 M to 1 M KCl in TGED. Fractions were collected and assayed as before. There appeared to be extra bands on the SDS-PAGE lanes which corresponded to the fractions with the highest activity. These fractions were once again pooled and loaded onto a heparin-agarose column. This time the protein was eluted over a gradient of 0.1M-0.6M KCl.

Transcription Assays during Purification. RNA was synthesized using either the *tac* promoter or the *blaZ* promoter from *S. aureus*. The assay mix contained 100 µl DNA with either the *tac* promoter or the *blaZ* promoter (1.5 mg/ml), 30 µl of 100 mM solutions of ATP, CTP and GTP, 6 µl of ^{32}P-UTP (10 µCi/µl), 0.3 µl

UTP, 3 ml transcription buffer (50 mM Tris (pH 7.9), 10 mM $MgCl_2$, 200 mM KCl, 10 mM DTT, 0.1 mM EDTA, 1.0 mM K_2HPO_4 (pH 7.5) and 100 µg/100 µl BSA), and 3.75 ml dH_2O. In a 96-well plate, 45 µl of the assay mix was added to 5 µl of each fraction. Reactions were carried out at 30°C for 30 minutes. The RNA transcripts were harvested on a printed Filtermat B with 5% cold trichloroacetic acid using a Tomtec Harvester 96. The filters were dried and ^{32}P was counted using a Wallac 1450 Microbeta Plus liquid scintillation counter.

Western Blots. A 4-15% gel (BioRad) was run using SDS-PAGE under manufacturer's specifications. The protein was transferred to a nitrocellulose membrane by electro-transfer (200 mA for 1.5 hr, transfer buffer - 25 mM Tris (pH 8.3), 192 mM glycine and 20% methanol). The membrane was blocked at room temperature for 0.5-1 hr in 3% BSA, 50 mM Tris (pH 7.5), 0.2 M NaCl and 0.1% Tween 20. The membrane was then incubated in an antibody to *E. coli* σ^{70} (0.2-0.5 µg/ml) in wash buffer (1% BSA, 50 mM Tris (pH 7.5), 0.2 M NaCl, and 0.01% Tween 20) at 4°C overnight or room temperature for 1 hr. The membrane was washed three times in wash buffer at room temperature. A secondary antibody was added to wash buffer and the membrane was incubated for 1 hr at room temperature. The membrane was washed three times in wash buffer at room temperature then developed in developing solution (0.1 M Tris (pH 9.5), 0.1 M NaCl, 5 mM $MgCl_2$, 66 µl NBT (0.38 mM - purchased from Promega) and 33 µl BCIP (0.38 mM - purchased from Promega).

Gel Retardation Assay. DNA containing the *hom* promoter was labeled with ^{32}P-dCTP in reactions using the Pharmacia Biotech Oligolabelling Kit. Serial dilutions of the RNAP was added to tubes containing the labelled DNA, 0.5X TBE and 5% glycerol. The reactions were allowed to equilibrate for 15 minutes at

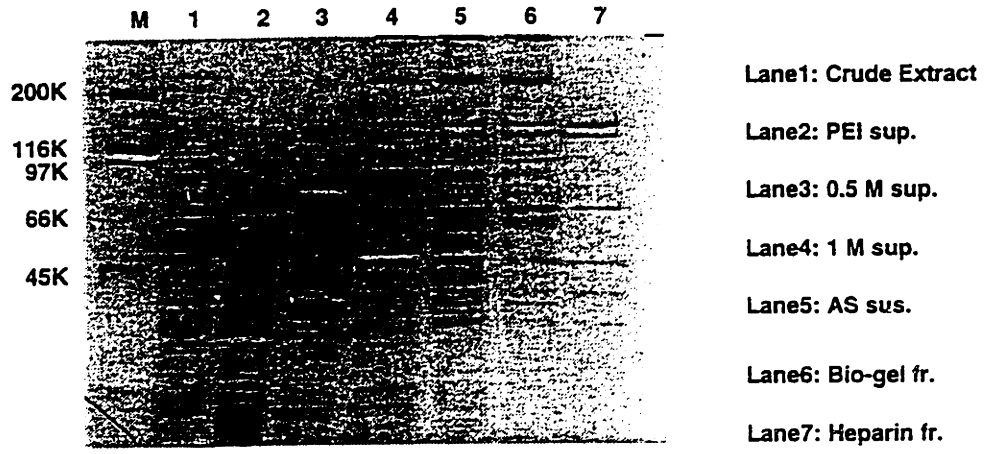
30°C before being loaded onto a 4% polyacrylamide gel run at 4°C. The voltage of the gel was around 300 V [102, 103].

***In vitro* Transcription Assay.** The purified RNAP was incubated with a DNA template, either a supercoiled plasmid or linearized plasmid, for 10 minutes at 30°C. Added to the final concentrations indicated were UTP, ATP, and CTP (0.4 mM), GTP (0.2 μM) and 10 μCi [^{32}P]-GTP. After 15 minutes, the reactions were terminated by adding 3xKCl-SDS stock solution (final concentrations of components of solution are 0.9% SDS, 50 $\mu g ml^{-1}$ tRNA, 130 mM KCl, and 50 mM EDTA). The tubes were incubated at 75°C for 15 minutes and then 10 minutes on ice to precipitate the protein. The precipitated protein was pelleted by centrifugation and the supernatant was removed and ethanol precipitated. The precipitated RNA was resuspended in sample buffer and loaded onto a 6% denaturing polyacrylamide gel [37].

RESULTS

RNAP Purification. SDS-PAGE analysis of the different steps of the RNAP purification are shown in Figure 2.1. After the first heparin column the *C. glutamicum* RNAP was purified over 2700-fold from the original crude extract. The subunit structure of *C. glutamicum* RNAP is similar to that of other prokaryotic RNAPs [37, 53-55]. It has two bands around 150,000 kd which are probably analogous to the β and β' subunits. There is another band around 66 kd which is most likely the *C. glutamicum* major σ factor, although another larger σ factor also is present (discussed below). It is not possible at this time to determine which band corresponds to the α-subunit. The α-subunit tends to be around 45 kd and there are a few bands in this region. The strongest band is

Figure 2.1: Purification of *C. glutamicum* RNA Polymerase - Below is the SDS-PAGE analysis of the different steps during the purification of *C. glutamicum* RNA polymerase. The protein was purified over 2700-fold.



Step	Protein Concentration (mg/ml)	CPM*/Protein (CPM/mg)	Fold-Purification
Crude Extract	21.5	3466	1
PEI Supernatant	5.05	10,245	3
1M Wash	3.6	59,170	17
NH_4^+ Suspension	54	30,164	8.7
Bio-gel Column	5.25	1,592,442	459
Heparin Column	2.1	9,374,896	2704

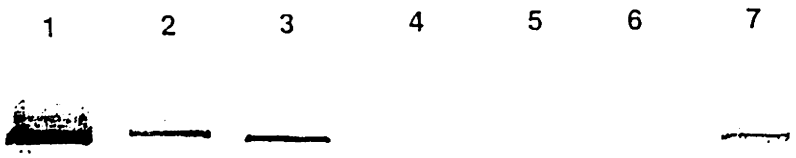
*Counts per minute in the RNA synthesized during the transcription assays used to test the fractions during the protein purification.

between 46-48 kd, so this probably is the α-subunit, but more tests will need to be done to ascertain this exactly.

After the heparin-agarose column step, SDS-PAGE analysis revealed the presence of several bands between 30 and 40 kd. The protein mixture was reloaded onto another heparin-agarose column using a shorter gradient (0.1M-0.6M KCl) in an attempt to separate these bands from the RNAP. SDS-PAGE analysis after the second heparin column showed that once again, the same proteins co-eluted. At this time it is not known whether these extra bands are contaminating proteins which co-eluted or alternative σ factors. The usual range for alternative σ factors is around 30 kd, so it is possible that some of these bands may be σ factors [62]. It would appear that *C. glutamicum* has at least two σ factors. From the SDS-PAGE analysis, the major σ factor is around 66 kd, athough the identity of this protein needs to be determined more rigorously to confirm this. A Western blot was done on *C. glutamicum* RNAP using an antibody to σ^{70} of *E. coli* (Figure 2.2). A component of the *C. glutamicum* RNAP is recognized by this antibody, so *C. glutamicum* has a minor protein which has homology to the *E. coli* major σ factor. This *C. glutamicum* protein appears to be slightly larger than the protein from *E. coli*, running around 80-90 kd in SDS-PAGE analysis. This band is very faint on the SDS-PAGE analysis of *C. glutamicum* RNAP. As this is the band which is recognized by the *E. coli* σ^{70} antibody, it is possible that this may be the *C. glutamicum* major σ factor and the band around 66 kd may be another contaminating protein. More analysis needs to be done to determine the identities of these bands.

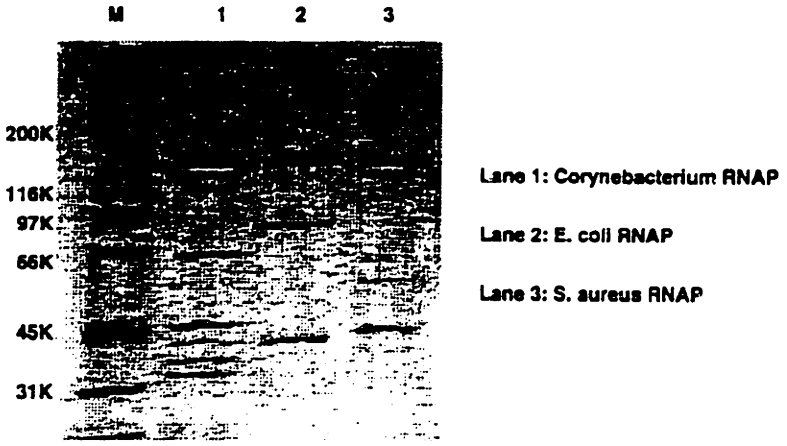
Comparison of *C. glutamicum* RNAP to Other RNAPs. Figure 2.3 shows an SDS-PAGE profile of *C. glutamicum* RNAP compared to a Gram-negative RNAP

Figure 2.2: Western Blot of RNA Polymerases from Various Prokaryotes - A Western Blot was done to test cross-reactivity of different RNA polymerases to an antibody raised against the major σ factor of *E. coli.* Of the seven prokaryotic RNA polymerases, only four had σ factors which were recognized by the antibody; *E. coli, E. cloacae, Pseudomonas aeruginosa,* and *C. glutamicum. E. faecium* also has a very faint band, most likely due to a poor protein preparation. The *C. glutamicum* band is also slightly faint, indicating that it may not be recognized by the *E. coli* major σ factor as well as the Gram-negative bacteria. All of the proteins cross-reacting are of similar size, running between 80-90 kd on an SDS-PAGE gel. All proteins tested were purified at Scriptgen.



Lane 1: *E. coli*
Lane 2: *E. cloacae*
Lane 3: *P. aeruginosa*
Lane 4: *S. aureus*
Lane 5: *B. subtilis*
Lane 6: *E. faecium*
Lane 7: *C. glutamicum*

Figure 2.3: Comparison of C. *glutamicum* RNA Polymerase to Those of *E. coli* and *S. aureus* - Below is an SDS-PAGE analysis of the RNA polymerases from C. *glutamicum, E. coli,* and *S. aureus*. All show a similar subunit pattern.



from *E. coli* and a Gram-positive RNAP from *S. aureus*. As can be seen, the profiles are all similar. All have β and β' subunits of similar molecular weight. The σ factors are in different ranges, with the *E. coli* σ factor a bit larger than those from the Gram-positive species. The α-subunits are also of similar weights.

Binding of RNAP to *hom* Promoter. The *hom* promoter expresses the operon which contains the genes for the enzymes homoserine dehydrogenase and homoserine kinase. A 1 kb piece of DNA which contains this promoter was used to demonstrate the binding of the *C. glutamicum* RNAP to a promoter in gel retardation assays. As shown in Figure 2.4, *C. glutamicum* RNAP effectively binds the DNA containing the *hom* promoter. Rough calculations of the apparent dissociation constant, K_d, can be made by a visual inspection of the gel to determine the protein concentration at which half the DNA is bound [103]. This estimation is only valid if the amount of DNA is small in comparison to the amount of protein at the midpoint [103]. In this case, the amount of DNA is approximately 100-fold less than the amount of protein. As can be seen from the gel, between the dilutions of 10^{-3} and 10^{-4}, the level of bound DNA drops precipitously, so the midpoint lies between these two concentrations of protein. This translates into a K_d of approximately 1.1×10^{-9} M.

Transcription Assays. The ability of the purified *C. glutamicum* RNAP to synthesize RNA transcripts was also determined by *in vitro* assays. The purified RNAP was able to transcribe RNA from both the supercoiled and the linearized plasmids, although the efficiency was greater using the supercoiled template (Figure 2.5). This was a little surprising because we expected that the *C. glutamicum* RNAP would not be able to transcribe using a linearized plasmid as a substrate. It has been reported that in *in vitro* assays using purified RNAP from

Figure 2.4: Gel Retardation Assay of *C. glutamicum* RNA Polymerase on the *hom* Promoter - In the assay below, different dilutions of *C. glutamicum* RNA polymerase were incubated with a radiolabeled fragment of DNA which included the *hom* promoter. At high concentrations of protein, the DNA is all held up in the well, but as the concentration of the DNA decreases, the amount of free DNA increases. Between the protein dilution of 10^{-3} and 10^{-4}, approximately half of the DNA dissociates from the RNA polymerase. This translates into a K_d of approximately 1.1×10^{-9} M.

Lane 1: Free DNA
Lane 2: Free DNA + RNAP X 10^{-2}
Lane 3: Free DNA + RNAP X 10^{-3}
Lane 4: Free DNA + RNAP X 10^{-4}
Lane 5: Free DNA + RNAP X 10^{-5}

Figure 2.5: *In vitro* Transcription from Linearized or Supercoiled Templates Using *C. glutamicum* RNA Polymerase - Below are the results of *in vitro* assays using a substrate which was either linear or supercoiled. Both substrates are utilized by the *C. glutamicum* RNA polymerase, but the supercoiled substrate is used more efficiently.

Lane 1: Supercoiled Plasmid
Lane 2: Linear Plasmid

the closely related *Mycobacterium smegmatis*, transcription was not obtained using linear plasmids [37]. Since this is the closest organism to *C. glutamicum* from which RNAP has been purified, we expected that they may share this dependence on supercoiling. All the promoters which were tested in *M. smegmatis* appeared to be heavily dependent on supercoiling [37]. This dependence in *M. smegmatis* has been hypothesized to be due to the high (G+C) content of the bacterium, about 65% [37]. Although only the *hom* promoter plus the promoters for the other genes on the plasmid (kanamycin resistance gene, ampicillin resistance gene, and genes of plasmid replication in *C. glutamicum*) have been tested for the effect of supercoiling, it would appear that the *C. glutamicum* RNAP is not as dependent on supercoiling as that of *M. smegmatis*.

Other *C. glutamicum* Promoters. Figure 2.6 shows a compilation of *C. glutamicum* promoters which have been described in the literature. Although the rest of the genome has a (G+C) content of 55.5-57.5% [1], most of the promoters are far more (A+T) rich. In these promoters, the (G+C) content was calculated for the first 40 base pairs of the promoter region. Only one of the promoters has a (G+C) content in the normal range or higher. Others, such as the *hom* promoter, have a (G+C) content far below the normal range.

DISCUSSION

C. glutamicum RNAP was purified from strain ATCC 13059 using a protocol similar to that described for the purification of other RNAPs [54, 55, 101]. SDS-PAGE analysis has revealed the presence of several bands which are similar in size to the subunits of other prokaryotic RNAPs. We are unable to positively identify all of the bands at this time, but using comparisons to other organisms, we can tentively assign subunits. *M. smegmatis* is the closest organism to *C.*

Figure 2.6: Comparison of (G+C) Content in *C. glutamicum* Promoters - Below are shown the apparent promoter sequences of 10 *C. glutamicum* promoters. All of the promoters listed except for the *pgk* promoter have (G+C) contents lower than the usual 55.5-57.5%. [35, 44, 47, 96, 99, 100, 104, 121, 136]

Promoter	+1	% (G+C)
hom	CTTTAAAGCAAAAATGAACAGCTTGGTCTATAGTCGGCTA	37.5
fda	TCACGACAAAAGTTGAGTGATGCAGGCATAATTGGCTATA	40.0
thrC	GGTGTGGGGGAGTATTGTGTCACCCCTTGGGATAGGGTTA	55.0
lysA	CGTATTCTGTGCGACGGGTGTACCTCGGCTAGAATTTCTC	52.5
askP1	CTGGCTAGGTAGACACAGTTTATAAAGGTAGAGTTGAGCG	45.0
pheA	TTCGGCGGGTTAAGCTGTGTAACCATGAGCGACGCACCAA	55.0
askP2	GTGCATTCAATGTGCCACTTCGCGTATGCTCGTCTTATAG	47.5
gap	ATCTGCTGCGAAATCTTTGTTTCCCCGCTAAAGTTGAGGAC	47.5
pgk	GGTTGCCAGCGCCACACAATGTGTGGCAATCTGGGACAG	60.0
gdh	TGTGGTCATATCTGTGCGACACTGCCATAATTTGAACGTG	45.0

glutamicum in which the RNAP has also been purified [37]. Its major bands in SDS-PAGE analysis are very similar, with the putative ß and ß' subunits around 150,000 kd, and the σ factor around 66 kd. In our preparation of RNAP, there are several bands between 33-46 kd, any of which may correspond to the α-subunit. We suspect that the band around 46 kd is the α-subunit because it has the strongest signal and because the α-subunit of *M. smegmatis* is also around 46 kd [37]. The other bands are probably contaminating protein which co-eluted in our purification. They may also represent alternative σ factors. Alternative σ factors have been found in all organisms in which they have been sought, so it is believed that *C. glutamicum* also has them. Further evidence of multiple σ factors is shown in the Western blot where a minor protein homologous to the *E. coli* major σ factor is detected. Since many *C. glutamicum* promoters are recognized in *E. coli* and vice versa, it is not surprising that *C. glutamicum* has a σ factor that is homologous to the *E. coli* σ^{70}. Some *E. coli* promoters, such as the *tac, trp*, and *lacUV5* are used almost as efficiently in *C. glutamicum* as they are in *E. coli* [52].

The binding assays of RNAP to a piece of DNA containing the *hom* promoter revealed a K_d of around 1.1×10^{-9} M. This is in the same range as the K_ds of some *E. coli* promoters [66]. As more *C. glutamicum* promoters are studied, the dissociation constants and rate constants can be put into context with data describing the strength of the promoters. At this time, however, the dissociation constant of one promoter alone does not have much meaning.

We have also shown that *C. glutamicum* is less dependent on DNA supercoiling for RNA synthesis than *M. smegmatis*. *C. glutamicum* RNAP is able to efficiently transcribe using a linearized template for a substrate in *in vitro* assays, whereas the closely related *M. smegmatis* cannot [37]. It must be pointed out that although

several promoters were tested in *M. smegmatis*, it was still a small subset and these promoters may just have been more dependent in supercoiling than others. Supercoiling does have an effect on *C. glutamicum* transcription, however, as the transcription efficiency was greater when a supercoiled substrate was used. The lack of total dependence on supercoiling which has been observed in *M. smegmatis*, may be due to the lower content of (G+C) in *C. glutamicum*. *C. glutamicum*, although considered a (G+C) rich organism, only has a content of 55.5-57.5% [1] while *M. smegmatis* has a content of 65% [37]. The extra energy provided by negative supercoiling to unwind the promoter region may not be as necessary in *C. glutamicum* to initiate transcription. Furthermore, the *C. glutamicum* promoters which have been identified have been relatively (A+T) rich. *C. glutamicum* may have opted to utilize this (A+T) richness as an alternative strategy to deal with its (G+C) content.

One caveat to these experiments is that we have not yet rigorously demonstrated that the RNAP is binding to the *hom* promoter, and is not instead binding and directing transcription non-specifically. To show that the RNAP binds to the *hom* promoter, we will make RNA with the RNAP and use primer extension analysis to map the start site of transcription.

Now that *C. glutamicum* RNAP has been purified, it can be used to analyze promoters in more detail. Using techniques such as DNA footprinting, the physical location of the RNAP on the promoter can be determined. This may lead to a clearer picture of a *C. glutamicum* promoter consensus sequence. The RNAP can also be used to validate the transcriptional strength of *C. glutamicum* promoters which have been determined by other methods such as reporter gene assays. As more information on *C. glutamicum* promoters is collated, it may be

possible to put together hybrid promoters which have special properties, such as the *E. coli tac* promoter.

CHAPTER 3

METHIONINE REPRESSION AT THE *HOM* PROMOTER OF *CORYNEBACTERIUM GLUTAMICUM*

Section I: A Common Element Mediates Repression at Both the *hom* and the *metA* Promoters in *Corynebacterium glutamicum*

SUMMARY

Methionine regulation in *Corynebacterium glutamicum* has been studied at two loci, the *hom* promoter and the *metA* promoter. At the *hom* promoter, which had been previously shown to be repressed two-three fold by methionine, we have shown that the site of repression is upstream of the transcription start site and is most likely due to a protein factor. In the course of the study, we created a *Corynebacterium glutamicum* strain which is derepressed for methionine at the *hom* promoter. This strain is also derepressed for methionine at the *metA* promoter, suggesting that a *Corynebacterium glutamicum* methionine regulator acts at multiple sites.

INTRODUCTION

Corynebacterium glutamicum is a Gram-positive, non-sporulating, non-motile, non-pathogenic soil bacterium [1, 4]. It is widely used in the industrial production of amino acids such as lysine [1, 4]. Because of this much study has been directed towards unravelling the pathways and control mechanisms of various amino acids, especially the aromatic and aspartate-derived amino acids [45]. As molecular biology techniques have been developed in *C. glutamicum*, many of the genes in these pathways have been cloned and characterized. While it is known that some of these genes are regulated at the transcriptional level, few

studies have been done to elucidate the regulatory mechanisms used in this organism.

Homoserine dehydrogenase and homoserine kinase are the first enzymes in the pathway to threonine, after the split at aspartate semialdehyde towards either threonine or lysine [44, 85, 104]. These two enzymes are encoded by the genes *hom* and *thrB* which are in a single operon [85]. This operon has been shown to be repressed two-three fold by methionine [85]. This repression had been previously thought to be mediated by a simple attenuation mechanism which occurs via a stemloop structure which can occur in the RNA transcript before the translation start site [44, 45]. In this paper we show that repression occurs whether or not this stemloop structure is present. Methionine has been reported to repress other genes in *C. glutamicum*, most notably the gene *metA*, which encodes O-acteylhomoserine transferase, the first enzyme of the methionine pathway after it splits from the threonine pathway at homoserine [88, 92].

In *Escherichia coli*, methionine repression is mediated by several regulators [70, 86]. Of these, *metJ* encodes a repressor which acts on several genes in the methionine pathway, including the genes for homoserine dehydrogenase and O-succinylhomoserine transferase (which is analogous to the *C. glutamicum* O-acetylhomserine transferase) [70, 86]. In this paper we present evidence that *C. glutamicum* also has a regulator which acts at at least two sites, the *hom* promoter and the *metA* promoter. To do this we have created a *C. glutamicum* strain, E12Rep-2, that is derepressed at both the *hom* and the *metA* promoters.

MATERIALS AND METHODS

Strains and Plasmids. *C. glutamicum* and *E. coli* strains are listed in Table 3.1. Plasmids used are listed in Table 3.1. Cells were usually grown in Luria-Bertani medium except where noted. *C. glutamicum* minimal medium composition is described in Colon, *et al.* [95]. Brain Heart Infusion (BHI) liquid medium was prepared to manufacturer's specifications (Difco) and recovery plates also included 40 g/l sorbitol and 10 g/l sucrose. *C. glutamicum* recovery medium after electroporation was composed of BHI plus 4% glucose.

DNA Manipulations. DNA manipulations in *E. coli* were performed as described previously [105, 106]. *C. glutamicum* plasmid DNA purification was done as described in Yoshihama, *et al.* and transformations were done as described in Follettie, *et al.* [35, 43]. Restriction enzymes were purchased from either New England Biolabs or Bethesda Research Laboratories. Chemicals were purchased from Sigma. DNA sequencing was done by the Sanger dideoxy-method using Sequenase and protocols from United States Biochemical.

Construction of pHomΔ+. Using the primers 5'-dGCGGGGCGACCGAGAAGT-3', which anneals to the pFS3.6 vector, and 5'-dATCGCGGTCGAGCGTTG-3', which anneals immediately before the ribosomal binding site of the *hom* gene, the wild type promoter was amplified by the polymerase chain reaction. This fragment was blunt-ended using Klenow and then cleaved with *XhoI*. The resulting fragment was cloned into pZIP which had been cleaved with *XhoI* and *SmaI*. The resulting candidate was checked by restriction enzyme mapping and sequencing.

TABLE 3.1: Strain and Plasmid List

STRAINS		Reference
C. glutamicum		
AS019	rif^R lab strain	43
E12	restriction deficient derivative of AS019	35
E12-Rep-2	derivative of E12 which is derepressed for methionine at the *hom* and *metA* genes	this paper
E. coli		
DH5α	*lacZΔM15, recA1*	BRL
PLASMIDS		
pFS3.6	Plasmid containing *hom-thrB* operon	89
pMTI	*C. glutamicum/E.coli* shuttle vector Ap^R in *E. coli* Km^R in *C. glutamicum*	35
pMT-SCM	pMTI with promoterless *cat* gene inserted in *SalI* site of polylinker - *cat* gene contains own ribosome binding site.	this paper
pZIP	pMT-SCM with *lacZ* gene inserted in frame in *PvuI* site of *cat* gene.	this paper
pHomΔ+	pZIP with native *hom* promoter starting at *SmaI* site and going through the region of hyphenated dyad symmetry inserted in polylinker	this paper
pHomΔ-	pZIP with *hom* promoter from *SmaI* site to *KpnI* site inserted in polylinker	this paper
pHomcm	pMT-SCM with *hom* promoter from upstream *XhoI* site to *KpnI* site	this paper

Construction of pHomΔ-. Plasmid pFS3.6 was cleaved with *XhoI* and *KpnI*. The resulting 0.7kb fragment was isolated from the gel using a Qiagen gel purification kit and cloned into pZIP which had been cleaved with *XhoI* and *KpnI*. The resulting candidate was checked by restriction enzyme mapping and sequencing.

Construction of pHomcm. Plasmid pHomΔ- was cleaved with *XhoI* and *KpnI*. The 0.7kb fragment was gel purified using a Qiagen kit and cloned into pMT-SCM which had been cleaved with *XhoI* and *KpnI*. The resulting candidate was checked by restriction enzyme mapping.

Preparation of Crude Extract. Cells were grown in LB or minimal medium and harvested. The pellet was washed in PBS and resuspended in PBS containing DTT. The cells were disrupted in a bead mill using glass beads from Sigma. The supernatants were collected after centrifugation and protein quantitation was done using the BioRad protein kit.

β-galactosidase Assay. β-galactosidase activity was measured by a modified version of the β-galactosidase assay described in Sambrook, *et al.* [106]. Briefly, crude extract was added to Z buffer (0.06 M $Na_2HPO_4 \cdot 7H_2O$, 0.04 M $NaH_2PO_4 \cdot H_20$, 0.01 M KCl, 0.001 M $MgSO_4 \cdot 7H2O$, and 0.05 M β-mercaptoethanol, pH 7.0) and 200 µl o-nitrophenyl-β-D-galactopyranoside (4 mg/ml) to a final volume of 1 ml. After a certain time, usually 90 s, 0.5 ml of 1 M Na_2CO_3 was added. The absorbance at 420 nm was then measured and specific activities per mg protein per minute determined.

Chloramphenicol Acetyl Transferase Assay. Chloramphenicol acetyl transferase was assayed by the spectrophotometric assay outlined in Shaw [107]. Briefly, 4 mg of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) was dissolved in 1 ml of 1 M Tris-HCl, pH 7.8. 50 µl of 20mM acetyl-CoA was added and the volume was brought up to 10 ml. Crude extract was added to this buffer to bring the total volume per assay to 980 µl. The reaction was monitored on a spectrophotometer at 412 nm, and then 20 µl of 5mM chloramphenicol was added. The rate of increase in absorption before the chloramphenicol was added was subtracted from the rate of increase after it was added. This net change in extinction per minute was divided by 13.6 (the molar extinction coefficient for 5-thio-2-nitrobenzoate is 13,600 at 412 nm) and the amount of protein added to give the µmoles per minute of CM-dependent DTNB reacted.

O-Acetylhomoserine Transferase Assay. O-acetylhomoserine transferase was measured using method II of Miyajima, *et al.* [92] In brief, the reaction mixture contained 100mM Tris-HCl, pH 7.5, 0.5 mM homoserine, 0.09 mM acetyl-CoA, and crude extract to a total volume of 1.5 ml. In parallel, a blank reaction mixture was prepared which had the same composition except that it did not contain homoserine. The rate of decrease in absorbance at 232 nm was measured, and this value was subtracted from the rate of decrease in the blank.

Construction of E12Rep-2. E12Rep-2 was isolated after nitrosoguanidine mutagenesis of E12(pHomcm). E12(pHomcm) was grown at 30°C in LB to exponential phase, harvested, and suspended in an equal volume of *C. glutamicum* minimal medium. Cells were mutagenized by the addition of 40 µg of NTG to 1 ml of cells and incubated without shaking at 30°C for 30 min [44]. These cells were then centrifuged, resuspended in 1 ml of fresh LB, and diluted

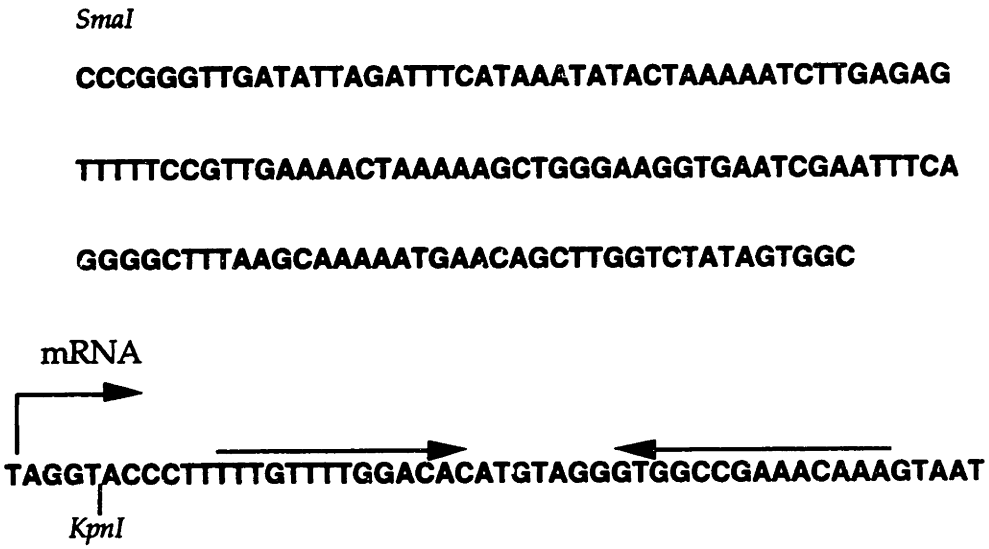
1/100 in 10 ml aliquots of fresh LB. These cells were then used to inoculate 100 ml cultures of minimal medium plus 750 mg/l of methionine. The cells were grown to exponential phase, diluted, and plated on minimal medium agar plates containing 825 mg/l methionine and 17 μg/ml chloramphenicol. Colonies which grew were tested for repression of the *hom* promoter in CAT assays with candidates grown in LB. Candidates which were derepressed were selected for further study.

Curing of pHomcm from E12Rep-2. The plasmid pHomcm was cured from E12Rep-2(pHomcm) by growing in BHI with no antibiotic selection. The cells were grown at both 37°C and 30°C in order to stress the cells [108]. Cells were diluted, plated on LB and replica-plated on LB/kanamycin (25 mg/l). After several rounds of reinoculation, colonies which had been cured of their plasmids were isolated.

RESULTS

Methionine Repression in E12(pHomΔ+) and E12(pHomΔ-). As stated in the introduction, it had previously been speculated that methionine repression in the *hom* promoter was mediated through a hyphenated dyad symmetry element which is located after the transcription start site (Figure 3.1) [44, 45]. In order to test this hypothesis, two vectors were made in which the *hom* promoter ± the leader sequence were cloned into pZIP and E12 was transformed with these vectors (Figure 3.2). The transformed cells were grown in minimal medium ± methionine and ß-galactosidase assays were done with the crude extracts. As a control, E12 harboring pZIP alone were assayed for ß-galactosidase activity and no activity was detectable (data not shown). In Figure 3.3 it is shown that methionine represses the native promoter 4.7± 1.9 - fold and the truncated

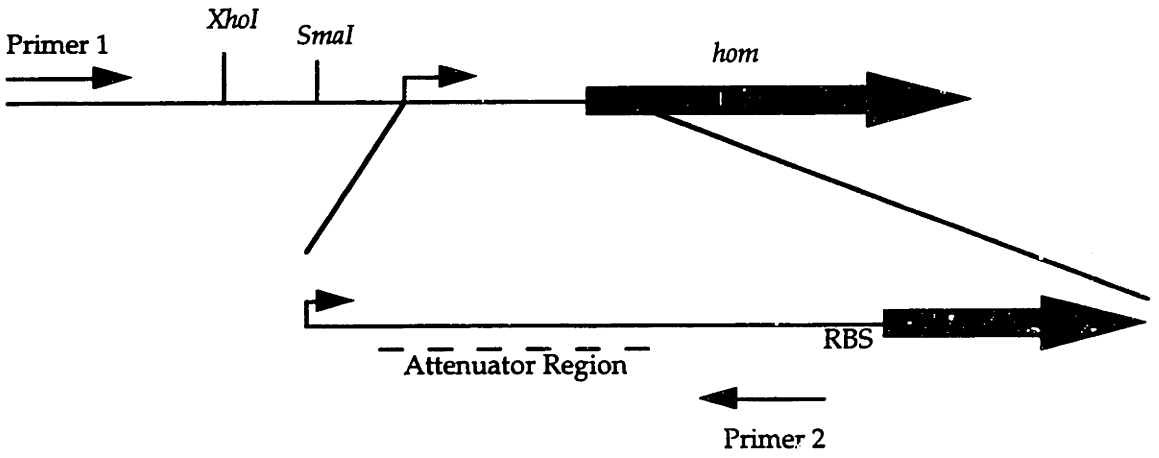
Figure 3.1: *hom* Promoter in Detail - Shown below is the *hom* promoter. The convergent arrows represent the region of hyphenated dyad symmetry. The *KpnI* site was used in cloning the pHomΔ- and pHomcm vectors.



AGGACAACAACGCTCGACCGCGATTATTTTTGGAGAATCA Translation Start Site

Figure 3.2: Construction of pHomΔ+ and pHomΔ- - The schematics of the strategy used to clone the modifications of the *hom* promoter are described below. a.) shows the strategy used to clone the full promoter. PCR was used to amplify the *hom* promoter. In pHomΔ+ the amplified fragment was cut at the *SmaI* site. b.) shows the strategy to clone the truncated *hom* promoter. In pHomΔ- the fragment from *SmaI* to *KpnI* is cloned into pZIP (marked by a dashed line).

a.) pHomΔ+



b.)pHomΔ-

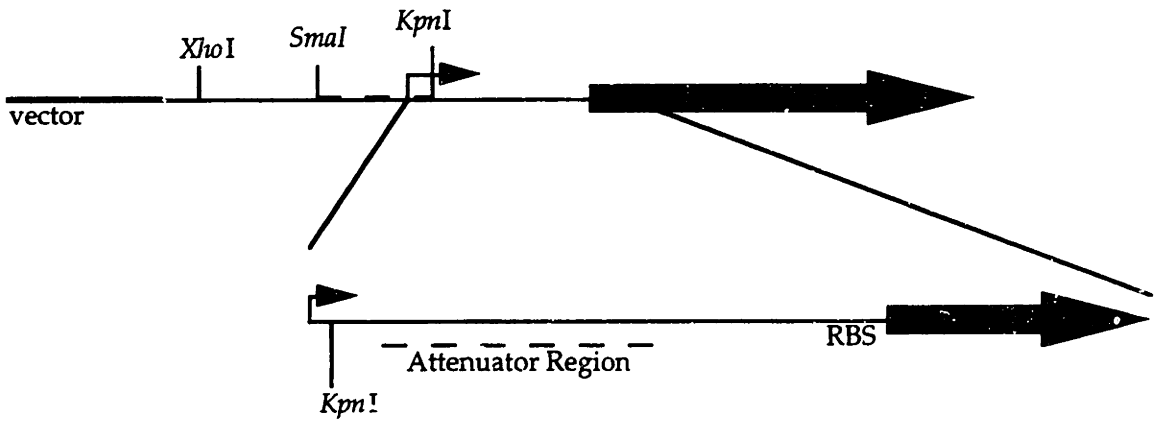
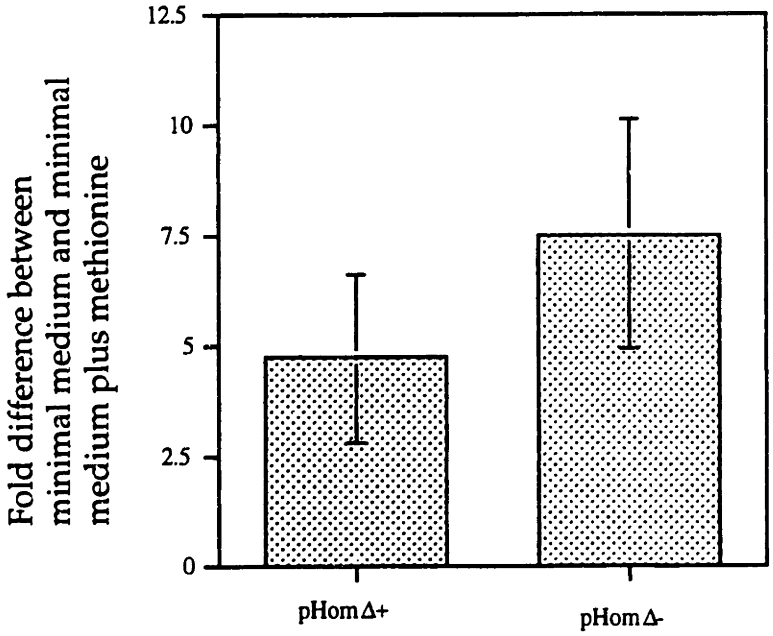


Figure 3.3: Methionine Repression at the *hom* Promoter - Methionine represses the *hom* promoter 4.7+1.9 fold in the native promoter (pHomΔ+) and 7.5+2.6 fold in the promoter without the stem loop structure (pHomΔ-). For pHomΔ+, the sample size was 11 and for pHomΔ-, the sample size was 17. The assays were done at comparable times in different experiments after the cultures were in exponential phase. In some cases, multuple assays may have been done on the same culture over a series of days.



promoter 7.5 ± 2.6 fold. Therefore, the leader sequence is not responsible for repression by methionine at this promoter. In fact, the promoter without the leader sequence is more strongly repressed by methionine. To investigate this further, we tested whether these promoters were differentially affected by the concentration of methionine in the medium. In Figure 3.4, the activity of ß-galactosidase in E12(pHomΔ+) and E12(pHomΔ-) are compared in minimal medium containing different concentrations of methionine. 10 mg/l of methionine has very little effect on either promoter construct, while 50 mg/l of methionine almost completely represses each promoter. Increasing the methionine concentration up to 1000 mg/l did not increase the level of repression when tested in E12(pHomΔ+).

Construction of pHomcm. To further study repression at the *hom* promoter, a strain which is derepressed for methionine at the *hom* promoter was sought. To do this, the *hom* promoter without the leader sequence was cloned into pMT-SCM, a vector which has a promoterless *cat* gene with its own ribosome binding site, to create pHomcm. Fortuitously, in this vector the truncated *hom* promoter was approximately 12-fold repressed by methionine (Figure 3.5).

Isolation of E12Rep-2. *C. glutamicum* strain E12 was transformed with pHomcm and this strain was mutagenized with NTG. The mutagenized cells were plated on minimal medium plates containing 825 mg/l methionine and 17 µg/ml chloramphenicol. These conditions were such that only those cells which are derepressed for methionine at the *hom* promoter should have enough expression of the *cat* gene to survive. Candidates were assayed for CAT activity after growth in LB. Those candidates which had activity comparable to E12(pHomcm) grown in minimal medium without methionine were kept for further study.

Figure 3.4: Comparison of E12(pHomΔ+) and E12(pHomΔ-) Grown in Different Concentrations of Methionine - Exogenous methionine has a similar effect on both E12(pHomΔ+) and E12(pHomΔ-).

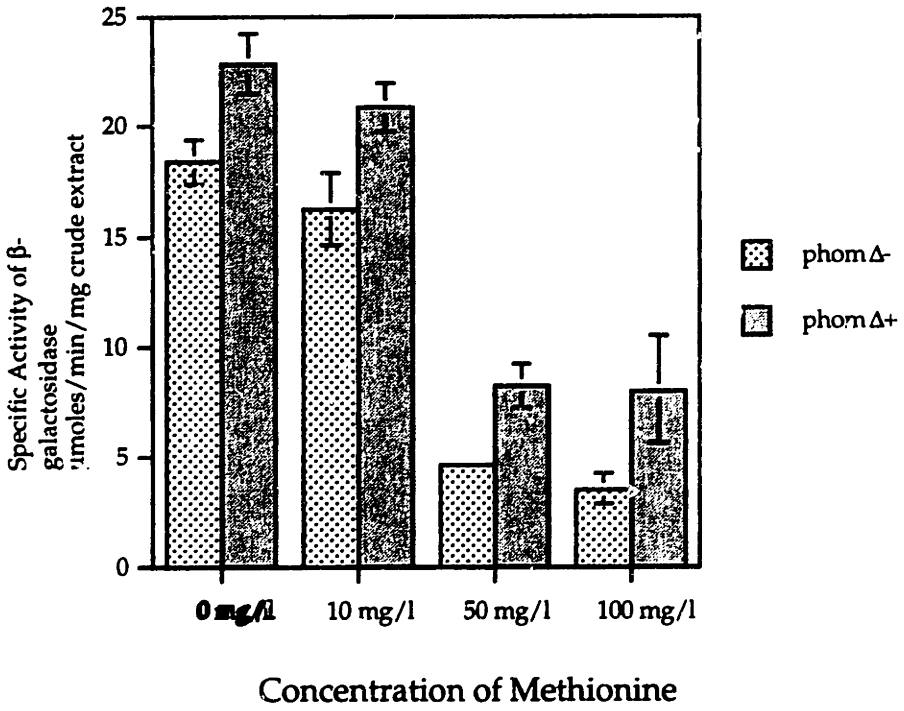


Figure 3.5: Methionine Repression at *hom* Promoter Using the *cat* Reporter Gene - The graph below shows the difference in chloramphenicol acetyl transferase activity in E12(pHomcm) when grown in minimal medium±methionine. Methionine represses the expression of the *hom* promoter : *cat* reporter gene 12.7-fold.

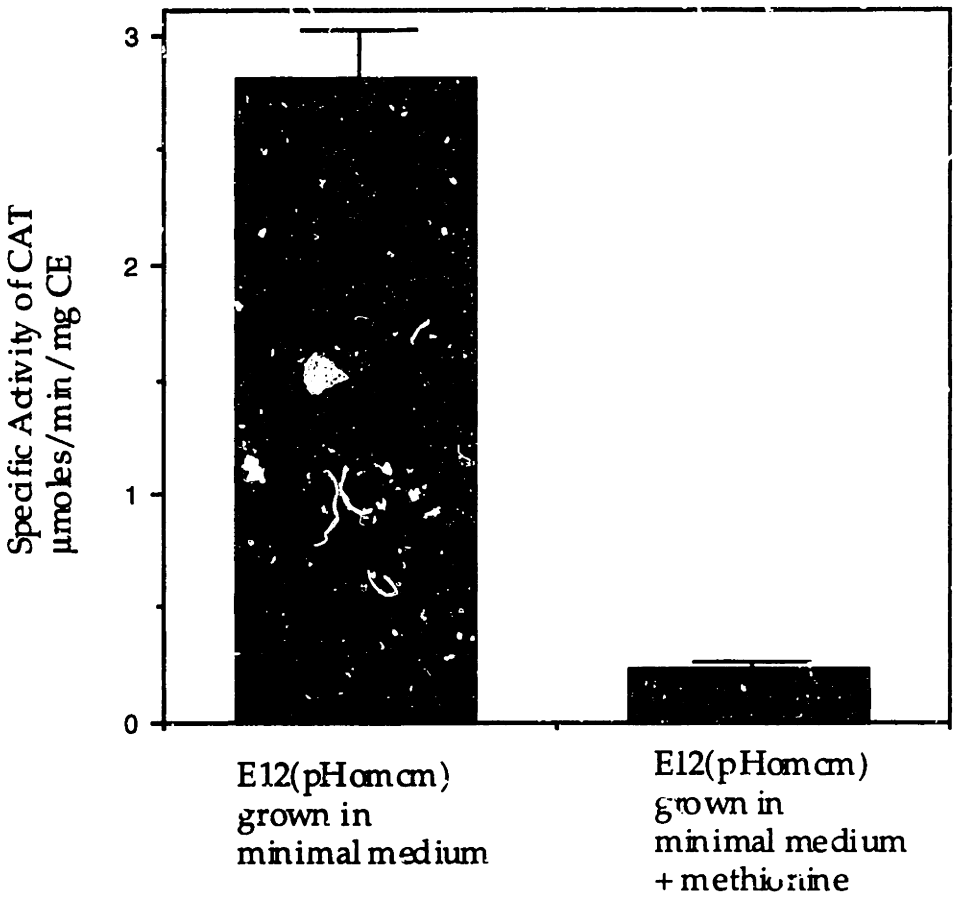


Table 3.2 shows the activities of E12Rep-2(pHomcm) in LB compared to E12(pHomcm). E12Rep-2(pHomcm) had much higher CAT activity in LB and when it was grown in minimal medium ± methionine, the repression at the *hom* promoter was only about 2-fold.

Since both the plasmid and the strain were mutagenized with NTG, we had to determine whether the mutation was in the plasmid or the genomic DNA. To do this, we isolated the plasmid from E12Rep-2 and transformed it into unmutagenized E12. This strain was fully repressed when tested in minimal medium ± methionine. In order to test the strain, we cured E12Rep-2 of the plasmid and retransformed it with pHomcm. Once again this strain showed the derepression that was present in the initial candidate. This indicates that E12Rep-2 has a mutation in either the *C. glutamicum* methionine regulator or some unknown element which controls the regulator.

Regulation at the *metA* Gene. The isolation of strain E12Rep-2 allowed us to test this strain to see whether other genes which are repressed by methionine are affected by the mutation in this strain. In other prokaryotes, such as *E. coli*, there is a methionine repressor which represses at several loci [70]. The *metA* gene encodes O-acetylhomoserine transferase, an enzyme which converts homoserine to O-acetylhomoserine in the first step of the methionine-exclusive biosynthetic pathway [92]. This step has been shown to be both inhibited and repressed by methionine in *C. glutamicum* [92]. Table 3.3 shows the comparison of O-acetylhomoserine transferase assays of E12(pHomcm) and E12Rep-2(pHomcm) in cultures grown in minimal medium ± methionine. E12(pHomcm) shows approximately 7-fold repression when grown in methionine. E12Rep-2(pHomcm), on the other hand, shows approximately 2-fold repression when

Table 3.2: Assay Results from E12Rep-2(pHomcm) - a.) The E12Rep-2(pHomcm) strain shows much higher chloramphenicol transferase activity in LB than E12(pHomcm). b.) E12Rep-2(pHomcm) is almost completely derepressed at the *hom* promoter for methionine. The amount of repression present, about 2-fold, is much less than in the wild type strain, which is about 12-fold.

a.) CAT assays of E12(pHomcm) and E12Rep-2(pHomcm) grown in LB:

Strain	CAT activity µmoles/mg/min
E12(pHomcm)	0.35
E12Rep-2(pHomcm)	2.16

b.) CAT activity of E12Rep-2(pHomcm) grown in minimal medium ± methionine:

E12Rep-2(pHomcm)	CAT activity µmoles/mg/min
- met	6.5
+ met	3.3

Table 3.3: Acetyl Homoserine Transferase Assays - E12(pHomcm) and E12Rep-2(pHomcm) were assayed for their acetyl homoserine transferase activity when grown in minimal medium±methionine. The E12Rep-2 strain has high activity under both conditions, with a slight repression when grown in methionine, while the E12 strain is highly repressed when grown in the presence of methionine.

Strain	Methionine	Transferase Activity DA/mg crude extract/min
E12(pHomcm)	-	0.10
E12(pHomcm)	+	0.014
E12Rep-2(pHomcm)	-	0.54
E12Rep-2(pHomcm)	+	0.25

grown in methionine. However, the activity of the enzyme is much higher in both the E12Rep-2(pHomcm) cultures with or without methionine than it is in the E12(pHomcm) culture without methionine. This indicates that the mutation in the E12Rep-2 strain also has an effect on the *metA* gene. Furthermore, since E12(pHomcm) has lower activity when grown without methionine than the mutant strain, it is suggested that endogenous levels of methionine can mediate repression at the *metA* promoter.

DISCUSSION

In this paper we have demonstrated that the methionine repression mechanism occurs upstream of the transcriptional start site in the *hom* promoter. It had been hypothesized before that the RNA leader transcript that was upstream of the translation start point was responsible for repression by methionine at this promoter [44, 45]. This leader transcript is approximately 90 bp long and has a region of extensive hyphenated dyad symmetry. By comparing the level of repression in ß-galactosidase assays between E12(pHomΔ+) which has the leader RNA and E12(pHomΔ-) which does not, we have shown that an element upstream of the transcription start site mediates the repression. Interestingly, repression by methionine is higher in pHomΔ-, 7.5 fold versus 4.7 fold. The leader sequence may be responsible for this decrease in repression. This phenomenon needs to be studied further before any conclusions can be made.

As stated above, the site of methionine repression in the *hom* promoter is upstream of the transcription start site. This strongly suggests that repression at the *hom* promoter is mediated by a regulator protein. We decided to use mutagenesis to create a strain of *C. glutamicum* E12 which has a mutation in the regulator gene, thereby causing derepression at the *hom* promoter. To do this, we

first constructed a vector which places the *cat* gene under control of the *hom* promoter (pHomcm). We were greatly aided by the fortuitous result that repression at the *hom* promoter in pHomcm is approximately 12-fold. We have not done any experiments at this time to understand why there is this difference in repression using a different reporter gene. We then treated E12(pHomcm) with NTG and screened for survival on minimal medium plus methionine and chloramphenicol. Only those candidates which are derepressed at the *hom* promoter would have enough CAT activity to survive. We isolated several candidates which had high CAT activity in LB. When these candidates were tested in cultures of minimal medium ± methionine, the activity in the methionine-containing medium was less than 2-fold lower, as compared to the wild type 12-fold repression. In order to ensure that the mutation that was causing the derepression was chromosomally located and not in the plasmid, we isolated the plasmids and retransformed E12. Of the three candidates we tested, none of the plasmids conferred derepression at the *hom* promoter. We then chose the strain which had the highest activity, E12Rep-2, and cured the plasmid from it. We transformed the cured strain with pHomcm and found that once again the *hom* promoter was derepressed. That we could cause derepression by mutagenesis of the strain was further evidence that repression is caused by a protein effector.

There are three major mechanisms of methionine regulation in *E. coli*: repression by vitamin-B_{12}, activation by MetR, and repression by MetJ [70, 86]. The MetJ repressor acts at several sites including those of *metL*, which codes the bifunctional aspartokinase and homoserine dehydrogenase, and *metA* which encodes O-succinylhomoserine transferase [70]. We decided to test whether *C. glutamicum* also has a methionine regulator like MetJ that acts at multiple

methionine genes, so we tested the expression of the *C. glutamicum metA* gene in both E12(pHomcm) and E12Rep-2(pHomcm) in minimal medium ± methionine. We found that E12Rep-2 was also derepressed at the *metA* locus, suggesting that *C. glutamicum* has a similar methionine regulator as the *E. coli* MetJ which acts at different operators. This is the first known example of a *C. glutamicum* regulon. Repression at the *C. glutamicum metA* operator is much higher than at the *hom* operator. For example, the expression of homoserine dehydrogenase is only repressed two-three fold by exogenous methionine [85]. We have demonstrated a 40-fold difference at the *metA* locus between the wild type E12 grown in methionine and the derepressed strain grown without methionine. By using methionine auxotrophs, a 70-fold repression has been reported [92]. Besides the difference in the magnitude of repression, the *metA* locus is also repressed by endogenous levels of methionine. This endogenous methionine repression has been reported in the literature and is confirmed by the difference in activity between E12 and E12Rep-2 grown in minimal medium without methionine. The E12Rep-2 has about two-fold greater activity of homoserine acetyl transferase than E12.

While methionine causes repression at the *hom* and the *metA* promoters, we do not know whether this repression is caused by a repressor protein or an activator protein. For instance, an activator protein would sense that methionine levels are low and the methionine genes need to be expressed. A repressor protein would sense that methionine levels are high and the genes need to be repressed. Once the regulator protein is cloned and/or purified, more experiments can be done to distinguish the mode of effect.

Although the E12Rep-2 strain is greatly derepressed at the *hom* and *metA* operators, there is still a residual amount of repression when the strain is grown in methionine. The exact cause of this behavior is unknown at present time. It may be that the defect in the regulator protein still allows some repression to occur. Or, the mutation may not be in the regulator protein itself, but in an element which affects its expression, such as its promoter. Once the regulator is cloned, PCR can be used to amplify the gene from E12Rep-2 to see what type of mutation causes the derepression.

Another hypothesis is that we have not isolated a regulator mutant, but rather we have isolated a methionine transport mutant. Data which suggests that this is not the case comes from the O-acetyl homoserine transferase assays. In these experiments, E12Rep-2 grown without methionine had higher activity than E12 grown without methionine. This suggests that the endogenous methionine is not repressing E12Rep-2 as it is in E12. Furthermore, if a transport mutation was not allowing methionine into the cell, then E12Rep-2 grown with or without methionine would be expected to give the same activity. These pieces of evidence together indicate fairly strongly that the defect is not in methionine transport.

We have not yet tested the activity of the other gene in the *C. glutamicum* methionine pathway which is repressed by methionine, the gene which encodes O-acetyl homoserine sulfhydrylase [88]. At this time we would predict that it also will be derepressed in strain E12Rep-2.

Methionine regulation in *C. glutamicum* has some similarities to regulation in *E. coli*. For instance, in both organisms, a single regulator operates at multiple sites

[70]. Furthermore, the repression at *metA* is much higher than it is at either *metL* (in *E. coli*) or *hom* (in *C. glutamicum*), although in both cases *E. coli* has a much greater level of repression [70]. For example, in *C. glutamicum*, repression at *hom* is two-three fold and at *metA* it has been reported as 70-fold [92]. Repression at *metA* in *E. coli* is also greater than at *metL*, about 300-fold repression versus 40-fold repression [70].

The variation in repression effect at *hom* (or *metL*) versus *metA* is probably due to the different locations of the enzymes in the pathway. Homoserine dehydrogenase is in the pathway which goes to several other amino acids besides methionine, such as threonine and isoleucine. In *E. coli*, since the homoserine dehydrogease is part of the bifunctional aspartokinase-homoserine dehydrogenase enzyme, the effect of methionine is even further up in the biosynthetic pathways [70]. The *metA* product, O-acetylhomoserine transferase in *C. glutamicum* and O-succinylhomoserine transferase in *E. coli*, is the first step in the pathway which only leads to methionine. This pathway needs to be more tightly regulated by methionine than the general pathway, especially at the initial step where carbon precursor is being siphoned off from the threonine pathway. After all, methionine is a costly amino acid for the cell to make and it is to the cell's advantage to conserve energy by not making more methionine than needed.

Section II: Integration of the *hom: cat* Reporter Gene into the *C. glutamicum* Genome

SUMMARY

A vector, pSupddhcat, was constructed in order to integrate a *hom* promoter: *cat* reporter gene cassette into the *Corynebacterium glutamicum* chromosome. The target site was the *ddh* gene. In *Corynebacterium glutamicum* strain E12, the *hom* promoter: *cat* reporter gene was successfully integrated in a single cross-over event so that a wild type copy of the *ddh* gene remained. In *Corynebacterium glutamicum* ATCC 21253, the *hom* promoter: *cat* reporter gene was integrated in a double cross-over event, thereby disrupting the *ddh* gene. The E12 strain with the integration, E12#3, was tested for repression by methionine at the *hom* promoter by comparing chloramphenicol acetyl transferase levels when the cells were grown with or without methionine. The level of repression by methionine at this promoter was approximately the same whether the promoter was on a multi-copy plasmid or in the chromosome.

INTRODUCTION

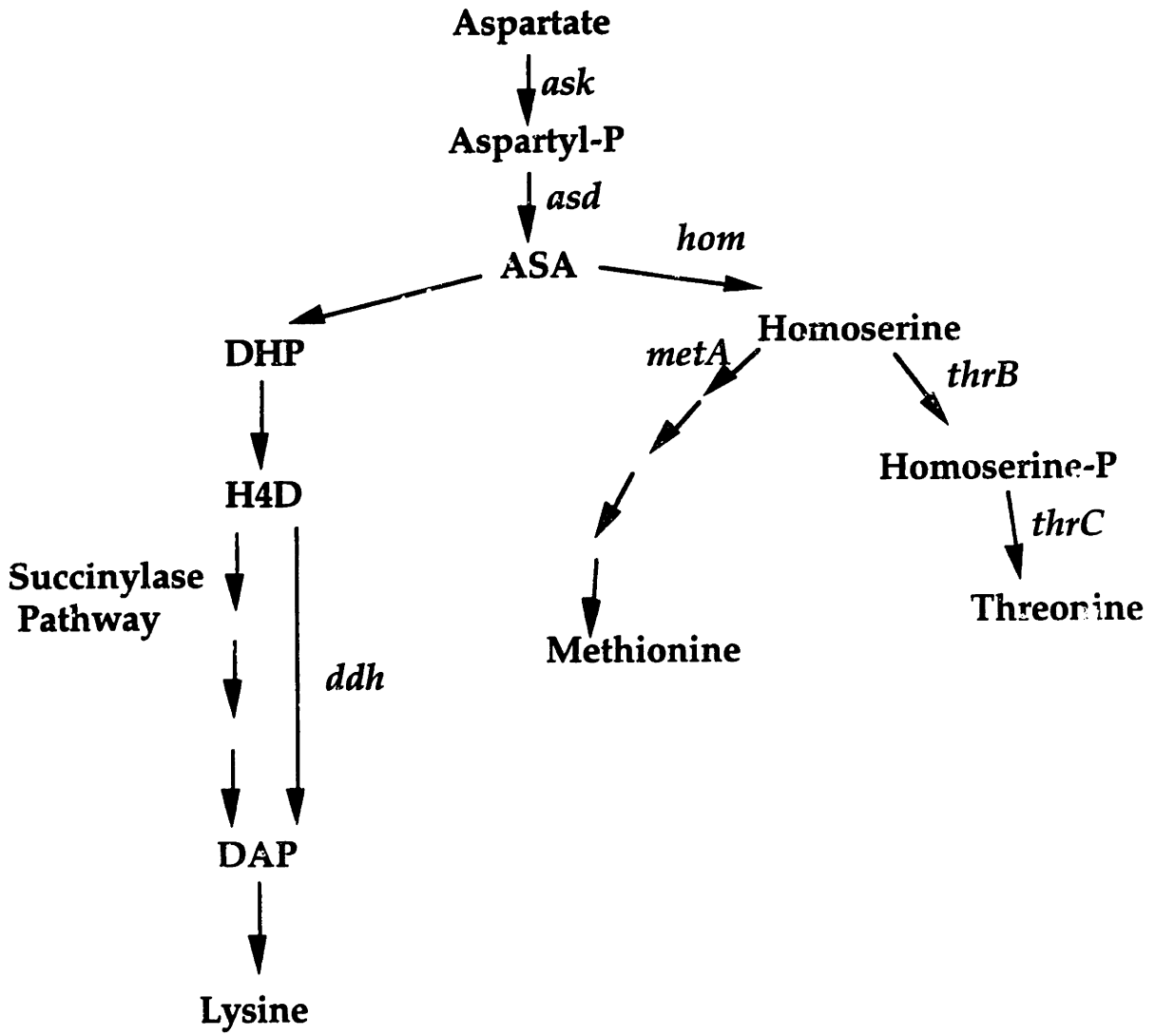
In an attempt to achieve higher levels of methionine repression at the *hom* promoter, a strain was constructed in which one copy of the *hom* promoter: *cat* reporter gene cassette was integrated into the genome. The vectors and methodology to do this type of targeted integration and gene disruption has been recently developed in *C. glutamicum* [48-50]. It involves using the vector designated pSUP301 which contains an *E. coli* origin of replication along with ampicillin resistance and kanamycin resistance markers. It also contains the *mob* site which is the P-type specific recognition site for mobilization from plasmid RP4 and also is believed to include the origin of transfer (*oriT*) [50]. A fragment

of the *C. glutamicum* DNA which is the target site for homologous recombination to take place is cloned into one of three unique sites in pSUP301, a *PstI* site in the Ap^R gene or an *XhoI* or a *HindIII* site in the Km^R gene. This new vector is used to transform the *E. coli* donor strain S17-1, which has had the transfer genes of a modified IncP-type (drug resistance plasmids with broad host ranges) plasmid RP4 integrated into its genome (complementing the *mob* site in pSUP301) [50]. The resulting strain is then mated with the *C. glutamicum* strain of choice. *C. glutamicum* has a natural resistance to nalidixic acid, which is toxic to *E. coli* so selection takes place on nalidixic plates plus whatever antibiotic or selectable marker is used in the vector [48-50].

The gene chosen for a target site in *C. glutamicum* was the *ddh* gene. *Ddh* encodes the enzyme diaminopimelate dehydrogenase (DDH), a bypass pathway in the biosynthesis of lysine (Figure 3.6) [36]. There are three different known pathways to lysine in prokaryotes, of which *C. glutamicum* has two. *E. coli* only uses the four step succinylase pathway described below [32]. For the two *C. glutamicum* pathways, the point of separation occurs at the metabolite L-Δ-tetrahydrodipicolinate [36]. At this point the carbon precursor can either take a four step pathway using the enzymes succinyl transferase, amino transferase, desuccinylase, and epimerase or the one step pathway using diaminopimelate dehydrogenase (DDH) to get to the intermediate *meso*-diaminopimelate (*meso*-DAP) [36]. *Meso*-DAP is a very important intermediate in that it is a crucial component of the *C. glutamicum* cell wall [1]. *Meso*-DAP is then converted by the enzyme diaminopimelate decarboxylase into lysine [36].

Since there is an alternative pathway to lysine, the *ddh* gene is a good target site for integrating the promoter: reporter gene cassette. The disruption of *ddh* in a

Figure 3.6: Pathways from Aspartate to Lysine, Threonine, and Methionine - *C. glutamicum* has two pathways from H4D to *meso*-DAP, through the succinylase pathway and through the *ddh* pathway. Abbrevations are: ASA, aspartate semialdehyde; DHP, dihydropicolinate; H4D, Δ'-tetrahydrodipicolinate; DAP, *meso*-diaminopimelate.



lysine-producing strain is also useful in a study of carbon flow to lysine which is another component of the Sinskey laboratory's *C. glutamicum* amino acid program [36, 47]. By disrupting *ddh* it should be discernible whether the four step pathway is robust enough to handle converting all the carbon precursor into lysine. A disruption strain is also a good control for NMR studies which have been done to determine the relative flux of carbon in each of the pathways. Many of these questions are being answered by Cathryn Shaw-Reid using the lysine-producing strain *C. glutamicum* 21253#10 which has the *ddh* disruption and whose construction is described below.

Two strains were chosen to integrate the *hom* promoter:reporter gene cassette. *C. glutamicum* strain E12 was chosen because all of the other promoter studies were done in that background and *C. glutamicum* strain 21253 was chosen because it is a high lysine producer and the lysine metabolic engineering studies needed to be done with that genetic background. Integrations of the promoter cassette were achieved in both strains. In E12, the *ddh* was not disrupted while in 21253 it was. Assays done in the E12 integration strain showed that the level of repression was consistent in both the strain with one copy of the *hom* promoter: reporter gene cassette and the strain with the cassette on a multi-copy plasmid.

MATERIALS AND METHODS

Strains and Plasmids. Strains and plasmids are listed in Table 3.4. *E. coli* strains were grown in Luria-Bertani broth. *C. glutamicum* strains were grown in LB, *C. glutamicum* minimal medium [95], or Brain Heart Infusion medium. Antibiotics used were ampicillin (50 mg/l) for selection in *E. coli*, kanamycin for selection of plasmids (25 mg/l) and transconjugants (6 mg/l) in *C. glutamicum* and nalidixic acid (25 mg/l) for selection of *C. glutamicum* transconjugants.

TABLE 3.4: Strain and Plasmid List

STRAINS		Reference
C. glutamicum		
AS019	rif^R lab strain	43
E12	restriction deficient derivative of AS019	35
E12#3	derivative of E12 in which the *hom* promoter: *cat* gene is integrated into the chromosome at the *ddh* gene	this paper
21253	lysine overproducing strain, *hom-*	ATCC
21253#6	derivative of 21253 in which the *ddh* gene is disrupted and a *hom* promoter: *cat* gene is inserted in its place	this paper
21253#10	derivative of 21253 in which the *ddh* gene is disrupted and a *hom* promoter: *cat* gene cassette is inserted in its place	this paper
E. coli		
DH5α	*lacZΔM15, recA1*	BRL
S17-1	mobilizable donor strain, MM294*recA*, which has a RP4 derivative integrated into the chromosome	48, 49
PLASMIDS		
pMTI	*C. glutamicum/E.coli* shuttle vector Ap^R in *E. coli* Km^R in *C. glutamicum*	35
pMT-SCM	pMTI with promoterless *cat* gene inserted in *SalI* site of polylinker - *cat* gene contains own ribosome	this paper

	binding site.	
pZIP	pMT-SCM with *lacZ* gene inserted in frame in *PvuI* site of *cat* gene.	this paper
pHomΔ+	pZIP with native *hom* promoter starting at *SmaI* site and going through the region of hyphenated dyad symmetry inserted in polylinker	this paper
pHomΔ-	pZIP with *hom* promoter from *SmaI* site to *KpnI* site inserted in polylinker	this paper
pHomcm	pMT-SCM with *hom* promoter from upstream *XhoI* site to *KpnI* site	this paper
pSUP301	pACYC177-*mob*, Km^R, Ap^R	48, 49
pddh-~~hind~~	*C. glutamicum ddh* gene cloned into pBluescript at the *PstI* site with the *HindIII* site of pBluescript removed	this paper
pddhcat	pddh-~~hind~~ with fragment from *HindIII* site to *SphI* site removed and replaced with the fragment containing the *hom* promoter: *cat* gene and Km^R gene on the *NdeI* to *SphI* fragment	this paper
pSupddhcat	The *Pst* fragment from pddhcat, containing the *ddh* gene interrupted with the *hom* promoter: *cat* gene and Km^R gene cloned into the *PstI* site of pSUP301	this paper

DNA Manipulations. General DNA manipulations were done as described previously [106]. Restriction enzymes, T4 DNA ligase, and other molecular biology enzymes were purchased from New England Biolabs and Bethesda Research Laboratories. DNA was isolated from agarose using the Qiagen kit for DNA purifications.

Plasmid Constructions

PCR Amplification of *ddh*. The *ddh* gene was PCR amplified using the primers 5'-dCCTCTTGCTGCAGCGAAGACAC and 5'-dCGGGATTTTCTGCAGCCCAGGT. Standard conditions were used as described previously [109]. Ampli-Taq polymerase was purchased from Perkin Elmer - Cetus. The template used was *C. glutamicum* E12 genomic DNA. The resulting amplified fragment was approximately 1.8 kb.

Construction of pddh-~~Hind~~. pBluescript was modifed to remove the *HindIII* site in the multiple cloning site by digesting pBluescript with *HindIII* then incubating with Klenow and dNTPs to create blunt ends. This was self-ligated and transformed into *E. coli*. Candidates were screened for the absence of a *HindIII* site. This modified pBluescript was digested with *PstI* and treated with alkaline phosphatase. The *ddh* gene was amplified using primers which had *PstI* sites included. The amplified fragment was treated with *PstI* and the resulting fragment was ligated into the the *PstI*-digested modified pBluescript. Candidates were screened by restriction patterns and also by the activity of DDH in enzyme assays.

Construction of pHomcm. Plasmid pHomΔ- contains the *hom* promoter minus a stemloop structure which occurs between the transcription start site and the ribosome binding site on an *XhoI-KpnI* fragment. This *XhoI-KpnI* fragment was

cloned into the corresponding *XhoI* and *KpnI* sites of plasmid pMT-SCM which has the promoterless *cat* reporter gene with its own ribosome binding site cloned into the *SalI* site of the *C. glutamicum/E. coli* shuttle vector pMT1. Candidates were screened by restriction fragment analysis and enzyme assays.

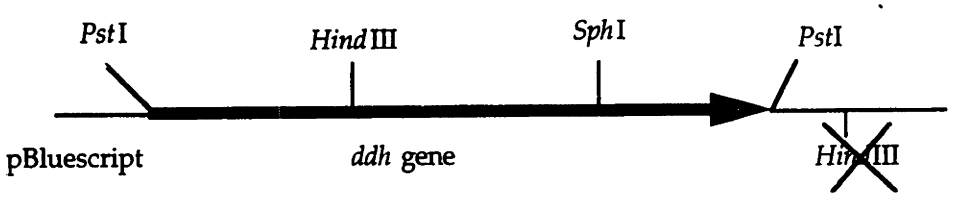
Construction of pddhcat. The *NdeI* (blunt-ended with Klenow) and *SphI* fragment of pHomcm which contains the *hom* promoter:*cat* gene and kanamycin resistance gene was cloned into pddh-~~Hind~~ at the *HindIII* (blunt-ended) and *SphI* sites. Candidates were screened by restriction fragment analysis.

Construction of pSupddhcat. The *PstI* fragment of pddhcat including the *hom* promoter: *cat* gene cloned inside of the *ddh* gene was cloned into pSUP301 at the *PstI* site (Figure 3.7 shows overall construction). Candidates were screened by restriction fragment analysis.

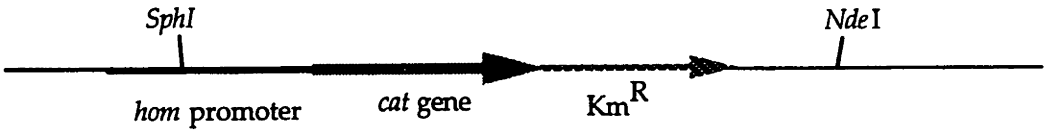
Transconjugation of pSupddhcat into *C. glutamicum* E12 and 21253. *E. coli* strain S17-1 was transformed with pSupddhcat. 10 ml LB cultures of *E. coli* S17-1(pSupddhcat) and *C. glutamicum* 21253 and E12 were grown overnight. 0.1-0.5 ml of the overnight cultures were used to inoculate 10 ml of fresh LB. These were grown to an OD_{600} of 1-2. Cells were harvested, resuspended in 5 ml PBS, spun again and resuspended in 5 ml PBS. 1 ml of the recipient strains, E12 and 21253, were aliquoted into Eppendorfs and heat treated for 10 minutes at 50°C, then placed on ice. These were spun for 30 sec and the supernatant removed. 0.5-1 ml of the donor strain, S17-1, was added and spun down. The cells were resuspended in 50 ml of LB and this was then plated in a big drop on LB-agar. The plates were put in a 30°C incubator overnight. The cells were scraped off the plate and diluted in 5 ml PBS. 200 ml aliquots were plated onto LB-agar with 25

Figure 3.7: Construction of pSupddhcat - Described below is the strategy used to construct the integration/disruption vector pSupddhcat.

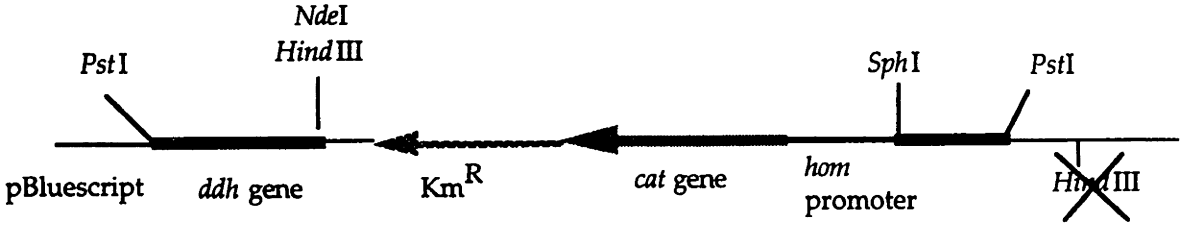
1. Used PCR to amplify *ddh* gene from *C. glutamicum* E12. Primers used had *PstI* sites at ends. Cloned into modified pBluescript (*HindIII* site was removed). Named it pddh-~~hind~~.



2. Constructed pHomcm. Cloned *hom* promoter minus the stemloop into a reporter gene vector containing *cat* gene.



3. Cut pHomcm with *NdeI* - blunt-ended it - and *SphI*. Cloned into *ddh* gene at *HindIII* site - blunt-ended- and *SphI* site. Named this pddhcat.



4. Cut pddhcat with *PstI* and cloned into the mobilizable vetor pSUP301 at *PstI* site. Named this pSupddhcat.

mg/l nalidixic acid and 6 mg/l kanamycin. After growth over 2-5 days at 30°C, candidates were restreaked on LB-agar with 25 mg/l nalidixic acid and 6 mg/l kanamycin. Candidates were screened by enzyme assays and Southern blot analysis.

***C. glutamicum* Genomic DNA Isolation.** 10 ml overnight cultures of the relevant strains grown in LB were harvested and resuspended in 1 ml TE plus 1 ml 1M sorbitol. 400 ml of lysozyme (100 mg/ml) was added and tubes were incubated at 37°C for 3 hours. The tubes were centrifuged and the supernatant poured out. The cells were resuspended in 4 ml TE and proteinaseK was added to a final concentration of 100 mg/ml. SDS was added to a final concentration of 0.5% and the cell mixtures were mixed well and incubated at 37°C for 3 hours. Sodium perchlorate was then added to a final concentration of 1M and the mixture was mixed gently and kept at room temperature for 30 minutes. The DNA was then extracted once with phenol/chloroform/isoamyl alcohol and once with chloroform. 2 volumes of ethanol was added and the DNA was collected by spooling or pelleting and resuspended in 1 ml of TE. The DNA was rocked at 4°C until it dissolved. RNaseA was added to a final concentration of 50 mg/ml and the tubes were incubated at 37°C for 30 minutes. The DNA was extracted once with phenol/chloroform/isoamyl alcohol and twice with chloroform and then ethanol precipitated [43]. DNA was finally resuspended in 0.5 ml TE and the amount of DNA was quantitated spectrophotometrically [106].

Southern Blots. Southern blots were done on the genomic DNA isolated from strains E12, 21253, E12#3, 21253#6 and 21253#10. DNA was cut with either *PstI* or *SphI*. The digested DNA was size fractionated on a 1% agarose gel. The DNA was transferred by capillary action to nitrocellulose. Probes of the *ddh* gene

was made using the digoxygenin kit of Boerhinger Mannheim according to protocols in the kit. The Southern blot was also done according to manufacturer's instructions and detection was done using the color substrate method.

Preparation of Crude Extract. Cells were grown in LB or minimal medium and harvested. The pellet was washed in PBS and resuspended in PBS containing DTT. The cells were disrupted in a bead mill using glass beads from Sigma. The supernatants were collected after centrifugation and protein quantitation was done using the BioRad protein kit.

Chloramphenicol Acetyl Transferase Assay. Chloramphenicol acetyl transferase was assayed by the spectrophotometric assay outlined in Shaw [107]. Briefly, 4 mg of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) was dissolved in 1 ml of 1 M Tris-HCl, pH 7.8. 50 µl of 20mM acetyl-CoA was added and the volume was brought up to 10 ml. Crude extract was added to this buffer to bring the total volume per assay to 980 µl. The reaction was monitored on a spectrophotometer at 412 nm, and then 20 µl of 5mM chloramphenicol was added. The rate of increase in absorption before the chloramphenicol was added was subtracted from the rate of increase after it was added. This net change in extinction per minute was divided by 13.6 (the molar extinction coefficient for 5-thio-2-nitrobenzoate is 13,600 at 412 nm) and the amount of protein added to give the µmoles per minute of CM-dependent DTNB reacted.

DDH Assay. DDH activity was measured by assaying the conversion of $NADP^+$ into NADH + H^+ in the reaction in which *meso*-DAP is converted to L-Δ-tetrahydrodipicolinate [36]. The decrease in absorbance at 340 nm was detected in an assay mixture which contained 0.8 ml $NaHCO_3$ buffer (8.4 g $NaHCO_3$, 0.5 l

H_2O, pH 10.5 with NaOH), 55 ml $NADP^+$(75 mg $NADP^+$ in 5 ml H_2O), 125 ml *meso*-DAP (250 mg *meso*-DAP in 5 ml H_2O) and 20 ml crude extract. The blank was dH_2O. The calculation used to determine the specific activity of DDH was $\Delta Amin^{-1}$ /(6.2X0.02Xmg protein/ml).

RESULTS

Cloning of the *ddh* Gene into pBluescript. The *ddh* gene of E12 was amplified using primers homologous to regions upstream and downstream of the gene. These primers also had *PstI* sites incorporated for ease of cloning into the *PstI* site of pBluescript. As a unique *HindIII* site in the *ddh* gene was to be used for a later step in the construction of the vector for transconjugation, the *HindIII* site in pBluescript was removed before the *ddh* gene was cloned into it. Enzyme assays were used to determine whether a functional *ddh* gene was amplified and cloned. *E. coli* does not have the *ddh* gene activity. Assays were done which compared the activity of DH5α(pBluescript), DH5α(pddh-~~Hind~~), and *C. glutamicum* 21253. Table 3.5 shows the results of these DDH assays. DH5α(pBluescript) has no measurable DDH activity, DH5α(pddh-~~Hind~~) has slight activity and *C. glutamicum* 21253 has wild type activity. The slight DDH activity in *E. coli* indicated that the *ddh* gene was cloned. There are many possible reasons why the activity is low in *E. coli*. The expression of the *ddh* gene is due to a *C. glutamicum* promoter which may not be very efficient in *E. coli*. The protein product may not be very stable or functional in *E. coli* either.

Cloning of pHomcm. The *hom* promoter had originally been cloned into the vector pZIP which used the *lacZ* gene as the reporter gene. The initial strategy was to use PCR to amplify the promoter: reporter gene and kanamycin resistance gene and clone this into the *ddh* gene. Additionally, the primer which lay

Table 3.5: *meso*-Diaminopimelate Dehydrogenase Activity in *E. coli* - The *ddh* gene was amplified by PCR and expressed in *E. coli*.

	Specific Activity μmoles/min/mg C.E
C. glutamicum 21253	0.52
E. coli (pBluescript)	-
E. coli (pskddh)	0.07

upstream of the *hom* promoter had a terminator structure incorporated into it so that once the cassette was incorporated into the genome, expression would have only been from the *hom* promoter. Amplification of this fragment, which was over 6 kb, was difficult to achieve and when small quantities were amplified, I was not able to clone the fragment. Another approach was taken in which the *cat* gene replaced the *lacZ* gene. The *cat* gene had the advantage that it was much smaller than the *lacZ* gene, approximately 0.7 kb versus 3.1 kb. The vector pMT-SCM which contained the promoterless *cat* gene with its own ribosome binding site in pMT1, an *E. coli/C. glutamicum* shuttle vector, already existed, and was what pZIP itself was derived from. The *hom* promoter was contained on a 0.7 kb *XhoI-KpnI* fragment. This included the promoter right up to immediately after the transcription start site, but not the 90 bp between the transcription start site and the translation start site. This fragment was cloned into the *Xho* and *KpnI* sites of pMT-SCM. The resulting plasmid, pHomcm, had the *cat* gene under the control of the *hom* promoter.

Activity of CAT. Since pHomcm contained the *C. glutamicum* origin of replication, it was possible to assay CAT activity in *C. glutamicum.* When assayed in minimal medium ± methionine, the activity was 12.7-fold higher in the culture which did not have methionine (Figure 3.5). This is a higher level of repression than that achieved for pHomΔ- under similar conditions. The reason for this difference was not explored.

Cloning of pSupddhcat. Initially it was attempted to amplify the promoter:reporter gene and resistance gene using the same primers used in the *lacZ* amplification attempt. Although in this case it was easier to amplify the fragment, which was now only a little more than 3 kb, it was still difficult to

clone. Another approach was taken in which a fragment of DNA containing the *hom* promoter: *cat* gene and kanamycin resistance gene was cloned into the *ddh* gene such that it was in the opposite orientation of the *ddh* gene (Figure 3.7). To prepare pddh-~~Hind~~ , a 0.6 kb fragment of the *ddh* gene was removed from the *HindIII* to the *SphI* sites. The resulting plasmid was named pddhcat. The entire modified *ddh* gene including the *hom* promoter:*cat* gene piece and the kanamycin resistance gene from pddhcat is contained on a *PstI* fragment. This *PstI* fragment was then cloned into the mobilizable vector pSUP301 at a *PstI* site to create pSupddhcat.

Transconjugation into *C. glutamicum* 21253 and E12. *E. coli* S17-1, which contains the transfer genes of plasmid RP-4 integrated into its genome, was transformed with pSupddhcat. This strain was then mated with *C. glutamicum* E12 and 21253. The mated cells were plated onto plates which contained 6 mg/l kanamycin to select for cells which had the kanamycin gene integrated and 25 mg/l nalidixate to kill the *E. coli* cells. Several candidates were obtained from each mating and these were restreaked on the same type of plate. Final candidates were tested by enzyme assays.

Enzyme Assays in *C. glutamicum* E12. For *C. glutamicum* E12, the goal of the transconjugation was to obtain a strain in which the *hom* promoter: *cat* reporter gene was integrated into the genome, so therefore a double integration in which the *ddh* gene is disrupted was not necessary. Strain E12#3 was obtained in which an integration did occur (Table 3.6). The strain retained its wildtype DDH activity and also picked up CAT activity. The level of CAT activity is about 14 times less than it is when the *cat* gene is present on a multi-copy plasmid. Furthermore, methionine represses the *cat* gene approximately 12-fold, which is

Table 3.6: DDH and CAT Assay Results. 21253#6 and #10 are disruption strains at the *ddh* locus. 21253#9 and E12#3 are integration strains in which a single copy of the *hom* promoter:*cat* gene has been integrated into the genome. Chloramphenicol acetyl transferase activity in E12#3 is about 14-fold less than it is on a multi-copy plasmid.

		DDH activity µmoles/mg/min
21253	#6	-
	#10	-
	#9	0.45
	no CE	-
E12	#3	0.3

E12 #3 CAT assays	µmoles/mg/min
-met	0.14
+met	0.018

The fold repression averages to about 12-fold

approximately the same amount of repression as there is when the *cat* gene is on a multi-copy plasmid.

Enzyme Assays in *C. glutamicum* 21253. For *C. glutamicum* 21253, the goal of the transconjugation was to obtain a strain which had the DDH activity removed, so therefore a double crossover needed to occur during the recombination event. Two candidates were obtained in which the DDH activity was no longer present, 21253#6 and 21253#10 (Table 3.6). These strains were also assayed for CAT activity after growth in LB. They had similar activities to E12#3 grown with methionine in the medium.

Southern Blot Analysis. Southern blots were done on the chromosomal DNA isolated from E12, E12#3, 21253, 21253#6, and 21253#10. The chromosomal DNA was digested with either *PstI* or *SphI* and size-fractionated by agarose gel electrophoresis. The probe used to hybridize the DNA was a fragment of the *ddh* gene labeled with digoxygenin-dUTP, and the bands were detected as per manufacturer's instructions. The results of the Southern blot were consistent with the results of the enzyme assays.

DISCUSSION

Techniques have recently been developed which allow the integration of DNA into the *C. glutamicum* genome [48-50]. Using these techniques, it is possible to both integrate a specific fragment of DNA at a targeted spot or disrupt a gene in the chromosome so that it no longer can make a functional protein. In this case, both options were desired. An integration of the *hom* promoter:*cat* reporter gene was needed in *C. glutamicum* E12 to study the effect of repression by methionine in the strain if only one copy of the promoter: reporter gene was present as

opposed to the promoter:reporter gene on a multi-copy plasmid. A double crossover event to produce a disfunctional DDH was necessary in *C. glutamicum* 21253 in order to study carbon flux in the pathway to lysine in a lysine-overproducing strain when the DDH overflow pathway is no longer present. In both cases, the necessary strains were obtained through the presented transconjugation strategy.

The cloning of the *ddh* gene from the *C. glutamicum* E12 genome was very straightforward. It was cloned into an *E. coli* vector and DDH activity was measured in *E. coli*. The strain with the *ddh* gene on a plasmid exhibited slight activity while the *E. coli* strain with no *ddh* had no activity. The DDH activity was very slight in *E. coli*, however. In comparison, the wild type level of the chromosomal copy of *ddh* in *C. glutamicum* is approximately 7.5 times higher than the activity from the multi-copy plasmid in *E. coli*. There are several possible reasons for this difference. One hypothesis is that since the *ddh* is being expressed from its own *C. glutamicum* promoter, that promoter is not recognized very well in *E. coli* and low activity is the result. Although many *C. glutamicum* promoters can be used in *E. coli* and vice versa, not all promoters work well and this may be one such promoter. A second hypothesis is that the protein product does not fold properly in the *E. coli* environment.

The choice of changing reporter genes from *lacZ* to *cat* proved fortuitous because the *cat* gene was more highly repressed by methionine than the *lacZ* gene; 12.7-fold repression versus 7.5 fold. The cause of this difference was never addressed, although differences in repression for different reporter genes has been reported elsewhere [80, 81]. A hypothesis for this difference is that the *lacZ* transcript is more stable than the *cat* transcript. As the level of *lacZ* transcript builds, there is

a saturating effect for the number of ribosomes translating it into protein. The level of repression is then lower because there is an upper boundary for the activity of the promoter when it is derepressed, but not for the repressed promoter. Therefore, the ß-galactosidase activities are closer together and the apparent level of repression is less at the protein level than it is at the transcript level. Data which corroborates this was produced when the cultures were grown in medium with all of the other amino acids, ± methionine. In this case, the specific activities of the reporter gene products (in this case ß-galactosidase) were a lot higher but the repression level stayed the same. The added amino acids helped the cultures grow faster, meaning that there would be more ribosomes present allowing for more protein synthesis and higher specific activities. In order to better understand this phenomenon, Northern blots should be done to examine the levels of RNA transcripts in each case. If the RNA analysis proves the hypothesis incorrect, the levels of protein should be examined by Western blots to see whether there is differential stability in the protein products.

In the cloning of the disruption vector it was desired to use PCR in order to place a terminator immediately upstream of the *hom* promoter so that there would not be any readthrough transcription from upstream in the genomic sequence. As this cloning proved difficult to do, it was decided to insert the *hom* promoter: reporter gene in the opposite orientation of the *ddh* transcription so that there would not be any readthrough from the *ddh* promoter. Readthrough from genes downstream of the *ddh* which are being transcribed in the same direction as transcription from the *hom* promoter would not be stopped in this strategy. From the specific activities of the CAT assays, however, the difference in activity from one copy of the gene versus the activity of multiple copies on a pSR1 derived

plasmid (which pMT1 is) is very similar to examples for other genes [85, 96]. Furthermore, the level of repression is also very similar whether in one copy or multiple. For these reasons, readthrough from promoters upstream of the *hom* promoter seem to have little effect on expression of the *cat* gene.

One of the difficulties in the cloning of these plasmids was that the fragment of DNA being inserted into the *ddh* gene included the *hom* promoter and both the *cat* gene and the kanamycin resistance gene. This was especially a hurdle when this fragment was being amplified via PCR. The kanamycin resistance gene needed to be included for selection purposes as it was not certain whether the level of CAT activity under repressed conditions would be high enough for selection of candidates after the transconjugation.

As stated earlier, one of the goals of the strain development was to see whether repression at the *hom* promoter would be more amenable to study if only one copy of the promoter:reporter gene was being expressed at a time. In this case, the level of repression for one copy versus multiple copies was comparable. The steps involved in reaching this answer, however, resulted in the use of a reporter gene system which, for unexplained reasons, demonstrates higher repression than the other system.

The other goal of the strain development was to create a *C. glutamicum* lysine-producing strain which no longer has a functional *ddh* pathway. Further study on this strain will be done by Cathryn Shaw-Reid. Preliminary studies indicate that this strain produces less lysine. An interesting phenomenon in this strain is that a much higher amount of glutamate is excreted.

Section III: Search for the Methionine Regulator

This next section describes experiments that have been done in an attempt to both find the gene for the methionine regulator and to find the site in the *hom* promoter to which the regulator binds.

Regulator Binding Site in *hom* Promoter

Two strategies have been used to find the operator site in the *hom* promoter. The first involved making nested deletions into the *hom* promoter using Exonuclease III and S1 nuclease (Promega Erase-A-Base method). The second strategy was to use a special strain of *E. coli*, XL1-Red (Stratagene), which is deficient in three DNA repair enzymes. Introduction of pHomΔ- into XL1-Red theoretically should produce random mutations in the plasmid. These plasmids could then be isolated and introduced into *C. glutamicum* E12 where screening is done for derepressed strains.

Neither strategy met with much success for perhaps both technical and theoretical reasons. Repressor sites are generally in three different sites of the promoter region; just upstream of the -35, in the spacer region, or between the -10 and the transcription start site and activator sites are generally upstream of and overlapping the -35 [68]. In almost all cases, a mutation which affects regulator binding may also have an effect on promoter strength. So, for instance, the nested deletions method would not have found the regulator site unless the site was upstream of the promoter region. The nested deletions would have been helpful in that a minimal length of DNA would have been identified which serves as the *hom* promoter. From this information, degenerate oligonucleotides could have been synthesized which correspond to the promoter region and

mutant promoters made from these degenerate oligonucleotides could have been screened for derepression.

Problems in working with *C. glutamicum* shuttle vectors include their size and their lack of unique restriction sites in opportune places. For example, in both pHomΔ- and pHomcm, there are no sites upstream of the *hom* promoter which can be used to protect the plasmid from Exonuclease III digestion in the nested deletions procedure. To get around this, I constructed a plasmid in which both the *hom* promoter: *cat* gene and kanamycin resistance gene were cloned into pBluescript. The extra regions were included because I thought it would be easier to later clone a large piece of DNA which had a nested deletion than a piece of DNA which only included the promoter and was less than 100 base pairs long. When I tried to make nested deletions in phomdel, however, I got large deletions (several kb) even at short time periods and I was not able to rectify this. I assumed that the plasmid DNA had nicks in it which were being recognized by the S1 nuclease. It was at this time that I was introduced to the XL1-Red method. Since I had little faith in the nested deletions method for finding a regulator site and really was using it mainly to find a minimal promoter region from which to design degenerate oligonucleotides, I decided to instead try the random mutagenesis method.

As mentioned above, I transformed XL1-Red with pHomΔ-. I chose to use pHomΔ- rather than pHomcm because the *hom* promoter and upstream region in pHomΔ- is only about 120 bp long while it is about 900 bp long in pHomcm and I thought it would be best to keep the promoter region to a minimum. After transforming XL1-Red, I collected the candidates, isolated the DNA and transformed *C. glutamicum* E12. The transformation efficiency, however, was not

very high, so I also transformed *E. coli* XL1-Blue (Stratagene) in order to make a more concentrated preparation of plasmid DNA. I transformed E12 with the plasmid DNA from XL1-Blue and got a higher transformation efficiency. I screened the candidates on plates containing Brain Heart Infusion Agar, 25 mg/l kanamycin and 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-Gal). I chose those candidates which turned brightest blue for further testing. Unfortunately, none of the candidates selected showed derepressed ß-galactosidase activity when assayed after growing in LB.

Using the blue screening did not work very well in this case because cells which had roughly the same amount of ß-galactosidase activity were different shades of blue. Furthermore, by screening in LB I was only screening for those candidates which were derepressed but also had full promoter activity.

The method of using XL1-Red to generate promoter mutations which are derepressed might work if pHomcm is used instead, or a new plasmid which has the shorter *hom* promoter region driving expression of the *cat* gene. Using antibiotic resistance as a selection would most likely yield fewer false positives than the *lacZ* screening. Once again, however, the only mutations which would be selected would be those that are derepressed but still have high promoter activity.

Another advantage to using the *cat* gene is that there is a higher level of repression in the *cat* gene. This might be important because if methionine repression in *C. glutamicum* is analogous to repression in *E. coli*, there might be several sites for the repressor. In *E. coli*, the promoters which are controlled by MetJ all have 2-5 "met boxes" which work in a cooperative fashion [70]. If there

are multiple binding sites in the *hom* promoter, a mutation in one might not have a significant effect if the overall repression level is low. By increasing the repression range, it might be easier to determine intermediate levels of repression as real.

Search for the Regulator Gene

Different approaches have been taken in the attempt of cloning the methionine regulator gene in *C. glutamicum*. The first efforts tried to capitalize on the methionine regulation story of *E. coli*. In *E. coli*, the *metJ* gene is immediately upstream of the *metB* gene [87]. The only methionine pathway genes we have cloned in our lab are two which complement *E. coli metB* and *metC* auxotrophs. Although these activities are presumed not to be important in the *C. glutamicum* methionine pathway [88], we were interested to see whether the methionine regulator gene was linked. Furthermore, although *metC* complementing clone was identified through sequencing to be the C-S lyase [94], the *metB* complementing activity could have potentially been the O-acetyl homoserine sulfhydrylase because if this is functional in *E. coli* it could potentially complement a *metB* auxotrophy. Unfortunately, neither clone complemented the regulator mutation. This does not prove that the methionine regulator gene of *C. glutamicum* is not linked to either of these other genes since the clones may not have had the complete regulator gene sequence. The genetic organization of *C. glutamicum*, however, is usually quite different from *E. coli*, so this was not unexpected.

The *C. glutamicum hom* promoter has been reported to not be functional in *E. coli* in part because of the inability of the *C. glutamicum hom* gene to complement an *E. coli* homoserine auxotroph [85, 90]. A possible explanation could be that the *E.*

coli MetJ so tightly represses the *hom* promoter that very little homoserine dehydrogenase is made. I decided to test this idea by transforming a *E. coli metJ-* strain and *E coli* DH5α with pHomΔ- to see whether the *hom* promoter was expressed in the *metJ-* strain. To my surprise, there was ß-galactosidase activity in both strains and this activity was higher in DH5α. Therefore, the hypothesis that the *E. coli* MetJ represses the *hom* promoter tightly and prevents transcription was incorrect. Furthermore, the belief that the *hom* promoter is not expressed in *E. coli* also appears to be wrong. There must be something in either the *hom* ribosome binding site or protein product that is not functional in *E. coli*. The idea that the *C. glutamicum* homoserine dehydrogenase is not functional in *E. coli* is not that far-fetched since *E. coli* has such a different structure for its homoserine dehydrogenase. In *E. coli* the homoserine dehydrogenase is part of the bifunctional aspartokinase/homoserine dehydrogenase [70]. Of course, there is the possibility that there are other regions either in the *hom* promoter or upstream in the vector which are recognized and used in *E. coli*. A control was not done to test these possibilities.

Regulator Screen

Since the methods described above did not yield a methionine regulator clone, a complementation strategy was devised. The initial purpose for creating the E12Rep-2 strain was to generate a genetic background in which to screen for the regulator gene. The strategy involves placing a toxic gene under the control of the *hom* promoter. In the derepressed strain, the toxic gene would be expressed and the cell would therefore die. If the regulator mutation is complemented, however, the *hom* promoter will be repressed and the toxic protein would not be synthesized. This strategy will only work if the regulator is a repressor. If the regulator is an activator, the mutation in E12Rep-2 has made the activator

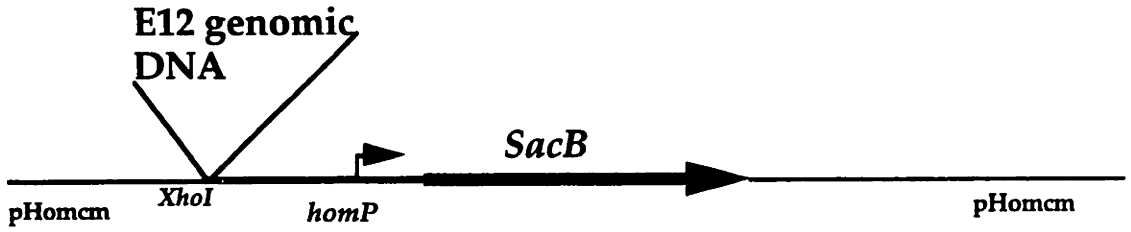
constitutive which could mean that a complementing regulator would not prohibit the expression of the toxic gene. The screen might still work if the regulator composition is as a multimer and that the presence of wild type regulator subunits stops the regulator from acting constitutively.

The *sacB* gene was used as the toxic element of the construction [110]. This gene, which is of *Bacillus* origin, encodes a levansucrase. When cells are grown on sucrose, the enzyme cleaves the sucrose and acts as a fructosylating agent, attaching fructose groups indiscriminately to different molecules. This proves fatal to the cell.

The initial construction is shown in Figure 3.8. A plasmid which carries the *sacB* gene, pUCD4121 [110], was partially digested with *KpnI* and fully digested with *XbaI*. The 1.8 kb fragment was isolated from an agarose gel and cloned into pHomcm which had been cut with *KpnI* and *XbaI*. The resulting plasmid, pHomSac, was then used to transform both E12 and E12Rep-2. The new strains, E12(pHomSac) and E12Rep-2(pHomSac) were tested on different concentrations of sucrose to see what concentration was toxic to E12Rep-2(pHomSac), but not to E12(pHomSac). A concentration of 0.27% sucrose in the agar was toxic to E12Rep-2(pHomSac), but not to E12(pHomSac).

The next step was to include a *C. glutamicum* genomic library into pHomSac. The site chosen to place this library was the *XhoI* site immediately upstream of the *hom* promoter. E12 genomic DNA was partially digested with both *XhoI* and *SacI* to fragments which were on average around 4 kb. These fragments were then ligated into pHomSac at the *XhoI* site (Figure 3.8). Different E. *coli* strains were transformed, such as DH5α and super competent XL1-Blue in an attempt to get

Figure 3.8: Regulator Gene Screening Vector - In order to screen for the *rep* gene, a library was made using the vector described below and this library was transformed into E12Rep-2. (The pHomSac vector is the vector shown without the E12 genomic library at the *XhoI* site.) If the *rep* gene is not included in the vector, the cells will die when grown on sucrose because of high expression of the *sacB* gene since the *hom* promoter is derepressed. If the *rep* gene is cloned into the vector, the promoter will be repressed and the cells will survive.



as many transformants as possible. The transformants were harvested and their DNA purified. The DNA was then used to transform E12Rep-2. These transformants were screened on Brain Heart Infusion agar which contained about 0.27% sucrose. Many transformants survived. Some were tested to see whether their O-acetyl homoserine transferase activity was now repressed when grown in complex medium. They all remained derepressed. The colonies were again screened on higher levels of sucrose, but the problem of false positives still remained. Rather than using replica-plating to test the colonies on sucrose, the transformation mixtures were plated directly onto the sucrose-containing agar. The problem of false positives remained.

There are several potential problems which may have thwarted the regulator screen. Most of them are in the library construction. The first problem is that the ligation and transformation efficiency has been low. This may have to do with the size of the vectors. PHomSac is approximately 10 kb already and the genomic DNA averages around 4 kb. It would be possible to decrease the size of pHomSac to about 8.6 kb, but that would involve more cloning steps and initially I thought it best to try the faster cloning method. It is also possible to decrease the size of the genomic DNA fragments. Since the cloning strategy necessitates the use of 6 base restriction enzyme cutters rather than 4 base cutters, sites occur on average once every 4100 base pairs. I have been hesitant to allow complete digestion to occur on the chance that either an *XhoI* or *SacI* site occurs in the methionine regulator gene which would render the library useless.

The false positive problem may be also due to the fact that the location of the genomic fragments is immediately upstream of the *hom* promoter. This may be interfering in some way with the expression of the *sacB* gene, although it is not

clear without further analysis how this interference would occur. The plasmids which have generated the false positives in E12Rep-2 have not been analyzed to see whether there are any noticeable characteristics which may be responsible for the survival phenotype.

I have begun to set up another strategy in order to isolate the methionine regulator gene. In this case, a C. *glutamicum* library which has already been constructed and tested would be used. Rather than including the *hom* promoter:*sacB* gene on the same plasmid with the library, the *hom* promoter:*sacB* gene is to be integrated into the genome of E12Rep-2. Screening can then be done by transforming this new strain with the C. *glutamicum* library. The advantage to this strategy, besides the use of a library which has already been tested and characterized, is that analysis of the resulting complementing plasmid will be easier. For instance, in the strategy described above, it would be very difficult to subclone and retest pieces of the initial DNA for complementation because of the constraints imposed by using such an unwieldy vector.

CHAPTER 4
THE ROLE OF METHIONINE REGULATION IN THE ASPARTATE-DERIVED AMINO ACID PATHWAYS

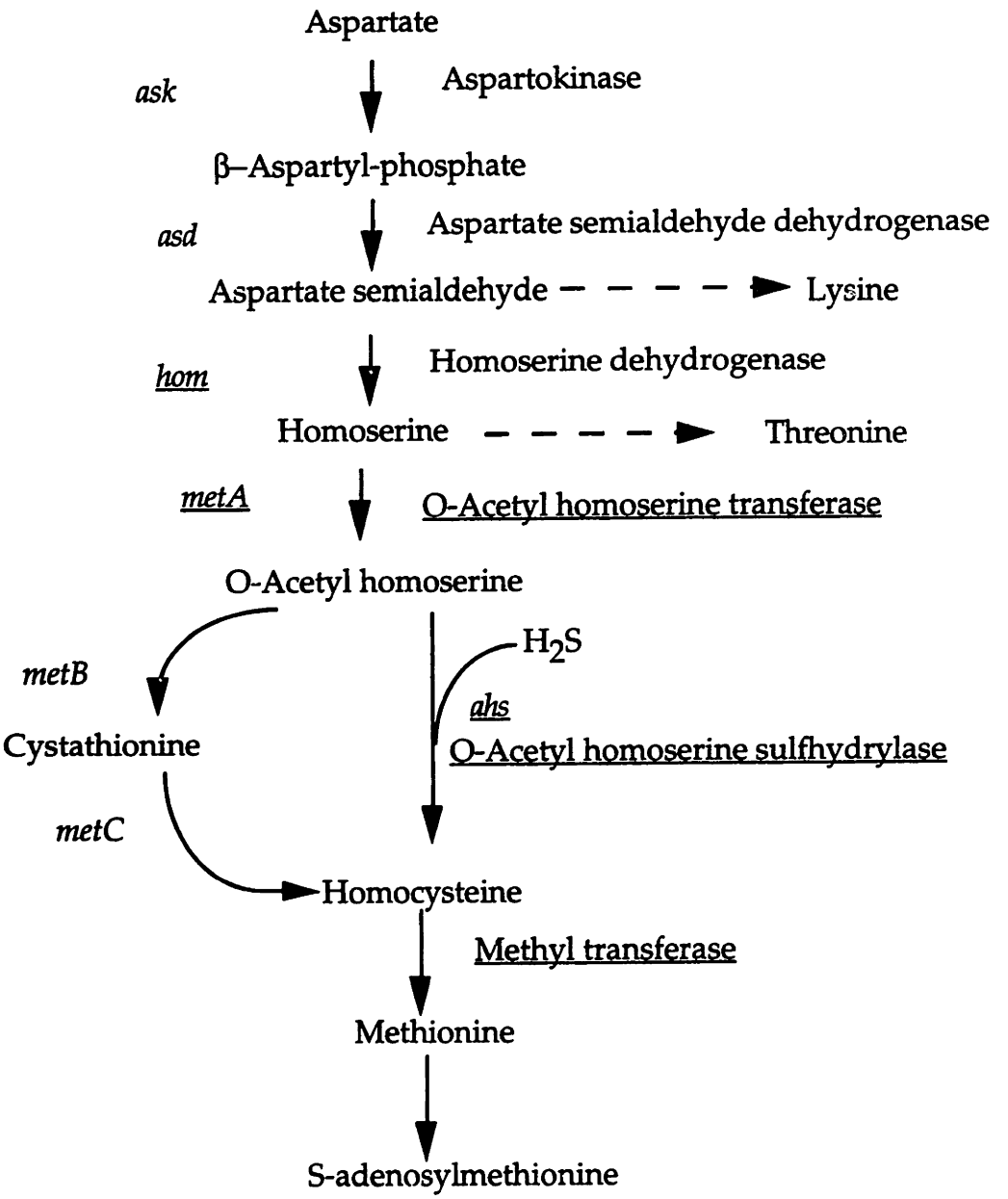
In this chapter, the role of methionine regulation in the pathways to methionine, threonine, and lysine will be discussed. In the first section, a summary of methionine regulation is given. Included in this summary is a qualitative discussion of what the presumed carbon flux is in different growth medium. The chapter ends with a theoretical discussion of why *C. glutamicum* evolved this pattern of regulation.

Section I: Pathway Regulation

There are few points of regulation in the amino acid pathways to threonine, methionine, and lysine (Figure 4.1) [1]. The key regulatory feature which controls the flow of carbon to the entire pathway occurs at the enzyme aspartokinase. This enzyme is inhibited by the concerted presence of both threonine and lysine [1]. Therefore, the intracellular pools of both threonine and lysine have to be large enough in order to shut down carbon to the entire pathway.

The second control point is at the branchpoint in which the carbon precursor either goes towards lysine or it goes towards threonine and methionine. The enzyme which directs the carbon towards threonine, homoserine dehydrogenase, has a 15 times greater affinity for aspartate semialdehyde than does the enzyme which directs carbon towards lysine, dihydropicolinate synthase. This greater affinity causes the majority of the substrate to be directed to the threonine and methionine branches [45].

Figure 4.1: Methionine Pathway in *C. glutamicum* - Below is the pathway from aspartate to methionine in *C. glutamicum*. Not as much is known about the pathway in *C. glutamicum* compared to *E. coli*. It appears that although the *metB* and *metC* enzyme activities are present in *C. glutamicum*, they may not be involved in methionine synthesis. Genes and enzymes which are underlined are regulated by methionine or S-AM. [88, 92, 111]



The next regulatory site in the threonine branch is homoserine dehydrogenase which is controlled at both the transcriptional and enzymatic level. The *hom-thrB* operon is repressed two-three fold by methionine in the medium [85]. The homoserine dehydrogenase enzyme in almost completely inhibited by 1-5 mM threonine [89]. In addition, the homoserine dehydrogenase activity is inhibited 50% by isoleucine [1]. There is also some inhibition of the second enzyme to threonine, homoserine kinase; 30 mM threonine competitively inhibits homoserine kinase 50% [95].

From homoserine, the carbon precursor can either go towards threonine via homoserine kinase or towards methionine via homoserine acetyl transferase. The first gene in the methionine pathway, *metA*, encodes the enzyme homoserine acetyltransferase [92]. This gene is repressed up to 70-fold by methionine and even endogenous levels of methionine are enough to repress its expression [92]. O-acetylhomoserine transferase is inhibited by both S-adenosyl methionine and methionine. The amount of methionine necessary for 50% inhibition, however, is large (4.8 mM) and therefore may not have any real physiological meaning [111]. There are conflicting reports in the literature about the affinity of O-acetylhomoserine transferase for homoserine. The most recent paper reports the K_M as very near or slightly under the K_M of homoserine kinase for homoserine while an older paper reports a much higher value [92, 111].

The next step in the pathway to methionine is O-acetylhomoserine sulfhydrylase [88]. Expression of the gene which encodes this enzyme has also been reported to be repressed by methionine. Although *in vitro* measurements of the sulfhydrylase activity shows inhibition by methionine, the concentration of

methionine which yields 50% inhibition is quite high and therefore probably does not have any physiological meaning [88].

The last step of the pathway is the conversion of homocysteine to methionine. The step is presumed to be repressed, but the literature is rather sparse for the specifics.

So what is the purpose of this regulation pattern? In general, bacteria must meet three challenges in order to survive; starvation or gradual depletion of nutrients, competition for surface sites and exposure to noxious chemicals [74]. Many of the regulatory strategies for the amino acid pathways were probably evolved in order to meet the first challenge, namely prevention of starvation. In nature, nutrient scarcity is probably the norm for most bacteria [112]. Bacteria may grow very slowly or have periods of rapid growth followed by long periods of no growth or starvation in their natural environment [112]. *C. glutamicum*, since it is a soil bacterium, most likely spends most of its lifetime in the stationary phase, although this has not been demonstrated experimentally. Most of the studies in *C. glutamicum* have been done while *C. glutamicum* is in the exponential phase, however. Whether the regulation which has been demonstrated in the exponential phase is relevant in the stationary phase is a subject which still needs to be addressed experimentally. Below is a discussion of how the known regulation controls the flux in the aspartate-derived amino acid pathways in different medium compositions during exponential growth. It is assumed in this discussion that this regulation has several purposes. One is to prevent the synthesis of too much of any particular metabolite. Another is to prevent the synthesis of a metabolite if the cell is growing in the presence of that metabolite.

Assume that the needs of the cell for the different amino acids can be roughly correlated to the percentage of each amino acid in the various proteins of *C. glutamicum*. This assumption is based on evidence that suggests that unless the pathways are manipulated, the cell does not excrete excess amounts of amino acids [95]. Table 4.1 is a break down of the amino acid compositions of fourteen proteins from *C. glutamicum*. Although the list is far from complete, is does provide a sense of the amino acid needs of the cell as required by its proteins. From the table, threonine and isoleucine make up 6.4% and 5.8% of these proteins. Methionine only composes about 1.9% of the protein. This branch also has to produce enough S-adenosyl methionine for one-carbon metabolism and spermidine, although this is most likely not that great of an extra burden. Lysine makes up approximately 4.2% of the protein. Once again, however, this branch must also produce all of the *meso*-DAP for the cell wall, which may add significantly to the amount of carbon needed. From these percentages of amino acids needed, *C. glutamicum* must put more carbon towards the threonine branchway to meet the needs of threonine, methionine, and isoleucine than down the lysine pathway, assuming the requirement for *meso*-DAP is less than two times the amount of lysine needed.

To test whether the assumption above, that a rough estimate of the amino acid needs of the cells can be derived from the amount of amino acids used, is plausible, we can examine the amino acid requirements of *E. coli*. *E. coli* has been analyzed to determine how much of each building block is present [74]. In Table 4.2, the amount of the aspartate-derived amino acids present per gram of dried cells is given. Although the amount of each amino acid used in *E. coli* is not exactly the same as in *C. glutamicum*, they are comparable. From these numbers,

Table 4.1: Amino Acid Compositions from *C. glutamicum* Genes - Amino acid compositions from 14 genes were analyzed and their frequencies were compiled (Phil Lessard, personal communication). Shown are the frequencies of the aspartate-derived amino acids as a percentage of the total number of amino acids.

Amino Acid	Percentage (%) of Total Amino Acids
Aspartate	6.5
Isoleucine	5.8
Threonine	6.4
Methionine	1.9
Lysine	4.2

Table 4.2: Amount of Building Blocks Derived from Aspartate Needed to Produce 1 g of *E. coli* Protoplasm [74]

Building Block	Amount present in *E. coli* (μmol/g dried cells)
Aspartate	229
Isoleucine	276
Methionine	146
Threonine	241
Lysine	326
Diaminopimelate	27.6

we see that the amount of lysine used is more than that estimated for *C. glutamicum*. However, the amount of methionine, threonine, and isoleucine needed is still double the amount of lysine. The amount of DAP present in *E. coli* is quite low. Given the differences is cell wall structure, this amount will be higher in *C. glutamicum*. How much more is unknown at this time. In order for there to be more carbon flow in the lysine path versus the threonine branch, the amount of *meso*-DAP needed in *C. glutamicum* would have to be twelve times more than the amount needed in *E. coli*, however. Much of the *E. coli* data is confirmed by an analysis done on the amino acid compositions of 207 unrelated proteins (Figure 4.3) [113]. Once again, the amount of lysine used in these proteins in higher than the analysis done in *C. glutamicum*. The other percentages of the aspartate-derived amino acids used in protein is fairly comparable to the compilation which was done for *C. glutamicum*.

Although the *C. glutamicum* regulatory pattern appears simple, the use of a few key control sites confers some level of regulation over the entire aspartate-derived amino acid network. If we consider lysine and threonine as the major branches of the pathway and methionine and isoleucine as sub-branches, we can see that *C. glutamicum* controls both its sub-pathways and its main pathways. Although not mentioned above, isoleucine regulates its own synthesis by inhibition of the first step in the isoleucine pathway from threonine and both inhibition and repression of the second step [83, 84]. Furthermore, isoleucine inhibits homoserine dehydrogenase approximately 50%, so that the flow of carbon through the threonine branch is decreased when there is an intracellular pool of isoleucine [1]. Methionine, as already discussed, also regulates its synthesis by repression and inhibition of the pathway leading to methionine. After the intracellular pools for these amino acids are filled, threonine itself can

Table 4.3: Occurrence of Amino Acids in Proteins - The following percentages indicate the frequency of occurrence of the aspartate-derived amino acids in 207 unrelated proteins [113].

Amino Acid	Occurrence in Proteins (%)
Aspartate	5.5
Isoleucine	4.6
Methionine	1.7
Threonine	6.0
Lysine	7.0

start to accumulate. As threonine builds up, its inhibition of homoserine dehydrogenase diverts increasing amounts of carbon precursor into the lysine pathway. As the internal concentration of lysine increases, the concerted action of both lysine and threonine act to feedback inhibit aspartokinase. As the intracellular pool of aspartate accumulates, at least one of the enzymes which provides oxaloacetate which may be fed into the system, phosphoenolpyruvate carboxylase, is repressed [75]. This diverts the carbon flux from aspartate back into the TCA cycle.

What is the purpose of directing the carbon flux preferentially to the threonine branch over the lysine branch? Or, perhaps more importantly, is it real? That homoserine dehydrogenase has a lower K_M for aspartate semialdehyde than dihydropicolinate synthase when tested *in vitro* does not automatically translate into any physiological relevance *in vivo*. For instance, the amount of aspartate semialdehyde available may be sufficiently high enough to satisfy the carbon flux to both homoserine dehydrogenase and dihydropicolinate synthase simultaneously. Alternatively, synthesis of dihydropicolinate may exceed that of homoserine dehydrogenase so that although dihydropicolinate synthase has a lower affinity for aspartate semialdehyde, there is so much of it that it can compete for the substrate. At this time, no one has studied the relative level of expression of *dapA* (encoding dihydropicolinate synthase) versus *hom*, so these questions remain unanswered.

Another possible explanation is that the difference in affinity for aspartate semialdehyde is representative of the level of carbon which needs to be directed towards each pathway. The amino acid analysis in Table 4.1 indicated that more carbon precursor needs to be directed towards threonine, methionine and

isoleucine than it does towards lysine and the cell wall (once again assuming that the amount of *meso*-DAP needed does not exceed two times the level of lysine). This distribution might be met by the amount and efficiencies of homoserine dehydrogenase and dihydropicolinate synthase.

One piece of evidence that is often given in support of the idea that threonine is synthesized preferentially is that in lysine overproducing strains, lysine accumulates late in the fermentation [6]. The delay may be partly due to the inhibition of homoserine dehydrogenase by threonine, so that all of the carbon is now directed towards lysine. In wild type strains, this large accumulation would not be seen because the flux of carbon through aspartate would be controlled by aspartokinase. Whether carbon goes towards threonine or lysine first is a moot point, except in overproducing strains. Regardless of whether there is a preference one way or the other, the carbon precursor has to be processed via both branches because the cell needs threonine, methionine, and isoleucine as well as lysine and *meso*-DAP all of the time when its growing or producing proteins.

Another site of interest is at homoserine. Homoserine can either be processed via the threonine and isoleucine pathway or via the methionine pathway, so how does the cell ensure that enough carbon is directed either to threonine and isoleucine or to methionine? O-acetyl homoserine transferase has either a similar affinity or less affinity for homoserine than homoserine kinase, depending on which paper is believed in the literature [92, 111]. Therefore no case can be made that carbon is drawn preferentially towards methionine due to a greater affinity for homoserine, i.e. a lower K_M value. One report has suggested that there is a higher level of the enzyme O-acetylhomoserine

transferase achieved than homoserine kinase, so this might draw the carbon precursor towards methionine [92]. However, if we look at the amount of methionine the cell needs relative to how much threonine and isoleucine are required, we see that much less methionine is necessary. Therefore, the cell probably does not have a problem directing enough carbon precursor towards methionine. Even if the distribution of homoserine was four times more towards threonine and isoleucine than to methionine, the cell would be able to produce enough methionine for its needs. It has also been shown that homoserine can accumulate during a fermentation [95]. This may be because the reaction from homoserine to homoserine-phosphate is thermodynamically unfavorable, and the reverse reaction is much more favorable. This extra intracellular pool of homoserine can be used by the cell to produce methionine when needed.

In fact, it would appear that *C. glutamicum* is much more concerned with limiting the amount of methionine synthesized than having enough. Much of the regulation in these pathways is mediated by methionine. Methionine has been shown to repress the *hom-thrB* operon, the *metA* gene, the gene encoding acetylhomoserine sulfhydrylase, and, perhaps, the last gene in the pathway [85, 88, 92]. The activity of O-acetylhomoserine transferase is also inhibited by S-AM [111]. This concern for regulating methionine synthesis makes sense when the metabolic cost of methionine is considered. Methionine is the most expensive amino acid to make, costing the cell one oxaloacetate, one C-unit, one NH_3, one S-unit, seven ATPs, and eight NADPHs [70]. Therefore, the cell has a vested interest in making sure that only the minimal amount of methionine necessary is produced.

The next parts will look at the pathways under different growth conditions to see potential strategies *C. glutamicum* might use to meet its metabolic needs for maintenance and growth.

Growth in Minimal Medium

In minimal medium, the cell has to produce all of the amino acids it needs for protein synthesis. Therefore, there will be carbon flux to all branches of the aspartate-derived amino acid pathways since all of the amino acids are necessary. Regulation comes in as a means of fine tuning the carbon flux. For example, as the methionine requirement is met, the *metA* gene is repressed by the endogenous methionine. This, along with the other modes of regulation in the methionine pathway will prevent synthesis of more methionine until the cell needs it. As all of the regulation mechanisms described are negative, i.e. they prevent synthesis, the only times most are used would be in response to temporary conditions where too much of any particular metabolite has been produced. In a growing culture, however, protein synthesis is continuous and therefore the amino acid pools must also be continuously replenished. One can imagine that as stationary phase is reached, and protein synthesis is not as high, the lesser need for amino acids will result in more of the regulation being used.

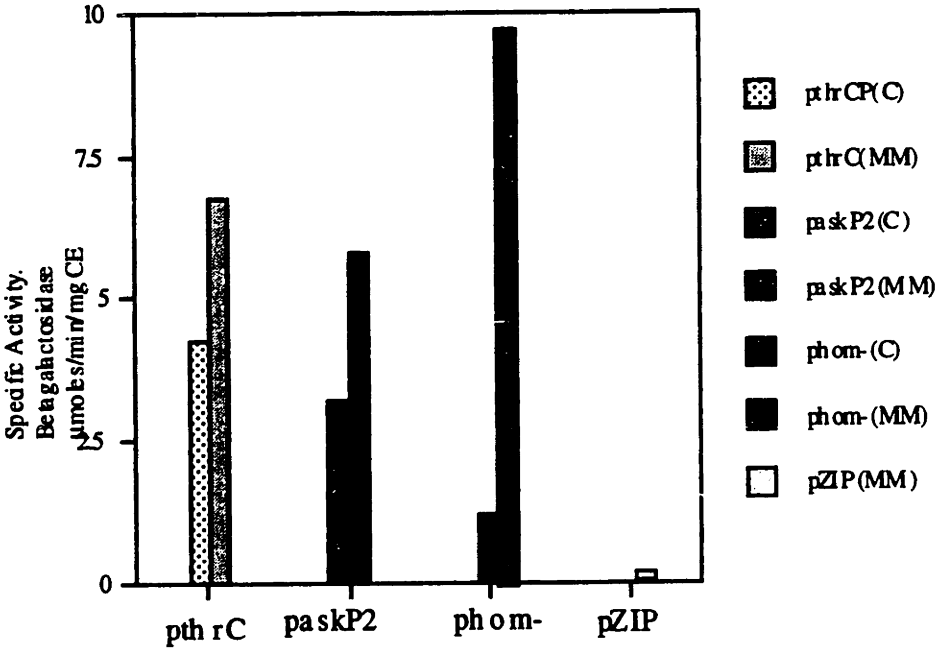
Growth in Complex Medium

Cells growing in complex medium enables the cells to utilize amino acids in the medium rather than synthesizing new amino acids. In this case, the question then becomes, how much, if any synthesis of amino acids takes place? Furthermore, how is the regulation mediated? We have shown that the promoters which are repressed by methionine are repressed when the cells are grown in methionine containing medium. However, all of the promoters which

we have tested have some activity when grown in complex medium (Figure 4.2). These transcripts are most likely translated into proteins. So where does the regulation take place? It must take place via the inhibition of the key enzymes, such as aspartokinase and homoserine dehydrogenase. The cell needs to import enough of the relevant amino acids so that inhibition can take place. For example, methionine represses *hom* so that less homoserine dehydrogenase is made. Both threonine and isoleucine will then inhibit the homoserine dehydrogenase so that there is very little synthesis on the threonine branch of the pathway.

On the lysine side, however, there are many unanswered questions on how regulation affects the production of *meso*-DAP. It would seem that there must be some carbon flow to the lysine branch because it has been reported that *meso*-DAP cannot be imported into the cell [36]. Therefore, the lysine pathway could not be completely shut down or else the cell wall would be affected. One potential solution is to synthesize *meso*-DAP from lysine using the reverse reaction. This reaction is extremely thermodynamically unfavorable, however (Colón, personal communication). To satisfy the thermodynamics, the only way to convert *meso*-DAP from lysine is to have very high concentrations of lysine inside the cell. Rather than building up inside the cell, lysine tends to be excreted, however (Colón, personal communication). It seems odd that *C. glutamicum* would have evolved in such a way as to inhibit aspartokinase when there are high concentrations of both threonine and lysine, yet not have a means to produce *meso*-DAP. There is most likely a piece of this puzzle which is still missing. This missing piece may be in some way related to the second pathway going from Δ-piperideine-2,6-dicarboxylate to *meso*-DAP. *Corynebacterium* is the only known genus (although a second pathway has also now been implicated in

Figure 4.2: *C. glutamicum* Promoters in Complex Medium - Below are the activities of the *thrC, askP2,* and *hom* promoters in complex (C) and minimal medium (MM). Although the promoter activity is lower in complex medium, there is still activity. The *askP1* promoter also shows activity in complex medium (data not shown).



Mycobacterium) which has two pathways to lysine [32]. Perhaps a reason for a second pathway is for the regulation of *meso*-DAP. For instance, *B. subtilis* has an isoenzyme of aspartokinase which is sensitive to the amount of diaminopimelate [114]; therefore, it is not unreasonable to consider that *C. glutamicum* may also have evolved a control mechanism to monitor the amount of *meso*-DAP. Alternatively, the next reaction which uses *meso*-DAP may be so thermodynamically favorable that it can drive the formation of *meso*-DAP from lysine.

In whatever way that *meso*-DAP is made, lysine and threonine do inhibit aspartokinase. This would lead to an accumulation of aspartate. Aspartate would then act at the *ppc* gene, encoding phosphoenolpyruvate carboxylase to repress the synthesis of more oxaloacetate [75]. This should result in less carbon coming into the aspartate pathways.

Growth in Lysine

If a culture is grown in conditions of high lysine, the situation would probably be much the same as in minimal medium except that when threonine concentrations were sufficiently high, both homoserine dehydrogenase and aspartokinase would be inhibited. This inhibition would be transient, however, since the intracellular threonine pool will decrease as the cell requires threonine, methionine, and isoleucine causing the inhibition to be relieved.

Growth in Methionine

Since methionine is so tightly regulated in *C. glutamicum*, growth in methionine results in less homoserine dehydrogenase being made and almost no O-acetyl homoserine transferase being synthesized. Furthermore, repression will occur at

the second gene in the methionine pathway and S-AM will inhibit the first enzyme. This will make the cell very dependent on using the exogenous methionine.

Growth in Threonine

Growth in threonine results in the inhibition of homoserine dehydrogenase. Carbon from aspartate will be directed towards lysine and eventually, as the lysine concentration increases, aspartokinase will also be inhibited. Isoleucine can be synthesized from the excess threonine. Methionine can potentially be synthesized from several sources too. In one case, since there is no methionine in the medium, the *hom* gene itself is not repressed so that the extra homoserine dehydrogenase being made may not all be inhibited by threonine. The homoserine that is produced can then be directed towards methionine. Furthermore, large amounts of threonine inhibit homoserine kinase so there may be a build up of homoserine which can be converted into methionine. Finally, all the reactions from homoserine to threonine are reversible so that presumably threonine itself can be converted into homoserine and from there to methionine.

Remaining Questions

The cases described above are a synthesis of what is known about regulation in the *C. glutamicum* aspartate-derived amino acid pathways. There are many questions which remain unresolved. One, as discussed above, has to do with *meso*-DAP and how it is made in the cell. Another question has to do with transcription. As stated above, of the promoters tested, those being the *ask-asd* promoter, the internal promoter in *ask* which drives the expression of both the ß subunit of aspartokinase and the *asd* gene, the *hom* promoter and the *thrC* promoter, all are used when cells are grown in complex medium. The synthesis

of all of these transcripts and their translation into protein seems like a tremendous waste of energy. Regulation of the aspartate-derived amino acids in *C. glutamicum* versus *E. coli* is very different in regard to regulation at the transcriptional level. In *E. coli*, a large number of the genes are repressed by the various endproducts, especially in the lysine and methionine pathways [1, 70]. As mentioned before, the only genes in C. *glutamicum* which have been shown to be repressed are some of the methionine genes and a gene in the isoleucine pathway [1, 83, 84]. Why *C. glutamicum* synthesizes all these transcripts and proteins which are not then used is a question which needs to be addressed.

Of course, in some cases the presence of these extra proteins may be very useful. For example, when there is too much threonine but not enough methionine, the synthesis of homoserine kinase and threonine synthase are important in order for threonine to be converted back into homoserine. This may also help explain another question arising from the pathway, why is the *thrB* gene, encoding homoserine kinase, part of an operon which is repressed by methionine? Perhaps the increased expression of the operon when no methionine is present is one way to increase the amount of homoserine kinase for the back reaction.

Section II: The *Corynebacterium glutamicum* Regulatory Strategy

Thus far, studies of the *C. glutamicum* biosynthetic pathways and regulation patterns suggest that *C. glutamicum* has opted for less complex strategies than the much better studied *E. coli*. Just in terms of isoenzymes, *E. coli* has three isoenzymes for aspartokinase. *Bacillus* has two, one which is sensitive to diaminopimelate and one which is inhibited by lysine and threonine [70, 114]. Only one aspartokinase has been found in *C. glutamicum* even though numerous attempts have been made to find an alternative isoenzyme [1]. This relatively

more simple approach to controlling its biosynthetic pathways has been found in all examples studied. Of course, it is still possible that there is another layer of complexity that just has not been discovered. This may be true in some cases, but the development of strains which are able to secrete large amounts of various amino acids does indicate that pathway regulation is easily bypassed in *C. glutamicum* [1]. This section will be a discussion of why *C. glutamicum* chose such a simple method of pathway regulation.

The obvious answer to why *C. glutamicum* evolved this regulatory pattern is that there must be some selective advantage in having it. The question then becomes what it the selective advantage? Perhaps the energy the cell saves in not having complex regulatory systems is greater than the energy it expends by transcribing genes and synthesizing proteins it does not need because they are in the growth medium. Or perhaps, the cell does not need complex regulatory systems like prokaryotes such as *E. coli.*

In discussing the regulatory differences between *C. glutamicum* and *E. coli* it is important to think about the many other differences. *C. glutamicum's* habitat is soil, animal feces, vegetables, and fruits [1] whereas *E. coli* is an enteric bacterium. The environmental conditions both face are dramatically different. Other differences include motility and growth rate. *C. glutamicum* does not sporulate either, which makes it different from other soil bacterium such as *Bacillus.*

C. glutamicum is a saprophytic bacterium, so it is scavenging its nutrients from soil, feces, and other organic material. Most of its habitats are usually solid, which may be important since *C. glutamicum* is non-motile [1]. One hypothesis as

to why *C. glutamicum* lacks a lot of biosynthetic regulation is because in its natural habitat it generally needs to make its own. When *C. glutamicum* does change its location it is due to external forces such as rain or earthworms so it does not have much of a grazing choice. If the cell cannot move to find food, sooner or later a lot of the nutrients around it will be depleted. Rather than spending a lot of energy developing regulation strategies, this soil bacterium may have instead evolved ways to utilize the energy sources which are available. For instance, some types of *Corynebacterium* and many types of *Rhodococcus* and *Nocardia* have evolved enzymes which are able to use different aromatic compounds as carbon sources [10, 18, 19, 115, 116].

Another reason to evolve complex regulatory systems is because it gives a growth advantage. For instance, *E. coli* have a lot of competition in their habitat; over 300 different species of bacteria have been isolated from human feces, and *E. coli* is not usually the majority species [74]. The dynamics of their habitat, while generally fairly constant, also mean that there will be periods of feast and periods of famine; the valve that separates the two segments of the gut opens about twenty times per day. If *E. coli* can find a way to shut down many of their metabolic pathways if lots of food is present and just concentrate on growing, they might have an advantage. Furthermore, many of the bacteria are most likely swept out of the system as the meal makes its journey so it may be important that the *E. coli* which are left can recolonize the habitat quickly.

A growth advantage may not be one of the goals of *C. glutamicum*, however. Soil is a wonderfully diverse and dynamic ecosystem in which many species are present even though the environment may be inhospitable at most times. In complex medium, at the optimal growth temperature of around 30°C, with good

aeration, the doubling time of *C. glutamicum* is between one hour and two hours. In soil, which for most of the year is probably not near 30°C in most places, the doubling time of *C. glutamicum* is much longer. For instance, the mean doubling time for bacteria in a deciduous woodland soil is approximately twenty days [74]. It would appear then, as had been mentioned earlier, that *C. glutamicum* spends most of its time in the stationary phase. Although the stationary phase has not been studied in *C. glutamicum*, it has been studied in many other microorganisms. While the stationary phase is marked as a period of no growth, this certainly does not mean that the cell metabolism is shut down during this period. In fact, it has been shown that as *E. coli* enters the stationary phase, 40-80 genes are expressed at specific times [112]. Furthermore, when in stationary phase, many bacteria undergo morphological changes and differences in cell wall composition [117]. There are also changes in the topology of the chromosome [117]. In stationary phase, the metabolic rate does decrease, but it is important that it does not shut down so that substrates can still be imported when they eventually become available so that the cell can resume growth. In order to maintain this level of endogenous metabolism, protein turnover increases and RNA stability decreases [117]. The turnover of ribosomes must also be controlled, however, since starved cells can resume high rates of protein synthesis immediately after nutrients become available [117]. Rather than expending energy on regulation during growth, *C. glutamicum* may have focused its energy on strategies to survive its environment.

If all of this is the case, then why does any regulatio in *C. glutamicum* exist? It might be a compromise position. For instance, for much of its life, *C. glutamicum* is probably in stationary phase. For those times when it is growing, there must

be an advantage to having some type of regulation so as to not waste lots of energy; therefore it evolved simple controls.

The conclusion of this discussion is that while we mostly study the exponential phase when we study *C. glutamicum*, to really understand this organism we need to start exploring the stationary phase. In doing so, we may discover that regulation in *C. glutamicum* is not as simple as we generally claim.

CHAPTER 5
RECOMMENDATIONS FOR FUTURE RESEARCH

The work that has been presented in this thesis raises many questions and opportunities which can be addressed in the future. In this chapter, some of these directions will be discussed.

C. glutamicum RNAP

In Chapter Two the purification of *C. glutamicum* RNAP polymerase is described. Earlier small scale purifications of *C. glutamicum* RNA polymerase has yielded small quantities of slightly purified enzymes. In a collaboration with Scriptgen, we were able to achieve a large scale preparation of purer enzyme. One main reason that prevented our success before in the large scale purification was the preparation of crude extract. Prior to our Scriptgen experience, we used sonication to lyse the cells. For *C. glutamicum*, 10 minutes of sonication was generally used, broken up into 30 second spurts. The crude extract after this procedure was usually about 4-10 mg/ml. There often seemed to be a lot of protein damage because it was difficult to keep the temperature of the crude extract low during the procedure. In contrast, in the procedure described in Chapter Two in which a Bead-Beater mill is used, we obtained protein concentrations of 21.5 mg/ml and we were able to keep the crude extract cold during the procedure. It is recommended that for large scale production of crude extract, the lab use a similar bead mill.

A preparation of *C. glutamicum* RNAP will not be necessary for a while, however, since we purified over 20 mg of it. It still needs to be rigorously established that the purified RNAP is functional. This can be done by analyzing RNA transcripts

made by the purified RNAP to demonstrate that the transcription start site is similar to what has previously been established *in vivo* or doing DNA footprinting to show that the RNAP is bound in the promoter region. Potential uses for this protein are to characterize the *C. glutamicum* promoters which have been identified in more detail. In Chapter Two it was mentioned that these apparent promoters were identified by procedures such as S1 nuclease and primer extension mapping. The RNAP can be used in footprinting assays and transcription assays to determine where the enzyme is placed on the DNA and to determine the exact transcription start site. In this way, the question of whether the *C. glutamicum* RNA transcripts are post-transcriptionally modified can be addressed. Furthermore, determining which base pairs of the promoter may be in contact with the RNAP may help sort out questions about the *C. glutamicum* consensus sequence.

Chapter Two also demonstrated that the *C. glutamicum* RNAP was able to transcribe using linear DNA as a substrate. The argument was made that in *C. glutamicum*, transcription occurred readily from the substrate, while in *M. smegmatis* it had been reported not to occur at all. While the linear template was transcribed, transcription was less efficient than it was from the supercoiled template. More quantitative experiments need to be done to study this in more detail. It would be interesting to see whether supercoiling has more of an effect on the more (G+C) rich promoters, such as *pgk* over the more (A+T) rich such as *hom* and *fda*.

While it is presumed that *C. glutamicum* has multiple σ factors, none have been identified yet. The promoter probe vectors, pZIP and pMT-SCM, can be used to

screen for promoters which are active under certain environmental conditions such as heat shock.

On the strain development side, the knowledge of *C. glutamicum* promoters should be considered when designing strategies to manipulate different pathways. For example, in some of the threonine strains, the *hom* gene is amplified. When fermentations are being run to produce threonine, a complex medium is used, so therefore the *hom* promoter is repressed two-three fold. To get around this the *hom* gene should be expressed from a constitutive, strong promoter. Once promoters which are recognized by alternative σ factors are identified, it might be of interest to construct multiple promoters which are expressed constitutively and then also under situations such as stationary phase or heat shock. Placing key genes which may be bottlenecks at particular times in a fermentation under control of these new synthestic promoters may help bypass the bottleneck.

***hom* Promoter**

In Chapter Three, regulation at the *hom* promoter is discussed. It is demonstrated in this chapter that the regulator site in the promoter is located upstream of the transcription start site. It had been previously thought that the leader RNA of the mRNA transcript was responsible for the regulation. In our comparison of the promoter with this leader RNA versus a truncated version, the truncated version was more repressed. This leader RNA has a region of extensive hyphenated dyad symmetry. While the regulator site is located upstream of the transcription start site, this region might be responsible for some other type of regulation. This hyphenated dyad symmetry element can form a stemloop structure. Another regulatory element which also makes use of a single stemloop

is growth rate control at the *bla* promoter in *E. coli* [118]. Another parameter to test this element then would be in media in which *C. glutamicum* grows at different rates. This hypothesis can be tested by placing the region of hyphenated dyad symmetry immediately downstream of the transcription start site of a constitutively expressed promoter, such as the *ask* promoter. A reporter gene can then be used to monitor differences in expression. After transforming *C. glutamicum* E12, this construction can be tested under different medium conditions such as ± methionine. As a control, a strain which harbors a plasmid with the *ask* promoter by itself expressing the reporter gene can be used.

Reporter genes were used to determine promoter strength. The use of reporter genes is not an entirely accurate method of determining promoter strength because of fluctuations which may occur in the plasmid copy number. Earlier on I had attempted to determine the plasmid copy number by also assaying the activity of the kanamycin resistance gene product and the amount of plasmid DNA present in each culture. Each of these methods had a certain amount of error associated with them. Since the range of promoter activities I was testing was narrow, the amount of error rendered the differences in promoter activity meaningless. I continued to use the reporter gene system for several reasons. Although the vector system that pZIP and pMT-SCM is based on is multi-copy in *C. glutamicum*, the number of plasmids is small, around 15, so big fluctuations that might happen in *E. coli* which can have 400 plasmids would not occur. Furthermore, the fluctuations in copy number is usually inversely proportional to promoter strength [119]. For example, a strong promoter usually is associated with a lower copy number [119]. Therefore, the strengths given for the promoters are on the conservative side. In addition, for the *hom* promoter studies, the same plasmid is tested in different medium. Growing cells in

different medium typically does not have much of an effect on plasmid copy number unless there is a limiting nutrient [119]. Furthermore, using the *hom* promoter:*cat* reporter gene integration strain, E12#3, I was able to compare *hom* promoter strength from a multi-copy plasmid versus one copy in the genome. In this case, the repression levels were approximately the same. Now that *C. glutamicum* RNAP is purified, it will be possible to test promoter strength by doing transcription assays.

A topic which was not addressed in this thesis was why there were differences in the repression rate for the *hom* promoter expressing the *lacZ* gene versus expressing the *cat* gene. A hypothesis is given in Chapter Three, Section II, about why this is. To further address this question, Northern blots could be done to see what the different RNA levels are. While this may be interesting, the variation in repression using different reporter genes probably would not shed more light on the basic biology of the organism.

There is a chance that some of the differences in promoter activity are due to translational differences. I have attempted to address this question by Northern blot analysis. Unfortunately, these attempts did not yield any valuable information due to technical difficulties. Repression by methionine at the *hom* promoter has been previously shown by RNA analysis, however [85]. Furthermore, the ribosome binding site used in all the promoter: reporter gene constructions was the same. I was also careful not to include any ribosome binding sites from each promoter's corresponding gene. Unless cryptic ribosome binding sites are present, translation should be the same in each vector.

A final recommendation revolving around the *hom* promoter would be to locate the regulator binding site in the promoter. In Chapter Three, Section III, I describe some of the attempts I have tried to locate this site(s) and the difficulties I have had. I think using the *E. coli* XL-1 Red strain to generate random mutations is a good method. Rather than using the *lacZ* gene, I would use the *cat* gene because it probably will not generate as many false positives and there is a greater range of repression which can be exploited in order to look for mutations which may only be partially repressed. As discussed in Chapter Three, Section III, another method would be to synthesize degenerate oligonucleotides which correspond to the *hom* promoter. This would have the advantage in that the mutations generated would be restricted to the promoter region.

Methionine Regulator

In Chapter Three, Section III, some strategies are outlined to isolate the methionine regulator gene. At this time, I think that the strategy in which the *hom* promoter: *sacB* gene is integrated into the E12Rep-2 strain and then a *C. glutamicum* library is used to try to complement the mutation is the best strategy. Another would be to try to reisolate a derepressed strain using transposon mutagenesis. The regulator gene could then be isolated by PCR using primers which recognize sequences in the transposon region. This may prove the best strategy, especially if the regulator turns out to be an activator. This system is not functional yet in our lab, but it will be soon.

Since the *metA* gene is more strongly repressed than the *hom* gene, it would be easier to isolate the regulator using the *metA* promoter. Before that can be done the *metA* gene has to be isolated. *MetA* can be potentially isolated in several ways. *C. glutamicum* methionine auxotrophs can be generated and screened for

metA mutations. *MetA* could be isolated from these by complementation with a *C. glutamicum* library. Alternatively, the protein could be purified and its N-terminus sequenced. Degenerate oligonucleotides which correspond to the protein sequence could be synthesized and used to probe a library for the gene.

The other enzymes in the *C. glutamicum* methionine pathway can be isolated in a similar fashion. The promoters of the other genes can also be tested in E12 to see whether they are repressed and whether that repression is derepressed in E12Rep-2. The O-acetyl homoserine sulfhydrylase gene, for instance, has been reported to be repressed by methionine and this could be tested by enzyme assays. To do the enzyme assays, however, O-acetyl homoserine needs to be synthesized.

Methionine Model

In the model presented for methionine regulation in Chapter Four, some conjectures are made which could be tested. For instance, the questions about the direction of carbon flux from aspartate semialdehyde towards either the threonine or the lysine branches can be studied in more detail. The promoter of the *dapA* gene needs to be identified and cloned into one of the reporter gene vectors. The promoter activity could then be measured and compared to the activity of the *hom* promoter. Furthermore, the dipicolinate synthase and homoserine dehydrogenase enzyme activities could be measured throughout a fermentation to see what the relative amounts of each is during the course of a fermentation.

There are many unresolved issues in the lysine pathway, especially in regards to *meso*-DAP. Some of these questions are being addressed by Cathryn Shaw-Reid

using 21253#10, the *ddh-* strain. The question of whether lysine can be converted into *meso*-DAP is one question. How much *meso*-DAP the cell needs is another.

An assumption made in Chapter Four is that the cell's production of metabolites, and therefore the carbon flux down each pathway, can be correlated to the proportion of each amino acid in final products such as protein or the amount of metabolites in other products, such as *meso*-DAP in the cell wall. To prove this hypothesis true, the amount of such products as *meso*-DAP and S-AM that the cell needs must be determined. As more *C. glutamicum* genes are sequenced, the relative percentage of each amino acid used will become more reliable. In order to measure the carbon flux, glucose can be radiolabeled and the amount and location of the isotopes can be measured and quantitated.

In order to test some of the other hypotheses, such as whether methionine can be made from the threonine pool, strains can be made. For example, to test this hypothesis, the aspartokinase can be disrupted, creating a lysine, threonine, methionine, and isoleucine auxotroph. If minimal medium is supplemented with lysine and threonine only and growth occurs then there is a good possibility that methionine is able to form from excess threonine and *meso*-DAP can form from lysine. If growth does not occur, the methionine and *meso*-DAP deficiencies would need to be tested individually. In one case, homoserine dehydrogenase could be disrupted, as long as homoserine kinase is left functional. In this way the methionine hypothesis could be tested. The conversion of *meso*-DAP from lysine could be tested by disrupting the *dapA* or *dapB* gene.

Final Remarks

When I first started studying transcription and transcriptional regulation in *C. glutamicum*, I expected that much of what I would find would be similar to *E. coli*. After all, when prokaryotic examples are given in text books, they are usually taken from *E. coli* and presented as being the "bacterial model." Although I knew that the bacterial world is diverse, I thought that this diversity was manifested more in morphology, pathology and habitat than in basic biology - and I did not think about it much.

So at the beginning of the project my approach to *C. glutamicum* was that it was just another variation of the *E. coli* prototype. My mission, therefore, was to find the differences. My experiments were designed to look for these differences; my presentations were given in a style which stated, "This is *E. coli*, and this is how *C. glutamicum* is different." Several things happened which gradually changed my approach to my work. Once when I gave a talk, Professor Arnie Demain rather bluntly pointed out that I was comparing apples to oranges and should start studying bacteria which are more closely related to *C. glutamicum*. Then I went to an American Society for Microbiology General Meeting in which Professor John Ensign from UW-Madison had won an award and gave a lecture on diversity in the prokaryotic world which was very inspiring. There is also Professor King who at every committee meeting from the initial preliminary exam asked me to address what my data meant when *C. glutamicum* was in its natural habitat. As I read more about other bacteria, I found the prokaryotic world quite exciting. I found that diversity was present in many forms, from transcriptional regulation to ability to use different carbon sources. I no longer think about *C. glutamicum* as an early model *E. coli*, but as an organism which evolved in its own right. In many ways, it is now more interesting to me to think

about why *C. glutamicum* and *E. coli* are similar in some ways rather than how they are different. While I still compare *C. glutamicum* to *E. coli*, it is because *E. coli* is still the best studied bacteria, so the most is known about its physiology.

During the course of this project I have endeavored to understand transcription in *C. glutamicum*. This has been aided greatly by the comments and suggestions from Professor Magasanik and Professor Grossman during committee meetings. On the experimental side of my education, I spent some time purifying RNA polymerase. This was a very useful exercise in that I was able to gain some biochemistry experience. The value it added in my understanding of transcription is a little more ambiguous due to the lateness in my graduate career in which it was purified. Hopefully there will be others who will be able to use the purified RNAP in a more effective manner.

The bulk of my research dealt with understanding regulation at the *hom* promoter. The main conclusion I reached is that the methionine repression at the *hom* promoter is mediated by the same effector as repression at the *metA* promoter. This conclusion addressed a question which I had long had: why did *C. glutamicum* bother to repress the *hom* promoter only two-three fold? Before this study, repression at the *hom* promoter had been considered independent from repression in the rest of the methionine pathway. Finding out that this repression is part of an overall strategy makes more sense. Now the challenge for the next person is to delve deeper into the problem and find out just how extensive methionine regulation is in *C. glutamicum*

I have not been able to answer all of the questions regarding repression at the *hom* promoter or methionine regulation in general. I have answered a few.

Perhaps more importantly, I took the project to a level in which better questions can now be addressed.

APPENDIX A: ISOLATION OF *CORYNEBACTERIUM GLUTAMICUM GLNA*

SUMMARY

Using an *Escherichia coli* strain which lacks a functional glutamine synthetase, we were able to isolate a section of *Corynebacterium glutamicum* DNA which contains the *Corynebacterium glutamicum glnA* gene. Preliminary sequencing indicates that the *glnA* gene is immediately downstream of the *thrC* gene. Enzyme assays demonstrate that glutamine synthetase is regulated by post-translational modification. A transcriptional analysis reveals that the gene has at least one promoter which is constitutively expressed.

INTRODUCTION

Glutamine synthetase is an enzyme which is involved in nitrogen regulation along with the enzymes glutamate dehydrogenase and glutamate synthase. In the cell 85% of nitrogen is derived from the amino nitrogen of glutamate while the other 15% comes from the amino nitrogen of glutamine [120]. Under conditions of nitrogen excess, glutamate dehydrogenase (GDH) is used to produce glutamate while under conditions of limiting nitrogen, glutamine synthetase (GS) and glutamate synthase (GOGAT) concertedly act to produce glutamate in the following reactions [121]:

$$\alpha\text{–ketoglutarate} + NH_4^+ + NADPH + H^+ \xleftarrow{GDH} \text{L-glutamate} + H_2O + NADP^+$$

$$\text{L-glutamate} + NH_4^+ + ATP \xleftarrow{GS} \text{L-glutamine} + ADP + P_i + H_2O$$

L-glutamine + α–ketoglutarate + NADPH + $H^+ <\underline{GOGAT}>$ 2 L-glutamate + $NADP^+$.

In general the genes and enzymes involved in these conversion are heavily regulated [120]. For example, the gene for glutamine synthetase *glnA* has been shown to be regulated in several ways, from an enhancer-like activation in *E. coli* and *Klebsiella aerogenes* using σ^{54} [71, 72], to a repressor in *Bacillus subtilis* [122], to anti-sense repression in *Clostridium acetobutylicum* [123], to an activator in *Streptomyces coelicolor* [124, 125]. Glutamine synthetase itself is often regulated by adenylating the enzyme in order to inhibit it [126].

Three different glutamine synthetases are known to exist in bacteria. GSI (*glnA*) is common in bacteria; it is relatively heat stable and it consists of twelve identical subunits. GSI is usually post-translationally modified by adenylation. GSII is more typical of eukaryotes but it has been found in bacteria, also. It is composed of eight smaller subunits and it is heat-labile. No adenylation is known to occur on GSII. The final example of GS is found in *B. fragilis*: it has six larger subunits and no sequence similarity to GSI or GSII. It is not regulated by adenylation [127]. Both *Rhizobiaceae* and *Actinomycetes* have been found which have both the GSI and the GSII activity [127]. In *Bradyrhizobium japonicum* and *Agrobacterium tumefaciens* the *glnA* homologous gene is expressed constitutively, but its enzyme is modified by adenylation. The gene for GSII, however, is regulated in response to nitrogen [127]. In *Streptomyces*, regulation of the *glnA* gene is controlled by the activator GlnR and its protein is controlled by adenylation [124, 125, 128]. Both GSI and GSII activity have been studied in *S. viridochromogenes* [127]. While GSI activity is regulated by adenylation, GSII is not [127].

C. glutamicum is a natural glutamate-producing bacterium under certain culture conditions such as biotin limitation [1]. The enzymes for all three enzymes, GDH, GS, and GOGAT have been purified and some of their characteristics studied [129, 130]. In addition the gene for GDH, *gdh*, has been cloned [121]. Because *gdh* mutants of *C. glutamicum* are glutamate auxotrophs, it is believed that glutamate formation and ammonia assimilation are mainly performed by GDH [121]. No transcriptional or translational regulation of *gdh* has been found when strains are grown under different carbon and nitrogen sources [121]. It is thought that this absence of regulation may contribute to the organism's ability to produce large amounts of glutamate [121].

Using the *E. coli* strain, YMC21, which lacks a functional GS [131], we were able to isolate a 5 kb piece of DNA which contains the *C. glutamicum* GS activity. Preliminary sequencing demonstrates that this fragment has homology to *glnA* genes of other organisms. The gene appears to have at least one promoter which is constitutively expressed. Furthermore, the protein product is post-translationally modified.

MATERIALS AND METHODS

Strains and Plasmids. Strains and plasmids are listed in Table A.1. Cells were grown in Luria-Bertani broth, *E. coli* M9 medium [106], or *C. glutamicum* minimal medium [95]. *C. glutamicum* transformations were plated on Brain Heart Infusion medium plus 4% sorbitol and 1% sucrose. Recovery medium was BHI plus 10% glucose. Chemicals were purchased from Sigma.

Table A.1: Strain and Plasmid List

STRAINS		Reference
C. glutamicum		
AS019	rif^R lab strain	43
E12	restriction deficient derivative of AS019	35
E. coli		
DH5a	*lacZΔM15, recA1*	BRL
YMC21	*Δ(glnA-G)2000*	131
PLASMIDS		
pMTI	*C. glutamicum/E.coli* shuttle vector Ap^R in *E. coli* Km^R in *C. glutamicum*	35
pMT-SCM	pMTI with promoterless *cat* gene inserted in *SalI* site of polylinker - *cat* gene contains own ribosome binding site.	this paper
pZIP	pMT-SCM with lacZ gene inserted in frame in PvuI site of cat gene.	this paper
pGln	pMT1 with *C. glutamicum* DNA fragment which complements *glnA* auxotrophy in YMC21	this paper
pGlnlib	pZIP with promoter region of the *C. glutamicum glnA* gene inserted in multiple cloning site	this paper

DNA Manipulations. DNA manipulations such as restriction enzyme digestions, ligations, modifications of DNA fragments, transformations, and purifications were done by standard methods [105, 106]. DNA fragments were isolated using a Qiagen kit. Enzymes were purchased from either New England Biolabs, Bethesda Research Laboratories or Boehringer Mannheim.

DNA Sequencing. DNA sequencing was done by the dideoxy method of Sanger. A kit and Sequenase enzyme from United States Biochemicals as well as the manufacturer's protocol were used.

Preparation of Crude Extract. Cells were grown in LB or minimal medium and harvested. The pellet was washed in PBS and resuspended in PBS plus DTT. Sigma glass beads were added and the cells were disrupted in a bead mill. The supernatants were collected after centrifugation and protein quantitation was done using the BioRad protein quantification kit.

γGT Assay. The γGT assay was used to measure the total amount of glutamine synthetase present and was done according to standard procedures [132]. In brief, a fresh assay mixture was composed of 7.53 ml dH_2O, 2.25 ml 1.0 M imidazole-hydrochloride (pH 7.15), 0.37 ml 0.80 M hydroxylamine-hydrochloride, 0.045 ml 0.10 M $MnCl_2$, 1.5 ml 0.28 M potassium arsenate (pH 7.15), 0.15 ml 40 mM sodium ADP (pH 7) and either 1.5 ml hexadexyltrimethylammonium bromide (CTAB, 1 mg/ml) or 1.5 ml dH_2O depending on whether the assay is being done on whole cells or crude extracts. A blank is prepared in a like manner but the arsenate and ADP are replaced with water. In a standard reaction in *C. glutamicum*, the pH of the assay solution is raised to 8.55. Sample and water are added to 0.4 ml of the assay solution to

bring the volume to 0.45 ml. This is equilibrated for 5 minutes at 30°C and the reaction is started by the addition of 0.05 ml of 0.20 M L-glutamine. The reaction is terminated by the addition of 1 ml of a stop solution containing 55 g $FeCl_3 \cdot 6H_2O$, 20 g of trichloroacetic acid and 21 ml of concentrated HCl per liter. Reactions are then centrifuged to remove any precipitate and absorbance at 540 nm is measured.

Forward Reaction Assay. The forward reaction measures the ability of GS to form glutamine [132]. Its assay mixture is composed of 7.2 ml dH_2O, 2.0 ml 1.0 M imidazole-hydrochloride (pH 7.15), 1.25 ml 0.80 M hydroxylamine-hydrochloride, 0.40 ml 3.0 M $MgCl_2$, 4.2 ml 0.85 M monosodium L-glutamate and either 2 ml CTAB (1 mg/ml) or dH_2O depending on whether whole cells or crude extracts are used for the assay. Freshly made assay mixtures are used for experiments. The assay is done at a pH of around 7.7. Sample and water are added to 0.4 ml of the assay mixture to bring the total volume to 0.44 ml and equilibrated at 30°C for 5 minutes. The reaction is then started by adding 0.06 ml of 0.2 M ATP. The reaction is terminated by addition of 1 ml stop mix. The reactions are centrifuged to remove any precipitate and absorbance is measures at 540 nm. The blank does not contain ATP.

β-galactosidase Assay. β-galactosidase activity was measured by a modified version of the β-galactosidase assay in Sambrook, *et al.* [106]. Briefly, crude extract was added to Z buffer (0.06 M $Na_2HPO_4 \cdot 7H_2O$, 0.04 M $NaH_2PO_4 \cdot H_2O$, 0.01 M KCl, 0.001 M $MgSO_4 \cdot 7H2O$, and 0.05 M β-mercaptoethanol, pH 7.0) and 200 µl o-nitrophenyl-β-D-galactopyranoside (4 mg/ml) to make 1 ml. After a certain time, usually 90 s, 0.5 ml of 1 M Na_2CO_3 was added. The A_{420} was then measured and specific activities per mg protein per minute determined.

Whole Cell Assay Method. 10 ml cultures were grown in 125 ml Erlenmeyer flasks. When the cultures were ready for harvesting, 1 ml of CTAB (1 mg/ml) was added and shaking was continued for 1 to 3 minutes. The cells were then harvested and washed in PBS, and resuspended in 1 ml PBS and kept on ice until use in the assays [132].

RESULTS

Cloning of *C. glutamicum glnA* Gene. The *C. glutamicum glnA* gene was isolated by screening a *C. glutamicum* library in *E. coli* strain YMC21, a *glnA*- strain generously donated by Prof. Boris Magasanik. This strain was screened for the ability of the library to complement a glutamine deficiency when grown on M9 medium without glutamine. The DNA from the initial candidates was purified and YMC21 was retransformed. Seven candidates retained the phenotype of *glnA*+ upon retransformation. A restriction enzyme mapping of the plasmid DNA showed that all had a similar restriction pattern. The plasmid with the shortest insert, approximately 5 kb, was taken for further analysis and named pGln.

Enzyme Assays. In order to further verify that the plasmid contained a gene which encoded a functional glutamine synthetase, enzyme assays measuring the amount of glutamine synthetase active in the forward reaction were done in both YMC21 and again in *C. glutamicum* E12. In YMC21, those cells harboring pGln had an activity of 0.5 (A_{540} /protein concentration) in comparison to YMC21(no plasmid) which had a background activity of 0.07 (A_{540}/protein concentration) for the same time period. The fact that the *C. glutamicum* copy of *glnA* is able to complement the glutamine auxotrophy and that there is measurable enzymatic

activity in *E. coli* indicates that the *C. glutamicum* promoter for *glnA* is recognized in *E. coli*.

After *C. glutamicum* E12 was transformed with pGln, enzyme assays were done to examine GS activity under different conditions. In order to determine what time range gave a linear response for the assays, a time course of the forward reaction was done (Figure A.1). The curve was linear over the range tested, so all assays were then done over time periods of 5 to 30 minutes. The activity of GS was examined at different temperatures and after growth in either high or low ammonia (Figure A.2). *C. glutamicum* GS is over eight times more active when grown under nitrogen limitation. Interestingly, *C. glutamicum* GS is slightly more active at 37°C than at 30°C even though the growing temperature for *C. glutamicum* is 30°C.

One means of regulating GS in through a post-translational adenylation modification. GS is active in the non-adenylated form and inactive in the adenylated form [133]. In order to measure the total amount of GS present in the cell, a γ-glutamyl transferase assay is done and in order to measure the amount of GS present which can make glutamine, a forward reaction assay is done [132]. The pH profile of GS changes dramatically for different states of adenylation. There is a pH isoactivity point, however, in which the measured activity of the glutamine synthetase is the same no matter what the state of adenylation [132]. In experiments in which E12(pGln) was grown in excess or limiting amounts of ammonia and the specific activities of the GS under these conditions compared, it was apparent that some type of modification of GS was taking place because the total amount of GS remained constant under those conditions but the amount of GS active in the forward reaction was much lower for those cultures in which

Figure A.1: GS Activity Over Time - GS activity using the forward reaction increases linearly over 5 to 30 minutes when grown in either excess or limiting ammonia.

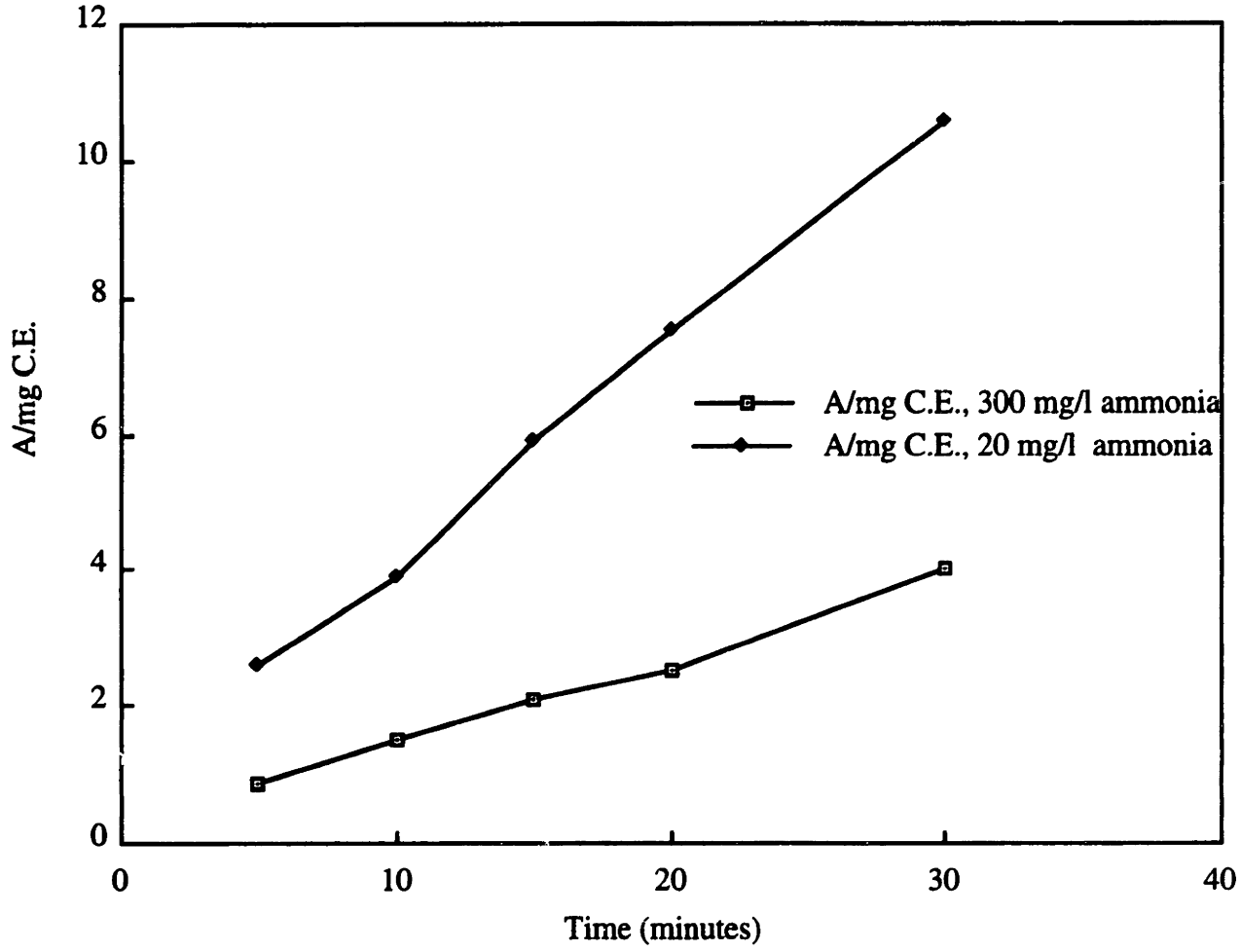
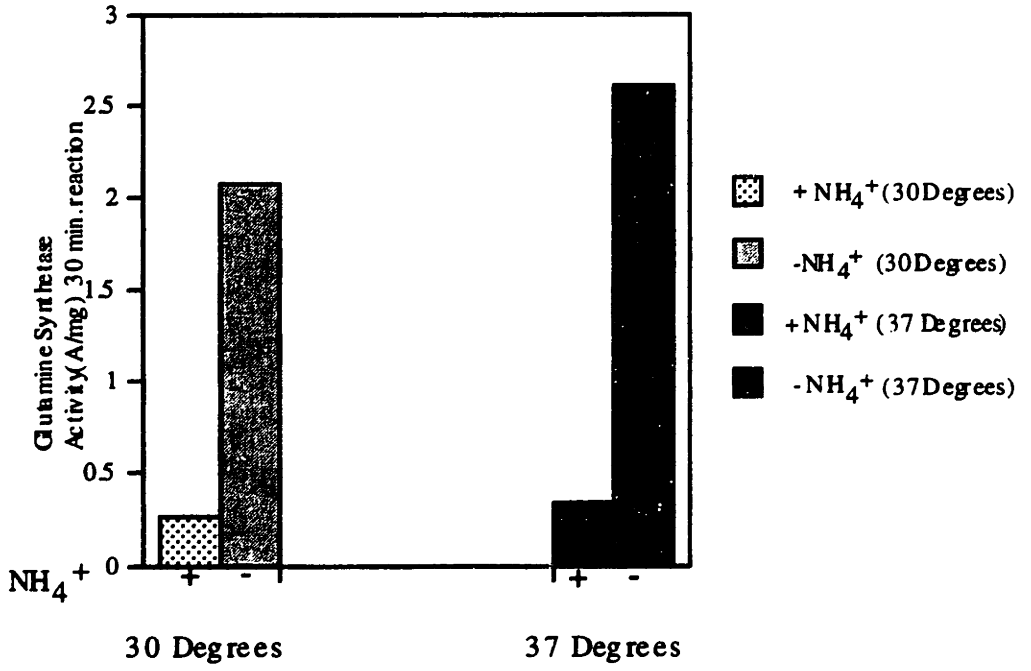


Figure A.2: GS Activity in the Forward Reaction at Different Temperatures - *C. glutamicum* GS has sightly higher activity at 37°C than it does at 30°C. When grown in high ammonia, the GS activity is much lower than when grown in limiting ammonia.



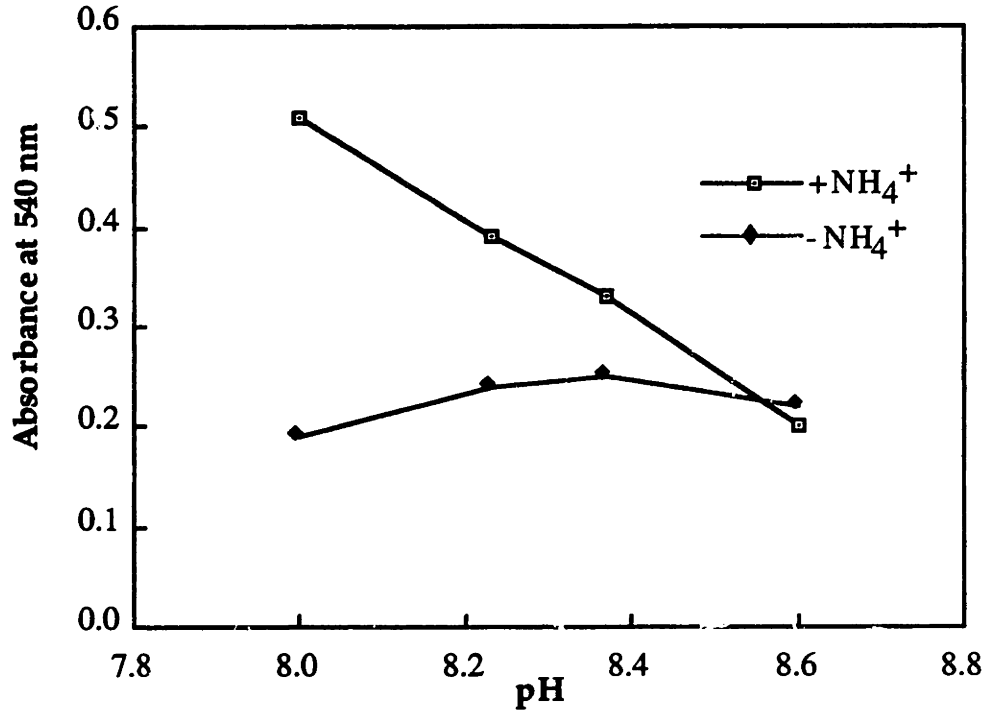
there was excess ammonia. A pH profile was done of the *C. glutamicum* GS in order to determine its isoactivity point. This was done for both crude extract and whole cells to ensure that the harvesting procedure did not alter the adenylation state of the protein. The isoactivity point determined by both methods was pH 8.55 (Figure A.3).

Sequencing of pGln. Several hundred base pairs of pGln have been sequenced. The sequence which has been determined so far shares much homology with the *glnA* gene of other bacteria such as *S. coelicolor, S. viridochromogenes,* and *Methylooccus capsulatus* [127, 134, 135]. The sequencing has also shown that the *C. glutamicum glnA* gene is immediately downstream of the *thrC* gene which encodes the enzyme threonine synthase [136].

Presence of a Constitutive Promoter for *glnA*. In order to determine whether the promoter of *glnA* is regulated, the 5 kb insert of pGlnA was digested with *BamHI* and these fragments were ligated into pZIP, a promoter probe *E. coli/C. glutamicum* shuttle vector. *E. coli* DH5α was transformed with the ligation mixture and the candidates were plated onto medium which contained X-GAL. The plasmids from colonies which turned blue were purified and sequenced. One plasmid, pGlnlib, contained a region of DNA which included the beginning of the *C. glutamicum glnA* coding region, as determined by homology to the *glnA*s of other organisms and 100 base pairs upstream of this coding region (Figure A.4). *C. glutamicum* E12 was transformed with pGlnlib and ß-galactosidase assays were done with cultures grown in different medium, including complex, minimal plus limiting amounts of ammonia and minimal plus excess ammonia. Under all conditions, the activity of the promoter was the same (Figure A.5).

Figure A.3: Determination of the Isoactivity Point for *C. glutamicum* Glutamine Synthetase - a.) Determination of isoactivity point using the whole cell method. b.)Determination of isoactivity point using crude extract. In both cases, the isoactivity point is around pH 8.55.

a..Whole Cell Method



b. Crude Extract

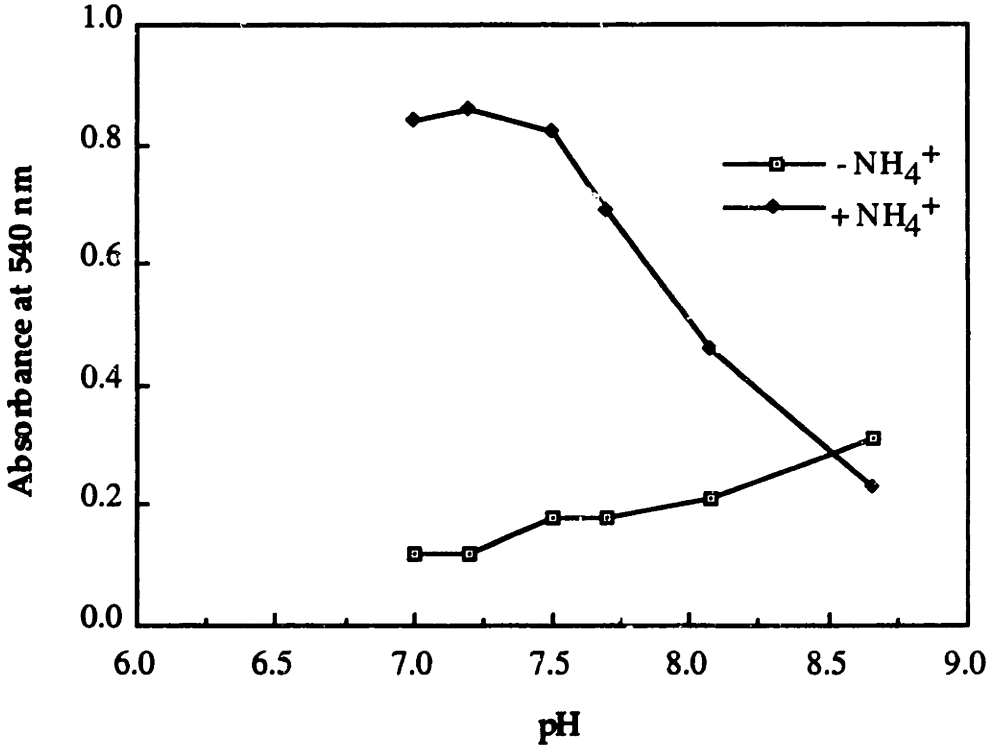


Figure A.4: Sequence of pGlnlib - The italicized nucleotides denote those which have been published with the sequence of the *thrC* gene. The stop codon of the *thrC* gene is 1320 base pairs upstream of this sequence. The underline nucleotides indicate where sequence homology to other *glnA* genes begins. The amino acid sequence for the homologous reading frame has also been deduced. The first valine codon could potentially be the start site. The entire pGlnlib sequence has not been determined yet.

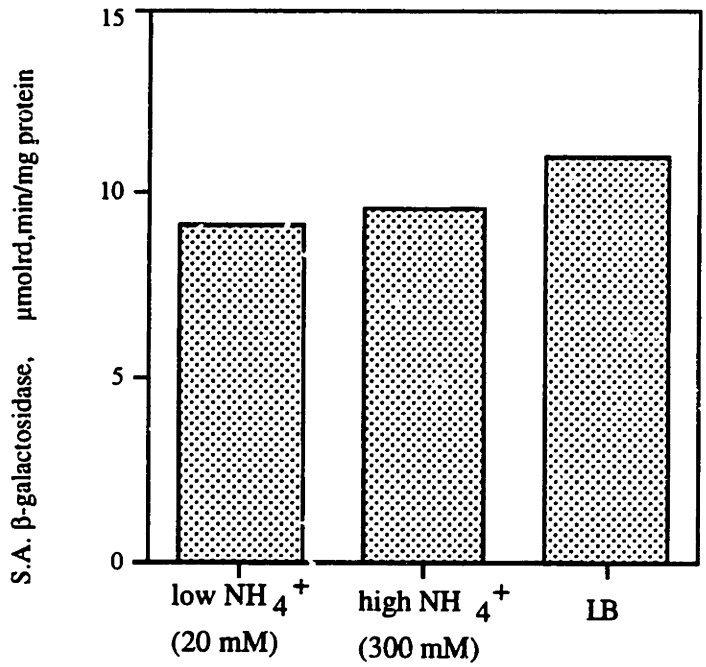
*ttttgccaaaaatctcgaattc*ttcaggtgtcgccagagccgatgcctaaaacgaaacgtcc

V A E S I D E S D M N L L P D L G T A T L
gtg gct gag tcg atc gac gaa tct gac atg aat ctc ctg cca gac ctc gga acg gcc acc ctt

D P P S W W I S P V R L R P T P Q E F E L
gat cct cca tca tgg tgg atc tcc cca gtt cgc ctg cgc cca acc ccg cag gaa ttc gaa ttg

Y F D C
tac ttc gac tgc

Figure A.5: β-galactosidase Activity of E12(pGlnlib) in Different Media - A fragment of DNA which includes approximately 100 base pairs upstream of the start site of the *C. glutamicum glnA* gene plus the first part of the *glnA* reading frame was cloned upstream of the *lacZ* gene in pZIP in order to monitor promoter expression during growth in different media. For the media tested, there was no significant difference in expression.



DISCUSSION

In this paper, the isolation of a 5 kb piece of *C. glutamicum* DNA which contains the *glnA* gene is reported. The *C. glutamicum glnA* gene has been partially sequenced. Its sequence shows homology with the GSI of other organisms such as *S. coelicolor* and *S. viridochromogenes* [127, 134]. Whether *C. glutamicum* has both the GSI and GSII types of glutamine synthetases is not known at this time. In order for this to be addressed, the *glnA* which has been cloned can be disrupted in the chromosome. If this *C. glutamicum* strain is now auxotrophic for glutamine, then only one *glnA* is present. A strain which would remain prototrophic would indicate that at least one other enzyme is present. Another way to test whether there are more than one form of GS is to assay E12 for its chromosomal GS activity after incubation at different temperatures. GSI is more thermostable than GSII so if the total GS activity drops at higher temperatures there may be both forms whereas if it stays the same, there is probably only one form [127]. This, of course, is not definitive proof, but merely evidence for one case or the other.

The results shown in Figure A.3 indicate that the *C. glutamicum* GS is post-translationally modified when grown in high ammonia. Although it has not been yet shown conclusively to be a modification through adenylation, many of the GSI enzymes are so modified. In order to further prove whether it is adenylation, experiments can be done in which the adenylated form of GS can be de-adenylated by treatment with snake venom phosphodiesterase [132].

It has also been shown that the *C. glutamicum glnA* promoter is constitutively expressed when cells are grown in complex medium, minimal medium plus excess ammonia, or minimum medium plus limiting ammonia. It has not been

shown conclusively that no transcriptional regulation takes place for this gene, however. Perhaps the small amount of ammonia present in the cultures is enough to repress the promoter so that activity in all media appears the same. In addition, the pGlnlib vector only has an upstream region of approximately 100 bp upstream of the last nucleotide which shows homology to the *glnA* of another organism. The actual promoter region has not been identified yet, so there is a chance that the whole promoter region including any operator sites is not present in this vector. For instance, a repressor or activator site may be just upstream of the stretch of DNA which is included in pGlnlib. The closest organisms to *C. glutamicum* in which *glnA* has been isolated are *Streptomyces* [65, 134]. The *glnA* of *S. coelicolor* is regulated by an activator, GlnR. If *C. glutamicum* is similar to *Streptomyces*, it would seem that the activator region should be present in order for there to be expression of the promoter. However, if *C. glutamicum glnA* is regulated differently, the regulatory region could be missing. Furthermore, *C. glutamicum glnA* may have multiple promoters so that pGlnlib may have the entire constitutive promoter but not all of a regulated promoter. These questions can only be answered upon further study.

APPENDIX B: ANALYSIS OF THE TGTGC REGION OF THE *THRC* PROMOTER OF *CORYNEBACTERIUM GLUTAMICUM*

SUMMARY

The nucleotide sequences of ten *Corynebacterium glutamicum* promoters, all for genes in biosynthetic pathways, have been analyzed. This analysis shows that the region of highest homology is a TGTGC motif located upstream of the transcription start site. A mutational analysis of this highly conserved region in the *thrC* promoter shows that single base pair substitutions in this region result in four-five fold range in promoter strength.

INTRODUCTION

Corynebacterium glutamicum is a Gram-positive, non-sporulating, non-motile soil bacterium commonly used for the production of amino acids because of a variety of unique physiological characteristics [1]. In recent years, the development of recombinant DNA technology has enabled the study of fundamental molecular biology questions regarding *C. glutamicum* and related species [43]. The main focus of study has been on different metabolic pathways involving genes in glycolysis and the biosynthesis of the aspartate-derived amino acids. Many of the genes in these pathways have been cloned; for some of the genes apparent promoters for the different operons have been identified by S1 nuclease mapping or primer extension and also by deletion analysis as RNA polymerase binding data is not available [35, 36, 44, 47, 85, 96, 98-100, 104, 136, 137]. Studies on the unique regulatory aspects of several of these genes are now being actively pursued [45]. However, little is yet known about basic regulation at the transcription level. Studies on *C. glutamicum* promoter structure and

transcriptional regulation are necessary to unravel control mechanisms in these central pathways.

Comparison of *C. glutamicum* Promoters

Ten *C. glutamicum* putative promoters have been identified and are shown in Figure B.1. In searching for common elements among these promoters, it was clear that although *C. glutamicum* recognizes *Escherichia coli* promoters, the paradigm of promoters for *E. coli* is not the same for these promoters in *C. glutamicum*. In *E. coli*, the major promoters for housekeeping genes have two hexameric motifs, one of TATAAT centered at -10 (where +1 is the transcription start site) and one of TTGACA centered at -35 [57]. These *C. glutamicum* promoters, which are also the promoters for housekeeping genes, do not seem to follow this pattern. Although a weak homology to the TATAAT motif can be found, a similar consensus at -35 is undetectable. Instead, the most prominent feature of these promoters is TGTGC which occurs around -20.

In order to further verify our interpretation of the promoter sequence information, a multiple alignment was done using the Eden Genesys Computer Software System (TEAM Associates, Westerville, OH, USA). This program allows for the maximization of information content within a prescribed window [138]. For this multiple alignment procedure, 41 nucleotides immediately upstream of the transcription start site for each apparent promoter were considered using a six nucleotide window. Shown in Figure B.2 are the results of the multiple alignment analysis. As indicated, the most conserved motif found is TGTGC. Although the motif is near -20 in many of the promoters, in others it is in variable positions. The first two positions, TG, are present in all of the

Figure B.1: Apparent *C. glutamicum* Promoters - Listed are the putative *C. glutamicum* promoters as determined by S1 nuclease mapping or primer extension. All promoters are for genes in either glycolysis or amino acid producing pathways. As shown, the most common motif in these promoters is TGTGC around -20, while the TATAAT box at -10 is also present. [35, 44, 47, 96, 99, 100, 104, 121, 136]

	-30	-20	-10	mRNA START
hom	TTTAAAGCAAAAA**TGAAC**AGCTTGGTC**TATAGT**CGGCTA			
fda	CACGACAAAAGTTGAG**TGATG**CAGG**CATAAT**TGGCTATA			
thrC	GTGTGGGGGAGTAT**TGTGT**CACCCCT**TGGGAT**AGGGTTA			
pheA	TCGGCGGGTTAAGC**TGTGT**AACCATGAGCGACGCACCAA			
lysA	GTATTCTGTGCGACG**GGTGT**ACCTCGGC**TAGAAT**TTCTC			
askP1	TGGCTAGGTAGACACAG**TTTAT**AAAGG**TAGAGT**TGAGCG			
askP2	TGCATTCAATGTGCCACTT**CGCGT**ATGCTCG**TCTTAT**AG			
gap	CTGCTGCGGAAATCTTT**GTTT**CCCCGC**TAAAGT**TGAGGAC			
gdh	TTGTGGTCATATC**TGTGC**GACACTGCCA**TAATTT**GAACGTG			
pgk	GGTTGCCAGCGCCACACAA**TGTGT**GGCAA**TCTGGG**ACAG			
		TGTGC	**TATAAT**	

Figure B.2: Multiple Alignment of *C. glutamicum* Promoters - A multiple alignment of the *C. glutamicum* promoters shows that the most conserved sequence is TGTGC. In this alignment the sequence occurs in variant positions from the mRNA start site.

askP1	GGCTAGGTAGACACAGTTTATAAAGGTAGAGT	**TGAGC**	G
askP2	TGCATTCAA	**TGTGC**	CACTTCGCGTATGCTCGTCTTATAG
fda	TATCACGACAAAAGTTGAG	**TGATG**	CAGGCATAATTGGCTATA
gap	GCATCTGCTGCGAAATCTT	**TGTTT**	CCCCGCTAAAGTTGAGGAC
gdh	TTGTGGTCATATC	**TGTGC**	GACACTGCCATAATTTGAACGTG
hom	GGGCTTTAAAGCAAAAA	**TGAAC**	AGCTTGGTCTATAGTCGGCTA
lysA	GTATGGGTCGTATTC	**TGTGC**	GACGGGTGTACCTCGGCTAGAATTTCTC
pgk	TACCGGTTGCCAGCGCCACACAA	**TGTGT**	GGCAATCTGGGACAG
pheA	TAGTTCGGCGGGTTAAGCTGTGTAACCA	**TGAGC**	GACGCACCAA
thrC	AGACGGTGTGGGGGAGTAT	**TGTGT**	CACCCCTTGGGATAGGGTTA
CONSENSUS		**TGTGC**	

promoters analyzed. The T in position 3, the G in position 4, and the C in position 5 are in six or more of the ten promoters.

Mutational Analysis of the TGTGC Motif

To determine whether the recurring TGTGC affects promoter strength, we undertook a mutational analysis of this region in the *thrC* promoter. The *thrC* gene encodes for threonine synthase, the last enzyme in the synthesis of threonine [136]. This promoter was chosen because it is fairly representative of the *C. glutamicum* promoters identified; it has a variation of the TGTGC, i.e. TGTGT around -20 and a sequence resembling TATAAT at -10. Furthermore, it is constitutively expressed and it is a housekeeping gene so is assumed to be recognized by the *C. glutamicum* equivalent of the primary sigma factor. In order to determine the effects of changes in the TGTGC motif, each nucleotide was substituted and the strength of the new promoter determined. Strengths of promoters were determined by inserting the promoters upstream of a promoterless *lacZ* gene and comparing the specific activities of ß-galactosidase.

The single substitutions in this region were generated either randomly or by site-directed mutagenesis. In the random method, the coding strand of the *thrC* promoter was synthesized with a misincorporation rate of 1/12 for the twelve base pair region surrounding the TGTGT motif. These mutant coding strands were annealed with wild type template strand and ligated into pZIP (Table B.1). In the site-directed mutagenesis method, a set of four primers were synthesized, each corresponding to the 5′ end of the coding strand of the *thrC* promoter:

5′-GAGGATCCATATAACCCTATCCCAAGGGGTGACA(ATG)AATAC-3′

5′-GAGGATCCATATAACCCTATCCCAAGGGGTGACAC(TGC)ATAC-3′

5′-GAGGATCCATATAACCCTATCCCAAGGGGTGA(GTA)ACAATAC-3′

Table B.1: Strain and Plasmid List

STRAINS		reference
C. glutamicum		
AS019	rif^R lab strain	43
E12	restriction deficient derivative of AS019	35
E. coli		
DH5α	*lacZDM15, recA1*	BRL
PLASMIDS		
pMTI	*C. glutamicum/E.coli* shuttle vector Ap^R in *E. coli* Km^R in *C. glutamicum*	35
pMT-SCM	pMTI with promoterless *cat* gene inserted in *SalI* site of polylinker - *cat* gene contains own ribosome binding site.	this paper
pZIP	pMT-SCM with *lacZ* gene inserted in frame in *PvuI* site of *cat* gene.	this paper
pZIP-TCP	pZIP with the *thrC* promoter cloned into the *KpnI/BamHI* sites in the polylinker. The promoter was constructed by designing oligonucleotides which correspond to both strands of the *thrC* promoter, incorporating a *KpnI* site at the 5' end and a *BamHI* site at the 3' end. After annealing the strands together, the dsDNA was kinased and ligated into pZIP.	this paper

5′-GAGGATCCATATAACCCTATCCCAAGGGGTGAC(GCT)CAATAC-3′.

Included at the 5′ end are extra base pairs in order to facilitate cleavage by *BamHI* (molecular biology enzymes and reagents were purchased from either New England Biolabs, Beverly, MA or Bethesda Research Laboratories, Gaithersburg, MD). Each primer contains one degenerate position corresponding to the positions of the first four nucleotides in the targeted motif TGTG. At this position, only the three non-wild type nucleotides were allowed to be incorporated during oligonucleotide synthesis. These primers were used for the PCR amplification of the promoter region. The other primer used for all amplifications anneals to the pMTI vector upstream of the polylinker region of the pMTI vector. PCR was done by standard methods as given by the manufacturer (Perkin Elmer Cetus). PCR products were digested with *KpnI* and *BamHI* and ligated into pZIP. Molecular biology techniques used were all standard protocols as given by manufacturer or Sambrook, *et al.* [106].

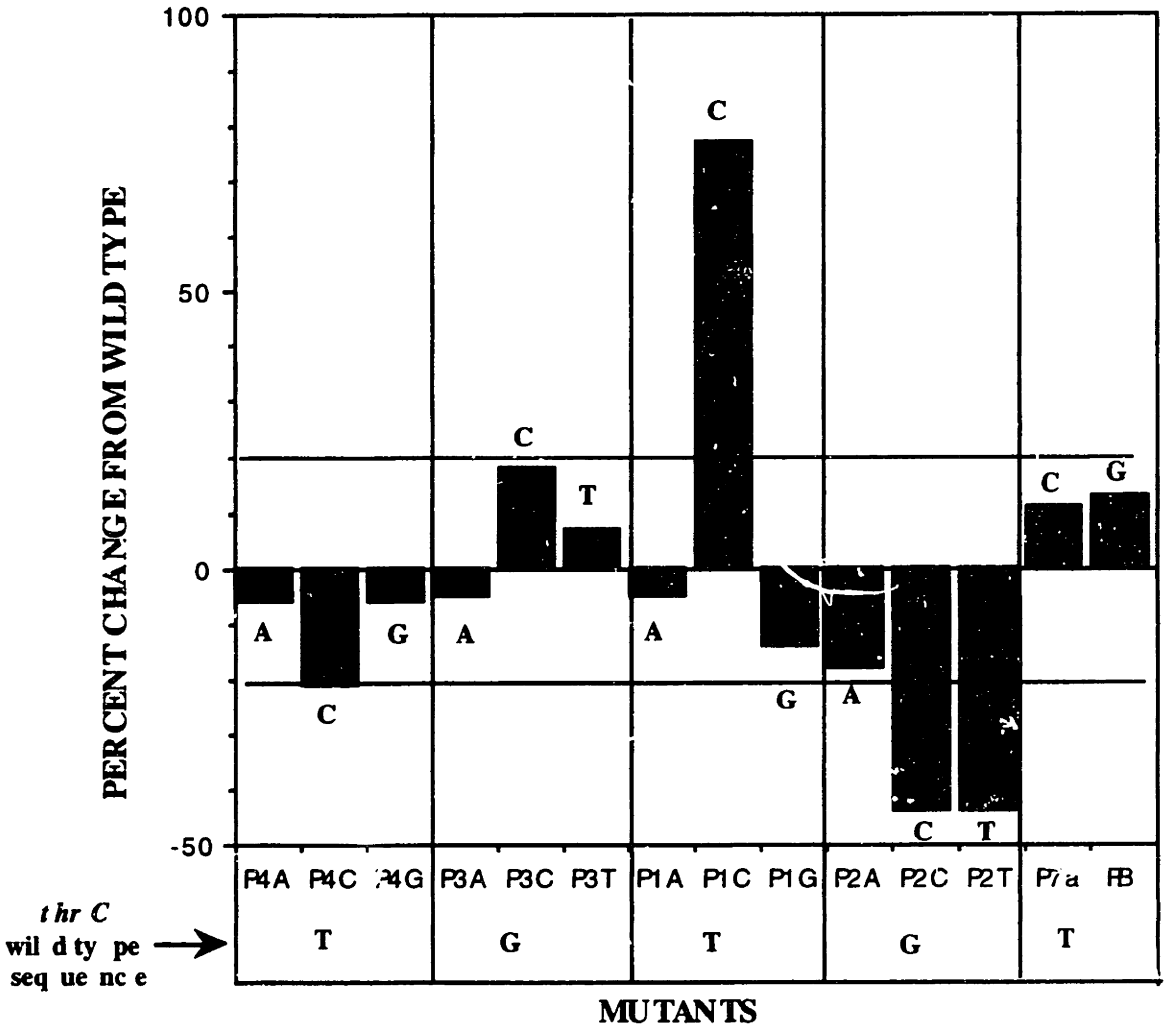
In order to screen for promoters with single substitutions, the plasmids, wild type and mutant, were transformed into *E. coli* DH5α using Ca++ competent cells and selecting for Ap^R [106]. Candidates were randomly selected and DNA purified by the method of Birmboin and Doly [105]. The promoter regions were sequenced by the Sanger dideoxy method using United States Biochemical (Cleveland, OH) Sequenase enzyme Version 2.0 and the dsDNA was denatured as described by Promega [139]. A total of fourteen plasmids with single base pair substitutions were obtained. These were chosen for further analysis and transformed by electroporation into *C. glutamicum* E12 as described previously [35].

To compare promoter strength, enzyme assays were done by harvesting cells after they grew to an OD_{600} between 1 and 2 in complex medium ((per liter) 5 g Bacto-Soytone, 5 g Yeast Extract, 15 g Bacto-Tryptone, 5 g NaCl). Cells were washed in Ringer's wash (per liter: 2.25 g NaCl, 0.105 g KCl, 0.120 g $CaCl_2 \bullet 6H_2O$, 0.5 g $NaHCO_3$) and then put in PES + 1mM dithiothreitol. The cells were then disrupted with glass beads, spun at 17K and the supernatant collected. Protein content was assayed by Bradford assays [140]. ß-galactosidase activity was measured as described previously [106]. The results are reported in Figure B.3. Substitutions at the most conserved residues, positions one and two, had very little effect on promoter strength as measured by b-galactosidase activity. Only the T -> C transition at position three resulted in a modest increase in activity of approximately 76%. G -> C and G -> T changes at position four both resulted in decreases in promoter strength of about 44%. All other single substitutions resulted in either very slight increases or decreases (less than 20% changes) or were virtually wild type in activity.

Conclusions

In this paper we have shown that the most conserved sequence of ten putative *C. glutamicum* promoters is TGTGC, which usually occurs around -20 but can occur either further upstream or downstream in the promoter. A mutational analysis of this region in the *thrC* promoter to determine how substitutions in these nucleotides affect promoter strength reveals that a four-five-fold range of promoter strengths as measured by ß-galactosidase specific activities can be achieved. Substitutions at the two most highly conserved residues, however, result in virtually wild type activity. From what is known of *C. glutamicum* promoter activity at this time, it is not known whether this is a significant change in promoter expression. In order to more clearly define whether the TGTGC

Figure B.3: Promoter Activities - The results of the β-galactosidase assays are shown. The wild type sequence of the *thrC* promoter is indicated and the substitutions at each nucleotide are represented by being labeled with the substituted base. Activities are given as the percent change from the wild type activity. The results are the average of at least two, but in most cases three or greater independent assays. The pZIP vector alone, with no promoter upstream of the *lacZ* gene, had negligible b-galactosidase activity.



motif directly interacts with RNA polymerase, other analyses such as DNA footprinting need to be done.

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